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Interactions between the human pathogen *Vibrio parahaemolyticus* and common marine microalgae

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ABSTRACT

Vibrio parahaemolyticus is a gastrointestinal pathogen that is abundant in coastal marine environments. Elevated numbers of *V. parahaemolyticus* cells have been correlated with marine microalgae blooms, particularly blooms of diatoms and dinoflagellates, but the nature of the relationship between *V. parahaemolyticus* and microalgae is unknown. We performed *in vitro* assays using 27 environmental *V. parahaemolyticus* strains and various phototrophs; a diatom, a dinoflagellate, unarmored and armored forms of a coccolithophore, and two species of cyanobacteria. The *V. parahaemolyticus* strains we employed contained different combinations of virulence-correlated genes, the hemolysin genes *tdh* and *trh*, the Type III Secretion System 2 (T3SS2) marker gene *vscC2*, and the Type VI Secretion System (T6SS) marker gene *vipA1*. We determined that all *V. parahaemolyticus* strains, even strains in which no virulence factor genes were detected, were able to cause decreases in diatom, dinoflagellate, and unarmored coccolithophore biomass *in vitro*. No correlation between content of any virulence gene and damage to microalgae was apparent. We hypothesize that marine microalgae represent a reservoir of nutrients that the copiotroph *V. parahaemolyticus* can utilize in salt marsh environments, which are often poor in labile carbon and energy sources. This helps to explain the recent correlations between *V. parahaemolyticus* and microalgae blooms in such environments.

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KEYWORDS: *Vibrio parahaemolyticus, tdh, trh,* T3SS2, T6SS, microalgae.

INTRODUCTION

Vibrio parahaemolyticus, a common organism in coastal environments, is a significant and sometimes pandemic human pathogen responsible for an estimated 34,000 cases of seafood-associated gastroenteritis per year in the United States [1]. Most cases of *V. parahaemolyticus*-induced gastroenteritis are self-limiting and relatively mild, but infections can be deadly in immunocompromised individuals. The common mode of transmission of this bacterium to the human host is ingestion of raw or undercooked shellfish, primarily oysters. In addition, some strains of *V. parahaemolyticus* can infect wounds and some produce systemic infections, while others are apparently non-pathogenic. Elevated densities of *V. parahaemolyticus* most often occur during the warm months and at warm locations [2, 3] but recently large vibriosis outbreaks have occurred at locations not considered typical for this organism [4, 5].

V. parahaemolyticus not only persists but can increase in population size very rapidly in coastal marine environments [3, 6, 7]. It is not understood how this copiotrophic organism acquires carbon and other nutrients in coastal marine ecosystems where levels of utilizable soluble (labile) carbon and energy sources are typically quite low [8-10]. Even considering the known catabolic versatility of this species [3], rapid growth opportunities in many coastal ecosystems would seem infrequent at best. The abundance of *V. parahaemolyticus* as

free-living cells in water is typically low (< 2,000 cells per liter) [6], but this organism can be very abundant in surficial sediment and in infaunal burrows [3, 6]. *V. parahaemolyticus* also occurs at higher levels in shellfish [2, 7, 11], and in association with algal blooms [12-16]. Thus, significant reservoirs exist even when no outbreak is underway [17]. The population expansion of *V. parahaemolyticus* that predicates an outbreak may be supported by means other than the typically low abundance, and largely refractory, dissolved organic carbon pool found in relatively low human impact coastal marine systems.

Potential for a *V. parahaemolyticus* outbreak has often been predicted on the basis of local temperature, salinity, turbidity, and chlorophyll *a* concentrations [18-20]. In addition, some correlations between *V. parahaemolyticus* densities and certain algal taxa, specifically diatoms and dinoflagellates [12, 21, 22], have been reported and elevated levels of *V. parahaemolyticus* can occur during dinoflagellate and diatom blooms [13, 14]. The interaction between microalgae and *V. parahaemolyticus* could be commensalistic, based on soluble exudates released from algal cells lysed by viruses [23, 24], inefficient grazing by zooplankton [25, 26], or from undamaged algal cells. Or, perhaps, the microalgae themselves serve as supplemental carbon sources that *V. parahaemolyticus* can utilize.

We examined the ability of *V. parahaemolyticus* to cause damage to healthy phototrophs. Chlorophyll *a* served as an indicator of phototroph biomass. Six phototrophs, including three species of microalgae and two species of cyanobacteria were incubated with several strains of *V. parahaemolyticus*. The phototrophs employed are abundant in marine environments and present a variety of cell wall surface structures and properties, providing insight into associations between susceptibility of the microalgae to *V. parahaemolyticus* predation and cell wall features.

MATERIALS AND METHODS

V. parahaemolyticus strain isolation and characterization

V. parahaemolyticus strains were isolated from the pristine North Inlet estuary near Georgetown, SC, USA (33°20'N, 79°12'W) in August and September 2011 as described previously [27]. The North Inlet-Winyah Bay National Estuarine Research Reserve protects the third largest watershed on the east coast of the United States; and North Inlet is a bar built oligotrophic salt marsh where human impact is negligible [28, 29]. Samples were diluted and plated directly onto Thiosulfate Citrate Bile Salts Sucrose agar (TCBS) (BD, NJ). The presumptive identification of all *V. parahaemolyticus* strains used in this study was confirmed by *recA* sequence analysis [27] using the PCR primers and protocols of Thompson *et al*. [30].

Virulence gene PCR screening

Two virulence-related hemolysin genes, *tdh* and *trh*, have been correlated with pathogenesis in *V. parahaemolyticus*, and these hemolysin genes are frequently used as molecular markers for strain virulence [27, 31]. Additional virulence factors, specifically secretion systems, have been discovered with recent sequencing of *V. parahaemolyticus* genomes [32-34]. The Type III Secretion System (T3SS2) has also been implicated in *V. parahaemolyticus* virulence [32] and the outer membrane protein gene, *vscC2* is a useful marker for this structure [35, 36].

