#### Abstract

Monitoring concentrations of bacterial pathogens and indicators of fecal contamination in coastal and estuarine ecosystems is critical to avoid 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 at precisely these times pathogenic bacteria such as Vibrio spp. and fecal indicator bacteria concentrations increase, posing significant risks to public health. Automated samplers, such as the rosette based "ISCO" sampler, are commonly used to conduct sample collection in aquatic systems. In our case, we have designed an autonomous vertical profiler (AVP) that is deployed in the Neuse River Estuary, NC USA with an automated ISCO sampler attached. The AVP can be remotely enabled by cell phone or internet to collect water samples during severe storm periods. Water samples are then stored in a rosette of proprietary bottles until conditions are safe enough, typically in less than 21 h, to collect the samples. There is a paucity of data regarding the effects of containment on bacterial concentrations of water stored over these short durations. Concerns exists with regards to autosampler methodology and associated sample holding times that might impact bacterial concentrations invalidating the subsequent laboratory analyses due to water samples' misrepresentation of original in situ conditions. Six experiments were conducted to address this concern, and data from the experiments were pooled into three sets for data analyses: short-term, long-term, and full-term. Estuarine water samples were collected in the fall of 2013, placed into an ISCO rosette and subsampled over relevant time frames and ambient temperature conditions. Vibrio spp. and fecal indicator bacteria were quantified over the course of the experiments using replicated culture-based methods, including Enterolert<sup>™</sup> and membrane filtration. The experiments demonstrated no significant impact of storage time when comparing concentrations of total Vibrio spp., Vibrio vulnificus, Vibrio parahaemolyticus, Enterococcus spp., or E. coli after storage as compared to original concentrations. Small scale variations in concentrations of specific targets were observed, particularly during midday hours that appeared to follow a diel cycle. Only culture-based analyses were conducted as part of this set of experiments, and further examination of bacterial community dynamics is warranted.

Based upon our experiments, holding times of up to 21 h appear to have a negligible effect on bacterial concentrations for estuarine waters. However, the findings also suggest 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, *V. vulnificus* and *V. 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 *E. 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 (Currieo 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 (Froelich et al., 2013; Burkholder et al., 2004; Currieo et al., 2003; Strom & Paranjpye, 2000).

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 sedimentary 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 (Wetz et al., 2008; Krometis et al., 2007; Characklis et al., 2005).

## 1.2. Autonomous Vertical Profiler and ISCO Automated Sampler

To study the dynamics of resuspension during storm events outside the limits of boatsampling, 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 at what is referred to as Station 60 in previous studies monitoring the NRE (Figure 1) (Fries et al., 2006; Paerl et al., 2006). Within the AVP, an ISCO Automated Sampler is programmed to fill proprietary bottles (1120 mL) with estuary water at a desired sampling scheme of varying time intervals and depths. The ISCO can be triggered at the beginning of severe weather events to collect water during a storm period.



**Figure 1.** Neuse River Estuary Modeling and Monitoring (MODMON) Project sampling stations. The autonomous vertical profiler (AVP) is located approximately at Station 60.

During sampling and the additional transport time between the AVP and laboratory, ISCO water samples are stored in bottles that are shaded but exposed to ambient temperatures. For example, a typical sampling regime in the NRE collects samples every 6 h for an 18 h period with an additional 3 h of transport time to the lab. Whereas long-term bottle effects of water samples has been sufficiently studied, most studies do not consider or do not provide evidence of potential short-term bottle effects; therefore, it was necessary to study short-term bottle effects especially in the context of ISCO autosampling during pulse stresses (i.e. storms lasting less than 24 h) in coastal marine environments. Environmental microbiologists expressed concern as to whether up to 21 h of bottle storage in the sun-protected but un-refrigerated ISCO affects bacterial concentrations and invalidates the sample from representing *in situ* conditions. This report claims 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 NRE experimentation.

#### 1.3. Methods in environmental microbiology: Bottle effects

Environmental microbiology studies the interactions of microbes in nature and under anthropogenic conditions. Finding a suitable method to collect field water samples and perform analysis is a challenge in the microbiology field especially considering the plethora of microbial habitats and interactions that influence methodology. Scientists utilize the term "bottle effects" to explain variability in experimental results, especially those that require transport and storage time before lab analysis.

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 & 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 change in genetic, biochemical and physical aspects of the sample may be triggered and pose concern as to the validity of experimental results (Madsen 2006). 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; Brozel & Cloete, 1991; Atlas & Bartha, 1998; Toranzos et al., 2002) studies do not provide direct supporting evidence. Some studies do not even mention effects of confinement on experimental results (e.g. Munn, 2004; Mimura et al. 2005).

