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Non-native macroalga may increase concentrations of *Vibrio* bacteria on intertidal mudflats

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ABSTRACT: We investigated whether the proliferation of a non-native macroalga, *Gracilaria vermiculophylla*, within the mid-Atlantic coast region, USA, could be related to concentrations of *Vibrio* bacteria in water, sediment, and oysters on intertidal mudflats where mats of the macroalga are found. *Vibrio* spp. are naturally found in a range of aquatic environments; in estuaries they are recognized as being biogeochemically and ecologically important. While most species are harmless, some pathogenic species (e.g. *V. parahaemolyticus* and *V. vulnificus*) can cause symptoms of disease in humans that range from gastrointestinal and wound infections to septicemia and death. Recent research efforts have focused on potential reservoirs and environmental conditions that can increase the incidence of human exposure to these species of bacteria. Our data indicated that *V. parahaemolyticus*, and *V. vulnificus* were commonly found on the macroalga in both summer and early fall. Summer and fall seasonal samplings indicated that mudflats with mats of *G. vermiculophylla* were associated with higher total *Vibrio*, *V. parahaemolyticus*, and *V. vulnificus* concentrations of proximal water, sediment, and oysters when compared with mudflats without macroalgal coverage. In addition, of all isolates confirmed to be *V. vulnificus*, regardless of source, 68% were confirmed as a highly virulent genotype, which indicated the presence of pathogenic forms of *Vibrio* across a range of matrices within the estuarine environment.

KEY WORDS: *Vibrio parahaemolyticus* · *Vibrio vulnificus* · *Gracilaria vermiculophylla* · Oyster · Non-native · Mudflat · Water quality · Sediment

INTRODUCTION

Vibrio bacteria are ubiquitous in coastal and estuarine environments, comprise as much as 40% of the culturable bacterial population, and reach coastal abundances as high as 10^7 cells per 100 ml (Thompson & Polz 2006, Urakawa & Rivera 2006). They are recognized for their importance in nutrient cycling, including N_2 fixation, carbon cycling, nitrate reduction, and phosphorus recycling (Thompson & Polz 2006, Urakawa & Rivera 2006).

While most members of this genus are harmless to humans, some pathogenic strains, such as *V. parahaemolyticus* (Vp), *V. vulnificus* (Vv), *V. cholerae*, and *V. alginolyticus*, can cause gastrointestinal illnesses, wound infections, or septicemia. Infection can occur via consumption of raw or undercooked seafood or via exposure of wounds to seawater (Oliver 2005). Typically, infections occur in warmer months, when *Vibrio* spp. densities are highest (Oliver 2005). A review of Florida infections over 12 yr found that in susceptible individuals, such as those with diabetes,

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liver disease, or the elderly, death occured in 44% of septicemia cases resulting from *Vibrio* spp. infections (Hlady & Klontz 1996).

Over the last several decades, reports of *Vibrio* spp. infections have been increasing, most likely due to climate change, the rising proportion of elderly people in the population, and increased human exposure to coastal waters via recreation and consumption of shellfish (Baker-Austin et al. 2010, 2013). Global climate change will increase sea level height, overall aerial extent of estuaries, and year-round sea surface temperatures, which could increase overall concentrations of warm-water loving *Vibrio* spp. (Baker-Austin et al. 2010, 2013). These increases in overall *Vibrio* concentrations could result in greater human exposure and infection from both Vp and Vv (Baker-Austin et al. 2013). Because of these increases in exposure and potential infection, it is important that researchers and managers understand the ecology of, and possible reservoirs for, Vp and Vv.

In estuarine waters, macrophytes, microalgae, invertebrates, and sediment may act as *Vibrio* reservoirs because researchers have found that they contain more *Vibrio* cells than the surrounding water column (Hayat Mahmud et al. 2006, 2007, 2008). While initial studies of the genus focused primarily on reservoirs of *V. cholerae*, emphasis is shifting toward expanding this knowledge to reservoirs of Vp and Vv. Benthic diatoms (Kumazawa et al. 1991a,b), zooplankton, copepods, sediments (Kaneko & Colwell 1973), estuarine snails (Kumazawa & Kato 1985, Kumazawa et al. 1991b), freshwater fish (Sarkar et al. 1985), and seaweed (Hayat Mahmud et al. 2006, 2007) can all be associated with Vp in coastal ecosystems. In addition, currently documented reservoirs of Vv include several size classes of zooplankton (Heidelberg et al. 2002), shellfish, crab, finfish intestines (DePaola et al. 1994), and algae (Hayat Mahmud et al. 2008).

