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Short Communication

High Numbers of *Vibrio vulnificus* in Tar Balls Collected from Oiled Areas of the North-Central Gulf of Mexico Following the 2010 BP *Deepwater Horizon Oil Spill*

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Abstract: The *Deepwater Horizon Oil Spill* was the largest oil spill in USA history releasing approximately 4.9 million barrels of crude oil into the Gulf of Mexico. Soon after the spill started, tar balls and other forms of weathered oil appeared in large numbers on beaches in Mississippi and Alabama. In this study, we analyzed tar balls for total aerobic bacterial (TAB) counts and also for the presence of *Vibrio vulnificus*, a human pathogen known to be abundant in the Gulf Coast environment and capable of causing severe wound infections by contact with contaminated surfaces. Our results showed that TAB counts were significantly higher in tar balls than in sand and seawater collected at the same location. In addition, *V. vulnificus* numbers were 10× higher in tar balls than in sand and up to 100× higher than in seawater. Densities of *V. vulnificus* were higher than 10^5 colony forming units/g of tar ball in all samples analyzed. Our data suggest that tar balls can act as reservoirs for bacteria including human pathogens.

Keywords: *Vibrio vulnificus*, Tar balls, *Deepwater Horizon oil spill*

The 2010 BP *Deepwater Horizon oil spill* (DHOS) released approximately 4.9 million barrels (779 million L) of Louisiana light sweet crude oil (from the MC-252 Macondo well) into the north-central Gulf of Mexico over a period from 20 April 2010 through 15 July 2010 (FISG 2010). An estimated 1.1 million barrels (22%) of DHOS oil may still exist in the Gulf of Mexico basin as (i) surface oil (light sheen), (ii) shoreline tar balls, and (iii) sediment oil or submerged ''oil mats'' associated with the benthos (FISG 2010). Because microbes utilize myriad organic compounds as sources of carbon and energy, soon after the DHOS event began it was widely publicized that endemic gamma-proteobacteria could significantly affect the environmental fate and ecological impacts of the spilled oil (Voosen 2010). Subsequently, and as expected, the large-scale influx of DHOS allochthonous carbon markedly increased the number of heterotrophic microbes in the water column and a high rate of hydrocarbon biodegradation was documented from within the DHOS oil plume (Hazen et al. 2010). The few existing DHOS-related microbial studies to date have focused on the interactions between the oil plume and the pelagic microbes (Hazen et al. 2010), but no published study has analyzed bacteria on the large amounts of shore-cast weathered oil (e.g., tar balls) from within DHOS-effected coastal zone habitats (i.e., marshes and beaches).

Among marine gamma-proteobacteria, *Vibrio* spp. are well-studied microorganisms because they (i) include

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human pathogens, (ii) are abundant in coastal ecosystems, (iii) are critical to the carbon cycle, and (iv) are highly effective at degrading varied carbon sources including some polycyclic aromatic hydrocarbons (Thompson et al. 2004). The human pathogen *Vibrio vulnificus* is of special concern for public health authorities in coastal areas of the southeastern United States because it is the leading cause of seafood-borne fatalities nationwide (Mead et al. 1999). In addition to primary septicemia after ingestion of contaminated seafood, cutaneous exposure to seawater, fish, shellfish, or fishing gear contaminated with this bacterium can cause severe wound infections. Signs of infection include inflammation and necrotizing fasciitis plus cellulitis that can lead to secondary septicemia. The fatality rate for patients contracting wound infections caused by *V. vulnificus* is 20–30% (Strom and Paranjpye 2000).

Since the DHOS, many citizens have encountered tar balls by stepping on them or inspecting them while recreating on beaches; therefore, quantifying bacteria in tar ball is epidemiologically relevant since very little information is available regarding weathered oil as source of bacterial contamination. The objective of this study was to quantify total bacterial numbers in tar balls that appeared in great numbers on Mississippi and Alabama beaches after the DHOS. In addition, and because of the epidemiological relevance of *V. vulnificus* in the Gulf Coast, the presence of this human pathogen was also evaluated.