The Type VI Secretion System (T6SS) has also been detected in some *V. parahaemolyticus* isolates [37]. This secretion system has not been implicated in the pathogenicity of *V. parahaemolyticus* to humans, but T6SS producing *V. parahaemolyticus* strains have been shown to cause damage to other prokaryotes *in vitro* when incubated on a surface [37]. Its impacts on eukaryotic microalgae are presently unknown. Strains were screened for the T6SS marker gene *vipA1* using the PCR primers and protocols of Salomon *et al*. [37].

V. parahaemolyticus strains were grown overnight at 37 ºC in Saline Luria-Bertani Broth (SLB; per L 27 g NaCl, 10 g Tryptone, 5 g Yeast Extract) and boiled extracts (15 min at 95-100 ºC) were prepared. All PCR reactions were completed within three days of DNA extraction and 1 µl of boiled DNA extract was used per reaction. PCR products were resolved on a 1.5% agarose gel and sequenced using an ABI Prism 3730 DNA analyzer to confirm gene identity. Sequences were analyzed using the Kimura 2 parameter model with Mega version 7 [38]. Sequence data obtained from this work were

submitted to the NCBI GenBank and assigned the accession numbers KX171447- KX171449.

Cultivation of microalgae and cyanobacteria

Phototroph cultures were obtained from the Bigelow National Center for Marine Algae and Microbiota (Bigelow Center, East Boothbay, ME). Three species of eukaryotic microalgae were used in this project, the diatom *Thallasiosira pseudonana* (CCMP 1335), the dinoflagellate *Prorocentrum minimum* (CCMP 695), and two strains of the coccolithophore, *Emiliania huxleyi* (CCMP 371 and CCMP 373). *T. pseudonana* and *P. minimum* are common in North Inlet and *E. huxleyi* CCMP 371 is a coccolith-producing (armored) form that causes extensive blooms. *E. huxleyi* CCMP 373 is an unarmored mutant phenotype. Two species of cyanobacteria were also used, *Prochlorococcus marinus* (CCMP 1986) and *Synechococcus bacillaris* (CCMP 1333).

All of the phototrophs employed have ubiquitous distributions worldwide and are frequently found in the same environments where *V. parahaemolyticus* occurs [39-41]. The phototrophs chosen had a variety of cell wall surface structures [39-42] and each required its own growth medium (S1). Microalgae and cyanobacteria were grown at 23 °C with an 11h light, 13h dark cycle.

In vitro experiments

V. parahaemolyticus strains were grown in SLB at 37 °C with shaking. At 5 h, cultures were in exponential growth phase and yielded approximately 2 x 10^7 cells mL^{-1}. Cultures were centrifuged (600 x g), the supernatants discarded, and cell pellets resuspended in a mixture of artificial sea salts (Instant Ocean, 33 ppt). Phototroph cultures were grown for 5 days, reaching approximately 2.0 x 10^5 cells mL^{-1}, then harvested by centrifugation (1075 x g) for 10 min, and the supernatants removed. Cells were resuspended in 33 ppt Instant Ocean. Bacterial strains and phototroph cultures were combined in 96 well microplates with a multiplicity of infection (MOI) of approximately 100:1. Chlorophyll *a* fluorescence was used as an indicator of phototroph condition, and was measured immediately after co-inoculation ($T_{initial}$) using a SpectraMax Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA). Microplates were then incubated with light for 24 h at 25 °C. After incubation, chlorophyll *a* fluorescence was measured (T_{final}). The percent difference between final and initial time points was determined by the formula: $((T_{final} - T_{initial}) / (T_{initial})) \times 100$. Before and after incubation, aliquots were observed under a Nikon Eclipse TS100 microscope to determine the effect of *V. parahaemolyticus* clinical strains ATCC 17802^T, ATCC 33846, and environmental strain 5-10-J5-4 on unarmored *E. huxleyi*. Microalgal cell counts were performed using a hemocytometer after the 24-h co-incubation.

Each *V. parahaemolyticus* strain was tested in 12 wells per 96 well plate. For true replication, each 96 well plate was repeated three times. Controls included replicates of phototrophs in appropriate media and replicates of phototrophs in artificial seawater (with no *V. parahaemolyticus* added), against which experimental replicates were compared. *Vibrio pacinii*, an avirulent *Vibrio* [43], was used as a non-*V. parahaemolyticus*, heterotrophic bacterial control. Changes in chlorophyll *a* fluorescence were compared to controls (phototrophs suspended in artificial seawater without *V. parahaemolyticus*) using one way analysis of variance (ANOVA). Multiple comparisons were made versus the control group using the Holm-Sidak method (SigmaPlot, 2016). The significance level used was 0.05.

All 29 *V. parahaemolyticus* strains were tested against the eukaryotic algae. A subset of these strains, some having the antibacterial mechanism T6SS (*vipA1*-positive) and some lacking it, was also tested against the cyanobacteria. We also used the T6SS-bearing POR1 strain, a derivative of the *V. parahaemolyticus* RIMD 2210633 reference strain, and two POR1 derivatives. The POR1 derivatives were the T6SS-knockout strain, POR1Δhcp1, and the T6SS de-repressed strain, POR1Δhns.

Dose response

To determine the effect of *V. parahaemolyticus* dosage on the *in vitro* experiments, bacterial cultures were serially diluted (10^7 to 10^2 cells mL^{-1}) in artificial seawater. These dilutions were incubated with unarmored *E. huxleyi*, the concentration of which was not altered.

RESULTS

Eukaryotic microalgae *in vitro* experiments

Twenty nine *V. parahaemolyticus* strains, 27 of which were environmental strains isolated from

North Inlet estuary, were incubated with microalgae. These environmental strains were previously confirmed to be *V. parahaemolyticus via recA* sequence analysis [27, 30]. The two non-environmental strains were clinical isolates, the *trh*-bearing *V. parahaemolyticus* type strain ATCC 17802^T and the *tdh*-bearing reference strain ATCC 33846. Nineteen of the *V. parahaemolyticus* strains contained varying combinations of the virulence-related genes *tdh*, *trh*, *vscC2*, and *vipA1*; no virulence factor genes were detected in ten of our strains (Table 1).

Chlorophyll *a* fluorescence is a strong indicator of phototroph health and biomass and was used

Table 1. Distribution of virulence-related genes in *Vibrio parahaemolyticus* strains[1].

