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The effect of storage time on *Vibrio* spp. and fecal indicator bacteria in an Isco autosampler

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ABSTRACT

Monitoring concentrations of bacterial pathogens and indicators of fecal contamination in coastal and estuarine ecosystems is critical to reduce adverse effects to public health. During storm events, particularly hurricanes, floods, Nor'easters, and tropical cyclones, sampling of coastal and estuarine waters is not generally possible due to safety concerns. It is particularly important to monitor waters during these periods as it is at precisely these times that pathogenic bacteria such as *Vibrio* spp. and fecal indicator bacteria concentrations fluctuate, potentially posing significant risks to public health. Automated samplers, such as the Isco sampler, are commonly used to conduct remote sample collection. Remote sampling is employed during severe storm periods, thereby reducing risk to researchers. Water samples are then stored until conditions are safe enough to retrieve them, typically in less than 21 h, to collect the samples. Concerns exist regarding potential "bottle effects", whereby containment of sample might result in altered results. While these effects are well documented in samples being held for 24 h or more, there is little data on bottle effects occurring during the first 24 h of containment, and less still on the specific effects related to this type of sampling regime. Estuarine water samples were collected in the fall of 2013, placed into an Isco autosampler and subsampled over time to determine the effects of storage within this type of autosampling device. *Vibrio* spp. and fecal indicator bacteria were quantified using replicated culture-based methods, including Enterolert™ and membrane filtration. The experiments demonstrated no significant impact of storage time when comparing concentrations of total *Vibrio* spp., *Vibrio vulnificus*, *Vibrio parahaemolyticus*, or *Enterococcus* spp. after storage compared to original concentrations. However, the findings also suggested that increased variability and growth can occur during the middle of the day. Therefore, if at all possible, analysis schedules should be modified to account for this variability, e.g. collection of samples after overnight storage should occur as early in the morning as practicable.

1. Introduction

1.1. Vibrio spp. and fecal indicator bacteria

Bacteria of the genus *Vibrio* are abundant in, and autochthonous to, estuarine ecosystems. The genus contains two human pathogens of importance to North Carolina coasts and estuaries, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Both are known to cause disease from ingestion or wound infection (Tantillo et al., 2004). Allochthonous bacteria also exist in estuary ecosystems and can include *Enterococcus* spp. and *Escherichia coli* which are used as a proxy of fecal contamination. Fecal contamination demonstrated by high levels of *Enterococcus* spp. is identified as a causal factor for gastrointestinal illnesses (Curriero et al., 2001; Fries et al., 2006). Urban and agricultural growth in coastal watersheds can increase microbial concentrations through stormwater runoff resulting in a decrease in water quality at locations where recreational and commercial fishing is prominent (Fries et al., 2008). Monitoring bacterial concentrations in coastal systems is therefore critical to avoid adverse effects to public health (Strom and Paranjpye, 2000; Curriero et al., 2001; Burkholder et al., 2004; Froelich et al., 2013).

Studies have documented increases in bacterial pathogens such as *Vibrio* spp. and fecal indicator bacteria after storm events in the Neuse River Estuary (NRE), North Carolina, USA (Fries et al., 2008; Hsieh et al., 2008). Storm activity in the NRE resuspends benthos-associated populations of *Vibrio* spp. into the water column (Wetz et al., 2008). Due to their affinity for fine particles, resuspension events also increase fecal indicator bacteria concentrations from contaminated stormwater runoff sources (Characklis et al., 2005; Krometis et al., 2007; Wetz et al., 2008).

Abbreviations: NRE, Neuse River Estuary; AVP, autonomous vertical profiler; TCBS, Thiosulfate Citrate Bile Sucrose agar; VIB, total *Vibrio* spp.; VV, *V. vulnificus*; VP, *V. parahaemolyticus*; CFU, colony forming units; ENT, *Enterococcus* spp.; MPN, most probable number.

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1.2. Autonomous vertical profiler and Isco automated sampler

During storm events, particularly hurricanes, floods, Nor'easters, and tropical cyclones, sampling of coastal and estuarine waters is not generally possible due to safety concerns. To study the dynamics of resuspension during storm events, outside the limits of boat-sampling, the autonomous vertical profiler (AVP) was created for *in situ* collection of water samples. The AVP floats in the upper NRE region near New Bern, NC (e.g. Fries et al., 2006; Paerl et al., 2006). Within the AVP, an Isco automated sampler is programmed to fill proprietary bottles (1120 mL) with estuarine water at a desired sampling scheme of varying time intervals and depths. The Isco can be triggered remotely at the beginning of severe weather events to collect water samples and environmental data during a storm period (e.g. Froelich et al. 2013).

