High salinity-induced loss of *Vibrio vulnificus* populations in North Carolina oysters

B.A. Froelich*, T.C. Williams, and J.D. Oliver
The University of North Carolina at Charlotte, 9201 University City Blvd. Charlotte, NC; 28223.
(bafroeli@uncc.edu)

Introduction

The human pathogen, *Vibrio vulnificus*, is responsible for nearly 95% of all seafood-related deaths, usually following consumption of raw or undercooked oysters (Jones and Oliver, 2009). This organism is routinely found in the oysters and water of estuarine environments as part of the normal microflora (Oliver, 2006). Using the selective and differential medium, CPC+, we are able to isolate *V. vulnificus* from the environment without the use of an enrichment step, allowing for direct counts from water and shellfish (Warner and Oliver, 2007b). In studies conducted by our laboratory in 2005/2006, 25-50% of all presumptive colonies collected from oysters using CPC+ were confirmed via PCR to be *V. vulnificus*. In July of 2007, we harvested oysters from the North Carolina coast that yielded only six *V. vulnificus* positive isolates out of 1401 presumptive samples. This was a most unexpected result, considering that the summer months are traditionally the months with the greatest *V. vulnificus* presence (Warner and Oliver, 2007a). In this report, we present a three year study in which we harvested oysters from North Carolina estuaries and sampled for *V. vulnificus*, comparing the data with oysters harvested from Gulf coast waters. Using identical methodology, we observed a loss of *V. vulnificus* from NC oysters corresponding with drought conditions, while simultaneously isolating high numbers from Gulf coast oysters. Additionally, we treated oysters harvested from NC estuaries with exogenous bacteria to measure the ability of *V. vulnificus* to reestablish itself as a significant part of the normal oyster flora and found it unable to do so.

Materials and Methods

Oyster collection and maintenance

Oysters were collected by hand from North Carolina estuaries and either shucked within one hour, or brought to the laboratory and placed into holding tanks containing artificial sea water (ASW). Oysters were regularly fed an algal mixture. Occasionally, oysters from Gulf Coast waters were also examined for *V. vulnificus* levels.

Bacterial strains and culture conditions

Unless otherwise noted, *V. vulnificus* strains were grown in heart infusion broth (HI) from freezer stock. Strain CVD713 is a *V. vulnificus* strain containing a TnphoA transposon conferring stable antibiotic resistance and alkaline phosphatase activity, allowing selection and differentiation on Tn Agar (Morris *et al.*, 1987; Murphy and Oliver, 1992; Wright *et al.*, 1990). Strain VVL1 is naturally bioluminescent strain of this species (Oliver *et al.*, 1986), and is therefore differential on non-selective solid media. Strain pGTR-Env1 contains a stable pGTR plasmid that confers kanamycin resistance (Ochi *et al.*, 1997), allowing its selection on media containing kanamycin and L-arabinose.

Oyster infection

Oysters were placed into tanks spiked with the "marked" strains of *V. vulnificus*. Using the selective media described above, we were able to differentiate these strains from the naturally occurring bacteria present within these oysters.

Oyster and water sampling

Oysters were removed (either from natural environment or aquaria) and aseptically shucked, dissected, and homogenized. After dilution, samples were plated onto media (TCBS, CPC+, HI, Estuarine Agar, Tn Agar, pGTR agar, or CHROMagar Vibrio).

Water samples were collected in sterile bottles and passed through a 0.2 micron filter. This filter was then placed onto CPC+, incubated overnight, and resultant colonies transferred to another CPC+ plate to allow for complete formation of identifying colors.

Results and Discussion

Using the *V. vulnificus*-selective medium CPC+, our lab regularly collects oyster and water samples from the NC coast. Colonies presumptive for *V. vulnificus* are examined via PCR (for *vvhA*, a hemolysin gene) to confirm identity. In both 2005 and 2006, our lab confirmed 41% of all presumptive colonies from CPC+ as *V. vulnificus* (Table 1). In 2007, two members of our lab independently harvested and sampled NC coast oysters, and collectively confirmed only 0.6% of presumptive *V. vulnificus* colonies out of 1041 total samples (Table 1). This failure to isolate *V. vulnificus* from oysters harvested from NC estuaries has continued to the present, with positive isolation rates from 0.6-0.7% (Table 1). An alternative medium, CHROMagar Vibrio, was utilized in conjunction with the CPC+ isolations. This medium, also selective and differential for *V. vulnificus*, provided presumptive colonies which were rarely positively identified as *V. vulnificus* (4% positive).