Strain designation	*tdh*	*trh*	*vscC2*	*vipA1*
TS 8-11-4	+	+	+	-
JBW 8-11-2	-	+	+	-
CW 9-11-2	+	-	+	-
JPW 9-11-9	+	-	+	-
JPW 9-11-10	+	-	+	-
JPW 8-11-1	+	+	-	+
5-10-J5-4	+	+	-	+
ATCC 17802^T	-	+	-	+
JPW 8-11-9	-	-	+	+
JBW 9-11-5	-	-	+	+
JPW 9-11-13	-	-	+	-
TBS 9-11-7	-	-	+	-
TS 9-11-6	-	-	+	-
JS 8-11-1	+	-	-	-
TBW 9-11-1	+	-	-	-
JBS 8-11-4	+	-	-	-
ATCC 33846	+	-	-	+
JS 8-11-5	+	-	-	+
JS 8-11-6	-	-	-	+
JBS 9-11-1	-	-	-	-
JPW 9-11-4	-	-	-	-
JS 9-11-3	-	-	-	-
TBS 8-11-3	-	-	-	-
JBW 9-11-4	-	-	-	-
JS 8-11-7	-	-	-	-
JS 8-11-9	-	-	-	-
TS 9-11-5	-	-	-	-
TPW 9-11-2	-	-	-	-
JS 8-11-2	-	-	-	-
POR1	-	-	+	+
POR1 Δhcp1	-	-	+	-
POR1 Δhns	-	-	+	+

[1]*tdh* and *trh* are hemolysin genes, *vscC2* is a marker gene for the Type 3 Secretion System II (T3SS2), and vipA1 is a marker gene for the Type 6 Secretion System (T6SS).
All strains were isolated from North Inlet estuary except two ATCC reference strains and three RIMD 2210633 derivative strains, all of which are indicated in bold.
T6SS is constitutively expressed in POR1Δhns.

to quantitatively measure the condition of each alga when exposed to *V. parahaemolyticus*. The unarmored coccolithophore *E. huxleyi* was most susceptible to *V. parahaemolyticus*, with chlorophyll *a* decreasing by 71.5-96.3% (Fig. 1A) after incubation. Variability among replicates was extremely low. No correlations could be made between content of virulence gene(s) and unarmored *E. huxleyi* biomass loss. Instead, consistently strong decreases in chlorophyll *a* fluorescence were observed in the presence of all *V. parahaemolyticus* strains (One Way ANOVA, all p values < 0.001). Chlorophyll *a* decreases were accompanied by strong decreases in microscopically visible cells (S3). The avirulent *Vibrio* control, *V. pacinii*, did not cause any decreases in chlorophyll *a* in unarmored *E. huxleyi* or any other microalga.

All *V. parahaemolyticus* strains tested caused a significant loss in the diatom *T. pseudonana* and the dinoflagellate *P. minimum* biomass (Figs. 1B and 1C). The decreases in chlorophyll *a* from these species were not as extreme as those of unarmored *E. huxleyi*, amounting to 15-50% (*T. pseudonana*: 15.3 to 48.0%; *P. minimum* 14.7 to 53.3%). Compared to controls, all *V. parahaemolyticus* strains tested caused significant chlorophyll *a* decreases in both algae (One Way ANOVA, all p values <0.001), regardless of virulence gene content.

The armored *E. huxleyi* showed highest variability when incubated with *V. parahaemolyticus* (Fig. 2), with three different results observed: (a) Some *V. parahaemolyticus* strains had no effect (13 strains, One Way ANOVA, p values 0.09-0.88, no growth stimulation). (b) Some produced significant inhibition of armored *E. huxleyi* growth (8 strains, One Way ANOVA, p values < 0.001 to 0.009). When exposed to these strains, the armored *E. huxleyi* grew (i.e.: the chlorophyll *a* fluorescence (biomass) increased) but this growth was significantly less than the control with no *V. parahaemolyticus.* (c) Significant losses in *E. huxleyi* biomass, as seen by decreases in chlorophyll *a* fluorescence (8 strains, One Way ANOVA, all p values < 0.001). Once again, we found no correlation between content of virulence factor gene(s) and decreases in chlorophyll *a* fluorescence. For example, strain TBS 8-11-3 caused significant armored *E. huxleyi* biomass loss, yet no virulence factor genes were detected in this strain.

Dose response

We conducted dose response experiments (Fig. 3), challenging unarmored *E. huxleyi* with three of our environmental *V. parahaemolyticus* strains at levels of 10^7 to 10^2 cells mL^{-1}. We found that even at low *V. parahaemolyticus* concentrations, this pathogen was able to reduce the biomass of unarmored *E. huxleyi*. As expected, the degree of chlorophyll *a* loss decreased at lower *V. parahaemolyticus* concentrations. For example, *V. parahaemolyticus* strain TBW 9-11-1 caused an 83.1% decrease in unarmored *E. huxleyi* chlorophyll *a* fluorescence at 10^7 cells mL^{-1}, while at 10^2 cells mL^{-1} a 55.2% decrease in chlorophyll *a* fluorescence was observed.

***vipA1* in environmental *V. Parahaemolyticus* strains**

Six of our 27 (22%) environmental *V. parahaemolyticus* strains carried the T6SS marker gene *vipA1*. *vipA1* was also detected in the clinical reference strains ATCC 17802^T and ATCC 33846. The T6SS is only expressed when *V. parahaemolyticus* is also expressing lateral flagella on a surface [36]; however, we did not find the T6SS marker gene *vipA1* exclusively in strains isolated from surfaces. Three environmental strains that contained *vipA1* were isolated from sediment (JS 8-11-5, JS 8-11-6, 5-10-J5-4); the other three were isolated from water (JPW 8-11-1, JPW 8-11-9, JBW 9-11-5). Sequence data recovered from these amplicons confirmed amplification of the *vipA1* gene.

