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Pathogenic and non-pathogenic strains of *Vibrio parahaemolyticus* in Long Island Sound: Biotic and abiotic factors promoting abundance

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Abstract of the Thesis

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Abstract

Food safety is a major concern in the shellfish industry, as severe illness can result from consuming shellfish that have accumulated waterborne pathogens. Shellfish harvesting areas are typically monitored for indicator bacteria such as fecal coliforms that serve as proxies for enteric pathogens introduced from surrounding waterways, but these indicators have shown little relation to some naturally occurring pathogenic bacteria such as *Vibrio parahaemolyticus* (Vp). To examine the dynamics and ecology of pathogenic and non-pathogenic strains of Vp and address the relevance of indicator bacteria in predicting Vp concentrations, field surveys and experiments were carried out in western Long Island Sound, NY, a region that has experienced recent outbreaks of shellfish contaminated with Vp. Pathogenic and non-pathogenic strains were

quantified via PCR detection of marker genes and most probable number techniques. Field survey data showed little correspondence between fecal coliforms and Vp, but significant correlations between Vp and an alternative indicator, enterococci, as well as short-term (48 h) rainfall were observed. Experiments demonstrated that enrichment of seawater with phytoplankton-derived dissolved organic matter significantly increased the concentration of total Vp and the presence pathogenic Vp, but higher temperatures did not. Collectively, my thesis results suggest that fecal coliforms may fail to account for the full suite of important shellfish pathogens and that enterococci could provide a potential alternative or supplement to shellfish sanitation monitoring. Given the ability of algal-derived dissolved organic matter to promote the growth of pathogenic Vp, my results suggest that restricting nutrient inputs into coastal water bodies, which promote primary production and have detrimental secondary effects such as hypoxia, may additionally decrease the proliferation of Vp. As molecular detection methods for Vp and other pathogens are refined, monitoring efforts may be refined to more closely target specific pathogens of concern to protect human health.

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Introduction

Shellfish harvesting is a billion dollar industry in the US. Due to the health risks, including gastroenteritis and in extreme cases mortality, associated with consuming shellfish containing pathogenic microbes, food safety is a high priority in the industry (Rippey 1994, Wittman and Flick 1995). To ensure the safety of shellfish for human consumption, water quality standards are used to designate areas of coastal zones that are fit for shellfish harvest based on potential shellfish exposure to pathogenic microbes.

When filtering particles from water bodies, bivalves may accumulate microbial pathogens present into their tissues at concentrations many times greater than ambient levels (Metcalf et al. 1979). Pathogenic microbes often enter shellfish habitat via sewage or wastewater contamination, but may also occur naturally in estuarine environments (Mallin et al. 2000, 2001). Due to the complexity, time, and expense of identifying and measuring multiple pathogenic microbes in coastal waters (Fong and Lipp 2005), fecal coliform bacteria are typically used as indicator organisms in water quality surveys. If fecal coliform bacteria are found at high concentrations, it is assumed that enteric pathogens that can accumulate in shellfish tissues and sicken consumers will also be present and thus may have contaminated local shellfish stocks (Cabelli et al. 1983, Field and Samadpour 2007). To function as effective indicator organisms, fecal coliform bacteria should not reproduce in contaminated environments and should also be correlated with the presence of pathogens (Field and Samadpour 2007).

While fecal coliform bacteria standards for shellfish harvest are common, such a metric does not necessarily capture the full spectrum of pathogenic microbes that can accumulate in shellfish tissues (Field and Samadpour 2007), and it may be a particularly poor proxy for *Vibrio*

parahaemolyticus (Vp), which occurs naturally in estuaries and has been identified as a pathogen of concern in recent years in coastal waters (Yeung and Boor 2004, Wang et al. 2015, Xu et al. 2015). Vp is a gram-negative, curved, rod shaped bacterium. The species is halophilic and broadly distributed, inhabiting marine and estuarine waters in the Atlantic (Zimmerman et al. 2007, Blackwell and Oliver 2008, Jones et al. 2014), Pacific (Johnson et al. 2012), and Indian Oceans (Deepanjali et al. 2005). Though not all strains of Vp are pathogenic, Vp has several potential virulence factors, such as hemolysin, which can lead to cytotoxicity in host cells and severe gastroenteritis if Vp is consumed by humans or animals (Honda and Iida 1993, Hiyoshi et al. 2010). Past studies have shown both minimal (Koh et al. 1994, Parveen et al. 2008, Jones et al. 2014) and significant positive correlations between fecal coliforms and Vp as well as other *Vibrio* species, including strains that occur naturally in estuarine environments (Pfeffer et al. 2003, Blackwell and Oliver 2008).

Specific toxins primarily associated with pathogenic Vp are thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) (Honda and Iida 1993, Hiyoshi et al. 2010). Thermolabile hemolysin (TLH) has also been identified, but is present in all isolated strains of Vp, both pathogenic and non-pathogenic (Wang et al. 2015). As Vp has grown as an issue of seafood safety, efforts have been made to identify gene markers associated with pathogenesis of Vp, and the genes encoding for TDH (*tdh*), TRH (*trh*), and TLH (*tlh*) have been identified (Makino et al. 2003, Xu et al. 2015), allowing for the development of molecular Vp detection techniques (Nordstrom et al. 2007, Garrido et al. 2012, Whistler et al. 2015). As *tlh* is found in all known strains of Vp, it is considered a presence/absence indicator for Vp, whereas *tdh* and *trh* are used to specifically identify pathogenic strains.

Vp has been measured in shellfish in many regions across the US (DePaola et al. 2003, Johnson et al. 2012, Jones et al. 2014), but pathogenic Vp contamination in shellfish is a relatively recent phenomenon in Northeast U.S. waters, with the first major identified outbreak observed in 1998, as Vp has traditionally been associated with warmer coastal waters (Jones et al. 2014, Xu et al. 2015). Increases in pathogenic Vp outbreaks are thought to be due to an invasion of a pathogenic strain indigenous to the Pacific Northwest, as well as elevated water temperatures during summer months in recent years (Xu et al. 2015). While studies of Vp concentrations in shellfish tissues have increased as food-safety concerns have grown, studies in which quantitative measurements of total and pathogenic Vp have been made directly from water samples have been more rare. Post-harvest temperature and handling practices have been shown to largely control levels of pathogenic Vp in shellfish, as microbial populations are capable of rapid growth *in vivo* even when exposed to high-temperatures for only a short time (DePaola et al. 2010, Oliveira et al. 2011). While the effect of temperature on Vp concentrations has been well described, the role of other environmental factors in controlling pelagic Vp concentrations remains limited, and such information could be used to develop management plans that ensure food safety (Yeung and Boor 2004, Wang et al. 2015).