Processing to accurately represent microorganisms should be completed as soon as possible, especially with estuary water samples, due to the ability of microorganisms to reproduce quickly (Atlas & 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.

4

#### 1.4. In depth: bottle effects

Freshwater and saltwater stored in containers can increase bacterial cell numbers up to three orders of magnitude, especially in samples stored for longer than a day (ZoBell & Anderson, 1936; O'Carroll, 1988). Another study showed a 5 fold decrease in *V. cholerae* after two days (Heinemann & 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 (1991) 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, Brozel & 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 a distance away from the sampling location, samples are shipped cold because refrigeration is thought to retain bacterial composition and decrease enzymatic reaction rates, cell division and death (Harrigan & McCance, 1976; Brozel & Cloete, 1991). Nevertheless, short-term effects of storage in refrigerated conditions can trigger bacteria into a "viable but not culturable" state, which is similarly induced during the winter months, preventing them from forming colonies during incubation (Roszak & Colwell, 1987). Even at refrigerated temperatures, the dying out of Protozoa and other marine organisms could increase bacteria survival (ZoBell & Anderson, 1936).

The effects of sample volume on bacterial growth were demonstrated in several papers (e.g. O'Carroll, 1988; Ferguson et al. 1984; ZoBell & Anderson, 1936; Whipple, 1901; 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 & 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 2 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. Even our own data from the proof of concept stage of this thesis' experiment where we compared sampling with big bottles vs. small bottles showed no significant difference in bacteria counts, except for total *Vibrio* spp. (Figure 2).



**Figure 2.** Grouped bar plots representing bacterial concentrations over 8 h proof of concept experiment sampled in big and small containers. Error bars indicate SEM. Significant difference denoted by "A" and "B".

Studies which have observed volume bottle effects have attributed them to adhesion and surface-associated aggregation of microorganisms on bottle surfaces. ZoBell & Anderson (1936) calculated approximately half of total bacteria in a 100 cc. of sterile seawater sample 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 & 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 & Andersson, 1936). Even differences in primary productivity in mesocosm experiments have been attributed to artifacts of enclosure which include periphyton growth on sample container walls; therefore, shape and size of the container are important to consider when quantifying bacterial

concentrations (Petersen et al., 1997). On the other hand, Fuhrman & 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 interact in cooperative and inhibitory ways, shaping bacteria community structure, for example by decreasing the number of species, in confined samples (Whipple, 1991; Lawrence et al., 2002). Prolonged assays also affect dominance in microbial communities of the sample (Christian & Capone, 2002; Ferguson et al., 1984).

## 2. Methods

## 2.1. Experiment 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 (Figure 3). 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 (Chung et al., 1996; Sanders, 1978). Temperature, salinity and microbial community conditions at Calico Creek are similar to conditions of the estuary where the AVP resides.



**Figure 3.** Calico Creek, North Carolina, USA. Location of water collection for short, long and full-term experiments (Chung et al., 1996; Google Maps, 2013).

#### 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<sup>®</sup>; Loveland, CO, USA).

Water samples were filtered through 0.45-µm pore, glass fiber filters and plated on Thiosulfate Citrate Bile Sucrose (TCBS) agar or CHROMAGAR VIBRIO to quantify total *Vibrio* spp. (VIB) and presumptive *V. vulnificus* (VV) and *V. parahaemolyticus* (VP), respectively. Two plates of different dilutions (1 ml, 5 ml, or 10 ml) using phosphate buffered saline for each sample were prepared. Plates were then incubated for 24 h at 37°C. Green and yellow colonies were counted from TCBS plates to quantify VIB in colony forming units (CFU). 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<sup>™</sup>. Quantitray/2000<sup>®</sup> (IDEXX Laboratories) trays were incubated at 41°C for 24 h and quantification of most probable number (MPN) per 100 ml was calculated based on the numbers of small and large positive wells (Hurley & Roscoe, 1983; Fries et al., 2006).

### 2.3. Experimental Set-up

A total of six experiments were conducted during September and October 2013 (Table 1). The six 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 (Figure 3). 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. These samples were labeled as T<sub>0</sub>. Remaining replicate sets would be retrieved from the ISCO after a specific number of hours after collection elapsed (i.e. T<sub>3</sub> after 3 hours elapsed in the ISCO).