Cyanobacteria *in vitro* experiments

Cyanobacteria were exposed to a subset of our environmental *V. parahaemolyticus* strains; some that contained the antibacterial mechanism T6SS and some that did not. We found that regardless of content of *vipA1*, all *V. parahaemolyticus* strains tested stimulated cyanobacterial growth (Fig. 4). No decrease of biomass was observed in either species of cyanobacteria, rather there was a significant increase in chlorophyll *a* fluorescence during co-incubation with *V. parahaemolyticus* (One Way ANOVA, all p values < 0.001). There was no significant difference in cyanobacterial stimulation between strains that contained T6SS and strains that did not (Student's t-test, p values 0.214 and 0.252). The de-repressed strain POR1Δhns,

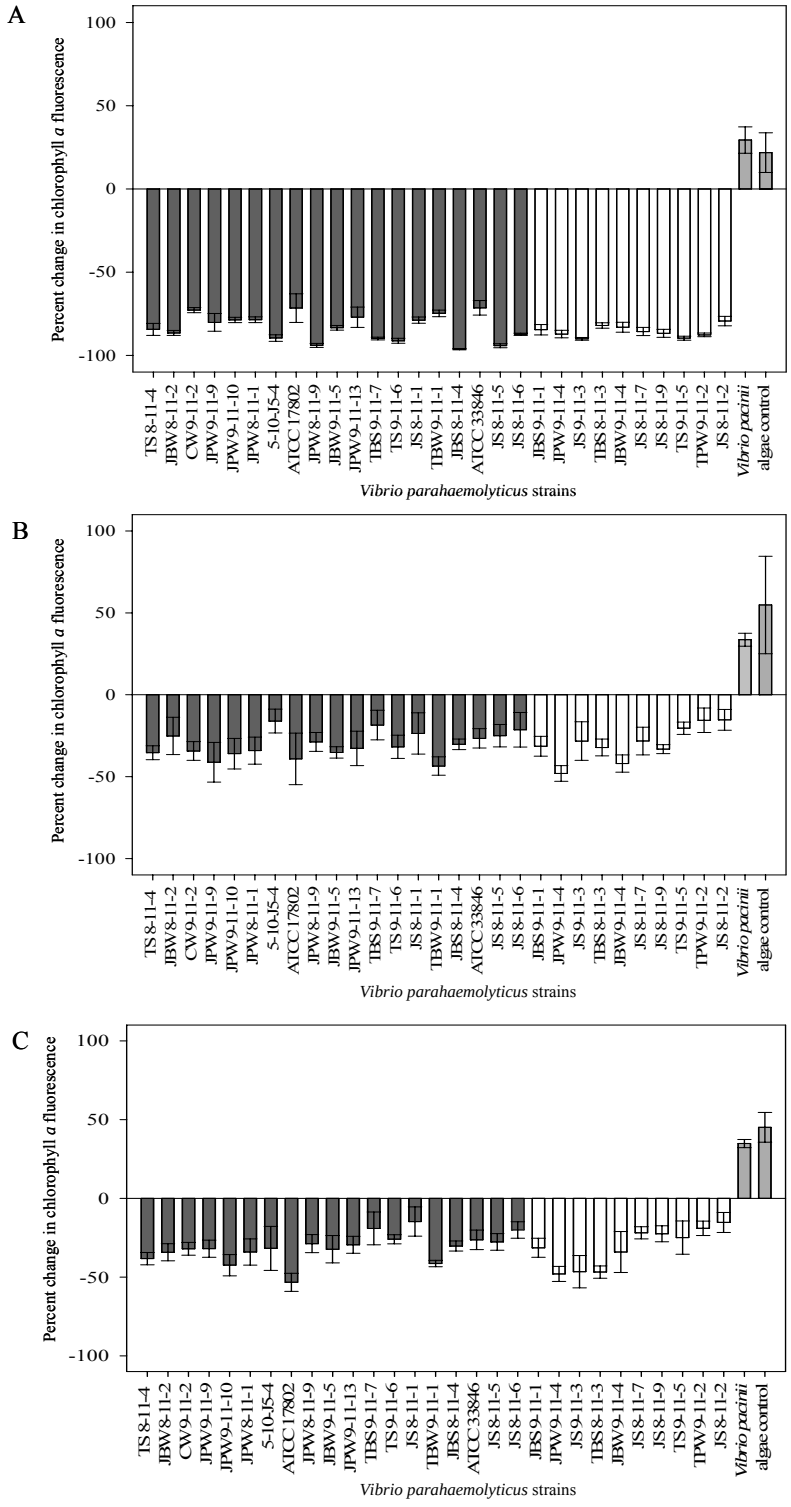

Fig. 1A-C. Changes in algal chlorophyll *a* fluorescence during 24-h incubation with *Vibrio parahaemolyticus* strains. Dark bars indicate *V. parahaemolyticus* strains containing at least one virulence factor gene, white bars indicate strains that had no virulence factor gene, and the light gray bars are the algal and *Vibrio pacinii* controls. Algae include (**A**) the unarmored coccolithophore *Emiliania huxleyi*, (**B**) the diatom *Thalassiosira pseudonana*, and (**C**) the dinoflagellate *Prorocentrum minimum*.