A number of factors can influence the fate and concentration of pathogenic microbes in coastal environments, including organic matter concentration, rainfall, light intensity, salinity, and water temperature (Campos et al. 2013). Increases in pathogenic microbes have also been linked to the density of human populations, the amount of developed land, and amount of impervious surfaces in catchments surrounding coastal bays (Mallin et al. 2000, Mallin et al. 2001), as well as the presence of septic systems (Lipp et al. 2001). Molecular techniques have been used in prior studies to measure Vp in water samples from areas along the east coast of the

U.S. and have found significant associations between Vp and water temperature, salinity, turbidity, and dissolved oxygen (Zimmerman et al. 2007, Parveen et al. 2008), although the relationship between these factors and pathogenic strains of Vp were not described. Organic matter concentrations, water temperature, and rainfall are generally believed to contribute to increases in concentrations of indicator bacteria (Campos et al. 2013), but their effects on total and pathogenic Vp in coastal waters have rarely been explored experimentally.

To more fully ensure the safety of shellfish, direct measurements of pathogenic Vp in contaminated harvest areas are warranted. Data assessing the concentration and distribution of Vp in coastal waters could prove to be an important management tool for ensuring shellfish safety as it could provide insight regarding the ecology of Vp as well as environmental factors that promote high concentrations. Here, I address this knowledge gap through surveys of total and pathogenic strains of Vp in estuaries across the north shore of Long Island, NY, USA, where outbreaks of pathogenic Vp-associated with human illnesses occurred in 2014. Beyond quantifying total and pathogenic strains of Vp, parallel measurements of multiple indicator bacteria and environmental variables were made. Further, experimental manipulations of temperature and dissolved organic matter were performed to assess their role in growth of total and pathogenic strains of Vp. Hypotheses proposed for the study included: 1) total and pathogenic Vp concentrations will show no significant correlation with indicator bacteria species and 2) total and pathogenic Vp concentrations will be significantly higher after experimental additions of dissolved organic matter and when incubated at temperatures higher than ambient levels.

Methods

Field Sampling of Vp, Environmental Parameters, and Indicator Bacteria

Field samples were collected weekly at four sampling sites located in areas currently uncertified for shellfish harvest within Hempstead Harbor, Oyster Bay Harbor, Cold Spring Harbor, and Northport Harbor, NY (Fig. 1), USA, from summer through fall 2015, the time period when Vp contamination had previously occurred in this region (Xu et al. 2015). Limited sampling was also performed at Huntington Harbor, NY, USA. A surface water sample was taken at each site using an autoclave-sterilized, 1-liter polypropylene Nalgene bottle. All samples were immediately transported in coolers and processed on the same day as they were collected. Dissolved oxygen levels were recorded *in situ* using HOBO U26-001 dissolved oxygen data loggers and YSI 5920 sondes. Salinity and water temperature were also measured on site using handheld the YSI 556 sonde and turbidity was evaluated using a Secchi disc. *In vivo* fluorescence-based quantification of chlorophyll *a* was determined using a bbe-Moldaenke FluoroProbe II (Catherine et al. 2012). Rainfall data was obtained from the National Weather Service monitoring station in Islip, NY, USA, located within 55 km of the sampling locations.

Fecal coliform and enterococci concentrations were measured using US EPA Clean Water Act Section 304(h) approved IDEXX Quanti-tray measurement system, which uses a Most Probable Number (MPN) approach (Eckner 1998). Using this method, samples are enriched with growth media, separated into sealed Quanti-trays, and incubated overnight. The MPN estimate is then based on the number of positive and negative wells of the Quanti-tray post-incubation.

Total and pathogenic Vp were also measured in terms of MPN using a multiplex PCR assay that quantifies both total and pathogenic strains of Vp via distinct genetic markers (Zimmerman et al. 2007, Parveen et al. 2008, Whistler et al. 2015). The Vp MPN procedure adapted from the USFDA Bacteriological Analytical Manual used volumes of 100 ml, 10 ml, and 1 ml in triplicate taken from field samples (Kaysner and DePaola 2004). Each volume was enriched with 10X

alkaline peptone water (10% peptone, pH 8.5), with additions of 11 ml, 1.1 ml, and 0.11 ml of alkaline peptone water to the 100 ml, 10 ml, and 1 ml sample volumes respectively. All volumes were then incubated overnight at 35ºC. Following incubation, 200 µl of the enriched samples were transferred to microcentrifuge tubes and placed into a boiling water bath for 10 minutes to lyse any Vp cells present (Zimmerman et al. 2007, Parveen et al. 2008). All samples were then immediately stored at -80ºC until PCR reactions were performed.

The multiplex PCR procedure used recently published primer sequences for *tlh*, *tdh*, and *trh* designed to aid in distinguishing between *tlh* and *trh* using a standard PCR procedure (Whistler et al. 2015). Forward and reverse primer sequences were as shown in Table 1. Multiplex primer mixtures were prepared with final concentrations of 1 µM *tlh* and 2 µM of *trh* and *tdh* to aid the amplification of pathogenic markers at low concentrations (Nordstrom et al. 2007). A 25 µl PCR mixture was prepared using 12.5 µl GoTaq Green Master Mix, 2.5 µl multiplex primer mixture, 2 µl template DNA, and 8 µl nuclease-free water. PCR temperature cycles began with an initial 3-minute denaturation at 94ºC then completed 30 cycles of denaturation at 94ºC for 1 minute, primer annealing at 55ºC for 1 minute, and extension at 72ºC for one minute. A final extension at 72ºC for 5 minutes completed the PCR procedure (Whistler et al. 2015). All PCR procedures used genomic Vp DNA or prior positive field samples as a positive control and nuclease-free water as a negative control. Amplicons were evaluated with gel electrophoresis, using 5 µl of sample on a 2% agarose gel with a current of 85 Watts run for 45 minutes. Total and pathogenic Vp were quantified based on the presence or absence of *tlh*, *trh*, and *tdh* at each dilution volume using a customized USFDA MPN procedure (Garthright and Blodgett 2003). Samples were considered to contain pathogenic strains of Vp if either *trh* or *tdh* were present. Samples below detection limits were reported as one half of minimum detectable levels to aid in visual

representation, and samples above the detection range were reported at the next most conservative MPN estimate.

Relationships Between Indicator Bacteria, Environmental Parameters, and Vp

Spearman's Rank Order regression analyses were performed on data from field samples to identify any correlation between fecal coliform bacteria, enterococci, water temperature, chlorophyll *a*, dissolved oxygen, salinity, turbidity, or rainfall and total or pathogenic Vp. Data on total and pathogenic Vp are also displayed as time series plots along with concentrations of indicator bacteria to visualize any temporal patterns present at each sampling location. Additionally, a logistic regression analysis was used to model the relationship between environmental parameters and the presence or absence of pathogenic Vp, with significance determined through a likelihood-ratio test. Confidence intervals for MPN field data were generated through the USFDA MPN procedure (Garthright and Blodgett 2003).