Both large and small bottles were filled at Calico Creek for Experiment 1. A single source for seawater stored in the large bottle provided water samples for analysis at  $T_0$ ,  $T_4$  and  $T_8$  points. Multiple small bottles were filled and retrieved as replicate sets of three from the ISCO at  $T_0$ ,  $T_4$  and  $T_8$  for analysis.  $T_0$  analysis for Experiment 1 was at 8:00.  $T_4$  and  $T_8$  analysis were conducted

at 12:00 and 16:00, respectively. VIB, VV and VP were quantified along with TC, ENT and EC concentrations. Temperature, salinity, pH and turbidity were recorded for each water sample at each time point. Because Experiment 1 results did not yield a significant difference between small vs. big bottle sample concentrations for VV, VP and ENT and since the ISCO autosampler uses only small bottles, we eliminated the use of big bottles in Experiment 2. We also did not measure pH or turbidity since they did not significantly affect bacterial concentrations during the course of Experiment 1. Except for number of replicates and time points, Experiment 2 was

conducted in the same manner as Experiment 1. Replicate sets of 4 were used for analysis at time points  $T_0$  (10:00),  $T_3$  (13:00) and  $T_6$  (16:00).

Water collection for Experiment 3 occurred in the evening.  $T_0$  (20:00) bottles were analyzed immediately while  $T_{12}$  (8:00),  $T_{15}$  (11:00),  $T_{18}$  (14:00) and  $T_{20}$  (16:00) bottles were analyzed the following day. Replicate sets of 4, small ISCO bottles were analyzed for temperature, salinity, VIB, VV and VP. Two of the four replicate bottles were analyzed for TC, EC and ENT. Experiment 4 was conducted in the same manner as Experiment 3 except for the last time point was  $T_{21}$ (17:00) and neither TC nor EC were quantified. ENT was calculated for all four replicates at each time point. Experiment 5 consisted of time points  $T_0$  (9:00),  $T_3$  (12:00),  $T_6$  (15:00) and  $T_9$  (18:00). This experiment analyzed the same parameters as Experiment 4 except only two replicates were analyzed for ENT.

EXPERIMENT	DATE	T <sub>ELAPSED</sub> (TIME OF DAY)	TERM	MICROBIAL PARAMETER & # OF REPLICATES					
				VIB	vv	VP	ENT	тс	EC
1*	9/20	T <sub>0</sub> (10:00) T <sub>4</sub> (12:00) T <sub>8</sub> (16:00)	short	Y 3	Y 3	Ү З	Ү З	Y 3	Y 3
2	9/27	Τ <sub>0</sub> (10:00) Τ <sub>3</sub> (13:00) Τ <sub>6</sub> (16:00)	short	Y 4	Y 4	Y 4	Y 4	Y 4	Y 4
3	10/3	T <sub>0</sub> (20:00) T <sub>12</sub> (8:00) T <sub>15</sub> (11:00) T <sub>18</sub> (14:00) T <sub>20</sub> (16:00)	long	Y 4	Y 4	Y 4	Y 2	Y 2	Y 2
4	10/17	T <sub>0</sub> (20:00) T <sub>12</sub> (8:00) T <sub>15</sub> (11:00) T <sub>18</sub> (14:00) T <sub>21</sub> (17:00)	long	Y 4	Y 4	Y 4	Y 4	N	N
5	10/18	T <sub>0</sub> (9:00) T <sub>3</sub> (12:00) T <sub>6</sub> (15:00) T <sub>9</sub> (18:00)	short	Y 4	Y 4	Y 4	Y 2	Ν	Ν
6	10/25	T <sub>0</sub> (9:00) T <sub>3</sub> (12:00) T <sub>7</sub> (16:00) T <sub>12</sub> (21:00) T <sub>23</sub> (8:00) T <sub>29</sub> (14:00)	full <sup>†</sup>	Ν	Y 4	Y 4	Y 2	Y 2	Y 2

\*Proof of concept experiment. Small vs. large bottles used.

<sup>T</sup>Experiment 6 T<sub>3</sub>, T<sub>7</sub>, T<sub>12</sub> used as T<sub>3</sub>, T<sub>6</sub>, T<sub>9</sub> to enhance short-term data set since match in elapsed time and time of day.

**Table 1.** Summary of six experiments conducted September to October of 2013 and their respective data analyses set and measured parameters.

Calico Creek water for Experiment 6 was collected in the morning of day 1 and analyzed at  $T_0$  and at intervals throughout the day. Leftover bottles were left to sit in the ISCO overnight until analysis continued in the morning and through early afternoon of day 2. The time points were  $T_0$  (9:00),  $T_3$  (12:00),  $T_7$  (16:00),  $T_{12}$  (21:00),  $T_{23}$  (8:00) and  $T_{29}$  (14:00). Each of the four replicate sample bottles was analyzed for VV and VP. Only two of the four were analyzed for TC, EC and ENT.