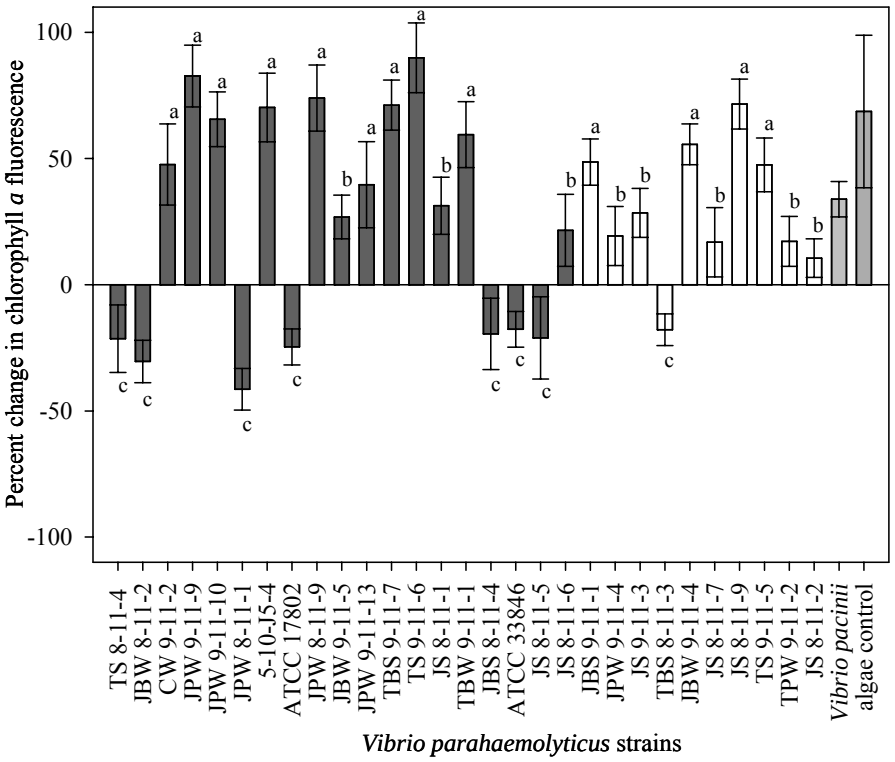

Fig. 2. Changes in the armored coccolithophore *E. huxleyi* chlorophyll *a* fluorescence during 24-h incubation with *Vibrio parahaemolyticus* strains. Dark bars indicate *V. parahaemolyticus* strains containing at least one virulence factor gene, white bars indicate strains that had no virulence factor gene, and the light gray bars are the algal and *Vibrio pacinii* controls. During incubation with the armored *E. huxleyi*, *V. parahaemolyticus* strains either, (a) had no effect on *E. huxleyi*, (b) significantly inhibited *E. huxleyi* growth or (c) significantly damaged *E. huxleyi*.

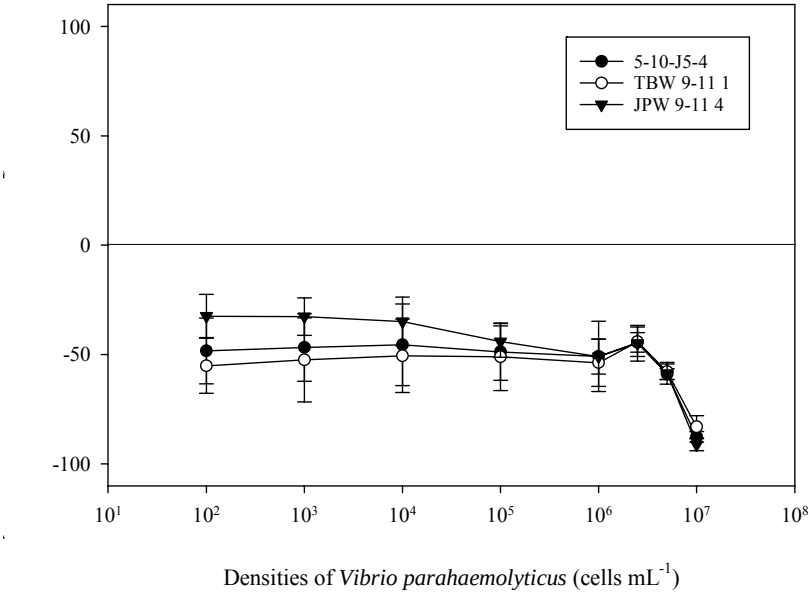

Fig. 3. Dose response curve of unarmored coccolithophore *Emiliania huxleyi* chlorophyll *a* fluorescence during 24-h incubation with *Vibrio parahaemolyticus* strains at varying concentrations.

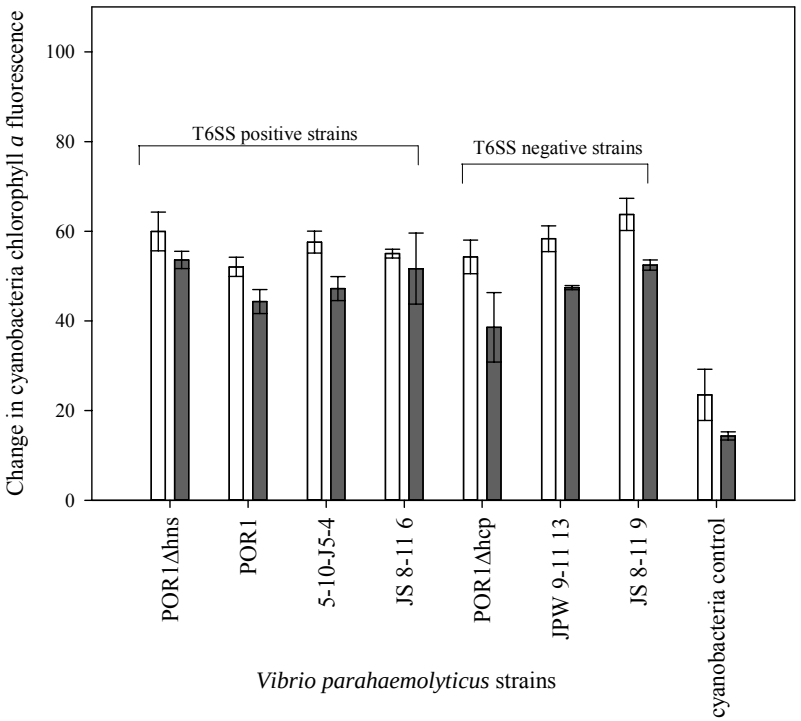

Fig. 4. Changes in cyanobacterial chlorophyll *a* fluorescence during 24-h incubation with *Vibrio parahaemolyticus* strains. White bars indicate the cyanobacterium *Prochlorococcus marinus* was used and dark bars indicate the cyanobacterium *Synechococcus bacillaris* was used. Several *V. parahaemolyticus* strains were used, some containing the antibacterial Type 6 Secretion System (T6SS) mechanism and some that did not. T6SS is de-repressed in POR1Δhns.

which constitutively expresses T6SS, also caused cyanobacterial stimulation. The T6SS may only be activated on surfaces [37], so we co-incubated cyanobacteria with *V. parahaemolyticus* strains on membrane filters incubated on agar plates. We found no evidence of cyanobacterial inhibition (S2). The cyanobacteria were the only phototrophs that were consistently stimulated by the presence of *V. parahaemolyticus*.