Experimental Incubations

Bottle incubations experiments were performed to understand the influence of temperature and dissolved organic matter (DOM) on the abundance of total and pathogenic strains of Vp. To produce DOM for experiments, phytoplankton cultures were utilized. Specifically, 1-liter of *Chaetoceros muelleri*, *Isochrysis galbana*, *Tetraselmis suecica*, and *Pavlova lutheri* grown in f/2 media at 24°C at 100 μEin m^{-2} s^{-1} at densities of 2 x 10^6 cells ml^{-1} (save for *Tetraselmis suecica* which had a density of 5 x 10^5 cells ml^{-1}; cells microscopically quantified via a hemocytometer) were centrifuged in 100 ml aliquots for 10 minutes at 10,000 rpm to pellet cells. After removal of the supernatant, the pellets were resuspended in a 50 ml Falcon tube using 10 ml of distilled water and then sonicated (Ultrasonic Power Corporation, Freeport, Illinois. Model 1000L) at 30% power for two one-minute intervals to lyse cells. A second round of centrifugation for 10

minutes at 10,000 rpm then took place to pellet any remaining particulate matter, and the supernatant was then filtered through combusted (2 h @ 450°C) Pall, GFF glass fiber filters (0.7 μm) using a syringe to obtain the final DOM for experimentation. The final stock DOM contained 1082, 642, and 427 mgL^{-1} of dissolved nitrogen, phosphorous, and organic carbon respectively. Four, 2-L polycarbonate bottles were filled with water from Cold Spring Harbor and 5 ml of the concentrated dissolved organic matter was added, mimicking the lysis of an algal bloom of ~ 3 x 10^5 cells ml^{-1}, a level previously observed in the sampling region (Hattenrath et al. 2010, Hattenrath-Lehmann et al. 2013, Hattenrath-Lehmann et al. 2015). Four separate 2-L bottles containing water from field sites without organic matter additions were used as a control. Bottles were placed in an outdoor, flow-through water bath at Stony Brook – Southampton, 75 km east of Cold Spring Harbor, which maintained water temperatures and light levels similar to those observed at a 1 m depth in Cold Spring Harbor at the time of sample collection. After 24 h, aliquots were removed to quantify densities of total and pathogenic Vp as described above. Two of these experiments were performed on September 15 and 29, 2015.

Temperature experiments were performed using water from Cold Spring Harbor divided into eight 2 L bottles, with four maintained at ambient temperature measured at the field site and four incubated at ~ 3ºC above ambient temperature. Temperature was manipulated by using heating wands in ambient outdoor water baths described above. After 24 h, aliquots were removed to quantify densities of total and pathogenic Vp as described for field samples. Two such experiments were performed on October 6 and 20, 2015. Differences in densities of total Vp between controls and treatments for both types of experiments were assessed through use of One-Way ANOVAs. Differences in the proportion of samples containing pathogenic Vp between

treatments were assessed using a two-sample test for equality of proportions. Confidence intervals for experimental results were generated via bootstrapping techniques.

Results

Field Surveys

Water temperatures followed expected seasonal patterns and were fairly consistent across sampling sites, with Northport Harbor showing higher temperatures in the early portion of the sampling period. Temperatures rose slightly in the first weeks of the sampling period before reaching sustained peak levels in late August, reaching or exceeding 25ºC at sampling sites. Thereafter, water temperatures steadily declined into fall, falling below 10ºC in the final weeks of sampling. Precipitation in the two days prior to sampling was limited during the sampling season. The largest rain event of 0.97 inches occurred in the first week of sampling and rainfall within the two-day window occurred only three additional times over the sampling period (Fig. 2).

Levels of total and pathogenic strains of Vp varied in space and time but were generally higher in summer than fall, highest at Cold Spring Harbor, and lowest at Northport Harbor. At Hempstead Harbor total Vp was detectable with week-to-week variation during summer months before a decline in late fall. Total Vp ranged from 0.4 to 240 MPN 100 mL^{-1}, with a mean concentration of 7.2 MPN 100 mL^{-1} (Fig. 3). Pathogenic strains were present in 63% of samples, primarily in the early and latter portions of the sampling period. When detected, pathogenic Vp concentrations ranged from 0.1 to 4.3 MPN 100 mL^{-1}, with a mean concentration of 0.5 MPN 100 mL^{-1} (Fig. 3). The proportion of total Vp that was pathogenic ranged from 0 to 28%, and averaged 4.2%. Indicator bacteria concentrations also showed temporal variation over the

sampling period with high concentrations in summer months before a decline in the latter portions of the sampling period (Fig. 3). Enterococci concentrations ranged from 2.5 to 1100 MPN 100 mL^{-1} with a mean of 51 MPN 100 mL^{-1}, while fecal coliform concentrations ranged from 6.0 to 1410 MPN 100 mL^{-1} with a mean concentration of 96 MPN 100 mL^{-1} (Fig. 3).

Vp was consistently present at Oyster Bay Harbor with the exception of one week late summer. When present, total Vp concentrations ranged from 0.4 to 240 MPN 100 mL^{-1}, with a mean concentration of 6.5 MPN 100 mL^{-1} (Fig. 4). Pathogenic strains were fairly persistent at this site, being found in 74% of samples. When detected, pathogenic Vp concentrations ranged from 0.03 to 7.6 MPN 100 mL^{-1} with a mean concentration of 0.4 MPN 100 mL^{-1} (Fig. 4). The proportion of total Vp that was pathogenic at Oyster Bay Harbor varied from 0 to 100%, though the majority of samples contained only a small proportion of pathogenic strains. Overall, the mean proportion of pathogenic Vp was 18%. Indicator bacteria showed more temporal consistency at Oyster Bay Harbor with an expected seasonal trend. Enterococci concentrations ranged from 2.5 to 280 MPN 100 mL^{-1} with a mean concentration of 11 MPN 100 mL^{-1}, while fecal coliform concentrations ranged from 1.1 to 32 MPN 100 mL^{-1} with a mean of 5.9 MPN 100 mL^{-1} (Fig. 4).

Cold Spring Harbor had consistently elevated total Vp levels, ranging from 2.3 to 240 MPN 100 mL^{-1} with the highest mean concentration among sites of 23 MPN 100 mL^{-1} (Fig. 5). Pathogenic Vp was found in 58% of samples and, when detected, concentrations ranged from 0.07 to 4.6 MPN 100 mL^{-1} with the highest mean concentrations among sites of 0.6 MPN 100 mL^{-1} (Fig. 5). Cold Spring Harbor also showed a broad range in the proportion of total Vp comprised of pathogenic strains, ranging from 0 to 100%, with the majority of samples having only a small proportion of pathogenic strains (0 to 10%). The mean proportion of total Vp

containing pathogenic strains was 7.9%. Indicator bacteria at the site were elevated for much of the sampling period, showing a peak in late summer months before declining in the later portions of the sampling period. Enterococci concentrations ranged from 2.5 to 350 MPN 100 mL^{-1} with a mean concentration of 8.1 MPN 100 mL^{-1}, while fecal coliform concentrations ranged from 2.0 to 310 MPN 100 mL^{-1} with a mean concentration of 14 MPN 100 mL^{-1} (Fig. 5).