During July through October 2010, sand (200 g/collection), tar balls (200 g/collection), and seawater (1 L/collection) were collected aseptically from the intertidal (swash zone) of three beaches in Alabama and two in Mississippi (Table 1; Fig. 1). Air temperature was measured in situ. Samples were placed in insulated coolers with ice packs immediately after collection and during transport to the Aquatic Microbiology Laboratory, Auburn University (internal temperature of the coolers remained between 15 and 20°C).

Upon arrival to the laboratory, seawater (500 ml) was centrifuged at 5,000×*g* at 20°C for 30 min. Pellets were re-suspended in 10 ml (50:1) phosphate buffered saline (PBS), and shaken vigorously to dislodge bacteria from suspended aggregates. Cell suspensions were 10-fold diluted in PBS to the 10^{-7} dilution. Sand (15 g of wet sand) was mixed with 15 ml of PBS and vortexed for 2 min to detach bacterial cells from sand grains. Large particles were allowed to settled by gravity and 2 ml of the supernatant was diluted into 8 ml of PBS. Ten-fold serial dilutions in PBS were carried out to the 10^{-7} dilution. Tar balls were prepared similar to sand samples, except that tar ball material (15 g) was broken down using a sterile inoculation loop prior to emulsification in PBS and a longer shaking time was employed (15 min). One hundred microliters of each dilution were plated onto T1N1 agar (1% tryptone, 1% NaCl, 1.5% agar) in triplicate and incubated for 48 h at 30°C. Total aerobic bacterial (TAB) counts were calculated as colony forming units (CFU) per ml (seawater) or gram (sand and tar ball) of undiluted sample. To quantify *V. vulnificus*, colonies were transferred to a 90-mm Whatman no. 541 filter paper (Whatman, USA), followed by colony-dot-blot DNA hybridization using a specific *V. vulnificus* probe (Wright et al. 1993) as described in Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) (FDA 2002). Positive dots were recorded from each filter paper as *V. vulnificus* counts (VVC) from that dilution.

Using Statistical Analysis System v. 9.2 (SAS Institute Inc., Cary, NC), both TAB counts and VVC were converted to base 10 logarithms to fit the model assumption of normal distribution. One-way analysis of variance (ANOVA) was used to determine the differences in VVC and Welch's ANOVA (allowing for unequal variance) in TAB in all samples. If either ANOVA or Welch's ANOVA was statistically significant, Tukey's method and Scheffe's method were applied to perform post hoc, pair-wise comparisons at $\alpha = 0.05$ for the means of log VVC or the Dunnett's T3 test (allowing unequal variance) as post hoc, pair-wise comparisons for TAB at $\alpha = 0.05$.

Table 1. Sample collection data

Collection date (dd/mm/yyyy)	Location	Geographic coordinates	Air temperature (°C)
27/07/2010	Dauphin Island, AL	30°14′54″N; 88°07′40″W	28.5
30/07/2010	Ship Island, AL	30°12′46″N; 88°58′08″W	30.7
06/09/2010	Gulfport, MS	30°22′07″N; 89°04′50″W	27.6
26/09/2010	Fort Morgan, AL	30°13′30″N; 88°00′33″W	26.5
17/10/2010	Gulf Shores, AL	30°14′55″N; 87°40′05″W	23.5