DISCUSSION

V. parahaemolyticus can cause varying degrees of marine microalgae biomass loss *in vitro*. The unarmored *E. huxleyi*, which was the most susceptible to *V. parahaemolyticus*, is rarely observed in the environment, yet grows well under laboratory conditions. It has been hypothesized that unarmored *E. huxleyi* rarely survives in the environment due to high susceptibility to predation [39]. Our data support this, as our environmental *V. parahaemolyticus* strains caused the greatest loss in unarmored *E. huxleyi* biomass. As microscopic observations demonstrated, intact unarmored *E. huxleyi* cells were not readily observed after incubations with *V. parahaemolyticus*. Conversely, the armored version of *E. huxleyi* was not very susceptible to *V. parahaemolyticus*; only eight of our *V. parahaemolyticus* strains reduced armored *E. huxleyi* biomass. We hypothesized that cell wall surface structures and properties may play a role in susceptibility of microalgae to *V. parahaemolyticus*. Our data confirm this; the unarmored *E. huxleyi* was consistently and severely damaged by all *V. parahaemolyticus* strains, while only a few strains had a negative effect on the armored version. The $CaCO_3$ coccoliths may protect the armored *E. huxleyi* cell and make this phenotype of *E. huxleyi* less susceptible to *V. parahaemolyticus*.

The dinoflagellate and diatom were also susceptible to *V. parahaemolyticus*. The intensity of biomass loss caused by *V. parahaemolyticus* was similar for both species, which have rigid cell wall structures (S1).

Cellulose thecal plates protect *P. minimum* and a silica frustule covers *T. pseudonana*. Although these microalgae are covered by cell walls composed of differing materials, they were similarly susceptible to *V. parahaemolyticus*. Correlations between elevated *V. parahaemolyticus* densities and dinoflagellate and diatom blooms have been reported [12, 21, 22]. This may be due to *V. parahaemolyticus* causing damage to these marine microalgae, leading to nutrient acquisition (predation) by *V. parahaemolyticus*. Certainly, release of dissolved organic carbon (DOC) from microalgae due to excretion may also contribute to the association between *V. parahaemolyticus* and algal blooms, but direct predation on algae by *V. parahaemolyticus* presents an interesting additional aspect to this association.

We found no correlation between content of *tdh*, *trh*, or *vscC2* and microalgae biomass loss. The hemolysin genes *tdh* and *trh* have long been used as molecular markers of *V. parahaemolyticus* virulence. However, our results are consistent with recent reports [44-46] that destruction of eukaryotic cells does not exclusively rely on these hemolysin genes. Content of the T3SS marker gene *vscC2* was also not correlated with algal loss. Ten of the *V. parahaemolyticus* strains used contained no known virulence factors, yet were able to cause decreases in algae chlorophyll *a* fluorescence. Our data suggest that another mechanism(s) by which *V. parahaemolyticus* damages these eukaryotes must exist.

Cyanobacteria were tested against a subset of our *V. parahaemolyticus* strains, some that contained the antibacterial mechanism T6SS, and some that did not. In addition, we used a T6SS knockout mutant and a T6SS de-repressed strain. All strains of *V. parahaemolyticus* caused cyanobacterial growth stimulation. We found no evidence that *V. parahaemolyticus* can decrease cyanobacteria biomass. Cyanobacteria grow better in non-axenic laboratory cultures (Lovell and many others, personal observations) and our finding of cyanobacterial stimulation when incubated with *V. parahaemolyticus* is consistent with this observation. Perhaps *V. parahaemolyticus* and other heterotrophic bacteria consume or neutralize some inhibitory byproducts of cyanobacterial growth. *In vitro*, the presence of *V. parahaemolyticus* results in cyanobacterial stimulation and microalgae biomass loss. In the environment, *V. parahaemolyticus* may affect phototrophic population dynamics. Further experimentation is needed to determine if *V. parahaemolyticus* can induce changes in marine phototroph populations, perhaps by selective predation upon phototrophs having more susceptible cell wall structures.

The *in vitro* experiments performed used high doses of *V. parahaemolyticus* to assure observation of damage if such occurred. We also determined what would happen when the unarmored *E. huxleyi* was incubated with varying concentrations of *V. parahaemolyticus*, including concentrations that more accurately mimic *V. parahaemolyticus* densities observed in the environment. As shown by our dose response curves, the unarmored *E. huxleyi* was susceptible to *V. parahaemolyticus* at low concentrations. These low concentrations (10^3 or 10^2 cells mL^{-1}) are similar to *V. parahaemolyticus* concentrations found in surficial sediment and shellfish [3, 6, 7, 11]. *V. parahaemolyticus* at low, "environmental" doses can still damage unarmored *E. huxleyi*, meaning that this interaction is certainly possible in specific environments.

High concentrations of marine microalgae and other phototrophs are often found in surficial sediment in salt marshes along the US east coast; in North Inlet estuary, where our *V. parahaemolyticus* strains were isolated, chlorophyll *a* in the sediment can reach as high as 101.5 mg chlorophyll *a* m^{-2} [47, 48]. *V. parahaemolyticus* concentrations in surficial sediment, particularly around fiddler crab burrows, can reach levels as high as 10^3 cells mL^{-1} [3]. Clearly, phototrophs and *V. parahaemolyticus* occur, and even bloom, in the same environments. Utilization of phototrophs as an additional nutritional resource in the sediment, as well as other areas rich in both *V. parahaemolyticus* and phototrophs, may be a mechanism supporting persistence of *V. parahaemolyticus*.