Vp was consistently present in Northport Harbor during late summer and early fall, with lower concentrations seen early in the sampling period. Total Vp concentrations ranged from 0.03 to 240 MPN 100 mL^{-1} while the mean concentration was the lowest among the sites at 1.7 MPN 100 mL^{-1} (Fig. 6). Pathogenic strains were found in 63% of samples at this site and, when detected, concentrations ranged from 0.06 to 0.7 MPN 100 mL^{-1} with a mean concentration of 0.2 MPN 100 mL^{-1}, the lowest average level among the study sites (Fig. 6). The proportion of total Vp containing pathogenic strains reached a maximum of 49% in September and the mean proportion of total VP that was pathogenic was 11%. Indicator bacteria concentrations in Northport were elevated late summer before declining. Enterococci concentrations ranged from 2.5 to 330 MPN 100 mL^{-1} with a mean concentration of 12 MPN 100 mL^{-1}, while fecal coliform bacteria concentrations ranged from 1.0 to 81 MPN 100 mL^{-1} with a mean concentration of 7.4 MPN 100 mL^{-1} (Fig. 6).

There were no significant correlations found between total Vp and any environmental parameters (Table 2). Pathogenic strains of Vp, however, showed significant positive correlations with enterococci densities ($r(56) = 0.35$, $p < 0.01$), and rainfall in the two days prior to sampling ($r(76) = 0.24$, $p < 0.05$). Together, these two parameters also produced a significant logistic regression model when applied to pathogenic Vp presence-absence data over all sampling sites, $p < 0.01$ (Table 3).

Experiments

In two experiments, the addition of dissolved organic matter to water samples from Cold Spring Harbor significantly increased total Vp concentrations in experimental trials (ANOVA, $F(1, 12) = 7.65$, $p < 0.02$; Fig. 7). Abundance of total Vp did not significantly differ between experiments ($F(1, 12) = 2.27$, $p > 0.05$). Dissolved organic matter additions also significantly increased the proportion of samples in experiments containing pathogenic Vp ($\chi^2 = 5.4018$, df = 1, $p < 0.05$; Fig. 8). In contrast, higher temperatures did not significantly alter levels of total Vp concentrations ($F(1, 13) = 0.43$, $p = 0.52$) and again no differences in abundance were seen between experiments performed (ANOVA, $F(1, 13) = 0.34$, $p > 0.05$; Fig. 9). Pathogenic strains were only seen in one experimental sample from temperature experiments, limiting statistical analyses of these microbes.

Discussion

Vibrio parahaemolyticus (Vp) is a marine bacterium of growing concern on international, national, and regional levels and yet little is known regarding the ecology or population dynamics of this microbe. During this study, the seasonal dynamics of Vp in multiple harbors across the north shore of Long Island were documented and contrasted with indicator bacteria species. Total Vp densities were dynamic and statistically unpredictable while the presence of pathogenic Vp was significantly correlated with recent rainfall and the indicator microbe, enterococci. Both total Vp concentration and the proportion of samples containing detectable levels of pathogenic Vp were significantly increased by the addition of dissolved organic matter derived from phytoplankton. Collectively, these findings bring new insight regarding the ecology of total and pathogenic Vp in temperate coastal ecosystems.

Indicator bacteria are often measured in coastal ecosystems to protect human health against pathogens. This study afforded the evaluation of efficacy of fecal coliform bacteria and enterococci as indicators of total and pathogenic Vp. While total Vp concentrations from field samples showed no significant correlation with indicator bacteria, pathogenic Vp showed a significant correlation with enterococci, contrary to my original hypothesis. This result was somewhat surprising as Vp is not an enteric pathogen and therefore is not typically associated with the sewage contamination indicator bacteria are intended to detect (Drake et al. 2007, Wang et al. 2015). Given that a direct indicator relationship between enterococci and pathogenic Vp is unlikely, enterococci may instead be associated with conditions favorable for the growth of pathogenic Vp. Given the record of large volumes of wastewater and stormwater that enters western Long Island Sound and surrounding harbors (Wolfe et al. 1991, O'Shea and Brosnan 2000), particularly within the interior of harbors where sampling took place, it is possible that enterococci serve as a proxy for nutrient and organic matter inputs, the latter of which has been correlated with bacterial production in the region (Anderson and Taylor 2001).

While significant correlations between total and pathogenic Vp and environmental parameters were largely absent, again contradicting initial hypotheses, a significant positive correlation was observed between pathogenic Vp and rainfall in the two-day period prior to sampling. This result also aligns with experiments showing increased Vp concentrations with DOM additions, as short-term increases in rainfall introduce organic matter from surrounding catchments into coastal water bodies (Mallin et al. 2001). Rainfall has also been shown to increase indicator bacteria concentrations in coastal bays (Kashefipour et al. 2006, Campos et al. 2013), and this overlap between rainfall, organic matter input, and levels of indicator bacteria may again explain the observed association of pathogenic Vp and enterococci.

During this study, the concentrations and prevalence of total and pathogenic Vp, respectively, increased significantly upon the exposure to DOM originating from phytoplankton. DOM is the primary energy source for bacteria in the ocean (Azam et al. 1994, Nagata 2008) and phytoplankton are the primary source of ocean organic matter (Baines and Pace 1991). Recent studies of Long Island coastal waters have documented a steady increase in nitrogen levels entering coastal waters and an increasing prevalence of algal blooms (Gobler et al. 2008, Hattenrath et al. 2010, Hattenrath-Lehmann et al. 2013). Furthermore, many of these algal blooms have been shown to be promoted by the loading of excessive nitrogen from land to sea (Hattenrath et al. 2010, Gobler et al. 2012, Hattenrath-Lehmann et al. 2015). Given that total Vp concentrations and the proportion of samples containing pathogenic strains of Vp increased following the addition of algal organic matter, it would seem that the issue of pathogenic Vp contamination in shellfish in Long Island coastal waters may be related, at least in part, to rising levels of nitrogen loading that are promoting algal blooms and higher levels of DOM.