During sampling periods, which is typically not longer than 18 h, and the transport time between the AVP and laboratory, which is typically not longer than 3 h, Isco water samples are stored in bottles that are shaded but exposed to ambient temperatures. Whereas long-term "bottle effects", defined as unreasonable variability between original and contained samples, of water samples have been sufficiently studied, most studies do not consider or do not provide evidence of potential short-term (less than 24 h) bottle effects. Therefore, it was necessary to study short-term bottle effects especially in the context of Isco autosampling during pulse stresses (e.g. storms lasting less than 24 h) in coastal marine environments. There was concern as to whether up to 21 h of bottle storage in the sun-protected but unrefrigerated Isco autosampler affects bacterial concentrations that potentially renders the sample as unrepresentative of *in situ* conditions. This report provides evidence that short-term bottle effects are not significant on total *Vibrio* spp. abundance, and *V. vulnificus*, *V. parahaemolyticus*, and *Enterococcus* spp. concentrations when using the Isco autosampling methodology specific to the currently employed approaches for NRE experimentation.

1.3. Methods in environmental microbiology: bottle effects

While attention is given to collecting samples under aseptic conditions and choosing appropriate construction material of sampling containers, few studies mention the artifacts of containment on experimental results. Pernthaler and Amann (2005) articulated the uncertainty around the apparent effect of variability in experimental studies: "Such investigations are often plagued by the mysterious 'bottle effect,' a hard-to-define concept that reflects the worry of whether phenomena observed in confined assemblages are nonspecific consequences of the confinement rather than a result of the planned manipulation." Hammes et al. (2010) summarized bottle effects to include changing cell concentrations, grazing and bacterivory, viability and cultivability, and population composition. As soon as a sample is removed from the field study site, artifacts of enclosure such as changes in genetic, biochemical and physical aspects of the sample may be triggered and pose concern as to the validity of experimental results (Madsen, 2011). Many published studies implicitly hypothesize a "safe period" of less than 24 h within which samples accurately represent *in situ* processes and while the general recommendation is to conduct immediate analysis or to minimize time of storage (e.g. Ferguson et al., 1984; O'Carroll, 1988; Brözel and Cloete, 1991; Atlas and Bartha, 1998; Toranzos et al., 2007), some studies do not provide direct supporting evidence. Other reports do not even mention the effects of confinement on experimental results (e.g. Munn, 2004; Mimura et al., 2005).

Analysis of samples should be completed as soon as possible to accurately represent microorganisms, especially with estuary water samples, due to the ability of microorganisms to reproduce quickly (Atlas and Bartha, 1998). However, most investigations on microbiological parameters under confinement were based on samples taken at daily, weekly, or monthly intervals. Very few studies have tested the effect of storage time within the first 24 h before analysis.

1.4. In depth: bottle effects

Freshwater and saltwater stored in containers can exhibit increases in bacterial concentrations up to three orders of magnitude, especially in samples stored for longer than a day (ZoBell and Anderson, 1936; O'Carroll, 1988). Yet another study showed a 5 fold decrease in *Vibrio cholerae* after two days (Heinemann and Dobbs, 2006). The doubling time of culturable bacteria is affected by containment in as few as 5 h of sample collection (Ferguson et al., 1984). Whipple (1901) saw a 10–15% increase in bacterial concentrations within the first 3–6 h of storage followed by an increase of several hundred percent. Conversely, Brözel and Cloete (1991) did not see a significant increase or decrease of culturable bacteria counts at 4, 10, 20, and 30 °C during 24, 48, 72, and 216 h.

When bacterial analysis is performed some distance away from the sampling location, samples are typically shipped cold because refrigeration is thought to retain bacterial composition and decrease enzymatic reaction rates, cell division and death (Harrigan and McCance, 1979; Brözel and Cloete, 1991). Nevertheless, short-term effects of storage in refrigerated conditions can trigger some bacteria to enter a "viable but not culturable" state, which is similarly induced during the winter months, preventing them from forming colonies during culture (Roszak and Colwell, 1987). Even at refrigerated temperatures, the death of Protozoa and other marine organisms could possibly increase bacteria survival (ZoBell and Anderson, 1936).

The effects of sample volume on bacterial growth were demonstrated in several laboratories (e.g. Whipple, 1901; ZoBell and Anderson, 1936; Ferguson et al., 1984; O'Carroll, 1988; Marrase et al., 1992) and all agreed that as sample volume increases, the effects of confinement on bacterial activity and growth are less prominent. While ZoBell and Anderson (1936) showed evidence of multiplication of bacteria in seawater within 8 h of storage, almost no difference was found in their density in different volumes during the first two days. Hammes et al. (2010) also found no correlation between six bottle sizes and total cell count of bacterial populations using three independent enumeration methods for up to five days of storage.