Acquisition of carbon and energy sources in estuaries having low availability of labile resources is imperative for *V. parahaemolyticus* survival and propagation. High densities of *V. parahaemolyticus* in oligotrophic environments prove that this copiotrophic human pathogen has mechanisms for gathering carbon under resource-poor conditions. We consider that marine microalgae represent a reservoir of

nutrients that *V. parahaemolyticus* can utilize. Primary producer biomass in salt marshes is dominated by cordgrasses, such as *Spartina alterniflora*; however, we do not think that *S. alterniflora*, and other macroflora, are the key to *V. parahaemolyticus* nutrient-acquisition in salt marshes. Up to 80% of vascular plant biomass is comprised of recalcitrant lignocellulose and its breakdown products [49]. Up to 40% of dissolved organic carbon in salt marshes also consists of recalcitrant lignocellulose [50]. Benthic marine phototrophs are responsible for almost half of the primary production in salt marsh ecosystems like the North Inlet estuary [47] and may be a preferred source of easily degraded carbon for heterotrophic bacteria. Salt marsh microphototroph populations primarily consist of cyanobacteria and diatoms and these organisms occur at highest biomass in the surficial sediment, where elevated *V. parahaemolyticus* densities are also found. We propose that it is no coincidence *V. parahaemolyticus* populations are correlated with marine microalgae as marine microalgae provide a nutrient-rich resource that *V. parahaemolyticus* can utilize in an otherwise nutrient-restricted system.

CONCLUSION

We performed *in vitro* assays co-incubating *V. parahaemolyticus* with various phototrophs and discovered that all *V. parahaemolyticus* strains, with or without known virulence-correlated genes, can cause significant decreases in marine microalgal biomass. There was no correlation between known *V. parahaemolyticus* virulence genes and microalgal destruction, suggesting other mechanisms of virulence. We hypothesize that marine microalgae can act as a source of nutrients for *V. parahaemolyticus* in the environment, providing an explanation for recent correlations found between increased *V. parahaemolyticus* abundance and microalgal blooms. Our study provides insight into how this copiotrophic organism is able to persist in an environment that may be poor in readily utilized organic carbon and energy sources.

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SUPPLEMENTARY MATERIAL

S1. Surface structure of microalgae and cyanobacteria

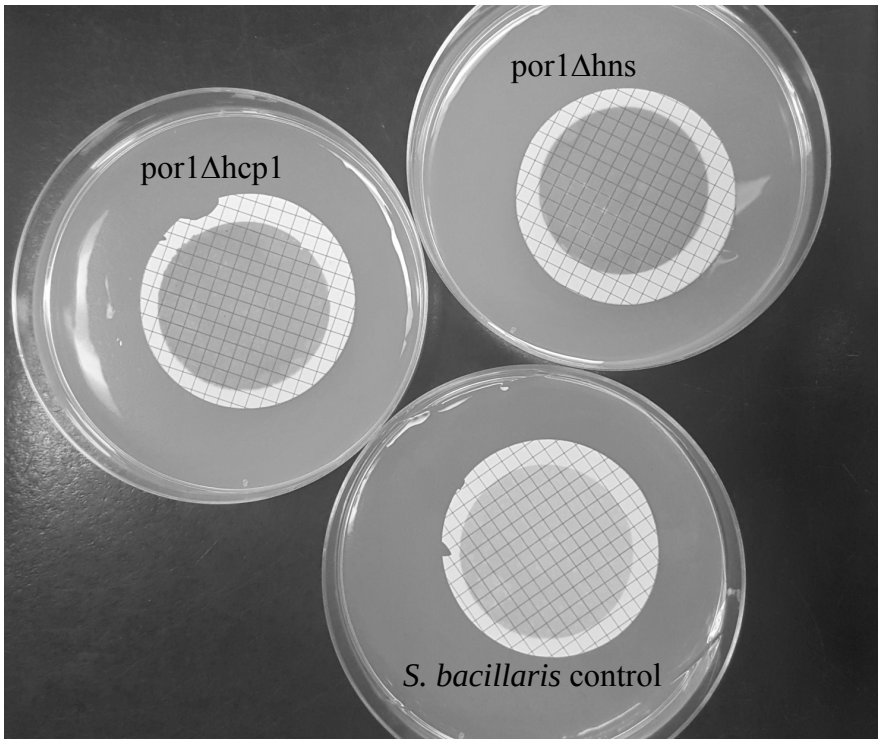
Two species of cyanobacteria were used in this study, *Prochlorococcus marinus* (CCMP 1986) and *Synechococcus bacillaris* (CCMP 1333). As is typical of gram negative prokaryotes, *P. marinus* and *S. bacillaris* have cell walls composed of peptidoglycan surrounded by an outer membrane and a glycocalyx of polysaccharides and polypeptides. Three species of eukaryotic microalgae were used, the diatom *Thallasiosira pseudonana* (CCMP 1335), the dinoflagellate *Prorocentrum minimum* (CCMP 695), and two strains of the coccolithophore, *Emiliania huxleyi* (CCMP 371 and CCMP 373). *E. huxleyi* CCMP 371 is a coccolith-producing (armored) form that often causes extensive blooms. *E. huxleyi* CCMP 373 is an unarmored mutant phenotype.

The bloom producing armored form can have as many as thirty $CaCO_3$ coccoliths forming multiple layers to protect the cell interior. The unarmored *E. huxleyi* lacks coccoliths and these strains are rarely isolated from the environment. Unarmored cells are thought to arise from armored cells *via* mutation; reversion back to the coccolith-forming morphology has not been reported [39].