The findings of this study have implications for current water quality monitoring strategies and the indicator bacteria paradigm as a whole. The use of fecal coliform bacteria as indicator organisms for monitoring the safety of shellfish growing or harvesting areas relies on the assumption that the bacteria will reliably co-occur with pathogens of concern in the region, and, by extension, that major pathogens are enteric in nature (Harwood et al. 2005). This study demonstrates that this paradigm does not hold for Vp, a non-enteric pathogen responsible for a significant amount of shellfish-related health issues, that showed no association with fecal coliform bacteria concentrations used to ensure food safety. Total Vp densities were also not associated with fecal coliform bacteria. This is not necessarily surprising as fecal coliform bacteria were designated as indicator organisms, in part, because they do not reproduce in

contaminated environments (Burkhardt et al. 2000, Field and Samadpour 2007) whereas Vp was shown to grow rapidly during incubations with elevated levels of algal organic matter. Pathogenic strains of Vp were, however, significantly correlated with enterococci densities, supporting the use of enterococci as indicator bacteria as for shellfish safety, rather than fecal coliforms, a finding consistent with prior studies of bathing beaches (Noble et al. 2003, Jin et al. 2004). However, Vp and enterococci have shown no significant relationship in other regions of the Gulf, East, and West coasts of the United States (Johnson et al. 2012). Given this uncertainty regarding the presence of different pathogens and their association with common indicator organisms, an alternative approach to water quality monitoring might be one tailored to pathogens present in regional water bodies, especially those that are not enteric in nature, as opposed to broad national standards based on proxies for enteric pathogens alone (Field and Samadpour 2007). Such a strategy for Vp monitoring has become more feasible in recent years given the advances in molecular detection techniques, exemplified by the assay presented within this study that employs a standard MPN-PCR procedure. Performing these more targeted assays in parallel with current indicator organism monitoring would provide more robust standards of shellfish safety with regard to non-enteric pathogens such as Vp that can pose equal or greater risks to human health than most traditionally monitored enteric pathogens, and examples of such strategies have been implemented in a number of shellfish harvest regions already.

Traditionally, concentrations of bacterial human pathogens in coastal waters parallels water temperatures (Cook et al. 2002, DePaola et al. 2003, Vezzulli et al. 2013). During this study, a clear temperature-dependent pattern in concentrations of total or pathogenic Vp was not apparent until perhaps late fall when multiple sampling sites showed a sharp decline in total and pathogenic Vp densities. This pattern is suggestive of a minimal threshold temperature effect on

Vp abundances rather than a simple linear relationship with temperature, and past studies have shown minimal detection below 15 ºC and optimum growth above 35 ºC (Yeung and Boor 2004, Su and Liu 2007). The relative uncertainty surrounding pathogenic Vp concentrations as well as the potential for rapid growth of Vp when encountering excessive DOM pose challenges for future management of shellfish growing areas affected by this microbe. It is important to note that the majority of pathogenic Vp growth in shellfish occurs post-harvest as the bacteria rapidly multiply *in vivo* if shellfish are exposed to warmer temperatures (DePaola et al. 2010). Thus, control measures designed to reduce the risk of pathogenic Vp buildup have focused on shellfish handling, including shading and refrigeration of shellfish once harvested (Su and Liu 2007, Wang et al. 2015). Given the complexities in growth dynamics of Vp strains, if such temperature control measures are employed effectively it is possible that future monitoring of Vp could be based around periodic presence/absence sampling, based on either limits of detection or threshold concentrations, to identify at-risk areas in which shellfish handling should be more closely controlled. The MPN assay presented here and in other studies could be employed in such a sampling regime, and it is likely that methods will continue to improve as molecular techniques continue to be refined. For example, more sensitive molecular techniques such as qPCR have also enabled analysis of the ecology of total and pathogenic Vp (Nordstrom et al. 2007, Garrido et al. 2012, Whistler et al. 2015), knowledge of which will prove critical in refining future management strategies. Quantitative models such as the one presented within this study, showing a significant relationship between enterococci concentrations, rainfall, and the presence of pathogenic Vp, provide insight into potential environmental drivers of pathogenic Vp. Future studies should aim to further refine such models to provide a predictive framework for management efforts.

The observed increase in total and pathogenic Vp in the presence of elevated DOM levels and the correlation of pathogenic strains with rainfall also highlights the need for holistic coastal management practices in reducing pathogen risk. Inputs of organic matter to coastal water bodies have been associated with detrimental ecological phenomena including hypoxia and harmful algal blooms (Paerl et al. 1998, Taylor et al. 2006, Diaz and Rosenberg 2008), and results within this study indicate that organic matter may also contribute to the proliferation of marine pathogens, further impacting coastal resources. Similarly, it has long been known that runoff within sundry coastal environments can enhance levels of multiple classes of pathogens (Mallin et al. 2000) as well as deliver high levels of nutrients that promote algal blooms (Paerl et al. 1998, Heisler et al. 2008) and in turn, enhance DOM levels (Baines and Pace 1991). Hence, coastal management efforts that seek to restrict excessive nutrient loading from run-off and other sources aimed at restricting algal blooms and hypoxia seem likely to also minimize abundance of Vp, although pathogenic Vp has been detected in pristine estuaries (Gutierrez West et al. 2013).

Tables

Table 1: Primer sequences used in the MPN PCR assay for identification of *tlh*, *trh*, and *tdh*.

Gene	Primer sequence	Amplicon size
Tlh	F: AGAACTTCATCTTGATGACACTGC	401 bp
	R: GCTACTTTCTAGCATTTTCTCTGC	
Trh	F: CATAACAAACATATGCCCATTTCCG	500 bp
	R: TTGGCTTCGATATTTTCAGTATCT	
Tdh	F: GTAAAGGTCTCTGACTTTTGGAC	269 bp
	R: TGGAATAGAACCTTCATCTTCACC	

Table 2: Field survey spearman rank correlations

	Total Vp			Pathogenic Vp		
	n	cor	*p*	n	cor	*p*
Enterococci	56	-0.03	0.83	**56**	**0.35**	**<0.01**
Fecal Coliforms	68	0.16	0.19	68	0.06	0.60
Water Temp.	76	-0.13	0.25	76	0.04	0.72
Rain: Week	76	0.16	0.16	76	0.17	0.15
Rain: Four Day	76	0.05	0.68	76	0.16	0.16
Rain: Two Day	76	0.11	0.35	**76**	**0.24**	**0.04**
Chl *a*	59	-0.10	0.46	59	-0.20	0.14
Secchi	36	0.01	0.95	36	0.12	0.50
Sal	40	0.14	0.39	40	0.08	0.61
DO min	56	0.15	0.97	56	-0.07	0.39

Table 3: Pathogenic Vp presence/absence logistic regression model.