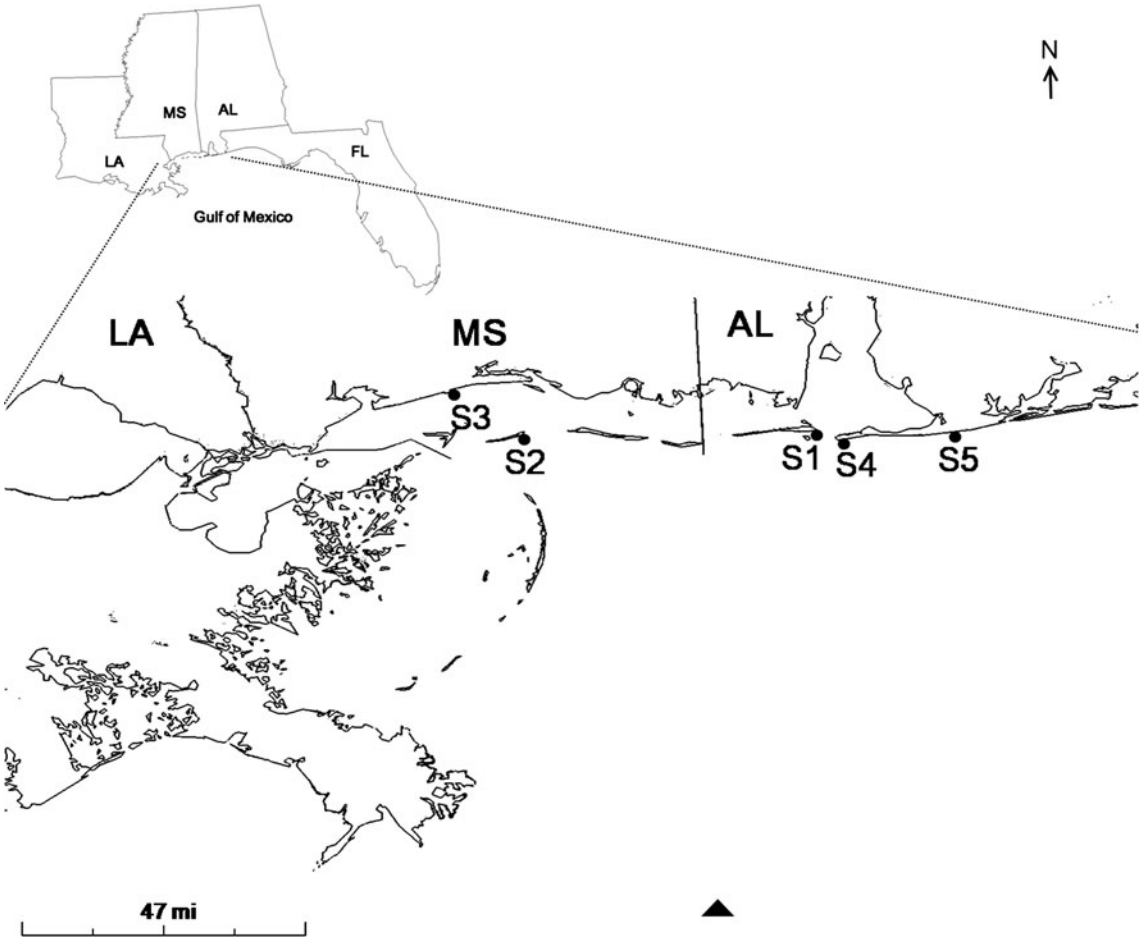

Figure 1. North-central Gulf of Mexico showing collecting sites: S1, Dauphin Island; S2, Ship Island; S3, Gulfport; S4, Fort Morgan; S5, Gulf Shores. Triangle indicates position of *BP Deepwater Horizon* wellsite, Mississippi Canyon Block 252 of the Gulf of Mexico.

Shoreline tar ball density and abundance is used to estimate the amount of spilled oil within an area (NOAA 2000) as part of the shoreline cleanup assessment team process (Goodman 2003). In our collection sites, tar ball density was in the range of 20–40 tar balls per m^2, with an average size of 3 cm. Tar balls are considered more of a nuisance than a public health concern with cutaneous allergic reactions due to hydrocarbons as primary hazard (NOAA 2010). Tar balls have not reportedly been identified as a source of infectious disease, but in this study we found TAB counts significantly higher in tar ball samples (5.1×10^6 to 8.3×10^6 CFU/g) than in seawater (3.5×10^3 to 3.1×10^4 CFU/ml) and sand (1.9×10^5 to 4.2×10^5 CFU/g) (Welch's ANOVA $P < 0.0001$; Dunnett's T3 $P < 0.05$) (Table 2). The levels of TAB we described are on the upper range of those previously reported (3×10^4 to 3×10^6 CFU/g) from tar ball samples collected in Nigeria (Itaha and Essien 2005), one of the few studies in where bacterial counts in tar balls have been measured. Interestingly, counts of the human pathogen *V. vulnificus* were significantly higher in tar ball samples than in any other sample analyzed (ANOVA $P < 0.0001$, Tukey's HSD $P < 0.05$). Numbers of *V. vulnificus* in tar balls were $>10^1$ higher than in sand and $>10^2$ higher than in seawater. *Vibrio vulnificus* numbers in tar balls were $>10^5$ CFU/g in all cases, reaching $>10^6$ CFU/g in 4 out of 5 samples analyzed (Table 2).