Studies which have observed volume bottle effects have attributed them to adhesion and surface-associated aggregation of microorganisms on bottle surfaces. ZoBell and Anderson (1936) calculated approximately half of total bacteria in a 100 cm^3 of sterile seawater sample that resided in the water itself while the other half remained attached to the glass surface of the bottle. Glass surface adhesion due to nutrient depletion in the water was described as a potential reason for the decrease in culturable count since nutrients become concentrated in films on solid surfaces (ZoBell and Anderson, 1936; Ferguson et al., 1984). Volume effects were found to disappear when organic matter was added to samples in more than a few milligrams (ZoBell and Anderson, 1936). Even differences in primary productivity in mesocosm experiments have been attributed to the artifacts of enclosure which include periphyton growth on sample container walls; therefore, the shape and size of the container are important to consider when quantifying bacterial concentrations (Petersen et al., 1997). On the other hand, Fuhrman and Azam (1980) showed that ATP on walls of glass containers of different surface to volume ratios rose to 3–5% of total ATP by 22 h, but bacterioplankton cell counts were within 5% of each other. Studies that did not observe surface wall growth admit that carbon adsorbs to clean glass surfaces but question how these effects can be dramatic enough to alter growth (Hammes et al., 2010).

During initial colonization on surface walls, microbes can interact in cooperative and inhibitory ways, shaping bacteria community structure, for example by decreasing the number of species, in confined samples (Whipple, 1901; Lawrence et al., 2002). Prolonged assays also affect

dominance in microbial communities of the sample (Ferguson et al., 1984; Christian and Capone, 1997).

In the study reported here, natural estuarine water samples were collected in duplicate to permit immediate sampling as well as storage in Isco collection bottles within the Isco autosampler housing. Water samples were analyzed, at time points, for up to 24 h for comparison with the initial control sampling.

2. Materials and methods

2.1. Experimental site

Sampling for this study was done at Calico Creek, a small tidal estuarine creek in the lower Newport River estuary, near Morehead City, NC, USA (Fig. 1). Calico Creek receives much of its water from the Newport River but also from the creek's watershed, stormwater runoff, and chlorinated secondary effluent from the Morehead City, NC treatment plant (Sanders, 1978; Chung et al., 1996). Temperature, salinity, and microbial community conditions at Calico Creek were very similar to the conditions of the estuary where the AVP is deployed.

2.2. Seawater parameters and bacterial analyses

Temperature and pH were recorded using an *Ultra*BASIC UB-5 pH meter (Denver Instrument; Bohemia, NY, USA). A HI96822 Seawater Refractometer (HANNA Instruments Inc.; Woonsocket, RI, USA) was used to determine salinity. Turbidity was recorded using a 2100Q Portable Turbidimeter (HACH®; Loveland, CO, USA).

Water samples were filtered through 0.45-μm pore, mixed cellulose ester filters and plated on thiosulfate–citrate–bile salts-sucrose agar (TCBS, BD, Franklin Lakes, NJ, USA) to quantify total *Vibrio* spp. (VIB) and CHROMagar VIBRIO (CHROMagar, Paris, France) to quantify presumptive *V. vulnificus* (VV) and *V. parahaemolyticus* (VP), respectively. Plates were incubated for 24 h at 37 °C. Green and yellow colonies were counted from TCBS plates to quantify VIB in colony forming units (CFU) per 100 ml of water. Purple and blue colonies were counted on CHROMagar plates to quantify VP and VV, respectively, also in CFU.

Fecal indicator bacteria *Enterococcus* spp. (ENT) were quantified using Enterolert™ (IDEXX Laboratories, Westbrook, ME, USA). Quanti-tray/2000® (IDEXX Laboratories) trays were incubated at 41 °C for 24 h and quantification of most probable number (MPN) per 100 mL was calculated as per manufacturer's instructions (Hurley and Roscoe, 1983; Fries et al., 2006).

2.3. Experimental set-up

Experiments were conducted during September and October 2013. The experiments addressed variations in bacterial concentrations according to bottle effects associated with water storage in an Isco autosampler and used estuarine water from Calico Creek as the source water (Fig. 1). Bottles were stored in the Isco autosampler compartment outside, with the exception of a single replicate set that was taken immediately to the lab for analysis. The remaining replicate sets were retrieved from the Isco for analysis at specific time intervals.

2.4. Evaluating short-term (<6 h) effects of water storage

Isco bottles were filled and held in the Isco housing, except one sample which was analyzed immediately. Initial sampling occurred between 9:00 and 10:00 (T_0) with time points at 12:00–13:00 and 15:00–16:00. Once a bottle was taken to the lab for analyses and subsampled, it was discarded. This experiment was performed 16 times measuring VIB, VV, and VP and eight times when measuring ENT. Temperature, salinity, pH, and turbidity were recorded for each water sample at each time point.