Researchers often classify Vv that are confirmed using molecular techniques into one of 2 genotypes: either the less virulent E-genotype, or the more virulent C-genotype that is commonly associated with human infection (Rosche et al. 2005). C-type strains show less down regulation of capsular polysaccharides than E-types, which potentially increases their virulence and/or decreases their ability to form biofilms (Hilton et al. 2006, Joseph & Wright 2004). To date, most studies report Vv isolates collected from environmental samples to be primarily of the E-genotype (Rosche et al. 2005, Warner & Oliver 2008b, Froelich & Oliver 2013). C-type strains of Vv appear to show greater seasonal effects when compared with the E-types; while both genotypes are increasingly abundant in warmer summer months, the C-types comprise a larger percentage of the total population (Warner & Oliver 2008a). In addition, it is proposed that E-genotype strains are more commonly found in oysters because they more readily integrate into marine aggregates, which are large enough to be separated out and ingested by oysters during water filtration (Froelich et al. 2013).

Gracilaria vermiculophylla is a non-native, red macroalga from East Asia that has been introduced to temperate estuaries around the world (Kim et al. 2010, Gulbransen et al. 2012). Currently, the known distribution of *G. vermiculophylla* on the east coast of the USA ranges from Massachusetts to South Carolina (Gulbransen et al. 2012). It was first confirmed in Virginia using genetic testing in 2004, but researchers hypothesize that it has been in the area since the 1970s, when it was unintentionally introduced attached to oyster shells (Thomsen et al. 2006, Gulbransen et al. 2012). *G. vermiculophylla* often accumulates on intertidal mudflats to form dense mats (up to 15 cm deep in Virginia) that can remain on a scale of months to years due to attachment to the tube building polychaete *Diopatra cuprea* (Thomsen & McGlathery 2005, Gulbransen & McGlathery 2013). Preliminary testing showed that Vp and Vv could be recovered from *G. vermiculophylla* thalli, a finding that led us to question whether this macroalga could be associated with higher levels of *Vibrio* bacteria on the intertidal mudflats where it persists.

Virginia epidemiological data sets support the paradigm previously described in Baker-Austin et al. (2013) of increasing *Vibrio* infections over time, with reported infections more than doubling in the past 20 yr (Pelton 2009). It is important that managers and watermen in the area understand how the habitat surrounding oyster reefs might affect *Vibrio* levels in harvested oysters. Prior work has found that attachment of *Vibrio* bacteria to particles, marine aggregates, or some type of biological substrate can enhance organic carbon availability and reduce environmental stress from sunlight/UV exposure, temperature, ozone, and nutrient limitation in the bacteria (Tang et al. 2011). Therefore, we hypothesized that *Vibrio* bacteria would colonize the surface of *G. vermiculophylla* thalli because it is a surface that provides shelter, organic matter, and protection from environmental fluctuations. The association of *G. vermiculophylla* with *Vibrio* spp. could have important consequences for densities of the bacteria found in sediment, water, and oyster tissue on mudflats. While it is possible that other species of macroalgae

in the area could have a similar association with *Vibrio* spp., we chose to investigate *G. vermiculophylla* because it is the most common species found in the region and it often forms mats that can be long-lived on mudflats (Thomsen et al. 2006).

The goals of this study were to (1) quantify concentrations of total *Vibrio*, Vp, and Vv associated with *G. vermiculophylla* thalli; (2) compare differences in total *Vibrio*, Vp, and Vv concentrations in sediment, water, and oysters on mudflats proximal to areas either with (vegetated) or without (bare) *G. vermiculophylla* mats; and (3) investigate the relative public health concern associated with the presence of Vv, by determining which genotypes of Vv were present in the samples (C-genotype or E-genotype).