#### 2.4. Compilation of Experiments into Short, Long and Full-terms

All raw data was converted into relative change by dividing a measured value by the average of the replicate samples measured at  $T_0$  within each experiment. As result, experiment data could then be compiled into three different data analyses sets: short, long and full-term categories (Table 1). Experiments 1, 2 and 5 were compiled into a short-term data set by time of day which was then enhanced by incorporating  $T_3$ ,  $T_7$  and  $T_{12}$  time points from Experiment 6 as  $T_3$ ,  $T_6$  and  $T_9$  time points since they matched in approximate elapsed time and time of day. Experiments 3 and 4 are long-term and Experiment 6 is our full-term experiment. The long term experiment was the only experiment that was conducted starting in the evening.

#### 2.5. Statistical Analyses

One-way ANOVAs with a Holm-Sidak post-test for multiple comparisons run by SigmaPlot determined significance ( $p \le 0.05$ ) between time points.

#### 3. Results

For each of the three compiled data analyses sets, the temperatures according to time of day were significantly different from one another over the course of the experiment (Figure 4). Conversely, salinity remained constant within each experiment (Figure 4).

In our short-term experiments, we observed no significant change in total VIB (p=0.189), VP (p=0.521) and ENT (p=0.080) concentrations within the first nine hours of collection. VIB concentrations revealed an increasing trend at  $T_3$  and decreasing through  $T_9$  (Figure 5a). VP concentrations increased through  $T_3$  and peaked at  $T_6$ , but decreased at  $T_9$  (Figure 5b). VV concentrations peaked at  $T_3$  and decreased through  $T_9$ . While these changes were noticeable, they were not significant.  $T_3$  and  $T_9$  VV concentrations were significantly different from each

other (p=0.001) (Figure 5c). However, neither  $T_3$  (p=0.243) nor  $T_9$  (p=0. 302) values were significantly different from  $T_0$ . ENT decreased through  $T_6$  but increased at  $T_9$  (Figure 5d).

Over the long-term experiments, the concentrations of VIB (p=0.521), VP (p=0.509), VV (p=0.334) and ENT (p=0.509) were also not significantly different at any time point over the 21 h as compared to the concentration at  $T_0$ . Total VIB peaked at  $T_{12}$  and  $T_{18}$  (Figure 6a). VP peaked at  $T_{15}$  and decreased through  $T_{21}$  (Figure 6b). Both VV and ENT peaked at  $T_{15}$  and followed downward trend to  $T_{21}$  (Figure 6c, d).

No significant difference was detected in microbial concentrations over time for the full length experiment for VP (p=0.109), VV (p=0.415) and ENT (p=0.053) concentrations at any point in time as compared to time zero. VP first decreased then peaked at  $T_7$  and  $T_{23}$  (Figure 7a). VV peaked at  $T_3$ , dipped at  $T_7$  and continued to increase through  $T_{29}$  (Figure 7b). ENT concentrations peaked at  $T_{12}$  and dipped at  $T_{23}$  subsequently increasing through  $T_{29}$  (Figure 7c).

When analyzing the variability of the VV concentrations associated with samples collected from morning through early evening, we observed a surprising trend (Figure 8). Changes in concentrations during sampling points close to noon indicated variability that was noticeable higher that during other periods of the day. VV concentrations were also compared to temperature for the short and long-term experiments (Figure 9).







**Figure 4.** Line plots representing fold change of temperature and salinity over (a) short, (b) long and (c) full-term experiments. Significant difference existed in temperatures over time, but did not exist for salinities. Error bars indicate standard error of means (SEM).



**Figure 5.** Line plots representing fold change of bacterial concentrations over short-term experiment. Time represented by total elapsed time and time of day. (a) Total *Vibrio* spp. (VIB), (b) *V. parahaemolyticus* (VP), (c) *V. vulnificus* (VV) and (d) *Enterococcus* spp. (ENT). Significance difference for  $T_3$  and  $T_9$  VV concentrations denoted by "A" and "B". Error bars indicate SEM.



**Figure 6.** Line plots representing fold change of bacterial concentrations over long-term experiment. Time represented by total elapsed time and time of day. (a) Total *Vibrio* spp. (VIB), (b) *V. parahaemolyticus* (VP), (c) *V. vulnificus* (VV) and (d) *Enterococcus* spp. (ENT). Error bars indicate SEM.







**Figure 7.** Line plots representing fold change of bacterial concentrations over full-term experiment. Time represented by total elapsed time and time of day. (a) *V. parahaemolyticus* (VP), (c) *V. vulnificus* (VV) and (d) *Enterococcus* spp. (ENT). Error bars indicate SEM.



**Figure 8.** Bar graph of fold change means versus time of day of combined short, long and full-term *V. vulnificus* concentrations. Significant difference denoted by "A" and "B". Error bars indicate SEM.