2.5. Evaluating extended short-term (<24 h) effects of water storage

Isco bottles were filled and held in the Isco housing, except one sample which was analyzed immediately when collected at 20:00 (T_0). Other samples were analyzed from 8:00–9:00, 11:00–12:00, 14:00–15:00 and 16:00–17:00 the following day. Temperature, salinity, VIB, VV, VP, TC, EC, and ENT were measured, and the experiment was repeated 12 times, except ENT which was analyzed eight times and TC and EC which were performed in quadruplicate.

A second, alternate run of this experiment, conducted to represent a different portion of the day and include more than one daily warming period, was performed with the initial (T_0) sample occurring at 9:00

Fig. 1. Calico Creek in Morehead City, NC, USA with inset showing creek location relative to surrounding area (Google Maps, 2014). Star shows water sample location.

and time points at 12:00, 16:00, 21:00 and then 8:00 and 14:00 the following day. This experiment was repeated four times measuring VIB, VV, VP, and twice when measuring ENT. Temperature, salinity, pH, and turbidity were recorded for each water sample at each time point.

2.6. Time of day analysis

All data from all experiments for *V. vulnificus* bacterial concentrations were combined by time of day, rather than by elapsed time (Fig. 6). This permitted the visualization of the effect of being exposed to ambient temperature and sun at a particular time of day (e.g. morning, noon, afternoon, evening, or night) regardless of how many hours that samples had been contained in the Isco bottles.

2.7. Statistical analyses

Data were checked for normality and subsequently log-transformed to reduce skewness. Data were then analyzed using a one-way ANOVA with a Holm-Sidak post-test for multiple comparisons using SigmaPlot (Systat Software Inc., San Jose, CA). An alpha of 0.05 was used to determine significance.

3. Results

For each of the experiments, the water temperatures increased or decreased significantly ($p < 0.05$) as the bottles warmed throughout the day or cooled over night (Fig. 2). Conversely, salinity remained constant ($p > 0.05$) within each experiment (Fig. 2), regardless of the time elapsed or time of day sampled.

In the short-term experiments, there were no significant changes observed in VIB, VP, or ENT concentrations within the first 9 h of collection (Fig. 3). VV concentrations at T_3 and T_9 were significantly different from each other ($p = 0.001$, Fig. 3C). However, neither T_3 ($p = 0.243$) nor T_9 ($p = 0.302$) values were significantly different from the initial sample at T_0.

Over the extended short-term experiments, the concentrations of VIB, VP, VV, and ENT were also not significantly different ($p > 0.05$) from the T_0 sample at any time point over 21 h. Total VIB peaked at T_{12} (Fig. 4A). VP peaked at T_{15} (Fig. 4B). Both VV and ENT peaked at T_{15} and followed a non-significant downward trend to T_{21} (Fig. 4C, D).

No significant difference ($p > 0.05$) was detected in microbial concentrations over time for the alternate extended short-term experiment for VP, VV, or ENT concentrations at any point in time compared to T_0. VP first appeared to decrease then peaked at T_7 and T_{23} (Fig. 5A). VV peaked at T_3, dipped at T_7 and continued to increase through T_{29} (Fig. 5B). ENT concentrations peaked at T_{12}, dipped at T_{23}, and subsequently trended upward through T_{29} (Fig. 5C).

When examined solely by time of day the sampling occurs, rather than by the elapsed time in containment, VV concentrations are observed to peak around noon and exhibit the lowest values at night (Fig. 6). While none of the samples were significantly different than the T_0 control samples, the samples that were contained and sampled at noon were significantly higher ($p < 0.05$) than those sampled at night. Bacterial concentrations during sampling points close to noon had more variability between repeated samplings than during other periods of the day.

When compared using Spearman Rank Correlation Analysis, the water temperature within the bottles had a significant negative correlation with VV, ENT, and VIB concentrations ($r = -0.56$, $p < 0.0001$; $r = -0.75$, $p < 0.0001$; and $r = -0.25$, $p = 0.04862$; respectively).

4. Discussion

The data collected in this study show that short-term (3–9 h) or extended short-term (3–24 h) bottle effects are non-significant for culturable *Vibrio* spp., *V. vulnificus*, *V. parahaemolyticus*, and *Enterococcus* spp. abundance. The short-term dataset reveals that any sample stored in Isco bottles and analyzed up to 9 h after collection are valid

