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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