**Figure 9.** Line plots representing fold change of *V. vulnificus* concentrations and temperature over short-term (a) and long-term (b) experiments. Time represented by total elapsed time and time of day. Significant difference denoted by "A" and "B". Error bars indicate SEM.

## 4. Discussion

In studying the short-term bottle effects associated with ISCO autosampling methodologies, our data shows that short-term bottle effects are non-significant for culturable total Vibrio spp., V. vulnificus, V. parahaemolyticus, and Enterococcus spp. abundance. Our short-term dataset reveals that any sample stored in ISCO bottles and analyzed up to 9 h after collection are valid representations of T<sub>0</sub> concentrations. Our long-term experiment 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 likely that temperature plays a role in the fluctuation of *V. vulnificus* concentrations as stored over the course of a day. Noon and evening V. vulnificus concentrations were significantly different from  $T_0$ concentrations, and the increased likelihood of variation is most likely due to high and low of temperature for samples that are analyzed at those times. A study conducted at 10, 20 and 37°C showed bacterial increasing to a maximum quicker at higher temperatures (12 h at 37°C, 22 h at 20°C and 50 h and 10°C), supporting the likelihood that V. vulnificus increased at noon due to increased temperatures (Butterfield, 1933). While V. vulnificus concentrations seemed to continuously decrease after peaking around midday, our full term experiments reveal 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 the seemingly diel pattern could be attributed to predation

interactions in response to temperature. Bdellovibrio and like organisms (BALOs) preferably consume *Vibrio* spp. (Williams & Pineiro, 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 causes *V. vulnificus* concentrations to rise and fall. Noon and evening 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.

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 ensures that bacteria are loosened from surface walls as Taylor & 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 & 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 or not the relationship of concentrating a sample through filtration on the observed bacteria activity causes variability is also interesting. The filtration used to 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 hours 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 likely-hood of variation we do not recommend analyzing samples in the noon and evening times of day. Our study provides evidence against bottle

16

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 & Capone, 2002). If keeping samples saturated with oxygen should become a priority, bottles should be kept partly filled and in contact with air with daily shaking (ZoBell & 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 period.

Changes to normal growth activity are unavoidable during laboratory measurement of bacterial populations. Relating of results back to the natural environment should fully disclose 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 & Capone, 2002). 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.

Note: The table below summarizes 11 bottle effect studies dated from 1936 to 2010 that were used as reference in this paper.

**Table 2.** Summary of 11 bottle effect studies dated from 1936 to 2010 that were used as reference in this paper.

AUTHOR(S) & YEAR	TITLE	WATER	CONTAINER	FACTORS	EXPERIMENT	RESULT(S) & CONCLUSION(S)
Fuhrman & Azam (1980)	Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California	Seawater	Glassware, baked 2 h at 400°C	15, 100 and 500 ml with 3, 10, and 5 3 cm long glass tubing added, respectively (for three different surface to volume ratios)	Samples taken periodically for 30 h for water cell biomass and surface cell biomass	<ul> <li>Cell counts from different surface to volume ratios within 5% of each other</li> <li>Substantial growth without large particles in water</li> <li>ATP on walls negligible within 15 h, increased 3-5% of total ATP by 22 h and was 8-14% of total by 30 h</li> </ul>
Brozel & Cloete (1991)	Effect of storage time and temperature on the aerobic plate count and on the community structure of two water samples	Cooling- water and tap water	Sterile 10 ml glass tubes	4, 10, 20, 30°C for 24, 48, 72 and 216 h	Aerobic plate count and bacterial community structure of each sample determined	<ul> <li>No significant trend for culturable bacteria count or change in community structure</li> <li>No temperature where culturable bacteria count after 24 h equal to initial count</li> </ul>
Ferguson et al. (1984)	Response of marine bacterioplankton to differential filtration and confinement	Seawater	4 L autoclaved polypropylen e bottles	Unfiltered, 0.2-µm and 3.0-µm filtered water	Subsamples taken at 0, 16 to 18 h, and 30 to 34 h	<ul> <li>Culturable cells increased from 0.08% of total cell number (TCN) upon collection, 13% at 16 h and 41% at 32 h</li> <li>Shift in dominance from non-culturable to culturable bacteria</li> <li><i>Vibrio</i> spp. only bacteria not observed initially</li> <li>Dissolved primary amines (DPA) increased then decreased during confinement</li> <li>41% of growth within 16 h attributable to bottle effect</li> <li>Bottle effect increased TCN 10% after incubation of approx. 15 h</li> <li>Confinement can affect estimate of TCN within as few as 5 h</li> </ul>
Zobell & Anderson (1936)	Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces	Seawater	Chemically clean and sterilized Pyrex bottles or flasks	<ul> <li>Bottles of differing volume</li> <li>Differing volumes in varying types of bottle</li> <li>Partially filled bottles of differing volumes</li> <li>Completely filled bottles of differing volumes</li> <li>Bottles with different initial oxygen concentrations</li> <li>Bottles with different volume to solid surface ratios</li> <li>Bottles of same volume stored without shaking</li> </ul>	Samples storage at 16°C and tested for bacteria concentration and oxygen content; storage in number of days	<ul> <li>Densest bacterial populations appear in smallest volume (or highest surface area) in low nutrient concentrations therefore related to surface area of underwater glass surface</li> <li>Bacteria detach from walls if a few mg of organic matter present</li> <li>Little to no difference in bacteria population within the first two days at any volume</li> <li>Bacteria population abundance not related to surface area of sample exposed to air</li> </ul>
Heinemann & Dobbs (2006)	Microbiological 'bottle effects' not to be ignored (a comment on Mimura et al., 2005)	Seawater	NA	<i>V. cholerae</i> in water sample stored at 4°C	Daily analysis for 1 week then 14 d after collection	<ul> <li>5-fold decrease in mean CFU after 2 d</li> <li>40-fold decrease in mean CFU after 7 d</li> <li>CFU remained low after 14 d</li> </ul>