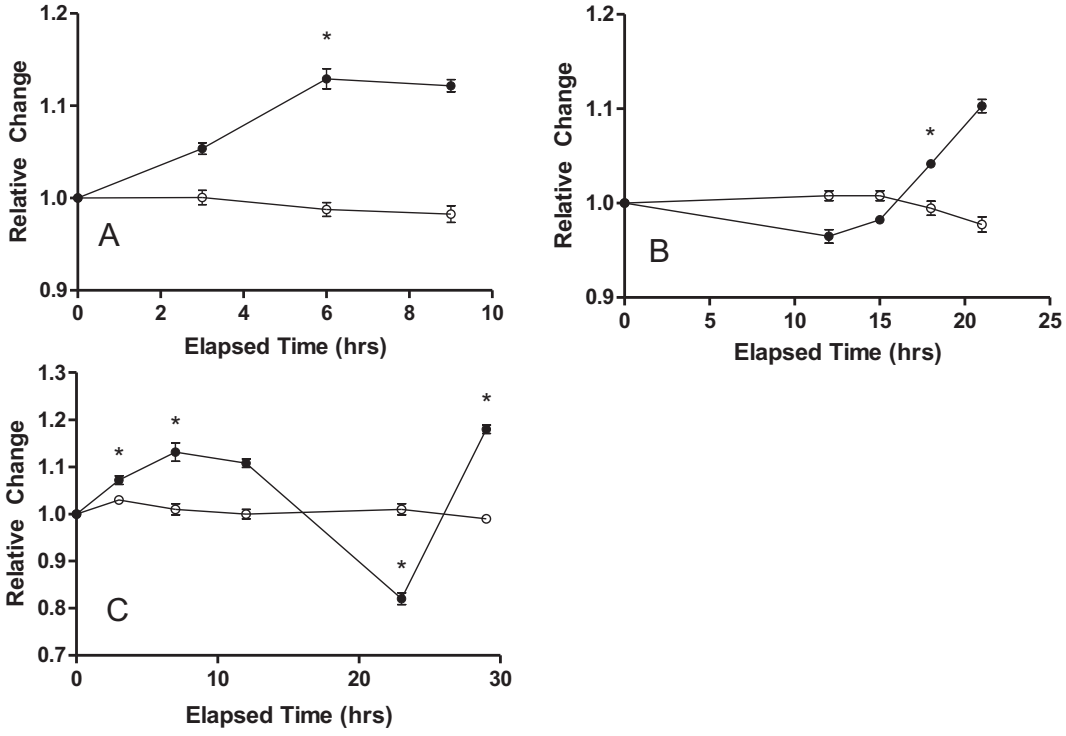

Fig. 2. Relative change of salinity (open circles) and water temperature (closed circles) of short-term (A), extended short-term (B) and alternate extended short-term (C) experiments. Asterisks indicate a value that is significantly different from the previous time point. Error bars represent SEM.

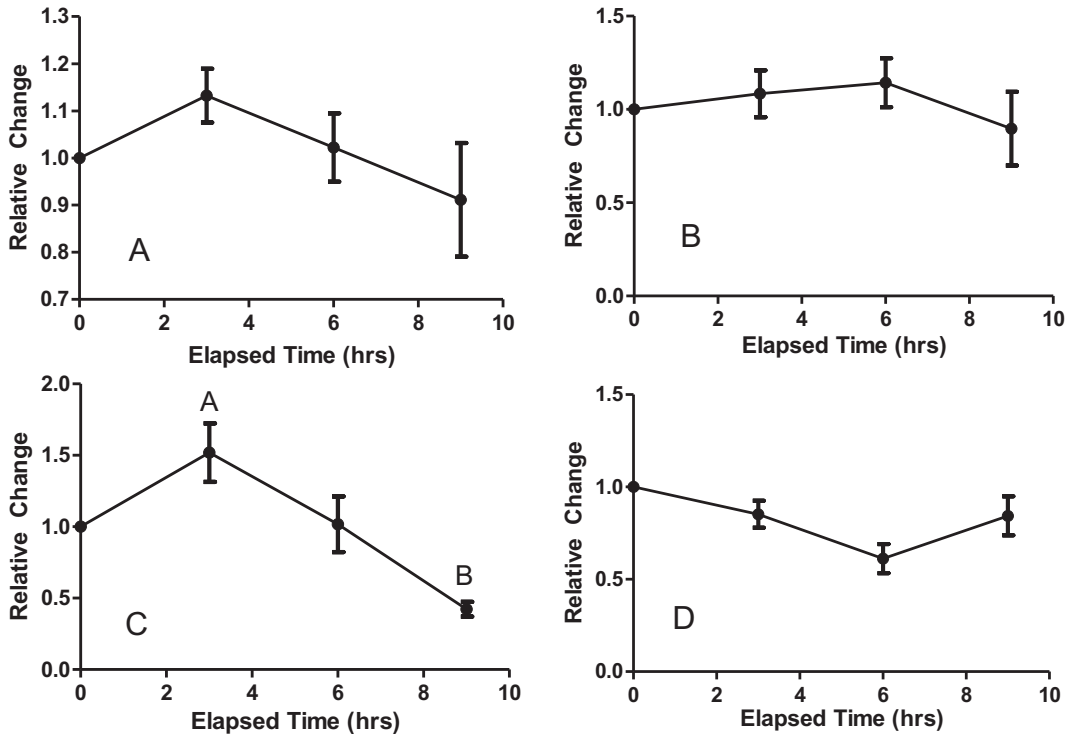

Fig. 3. Relative change of total *Vibrio* spp. (A), *V. parahaemolyticus* (B), *V. vulnificus* (C), or *Enterococcus* spp. (D) in short-term experiments. Points with different letters indicate values that are significantly different. Error bars indicate SEM.