	Coefficients			
	Estimate	Std. Error	z	*p*
Intercept	-0.091	0.350	-0.261	0.794
Enterococci	0.012	0.009	1.244	0.213
Rain: Two Day	11.43	11.092	1.030	0.303
Null Deviance	74.095 on 55 degrees of freedom			
Residual Deviance	63.297 on 53 degrees of freedom			
Likelihood-ratio	***p* = 0.005**			

Figures

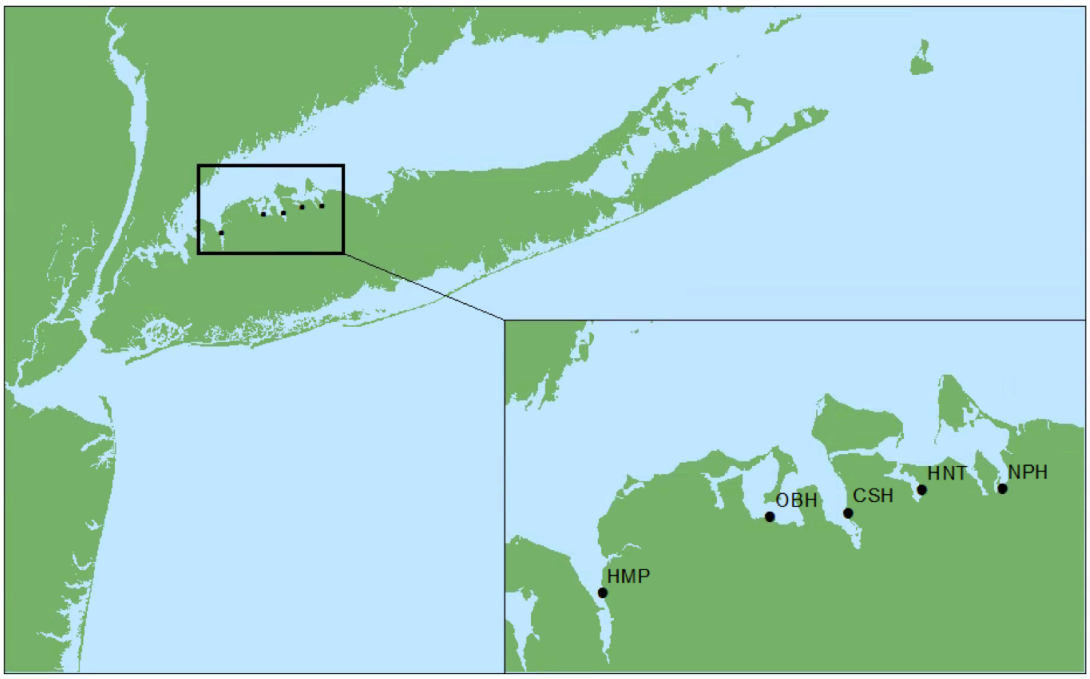

Figure 1: Sampling sites used in the Vp survey, located at Hempstead Harbor (HMP), Oyster Bay Harbor (OBH), Cold Spring Harbor (CSH), Huntington Harbor (HNT), and Northport Harbor (NPH).

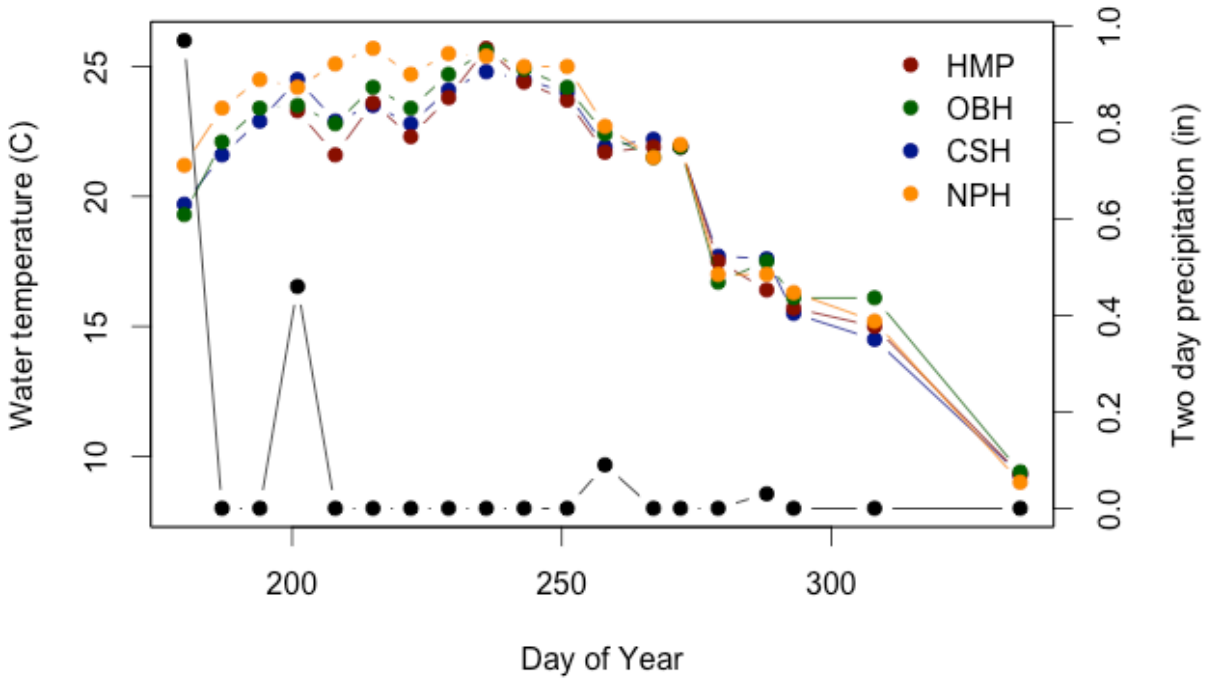

Figure 2: Water temperature recorded at individual sampling sites and precipitation accumulated in the two days prior to sampling recorded by the Islip, NY, National Weather Service station over the sampling period.