Petersen et al. (1997)	Scaling aquatic primary productivity: experiments under nutrient- and light- limited conditions	Seawater	Sun-Lite glazed (fiber glass- reinforced glazing material)	<ul> <li>0.1, 1, 10 m<sup>3</sup> samples of constant depth (1 m)</li> <li>Same volume samples in constant shape (0.56 radius to depth ratio)</li> </ul>	Nutrient concentration, primary productivity (PP) and respiration measured during spring, summer and fall	<ul> <li>Differences in PP at constant depth less extreme than at constant shape - wall area to unit volume ratio as artifact</li> <li>Rate of PP per volume and rate of PP per area increased as depth increased</li> <li>Radius effect on surface area to volume ratio - total ecosystem metabolism increased decrease in radius</li> </ul>
Hammes et al. (2010)	Critical evaluation of the volumetric "bottle effect" on microbial batch growth	Filter- sterilized river water and drinking treatment plant water	Cleaned glassware	30°C incubation until stationary phase reached (approx. 3, 4, 5 days) at 20, 40, 250, 500 and 1000 ml	Net growth quantified by total cell counting, total ATP analysis, and conventional plating with less than 5 day assays	<ul> <li>No growth on walls - questions how surface growth dramatic enough to make a difference on results</li> <li>No correlation between bottle size and net growth</li> <li>Rule out volume-related bottle effects</li> </ul>
Taylor & Collins (1949)	Development of bacteria in waters stored in glass containers	Windermere lake water	Bohemian glass, gutta percha, Pyrex glass and fused silica containers	<ul> <li>1 L Pyrex, 32 oz. glass vs. 1 L gutta percha containers</li> <li>1 L Winchester bottle vs. 1 L Pyrex flasks</li> <li>1 L Pyrex flask with differing agitation schemes</li> <li>150, 500 vs. 2000 ml Pyrex flasks</li> <li>Differing volume Pyrex flasks shaken before sampling</li> <li>25 vs. 1000 ml glass without shaking</li> </ul>	<ul> <li>Plates incubated for bacteria counts for 3, 4 or 10 days at 20°C</li> <li>Oxygen content measure for different volumes for different days</li> </ul>	<ul> <li>Greater numbers of bacteria when sample shaken before sampling</li> <li>Quiescent conditions (most similar to natural conditions) show no significance increase in bacteria</li> <li>Water-soluble substances of glass type stimulate bacterial activity</li> <li>Observed growth of bacteria and demonstrable organic matter on walls</li> </ul>
Butterfield (1933)	Observations on changes in numbers of bacteria in polluted water	River, creek and sewer waters	Glass	10, 20 and 37°C in the dark	Bacterial density determined at 0, 4, 8, 24, 48, 96 h, 6 d, 15 d and sometimes 41 d	<ul> <li>37°C samples reached max bacterial density in 12 h, 20°C in 22 h and 10°C in 50 h</li> <li>All subsequently decreased bacterial density</li> </ul>
Whipple (1901)	Changes that take place in the bacterial contents of waters during transportation	Tap water	Glass	Large vs. small bottles, non-agitated vs. agitated	Time factors vs. number of bacteria and number of species	<ul> <li>Number of bacteria decreased 10-25% within 3-6 h subsequently increased by many hundred percent</li> <li>Increase much greater in small than large bottles though effect reduced with agitation</li> <li>77 bacteria/ml initial gallon sample yielded 300 bac/ml in pint and 7020 in two ounces after 24 h</li> <li>Reduction in the number of species observed in all samples</li> </ul>
Marrase et al. (1992)	Seasonal and daily changes in bacterivory in a coastal plankton community	Sound water	Poly- carbonate containers	100, 200 and 500 ml and 1, 2 and 20 L at 19°C without stirring under darkness, light, and 12 h dark/light cycle	<ul> <li>Samples removed initially and every 6 h over 36 h</li> <li>Light regime samples collected initially, after 12, 24, 36 and 48 h</li> <li>Bacterial grazing rates measured disappearance of fluorescently labeled bacteria</li> </ul>	<ul> <li>Large variability in small bottles - larger samples more representative</li> <li>No effect of light on samples</li> <li>Higher rates of bacterivory at higher temperatures</li> </ul>