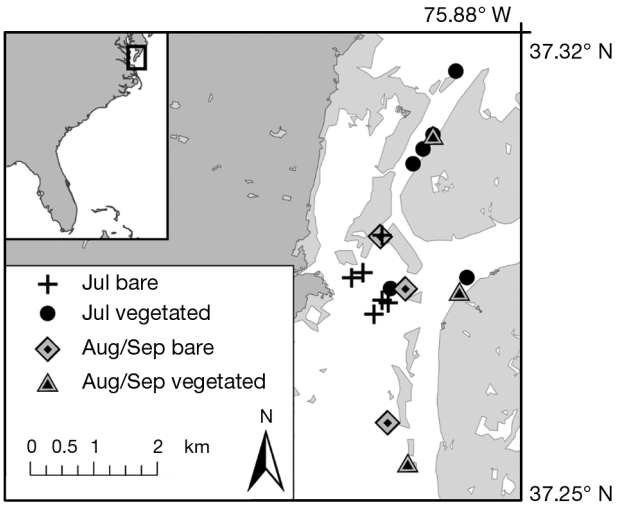

Fig. 1. Map of study sites visited during July, August, and September 2012 surveys

MATERIALS AND METHODS

Study site

Sampling was conducted on mudflats with and without *Gracilaria vermiculophylla* coverage, within the Virginia Coastal Reserve Long Term Ecological Research (VCR LTER) site (Fig. 1). All sample sites were within 6 km of one another, facilitating rapid sample collection.

Sample collection

Samples were collected once in July 2012, 3 times in August 2012, and 3 times in September 2012 (7 collections). Even though the sampling window was temporally narrow, we captured conditions during summer, when recreational water quality is of high importance, and during early fall, when shellfish harvesting commences in the Virginia coastal bays.

G. vermiculophylla samples were collected by hand to determine concentrations of total *Vibrio*, Vp, and Vv associated with the macroalgal thalli. On each sampling date, 10 g samples of *G. vermiculophylla* were collected and stored in sterile plastic bags until analysis.

Sampling performed on 2 July 2012 was conducted at a larger spatial scale and covered 6 bare and 6 vegetated sites, with 1 replicate sample of water, sediment, and *G. vermiculophylla* processed per study site. This sampling was focused on quantification of total *Vibrio*, Vp, and Vv concentrations in water and sediments in areas either associated with or not associated with *G. vermiculophylla*; oysters were not sampled. Sampling in August and September covered 3 bare and 3 vegetated sites, with 3 replicate samples each of water, sediment, oysters, and *G. vermiculophylla* at each study site. Because temperature and salinity demonstrably affect *Vibrio* concentrations (Thompson et al. 2006), we measured both variables at all sample sites, on each sampling date, using a multi-parameter sonde (6920 V2 YSI International).

Grab surface water samples were collected by filling autoclaved 1 l bottles and sediment samples were collected using a modified sterile 60 cc syringe. At each site, four 1 cm deep sediment cores were combined in a sterile Whirl-pak® bag. Five to 10 oysters were collected from each sample site in August and September, placed into a sterile plastic bag, and transported to the laboratory. All samples were stored in a cooler after collection and processed within 6 h of collection in the field.

Average *G. vermiculophylla* biomass was determined once in July, August, and September within a 100 m^2 section of each mudflat. Briefly, for each site that was determined to be *G. vermiculophylla* covered, all visible *G. vermiculophylla* found within ten 0.25 m^2 quadrats were collected to determine average dry mass of the alga m^{-2} at each site.

Laboratory processing

All samples were plated on thiosulfate-citrate-bile salts-sucrose (TCBS) medium (Oxoid) for total *Vibrio* enumeration and on CHROMagar™ Vibrio medium (CHROMagar) to determine presumptive concentrations of Vp and Vv. Water samples were filtered onto