To test if *V. vulnificus* biodegraded tar balls, we attempted to culture several strains of this bacterium on tar ball-enriched seawater agar (Itaha and Essien 2005) (data not shown). No visible growth was observed on culture plates after 7 days of incubation. It is plausible that *V. vulnificus*, although probably not actively consuming organic compounds directly from the tar balls, could benefit from byproducts of the microbes that do degrade weathered oil.

Table 2. TAB counts and VVC

Date	Location	Sample	Total bacterial count (CFU/ml or CFU/g)	*V. vulnificus* count (CFU/ml or CFU/g)
27-07-2010	Dauphin Island, AL	SW	$3.1 \pm 0.4 \times 10^{4a}$	$1.0 \pm 0.0 \times 10^{3a}$
		S	$4.2 \pm 0.9 \times 10^{5b}$	$3.0 \pm 1.4 \times 10^{4b}$
		TB	$5.1 \pm 1.2 \times 10^{6c}$	$2.8 \pm 1.0 \times 10^{6c}$
30-07-2010	Ship Island, MS	SW	$4.3 \pm 0.4 \times 10^{3a}$	$5.0 \pm 2.8 \times 10^{2a}$
		S	ND	ND
		TB	$8.3 \pm 0.2 \times 10^{6b}$	$3.5 \pm 0.7 \times 10^{5b}$
06-09-2010	Gulfport, MS	SW	$5.4 \pm 1.9 \times 10^{3a}$	$5.5 \pm 2.1 \times 10^{2a}$
		S	$3.7 \pm 0.5 \times 10^{5b}$	$1.6 \pm 0.4 \times 10^{5b}$
		TB	$7.1 \pm 1.2 \times 10^{6c}$	$2.8 \pm 0.4 \times 10^{6c}$
26-09-2010	Fort Morgan, AL	SW	$3.5 \pm 0.3 \times 10^{3a}$	$6.7 \pm 5.8 \times 10^{a}$
		S	$3.8 \pm 0.2 \times 10^{5b}$	$1.0 \pm 1.0 \times 10^{4b}$
		TB	$5.4 \pm 0.2 \times 10^{6c}$	$2.7 \pm 0.6 \times 10^{5c}$
17-10-2010	Gulf Shores, AL	SW	$1.9 \pm 0.7 \times 10^{4a}$	$8.3 \pm 0.6 \times 10^{3a}$
		S	$1.9 \pm 0.7 \times 10^{4b}$	$1.3 \pm 0.6 \times 10^{3b}$
		TB	$8.0 \pm 1.5 \times 10^{6c}$	$3.3 \pm 0.5 \times 10^{6c}$

Values represent average ± standard deviation of repeat plate counts of each sample. Significantly different means ($P < 0.05$) within each sampling date are noted with superscripts a, b, and c.
SW, sea water; S, sand; TB, tar ball; ND, not determined.

Our values for *V. vulnificus* in seawater and sand were comparable to those previously reported in Gulf of Mexico seawater, sediment, and oysters (Motes et al. 1998). However, the high number of *V. vulnificus* recovered from tar balls is noteworthy because environmental samples rarely contain more than 10^5 CFU of *V. vulnificus* per gram (DePaola et al. 1994). Therefore, the high number of *V. vulnificus* ($>10^6$ CFU/g) we document in tar balls from Alabama and Mississippi beaches has clear public health implications. Tar balls are sticky, especially during the warmer months, difficult to remove (NOAA 2010), and upon contact with skin abrasions may vector *V. vulnificus* and lead to severe wound infections. Persons who have immunocompromising conditions (such as liver disease) are particularly at risk for *V. vulnificus* infections (Strom and Paranjpye 2000) and should avoid contact with contaminated sources.

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