# References

- Atlas, R.M., & Bartha, R. (1998). Quantitative ecology: numbers, biomass, and activities (Chapter 7). In: Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings Science Publishing, Menlo Park, California, 218–280.
- Brözel, V. S., & Cloete, T. E. (1991). Effect of storage time and temperature on the aerobic plate count and on the community structure of two water samples.
- Burkholder, J., Eggleston, D., Glasgow, H., Brownie, C., Reed, R., Janowitz, G., ... & Springer, J. (2004). Comparative impacts of two major hurricane seasons on the Neuse River and western Pamlico Sound ecosystems.Proceedings of the National Academy of Sciences of the United States of America, 101(25), 9291-9296.
- Butterfield, C. T. (1933). Observations on changes in numbers of bacteria in polluted water. Sewage Works Journal, 600-622.
- Characklis, G. W., Dilts, M. J., Simmons III, O. D., Likirdopulos, C. A., Krometis, L. A. H., & Sobsey, M. D. (2005). Microbial partitioning to settleable particles in stormwater. Water Research, 39(9), 1773-1782.
- Christian, R.R., Capone, D.G., 2002. Overview of Issues in Aquatic Microbial Ecology (Chapter 28). In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology, second ed. ASM Press, Washington, DC, pp. 323-328.
- Chung, H., Jaykus, L. A., & Sobsey, M. D. (1996). Detection of human enteric viruses in oysters by in vivo and in vitro amplification of nucleic acids. Applied and environmental microbiology, 62(10), 3772-3778.
- Curriero, F. C., Patz, J. A., Rose, J. B., & Lele, S. (2001). The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948–1994. Journal Information, 91(8).
- Dawson, M. P., Humphrey, B. A., & Marshall, K. C. (1981). Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. Current Microbiology, 6(4), 195-199.
- Ferguson, R. L., Buckley, E. N., & Palumbo, A. V. (1984). Response of marine bacterioplankton to differential filtration and confinement. Applied and Environmental Microbiology, 47(1), 49-55.
- Fries, J. S., Characklis, G. W., & Noble, R. T. (2006). Attachment of fecal indicator bacteria to particles in the Neuse River Estuary, NC. Journal of Environmental Engineering, 132(10), 1338-1345.
- Fries, J. S., Characklis, G. W., & Noble, R. T. (2008). Sediment–water exchange of Vibrio spp. and fecal indicator bacteria: Implications for persistence and transport in the Neuse River Estuary, North Carolina, USA.Water Research, 42(4), 941-950.
- Froelich, B., Bowen, J., Gonzalez, R., Snedeker, A., & Noble, R. (2013). Mechanistic and statistical models of total Vibrio abundance in the Neuse River Estuary. Water research, 47(15), 5783-5793.
- Fuhrman, J. A., & Azam, F. (1980). Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Applied and Environmental Microbiology, 39(6), 1085-1095.

Hammes, F., Vital, M., & Egli, T. (2010). Critical evaluation of the volumetric "bottle effect" on microbial batch growth. Applied and environmental microbiology, 76(4), 1278-1281.