Table 1: *V. vulnificus* presumptive isolates obtained North Carolina using CPC+

Year	2005	2006	2007	2008	2009	2010
No. Tested	166	201	1041	1428	404	750
% Positive	40.7%	40.7%	0.6%	0.6%	0.7%	0.7%

During this period, we also examined oysters from the Gulf coast with >95% of presumptive colonies from CPC+ (Table 2) confirmed as positive. CHROMagar Vibrio (only 2010) confirmed 100% of Gulf coast isolates to be *V. vulnificus* via PCR.

Table 2: *V. vulnificus* presumptive isolates obtained Gulf coast oysters using CPC+

Year	2005	2006	2007	2008	2009	2010
No. Tested	30	ND	ND	80	31	20
% Positive	57% *			96%	98%	100%

*These isolates were collected using CPC (not CPC+), ND = No Data

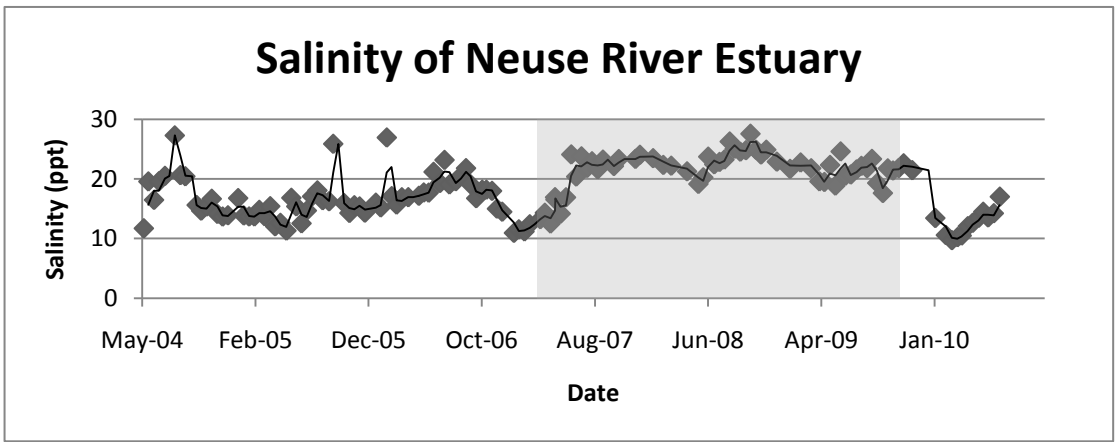

Figure 1: Biweekly salinity data from Neuse River estuary in NC. Black line = monthly moving average; Shaded area is period of drought. (from R. Noble and H. Pearl labs; UNC Institute of Marine Sciences)

This loss of *V. vulnificus* is possibly explained by a severe drought that affected North Carolina from May 2007 to September 2008. During this time, when salinities in this area are traditionally 15-18 ‰, the monthly moving average salinity was consistently well above 20 ‰ (Fig. 1), with anecdotal levels being as high as 40 ‰ or more. Several studies have shown that elevated salinities can cause a loss of *V. vulnificus* from oysters (Kaspar and Tamplin, 1993; Motes and DePaola, 1996; Motes *et al.*, 1998). This would seem to be a simple answer to the lack of identifiable *V. vulnificus* in 2007 and 2008. Once the estuary returned to nominal conditions, however, we would expect *V. vulnificus* to return to its "normal" levels in the estuary and its resident oysters. This was in fact the case with NC estuarine waters, as water samples collected in 2009 and 2010 resulted in numerous isolates which have been positively confirmed as *V. vulnificus* (>21%). Yet the oyster samples from these same waters have yielded few *V. vulnificus* (Table 1).

We simulated the return of *V. vulnificus* to the estuary *in vitro.* Oysters from NC estuaries were collected and placed into tanks at a salinity of 20‰. These oysters were treated with marked strains of *V. vulnificus*, as described above. We confirmed the results found by similar studies; exogenously added *V. vulnificus* cells are taken up by oysters, but are depurated rapidly (data not shown). It is generally felt that the existing oyster gut microflora prevents permanent colonization by added bacteria. **We thus propose that the long term salinity change to the NC estuarine environment, induced by prolonged drought conditions, resulted in a sharp decline of *V. vulnificus* from NC coast oysters, a change that has yet to return to pre-drought levels, even after the estuary salinity has decreased.**

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