0.45 µm sterile, gridded filters (Pall Corporation), which were placed onto each type of medium. Sediment samples were combined with equal parts of phosphate-buffered saline (PBS) (i.e. 10 g wet mass of sediment in 10 ml PBS; Amresco), vortexed for 5 min, and shaken for 1 min. This slurry was then immediately serially diluted in PBS and spread on TCBS and CHROMagar Vibrio plates. In order to control for variations in the initial water content of sediment samples, 2 ml of each sediment slurry were filtered onto duplicate, pre-dried and weighed glass fiber (GF) filters, dried in a 60°C oven for 48 h, and reweighed. This average dry mass of sediment was later used in calculations to determine total *Vibrio*, Vv, and Vp concentrations per gram dry mass of sediment. Each replicate of 10 g of *G. vermiculophylla* from vegetated sites was combined with 100 ml of PBS, vortexed for 5 min, and shaken for 1 min. Immediately after vortexing and shaking, subsamples of the resulting liquid were removed for serial dilutions and spread plating. Oysters were rinsed with distilled water to remove any excess sediment, then with ethanol, and patted dry. All shucking of oysters was done with an ethanol- and flame-sterilized knife. Once opened, the meat was rinsed with PBS, aseptically separated from the shell, and placed into sterile containers. Tissues from 5 oysters were combined and homogenized in a blender (Waring Commercial) with a 1:1 w:v ratio of grams of oyster meat to PBS (minimum of 25 ml PBS) using three 15 s long blending cycles separated by a 5 s pause. Three replicate, homogenized samples from each site were then serially diluted in PBS and spread on TCBS and CHROMagar Vibrio media.

All plated samples were incubated for 24 h according to the manufacturer's instructions (35°C for TCBS and 37°C for CHROMagar Vibrio). Colony forming units (CFUs) were counted on each plate after the incubation period in order to determine the presumptive CFUs per gram or milliliter of sample. Isolated colonies (300–400 per sampling period) were selected from CHROMagar Vibrio media using sterile loops into nuclease-free water and boiled for 10 min to release DNA for molecular typing. Presumptive Vp and Vv concentrations within each sampling period were multiplied by the proportion of isolates that were confirmed using molecular techniques to be Vp and Vv within the respective sampling period; this process produced an estimate of confirmed Vp and Vv concentrations. These values were used for statistical analyses, with reported values in tables and graphs displayed as means ± SE.

Molecular typing

Tubes containing released DNA were centrifuged at 10,000 × *g* for 10 min and the supernatant was then transferred to a fresh tube to be used as template DNA for PCR confirmation of species identification. Multiplex PCR reactions were used to confirm either Vp or Vv species identity by detecting amplification of species-specific DNA fragments. Vp isolates were confirmed using primers specific for *flaE* (McCarter 1995), with PCR conditions described in Hossain et al. (2013). Vv confirmation targeted a sequence located in the *vvhA* gene, which encodes for Vv-specific hemolysin, and had PCR cycling conditions as described in Warner & Oliver (2008b). The genotypes of confirmed Vv isolates were determined via multiplex PCR, examining for the *vcgC* or *vcgE* alleles (Rosche et al. 2005).

Statistical analysis

All statistical analyses to determine differences in total *Vibrio*, Vp, and Vv concentrations were performed on data separated by sample period (July, August, September) and sample type (water, sediment, oysters) in SAS 9.2. In July, *t*-tests were used to compare mean total *Vibrio*, Vp, and Vv concentrations at bare and vegetated sites. While all data collected from sediments did not need any transformations, Vv values from water had to be log transformed to satisfy ANOVA assumptions. Because transformations did not resolve homogeneity of variance issues with data for Vp levels in water, we used a nonparametric Wilcoxon test to analyze the data.

Total *Vibrio*, Vp, and Vv concentration data for August and September were analyzed using mixed model ANOVAs to determine differences between *G. vermiculophylla* coverage type (vegetated or bare), each of the 3 sample dates within each sample period, and the interaction of these 2 variables. All data satisfied ANOVA assumptions and were therefore analyzed without transformation.

RESULTS

Site conditions

Salinity and temperature were not significantly different between sites at each sampling period (Table 1). Average *Gracilaria vermiculophylla* biomass at vegetated sites was highest in July and

Table 1. Mean (±SE) salinity, temperature, and *Gracilaria vermiculophylla* biomass during each sample period

Date (2012)	Salinity (ppt)	Temperature (°C)	*G. vermiculophylla* biomass (g dry mass m^{-2})
02 Jul	31.86 ± 0.07	30.62 ± 0.34	112.02 ± 13.28
27, 28, 29 Aug	29.95 ± 0.16	27.66 ± 0.19	26.55 ± 3.46
19, 20, 21 Sep	31.36 ± 0.04	22.09 ± 0.27	15.27 ± 2.29

tapered off in August and September (Table 1). Total *Vibrio*, Vp, and Vv were found in relatively high abundances on *G. vermiculophylla* biomass in July, August, and September 2012 (Fig. 2, Table S1 in the Supplement at www.int-res.com/articles/suppl/m505p029_supp.pdf).