- Harrigan, W. F., & McCance, M. E. (1979). Laboratory methods in food and dairy microbiology. Metodos de laboratorio en microbiologia de alimentos y productos lacteos.
- Harvey, H. W. (1941). On changes taking place in sea water during storage. Journal of the Marine Biological Association of the United Kingdom, 25(02), 225-233.
- Heinemann, S., & Dobbs, F. C. (2006). Microbiological 'bottle effects' are not to be ignored (a comment on). Marine pollution bulletin, 52(10), 1310.
- Hsieh, J. L., Fries, J. S., & Noble, R. T. (2008). Dynamics and predictive modelling of Vibrio spp. in the Neuse River Estuary, North Carolina, USA.Environmental Microbiology, 10(1), 57-64.
- Hurley, M. A., & Roscoe, M. E. (1983). Automated statistical analysis of microbial enumeration by dilution series. Journal of Applied Microbiology, 55(1), 159-164.
- Krometis, L. A. H., Characklis, G. W., Simmons III, O. D., Dilts, M. J., Likirdopulos, C. A., & Sobsey, M. D. (2007). Intra-storm variability in microbial partitioning and microbial loading rates. Water Research, 41(2), 506-516.
- Lyons, M. M., Ward, J. E., Gaff, H., Hicks, R. E., Drake, J. M., & Dobbs, F. C. (2010). Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens. Aquatic Microbial Ecology, 60(1), 1.
- Lawrence, J.R., Neu, T.R., Marshall, K.C., 2002. Colonization, Adhesion, Aggregation, and Biofilms (Chapter 42). In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology, second ed. ASM Press, Washington, DC, pp. 466-468.
- Madsen, E. L. (2011). Environmental microbiology: from genomes to biogeochemistry. John Wiley & Sons.
- Marrase, C., Lin Lim, E., Caron, D.A. (1992) Seasonal and daily changes in bacterivory in a coastal plankton community. Marine Ecology Progress Series, 82, 281-289.
- Meyer-Reil, L.-A., Bolter, M., Liebezeit, G., & Schramm, W. (1979). Short-Term Variations in Microbiological and Chemical Parameters. Marine Ecology Progress Series, 1, 1-6.
- Munn, C.B., 2004. Methods in marine microbiology (Chapter 2). In: Marine Microbiology: Ecology and Applications. Garland Science/BIOS Scientific Publishers, New York, NY, pp. 19.
- O'Carroll, K., 1988. Assessment of Bacterial Activity (Chapter 13). In: Austin, B. (Ed.), Methods in Aquatic Bacteriology. John Wiley & Sons Ltd, New York, NY, pp. 347-354.
- Paerl, H. W., Valdes, L. M., Piehler, M. F., & Stow, C. A. (2006). Assessing the effects of nutrient management in an estuary experiencing climatic change: the Neuse River Estuary, North Carolina. Environmental Management, 37(3), 422-436.
- Pernthaler, J., & Amann, R. (2005). Fate of heterotrophic microbes in pelagic habitats: focus on populations. Microbiology and molecular biology reviews, 69(3), 440-461.
- Petersen, J. E., Chen, C. C., & Kemp, W. M. (1997). Scaling aquatic primary productivity: experiments under nutrient-and light-limited conditions. Ecology, 78(8), 2326-2338.
- Roszak, D. B., & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. Microbiological reviews, 51(3), 365.
- Sanders, J. G. (1978). The sources of dissolved manganese to Calico Creek, North Carolina. Estuarine and Coastal Marine Science, 6(2), 231-238.

- Strom, M. S., & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of Vibrio vulnificus. Microbes and infection, 2(2), 177-188.
- Tantillo, G. M., Fontanarosa, M., Di Pinto, A., & Musti, M. (2004). Updated perspectives on emerging vibrios associated with human infections. Letters in applied microbiology, 39(2), 117-126.
- Taylor, C. B., & Collins, V. G. (1949). Development of bacteria in waters stored in glass containers. Journal of general microbiology, 3(1), 32-42.
- Toranzos, G. A., McFeters, G. A., Borrego, J. J., & Savill, M., 2002. Detection of microorganisms in environmental freshwaters and drinking waters (Chapter 18). In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology, second ed. ASM Press, Washington, DC, pp. 249-264.
- Wetz, M. S., & Paerl, H. W. (2008). Estuarine phytoplankton responses to hurricanes and tropical storms with different characteristics (trajectory, rainfall, winds). Estuaries and Coasts, 31(2), 419-429.
- Williams, H. N. (1988). A study of the occurrence and distribution of bdellovibrios in estuarine sediment over an annual cycle. Microbial ecology,15(1), 9-20.
- Williams, H. N., & Piñeiro, S. (2007). Ecology of the predatory Bdellovibrio and like organisms. In Predatory prokaryotes (pp. 213-248). Springer Berlin Heidelberg.
- Yair, S., Yaacov, D., Susan, K., & Jurkevitch, E. (2003). Small eats big: ecology and diversity of Bdellovibrio and like organisms, and their dynamics in predator-prey interactions. Agronomie-Sciences des Productions Vegetales et de l'Environnement, 23(5-6), 433-440.
- Zobell, C. E., & Anderson, D. Q. (1936). Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. The Biological Bulletin, 71(2), 324-342.