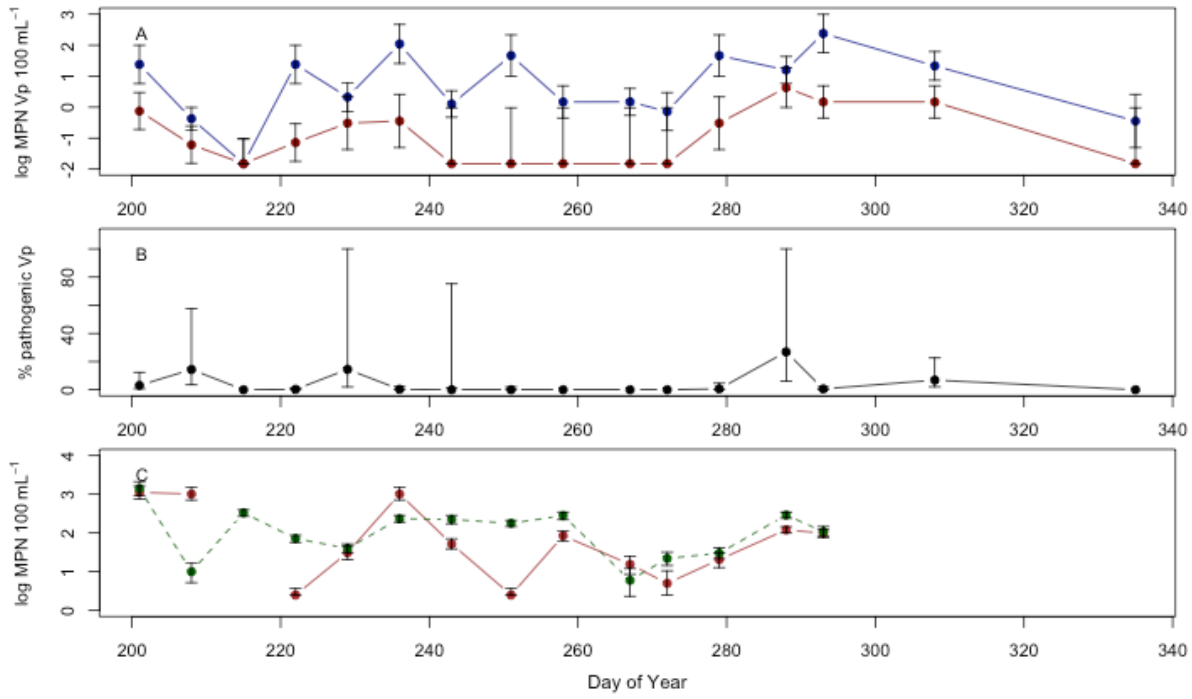

Figure 3: Field survey at Hempstead Harbor. A) Concentrations of total (blue) and pathogenic (red) Vp. B) Percent of total Vp identified as pathogenic. C) Concentrations of enterococci (solid) and fecal coliforms (dashed). Error bars represent 95% confidence intervals.

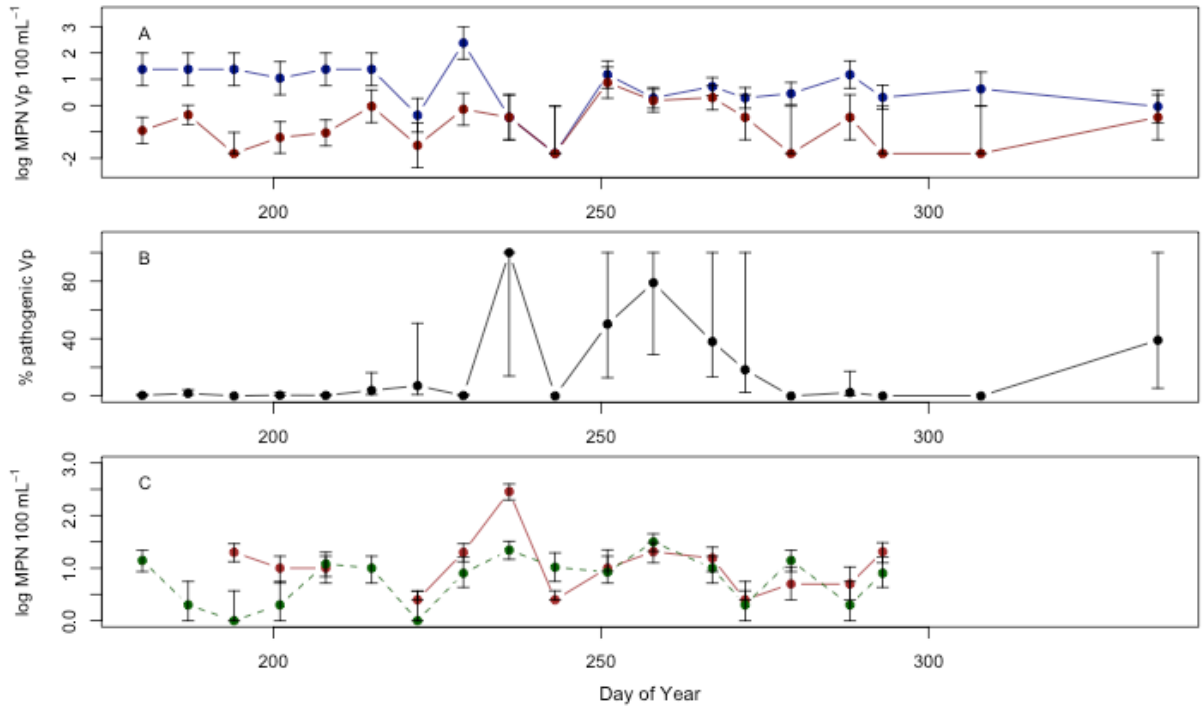

Figure 4: Field survey at Oyster Bay Harbor. A) Concentrations of total (blue) and pathogenic (red) Vp. B) Percent of total Vp identified as pathogenic. C) Concentrations of enterococci (solid) and fecal coliforms (dashed). Error bars represent 95% confidence intervals.

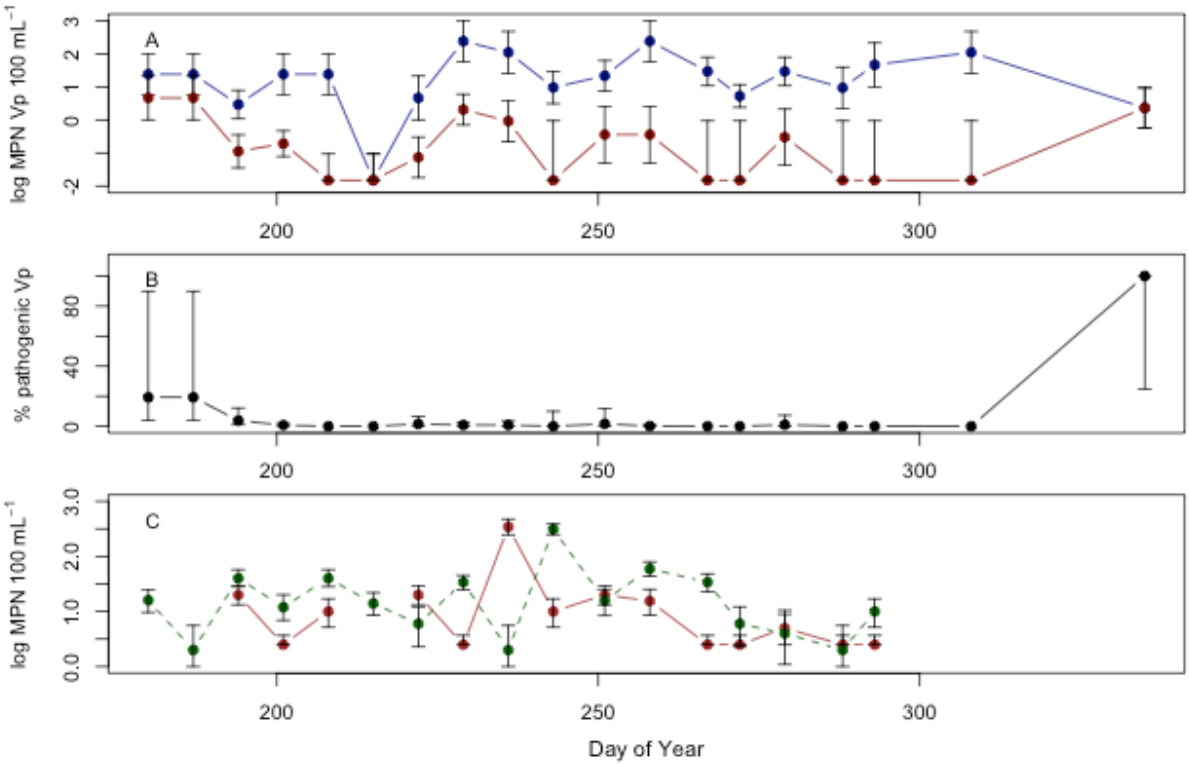

Figure 5: Field survey at Cold Spring Harbor. A) Concentrations of total (blue) and pathogenic (red) Vp. B) Percent of total Vp identified as pathogenic. C) Concentrations of enterococci (solid) and fecal coliforms (dashed). Error bars represent 95% confidence intervals.