representations of T_0 concentrations. The extended short-term experiments also demonstrates that water samples analyzed within 21 h of storage in the Isco autosampler are representative of the original samples at collection. However, it is also appears likely that temperature plays a role in the fluctuation of bacterial concentration, especially *V. vulnificus*, and that samples analyzed at noon exhibit more variation. Noon and night *V. vulnificus* concentrations were significantly different from each other, although still not significantly different from the T_0 control samples, and the increased variation is most likely due to high and low temperatures for samples that were analyzed at those times.

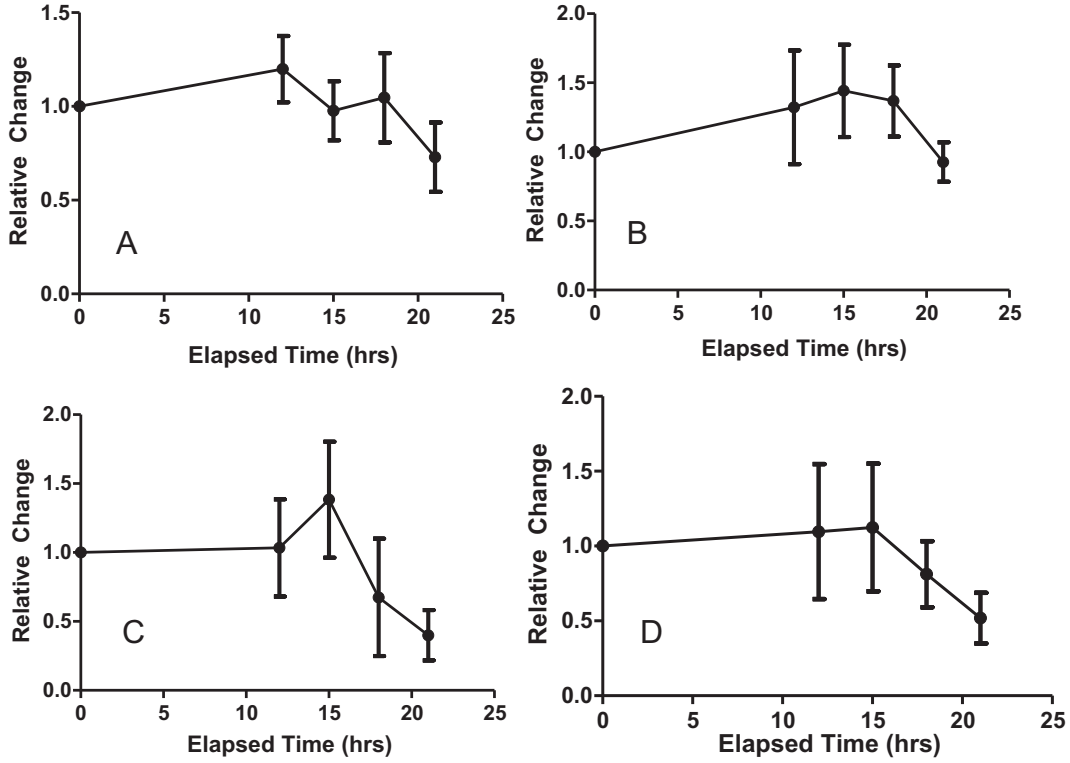

Fig. 4. Relative change of total *Vibrio* spp. (A), *V. parahaemolyticus* (B), *V. vulnificus* (C), or *Enterococcus* spp. (D) in extended short-term experiments. Error bars indicate SEM.