Surveys

2 July 2012. There was an overall trend of greater concentrations of total *Vibrio*, Vp, and Vv in water and sediment samples collected on vegetated rather than bare mudflats (Fig. 3). These differences were significant for total *Vibrio* concentrations in water (p = 0.0021) but not for sediment (p = 0.0592) samples collected on bare and vegetated mudflats (Table S1 in the Supplement). Vp levels in sediment (p = 0.0349), as well as Vv in water samples (p = 0.0020), were significantly different (Fig. 3, Table S1). No Vv was found in the sediment on either vegetated or bare mudflats (Table S1).

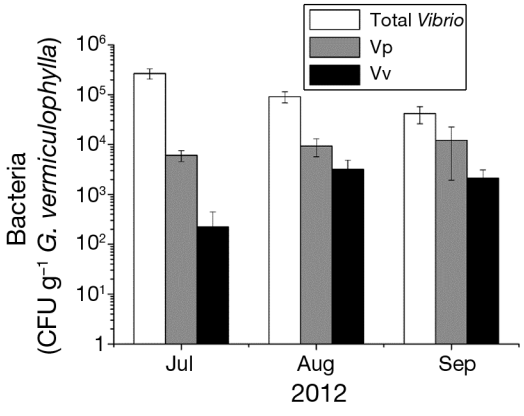

Fig. 2. Mean (±SE) total *Vibrio*, *V. parahaemolyticus* (Vp), and *V. vulnificus* (Vv) concentrations documented on *G. vermiculophylla* tissue in July, August, and September 2012. For specific values, see Table S1 in the Supplement. CFU: colony forming units

27–29 August and 19–21 September 2012. There was a trend in both August and September of higher levels of total *Vibrio*, Vp, and Vv in water, sediments, and oyster tissue collected proximal to mats of *G. vermiculophylla* when compared with concentrations from samples collected from bare mudflats (Fig. 4, Table S1 in the Supplement). Total *Vibrio* concentrations in water (p = 0.0877), sediment (p = 0.0325), and oyster tissue (p = 0.0875) samples were higher on vegetated mudflats in August (Table S1). In September, total *Vibrio* levels were not significantly different for water (p = 0.1345), sediment (p = 0.2478), or oyster tissue (p = 0.1686) samples on bare and vegetated mudflats (Table S1).

August water Vp (p = 0.0980) and Vv (p = 0.0887) levels showed a non-significant increase at *G. vermiculophylla* covered sites (Fig. 4, Table S1). Levels of Vp in sediment (p = 0.0422) and oyster tissue (p = 0.0382) were significantly higher when *G. vermiculophylla* was present (Fig. 4, Table S1). Vv levels showed a non-significant increase in oyster meat when *G. vermiculophylla* was present (p = 0.0589) (Fig. 4, Table S1). The interaction between *G. vermiculophylla* coverage state and sample date was not significant for total *Vibrio*, Vp, or Vv measurements in water or sediment during the August sampling period.