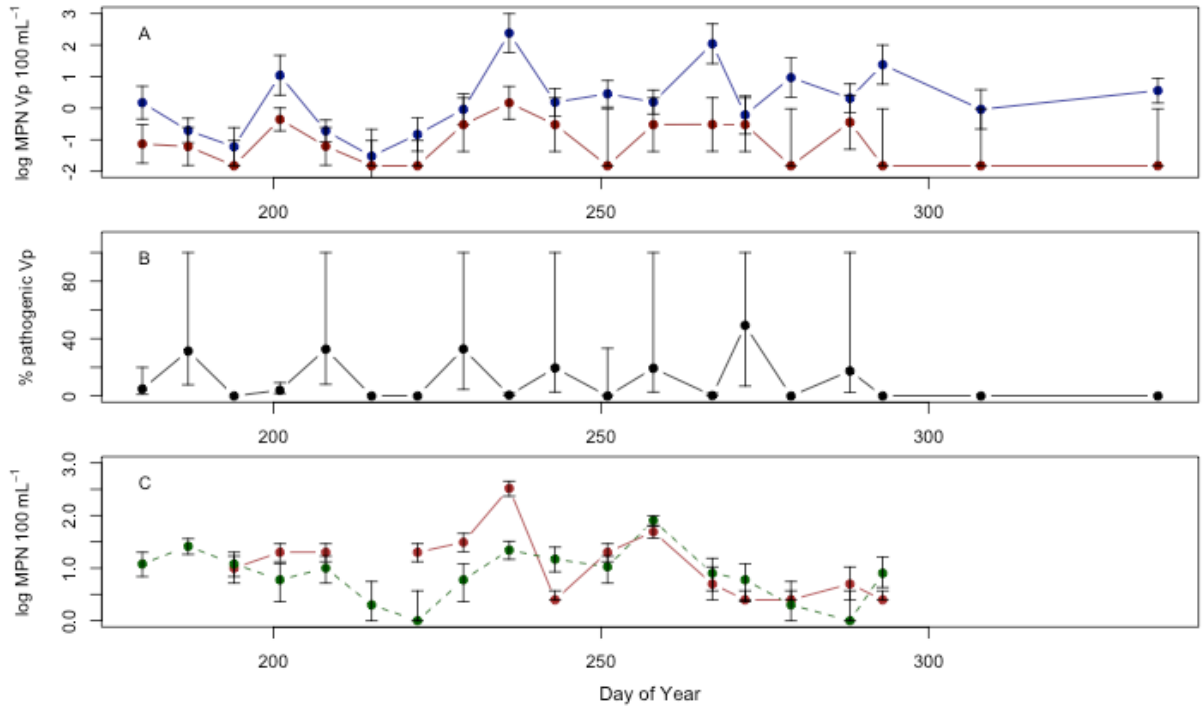

Figure 6: Field survey at Northport Harbor. A) Concentrations of total (blue) and pathogenic (red) Vp. B) Percent of total Vp identified as pathogenic. C) Concentrations of enterococci (solid) and fecal coliforms (dashed). Error bars represent 95% confidence intervals.

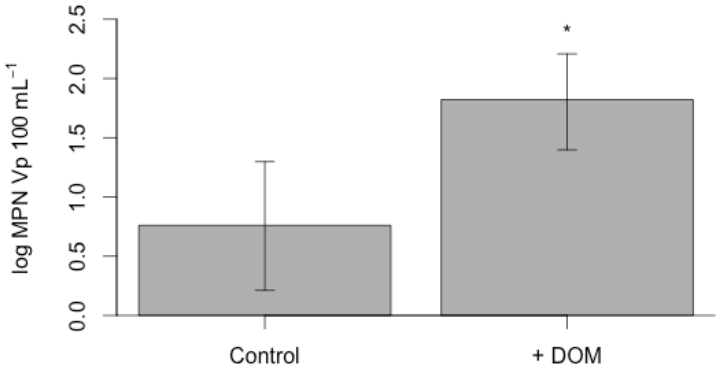

Figure 7: Concentrations of total Vp with and without dissolved organic matter additions. Error bars represent 95% confidence intervals. Significant differences (p<0.05) indicated with an asterisk.

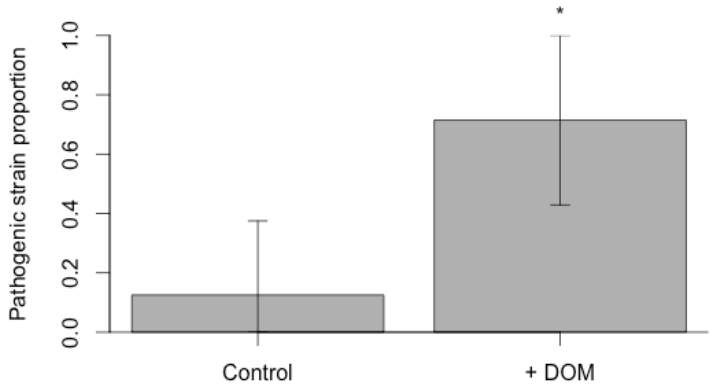

Figure 8: Proportion of samples containing pathogenic Vp with and without dissolved organic matter additions. Error bars represent 95% confidence intervals. Significant differences (p<0.05) indicated with an asterisk.

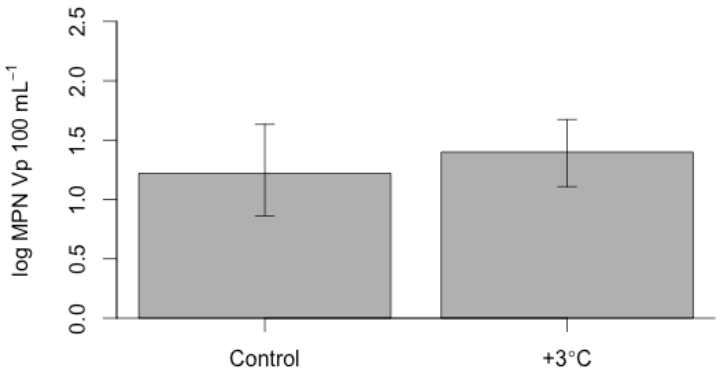

Figure 9: Concentrations of total Vp with and without a water temperature increase. Error bars represent 95% confidence intervals.

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