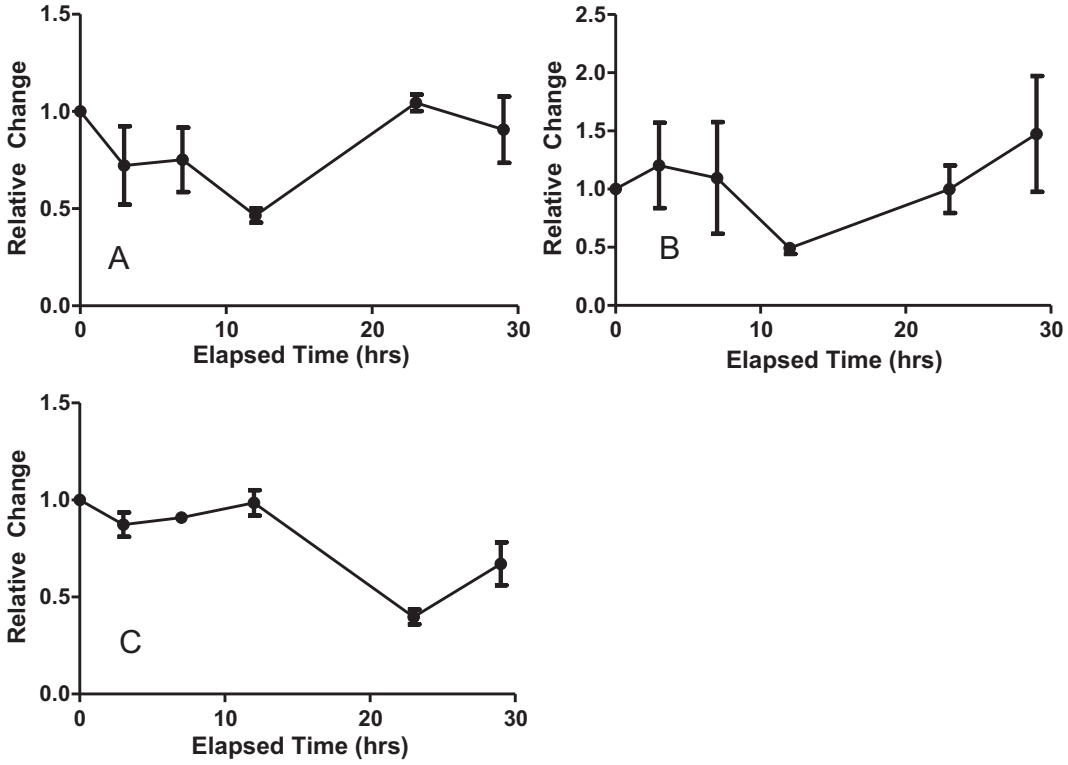

Fig. 5. Relative change in of total *Vibrio* spp. (A), *V. parahaemolyticus* (B), *V. vulnificus* (C), or *Enterococcus* spp. (D) in alternate extended short-term experiments. Error bars indicate SEM.

Bacterial growth rates can increase at higher temperatures, supporting the likelihood that *V. vulnificus* increased at noon due to increased temperatures (Butterfield, 1933), but this typically was not when the water temperature of the bottles was the warmest. Water temperature measured in the sample bottles was highest during the afternoon and early evening. While *V. vulnificus* concentrations appear to continuously decrease after peaking around midday, our alternate extended short-term experiment revealed concentrations increasing during the morning regardless of elapsed time in the Isco bottles. *V. vulnificus* is consequently exhibiting a diel pattern. This phenomenon deserves further study as there are several factors attributable to diel patterns that could hold implications for samples containing *V. vulnificus* stored in ambient environment or during transport conditions. An example of an indirect effect of the seemingly diel pattern could be attributed to predation interactions in response to temperature. Bdellovibrio and like organisms (BALOs) preferentially consume *Vibrio* spp. (Williams and Piñeiro, 2007). Not only are BALOs associated with surfaces which could increase their abundance in storage bottles, but they are also tightly coupled with temperature (Williams, 1988; Yair et al., 2003). The inverse relationship between temperature and *V. vulnificus* concentrations may have been a result of diurnal temperature change which could also affect BALO abundance in the water sample. We hypothesize the increase of BALOs with the increase in temperature following the time of day which causes *V. vulnificus* concentrations to rise and fall. Noon and night time points exhibit extremes in temperatures potentially increasing the effects of predation. Further research of predator–prey interactions of microbial communities in confinement would provide evidence for our hypothesis. Temperature itself could be playing an important direct role on bacterial growth, effecting metabolism directly through the production of enzymes that dictate growth substrate utilization.

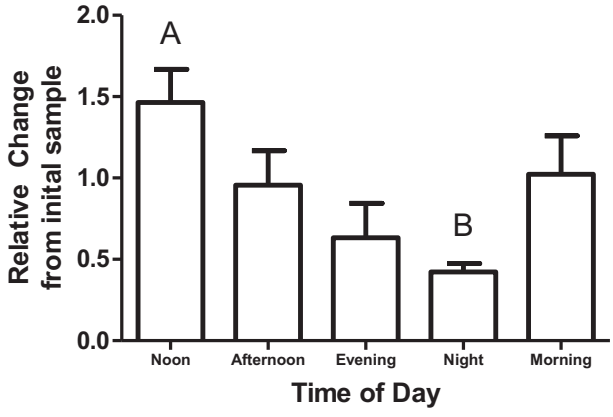

Fig. 6. Relative changes, from T_0 control samples, in *V. vulnificus* concentrations binned by time of day, regardless of elapsed time of containment. Data is pooled from all experiments. Bars with different letters are significantly different from each other. Error bars indicate SEM.