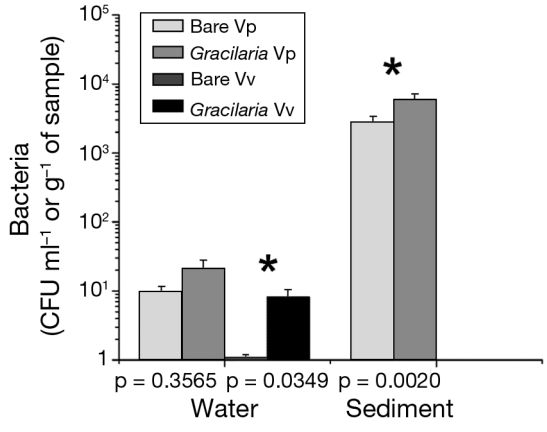

Fig. 3. Mean (±SE) *Vibrio parahaemolyticus* (Vp) and *V. vulnificus* (Vv) concentrations in water and sediment on mudflats with and without *Gracilaria vermiculophylla* coverage, from the widespread survey at 6 vegetated and 6 bare mudflats in July 2012. No Vv was found in the sediment on either vegetated or bare mudflats during this sample perod. Significant differences between concentrations on bare and vegetated mudflats are indicated by an asterisk between bars for each bacterial species. p-values for statistics between coverage types within each sample type are displayed on the *x*-axis. For specific values, see Table S1 in the Supplement. CFU: colony forming units

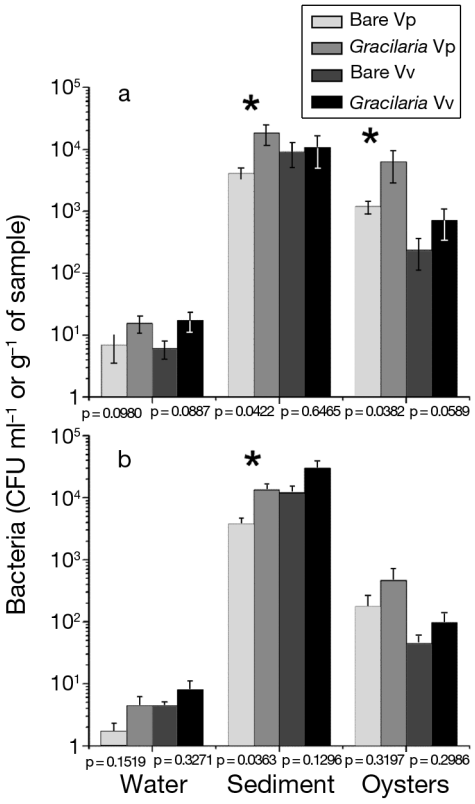

Fig. 4. Mean (±SE) *Vibrio parahaemolyticus* (Vp) and *V. vulnificus* (Vv) concentrations in water, sediment, and oysters, with and without *Gracilaria vermiculophylla* coverage nearby, on 3 sample days in (a) August and (b) September 2012. Significant differences between concentrations on bare and vegetated mudflats are indicated by an asterisk between bars for each bacterial species. p-values for statistics between coverage types within each sample type are displayed on the *x*-axis. For specific values, see Table S1 in the Supplement. CFU: colony forming units

In September, only Vp levels measured in sediments were significantly higher when *G. vermiculophylla* was present (p = 0.0363); all other densities of Vp and Vv were not significantly different between bare and vegetated mudflats (Fig. 4, Table S1). Sample date and the interaction between sampling date and coverage type were never significant for Vp, Vv, or total *Vibrio* measurements.

Molecular typing

Vp molecular analyses determined that, overall, the CHROMagar Vibrio medium correctly identified Vp colonies 81% of the time (total 846 isolates) from all 3 sampling periods. Specifically, 81% of the 348 isolates collected in July, 76% of the 271 isolates collected in August, and 89% of the 227 isolates collected in September were confirmed via PCR as Vp (Table S2 in the Supplement at www.int-res.com/articles/suppl/m505p029_supp.pdf).

PCR confirmation of Vv on 163 isolates over the course of all 3 study periods demonstrated that 36% of isolates (58 isolates) were confirmed as being this species. Specifically, 15% of 41 isolates in July, 36% of 36 isolates in August, and 44% of 86 isolates in September were confirmed as Vv (Table S2 in the Supplement). Of isolates confirmed to be Vv, regardless of source, 68% were C-genotype (the more virulent genotype, associated with human infections) and 32% were E-genotype (a relatively avirulent genotype, not typically associated with human infections). For a detailed breakdown of isolate confirmation and genotype, see Table S2.

DISCUSSION

Gracilaria vermiculophylla as a *Vibrio* reservoir

While other studies have looked at seaweeds in general as being reservoirs for Vp (Hayat Mahmud et al. 2006, 2007) and Vv (Hayat Mahmud et al. 2008), no studies have looked at the invasive macroalga *Gracilaria vermiculophylla* as a potential reservoir for *Vibrio* bacteria. Results from all sampling dates confirmed that *G. vermiculophylla* biomass was associated with levels of total *Vibrio*, Vp, and Vv that were higher than corresponding water samples (Fig. 2, Table S1 in the Supplement), thus confirming that this macroalga could be a reservoir of *Vibrio* bacteria.