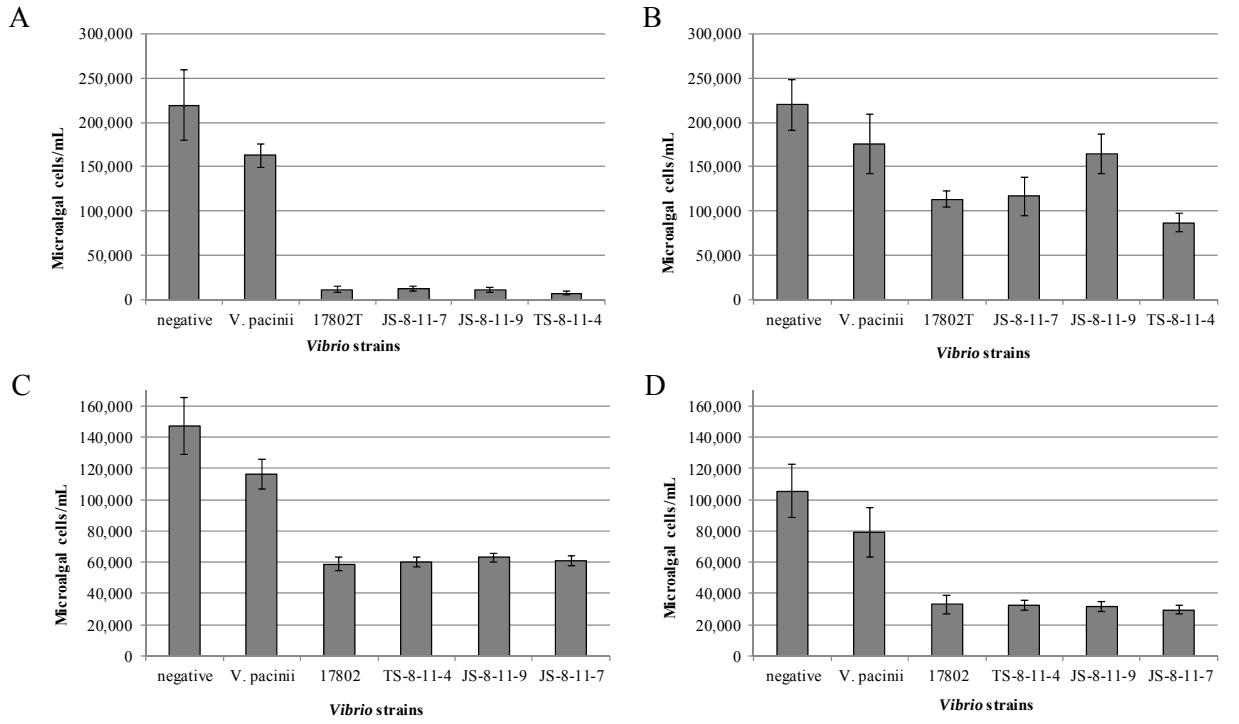
The centric diatom *T. pseudonana* is a model organism and was the first diatom chosen for genome sequencing [40]. As is the case for all diatoms, the cell wall, or frustule of *T. pseudonana*, is composed of amorphous hydrated silica in a species-specific three-dimensional structure [42]. The “petri dish” shape of centric diatoms is due to two unequal silicate halves (valves) that are connected by a series

of girdles. Additionally, to prevent silica dissolution, the frustule is covered by an organic casing made up of glycoproteins [42]. The dinoflagellate *P. minimum* is associated with harmful algal blooms (HABs) [41]. *P. minimum* cells are protected by overlapping thecal plates composed of cellulose.

Phototroph cultures were obtained from the Bigelow National Center for Marine Algae and Microbiota (Bigelow Center, East Boothbay, Maine). Each algal species required its own growth medium. Unarmored *E. huxleyi,* the dinoflagellate *P. minimum* and the cyanobacteria *S. bacillaris* were grown in F/2-Si (8.82×10^{-4} M $NaNO_3$, 3.62×10^{-5} M $NaH_2PO_4 \cdot H_2O$) supplemented with F/2 trace metals (1.17×10^{-5} M $Na_2EDTA \cdot 2H_2O$, 1.17×10^{-5} M $FeCl_3 \cdot 6H_2O$, 7.65×10^{-8} M $ZnSO_4 \cdot 7H_2O$, 4.2×10^{-8} M $CoCl_2 \cdot 6H_2O$, 9.1×10^{-7} M $MnCl_2 \cdot 4H_2O$, 2.6×10^{-8} M $Na_2MoO_4 \cdot 2H_2O$, 3.93×10^{-8} M $CuSO_4 \cdot 5H_2O$) and with F/2 vitamins (2.96×10^{-7} M thiamine·HCl, 2.05×10^{-9} M biotin, 3.69×10^{-10} M cyanocobalamin). Armored *E. huxleyi* was grown in F/50, which is a 1/25 dilution of F/2-Si. *T. pseudonana* was grown in F/2+Si (add 1.06×10^{-4} M $Na_2SiO_3 \cdot 9H_2O$ to F/2-Si recipe). The cyanobacterium *P. marinus* was grown in Pro99 medium (5×10^{-5} M $NaH_2PO_4 \cdot H_2O$, 8×10^{-4} M NH_4Cl) supplemented with pro99 trace metals (1.17×10^{-6} M $Na_2EDTA \cdot 2H_2O$, 1.17×10^{-6} M $FeCl_3 \cdot 6H_2O$, 8×10^{-9} M $ZnSO_4 \cdot 7H_2O$, 5×10^{-9} M $CoCl_2 \cdot 6H_2O$, 9×10^{-8} M $MnCl_2 \cdot 4H_2O$, 3×10^{-9} M $Na_2MoO_4 \cdot 2H_2O$, 1×10^{-8} M Na_2SeO_3, 1×10^{-8} M $NiSO_4 \cdot 6H_2O$). All media recipes can be found on the Bigelow Center website (https://ncma.bigelow.org/algal-recipes). Microalgae and cyanobacteria were grown at 23 °C with an 11 h light, 13 h dark cycle.



S2. Agar plate experiment Materials and Methods.
Cyanobacterial (*S. bacillaris*) cultures were grown for 5 days in F/2-Si medium (Bigelow) at 23 °C in an environmental chamber, with an 11h light, 13h dark cycle. *V. parahaemolyticus* strains were grown in saline Luria-Bertani Broth (SLB per L, 10 g tryptone, 5 g yeast extract, 27g NaCl) for 5 h at 37 °C with shaking. *V. parahaemolyticus* cultures were spun down and resuspended in a mixture of artificial sea salts (33 ppt). *V. parahaemolyticus* strains and cyanobacteria were combined (MOI of 100:1) and filtered onto a sterile 0.45 μm polycarbonate filter. Filters were then aseptically placed on solid F/2-Si (Bigelow recipe, add 1.5% agarose) and were left to incubate at 23 °C, with an 11h light, 13h dark cycle for 5 days. Pictures were taken after the 5-day incubation.



S3. Microalgal cell densities after a 24-h incubation with *Vibrio parahaemolyticus* strains (A-D).
Algae include (**A**) the unarmored coccolithophore *Emiliania huxleyi,* (**B**) the armored coccolithophore *Emiliania huxleyi*, (**C**) the diatom *Thalassiosira pseudonana*, and (**D**) the dinoflagellate *Prorocentrum minimum*.

CONFLICT OF INTEREST STATEMENT

None to declare.

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