According to Dawson et al. (1981) the number and size of marine *Vibrio* spp. increased and decreased dramatically, respectively, within 5 h of exposure to starvation and showed enhanced rates of adhesion to siliconized glass surfaces for survival. Future studies should apply sonication techniques to determine the quantity of *Vibrio* spp. on the surface and compare to *Vibrio* spp. in the water sample prior to agitation. Shaking will ensure that bacteria are loosened from surface walls as Taylor and Collins (1949) reconciled the increase in bacteria concentration to surface growth after conducting experiments between bottles that were shaken before sampling and not.

Although the Isco autosampler stores bottles in the shade, it is important to question what would happen if other methodology allowed bottles to be exposed to sunlight. Marrase et al. (1992) did not see an increase in bacterivory under different volumes within 24–48 h between light and dark conditions, just that higher rates of consumption were observed at higher temperatures. Would the oxygen content of water samples increase due to photosynthetic activity and in turn increase bacterial activity? ZoBell and Anderson (1936) found that bacterial activity increase in small volumes of seawater was not attributable to oxygen content in the water, but it would be ideal to monitor parameters such as oxygen, algae population, chlorophyll-A concentrations,

and zooplankton (grazer) counts to determine other variables that affect the concentration of bacteria in stored sample bottles.

Whether concentrating a sample through filtration causes variability on the observed bacteria activity or not is also interesting. The filtration approach used for abundance measurements in this study may pose a problem in that treatment of vacuum-filtering could have injured delicate cells. It has also been shown that *Vibrio* spp. and *E. coli* that attach to aggregates in the water persist longer than those in aggregate-free water and have a significant species-richness–area relationship (Lyons et al., 2010). Manipulating filter size may be of concern when aggregates are present or absent because of the possibility of altering cell count. To choose filter size, scientists must consider the natural conditions of the bacteria of interest to simulate nature as closely as possible.

There are a range of limitations to the study that was conducted. First, the study focused on culture-based quantification of *Vibrio* spp. and fecal indicator bacteria. Neither bacterial community structure nor population dynamics were studied in this experiment and would have been a valuable addition. For example, 16S rDNA sequence analyses would have been a useful addition to show the variation in species of eubacteria present in the water samples over time. Population dynamics could have been studied via either fluorescence *in situ* hybridization or qPCR analyses to determine interactions of *Vibrio* spp., fecal indicator bacteria, and other important bacterial players in the system in confined bottles. This could be done at very small time scales, for example, every 2 h for 20–24 h to observe small scale changes. Scrapping the sides of the bottle and collecting material that has settled to the bottom would allow us to understand the full array of particles attached and free living organism dynamics over time. Finally, ideally this experiment would be conducted over the course of a real extreme storm event, such as a hurricane, incorporating bacterial abundance, BALO abundance, viral analyses, community, and population dynamics and culture based analyses.

Regardless, our findings confirm that a sample analyzed at any point up to 21 h of storage in the Isco autosampler after collection is a valid representation of *in situ* concentrations; however, due to the increased likelihood of variation we do not recommend analyzing samples in the noon and night times of day. Our study provides evidence against bottle effects within 24 h of collection. In our collection regime, bottle effects experienced with samples that are stored for more than 24 h may be diminished by increasing sample size and decreasing surface area relative to volume. It is well known that surfaces provide substrate for many microorganisms and increase bacterial population, absorb substrates and metabolites, or release contaminants. An important consideration is the development of gradients and ability to agitate the sample to ensure homogenization (Christian and Capone, 1997). If keeping samples saturated with oxygen should become a priority, bottles should be kept partly filled, in contact with air, and shaken daily (ZoBell and Anderson, 1936).

As demonstrated by our study, the effect of temperature in the Isco autosampler during sampling period is also of note. It would be ideal to keep the Isco bottles in a water bath that filters water from the estuary as the sampling period progresses even though this could pose problems if water temperatures change dramatically, for example during an extreme storm event.

Changes to normal growth activity are unavoidable during laboratory measurement of bacterial populations. Relating results back to the natural environment requires full disclosure of the consequences of extrapolation (O'Carroll, 1988). Incubations monitoring short-term changes may provide direct information on a single variable pertaining to the confined community. Albeit, confinement disrupts the steady-state and/or flux system (e.g. production and consumption) experienced in the natural environment. Even *in situ* experimentation restricts the natural exchange of substrates (Christian and Capone, 1997). Artifacts of enclosure are unavoidable, and it is crucial environmental microbiologists understand the characteristics of bottle effects so that extrapolation of microcosm data to natural ecosystem is accurate.

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