Concentrations of total *Vibrio* documented on *G. vermiculophylla*, which ranged between 4.2 × 10^4 CFU g^{-1} in September and 2.7 × 10^5 CFU g^{-1} in July, fell within literature ranges of 2 to 6 log most probable number (MPN) g^{-1} for seaweed samples in Japan (Hayat Mahmud et al. 2008). In contrast, Vp levels documented in this study were on the order of 3 to 4 log CFU g^{-1}, which was much higher than the 21 to 110 MPN g^{-1} documented on seaweed in the literature (Hayat Mahmud et al. 2006, 2007). Similarly, Vv concentrations documented in this study were 1 to 2 log higher than values documented in the literature (Hayat Mahmud et al. 2008).

Effects of *G. vermiculophylla* on water, sediments, and oyster tissue

Data from all sampling periods show some support for the hypothesis that *G. vermiculophylla* presence

can be associated with an increase in total *Vibrio*, Vp, and Vv densities in water, sediment, and oyster tissue. These differences were significant for total *Vibrio* in water during July as well as in sediments during August. Vp in sediments during all 3 sampling periods as well as Vp in oysters in August had significantly higher levels on vegetated mudflats. In addition, Vv in water in July had higher concentrations when *G. vermiculophylla* was present.

Total *Vibrio* concentrations within water samples from this study (1 to 3 log CFU ml^{-1}) were similar to those documented in previous work (Kaneko & Colwell 1973, Hayat Mahmud et al. 2008); levels of Vp and Vv in water samples (all below 21 CFU ml^{-1}) (Kaneko & Colwell 1973, Hayat Mahmud et al. 2006, 2007, 2008, Staley et al. 2013) and sediment total *Vibrio* levels (4 log CFU g^{-1}) in this study were on the same order (Kaneko & Colwell 1973). Vp concentrations in the sediment, however, were 1 to 2 logs lower in the present study than documented in previous studies (literature values: 4 to 5 log CFU g^{-1}; Kaneko & Colwell 1973, Kumazawa & Kato 1985, Kumazawa et al. 1991b). For oyster Vp concentrations (present study: 2 to 3 log CFU g^{-1}) some studies report similar concentrations to ours (e.g. 3 log CFU g^{-1}; Kumazawa et al. 1991b), while others are much higher (5 log MPN g^{-1}; Kumazawa & Kato 1985). Similarly, when compared with Vv concentrations in oysters from the Gulf of Mexico, levels documented in the present study (between 1 and 2 log CFU g^{-1}), were on a par with (Staley et al. 2013) or much lower than (3 log MPN g^{-1}, Motes et al. 1998) literature values.

Our species-specific measurements, which indicated higher levels of Vp and Vv in water, sediments, and oysters near mats of *G. vermiculophylla*, may have public health implications that managers and watermen should be aware of. In the context of projected increases in concentrations of *Vibrio* bacteria due to climate change (Baker-Austin et al. 2010, 2013), it is important that future experimental work be conducted to explicitly test the transport and/or colonization of *Vibrio* bacteria from *G. vermiculophylla* biomass to nearby oyster reefs.

Molecular typing

In addition to overall *Vibrio* concentration trends, 68% of all Vv isolates collected, regardless of source, were C- rather than E-genotype strains (Table S2 in the Supplement). While most studies have reported a majority of environmentally collected Vv isolates to be of the E-genotype (Rosche et al. 2005, Warner & Oliver 2008b, Froelich & Oliver 2013), Yokochi et al. (2013) found as much as 91% of the Vv isolates from bay waters in Japan to be C-genotype. Since the sampling period for the present study was relatively short and Vv molecular analyses were somewhat limited, it would be interesting to determine the prevalence of C- versus E-genotypes of Vv over a range of seasons and matrices. In particular, additional work is warranted to investigate potential causes for higher levels of the C-genotype Vv isolates in Virginia.

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