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Potential effects of bacterial communities on the formation of blooms of the harmful dinoflagellate *Prorocentrum* after the 2014 Texas City "Y" oil spill (USA)

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ABSTRACT

The association between phytoplankton blooms and oil spills is still controversial despite numerous studies. Surprisingly, to date, there have been no studies on the effect of bacterial communities (BCs) exposed to crude oil on phytoplankton growth, even though crude oil changes BCs, which can then affect phytoplankton growth and species composition. Co-culture with crude oil-exposed BCs significantly stimulated the growth of *Prorocentrum texanum* in the laboratory. To gain more direct evidence, oil-degrading bacteria from oil-contaminated sediment collected after the Texas City "Y" oil spill were isolated, and changes in dinoflagellate growth when co-cultured with single bacterial isolates was investigated. The oil-degrading bacterial isolates significantly stimulated the growth of dinoflagellates (axenic and xenic cultures) through releasing growth-promoting substances. This study provides new evidence for the potential role of oil-degrading bacteria in the formation of phytoplankton blooms after an oil spill.

1. Introduction

Crude oil pollution and harmful algal blooms (HABs) are both growing environmental threats to marine ecosystems (Anderson et al., 2012; Hu et al., 2011). These devastating events may be associated, as HABs have been observed after oil spills in the sea (Özhan et al., 2014b; Almeda et al., 2018; Gemmell et al., 2018). In a recent example, a dense *Prorocentrum* dinoflagellate bloom occurred after the Texas City "Y" oil spill which occurred on March 22, 2014 as a result of a collision between a barge and another ship in Galveston Bay, Texas, resulting in a release of 168,000 gallons of marine fuel oil (Yin et al., 2015; Gemmell et al., 2018; Williams et al., 2017). However, the association between phytoplankton blooms and oil spills remains controversial due to conflicting results of numerous studies. Some studies demonstrated that oil enhanced phytoplankton growth (Parsons et al., 1976; Linden et al., 1979; Vargo et al., 1982; Özhan et al., 2014a), whereas others found that oil inhibited photosynthesis (Nuzzi, 1973; Miller et al., 1978). In more recent studies auto/mixotrophic dinoflagellates, which are common HAB species, were not only more tolerant of oil than other competing phytoplankton taxa (e.g., diatoms) and grazers (e.g., ciliates), but also their growth was stimulated under oil exposure (≤1200 parts per billion) (Özhan et al., 2014a; Almeda et al., 2018). In addition, some heterotrophic dinoflagellates can ingest crude oil (Almeda et al., 2014). Taken together, oil spills and phytoplankton (particularly dinoflagellates) blooms can be closely associated, but there is a significant knowledge gap regarding the mechanisms that link oil spills and phytoplankton blooms.

Bacteria are a major factor contributing to growth promotion and inhibition of phytoplankton, and there is growing evidence for changes in phytoplankton growth due to modification of associated bacterial communities (BCs) (Buchan et al., 2014 and references therein). Recently, BCs have been recognized to play a role in the formation of HABs in nature (Park et al., 2015; Park et al., 2016, 2017, 2018; Bolch et al., 2017). Although it is well known that oil spills strongly affect both bacterial abundance and community composition in marine environments (Meng et al., 2016; Bacosa et al., 2015, 2016; Gemmell et al., 2018), there is no study on the impact of oil-driven changes in BCs on the formation of blooms. Thus, this study aimed to explore the role of bacteria in the formation of HABs after oil spills. As a first step of this study, the effects of oil-degrading bacteria on the formation of a *Prorocentrum* bloom after the Texas City "Y" oil spill were examined. Gemmell et al. (2018) suggested that the loss of grazers after this oil spill might have contributed to the formation of the bloom. To better understand the factors causing this bloom, the impact of changes

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in BCs after crude oil exposure on *Prorocentrum* growth using laboratory cultures was investigated. In addition, to gain more direct evidence, oil-degrading bacteria were directly isolated from an oil-contaminated site 6 days after the Texas City "Y" oil spill, and their effects on growth of dinoflagellates (including *Prorocentrum*) growth were investigated.

2. Material and methods

2.1. Algal cultures

The axenic (*Amphidinium carterae*, UTEX LB 1561; *Peridinium sociale*, UTEX LB 1948) and xenic (*Karenia brevis*, SP3TOX; *P. gracile*, PATX-3; *P. minimum*, PATX-1; *P. texanum*, CCMP3349) algal cultures were obtained from the UTEX algal culture collection of the University of Texas at Austin, the National Center for Marine Algae and Microbiota (NCMA) culture collection, the University of Texas Marine Science Institute (the Erdner laboratory and the Villareal laboratory), and Dr. Darren W. Henrichs (Texas A&M, College station, TX, USA). The cultures were incubated at 20 °C in F/2 or L1 media (Guillard, 1975; Guillard and Hargraves, 1993) with a salinity of 32 psu under cool-white fluorescent lamps (photon flux of 60 μE m^{-2} s^{-1}) on a 12-h light:12-h dark photoperiod.

2.2. Isolation and phylogenetic identification of oil-degrading bacteria from field samples

Sediment samples (water depth 2–3 m) were collected from two sites (C1 and E1, near the site of the Texas City "Y" oil spill) in Galveston Bay, Texas on March 28, 2014 (6 days after the Texas City "Y" oil spill) (Fig. 1). To isolate pure cultures of oil-degrading bacteria from these samples, the protocol of Latha and Kalaivani (2012) was used with modification. Five grams (wet weight) of each soil sample were inoculated in R2B broth (Bio-world, OH, USA) and incubated at 37 °C for 2 days. After incubation, 100 μL of broth culture was plated onto 2% Bushnell-Haas agar (Sigma-Aldrich, MO, USA) media using the spread plate technique. A solution of crude oil (10% w/v) in ether was uniformly sprayed over the surface of a Bushnell-Haas agar plate. The ether immediately vaporized and a thin layer of oil remained on the entire surface. The plates were incubated at room temperature (ca. 20 °C) for 2 days. The colonies that formed clear zones around them were considered as crude oil degraders. Each colony was transferred into 1.5% LB agar (BD Difco, NJ, USA) media to establish a bacterial culture. A total of seven bacterial strains were established.

To identify the bacterial isolates based on the 16S ribosomal RNA (rRNA) gene sequence, genomic DNA was extracted using the DNeasy blood and tissue kit following the manufacture's protocol (Qiagen, Hilden, Germany). PCR was carried out with template DNA (5 μL), 0.5 μM of forward/reverse primers (27F/1492R, Suzuki and Giovannon, 1996), and 2X PCR premix (Promega, WI, USA), which is composed of dNTP mixture (0.2 mM of each dNTP) and 0.05 units *Taq* polymerase. Using an Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany), thermocycling was conducted as follows; 95 °C for 5 min, followed by 38 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. After denaturation, the final extension was completed at 72 °C for 10 min. The size of PCR amplicons was confirmed using 1.0% agarose gel electrophoresis by standard methods (Sambrook and Russell, 2001). Then, PCR amplicons were sent to the Genomics core laboratory of Texas A&M University at Corpus Christi for sequence analysis. After obtaining partial 16S rRNA sequences (481 base pairs, the V1–V3 regions), phylogenetic positions of bacteria cultures were analyzed via the use of MEGA-X software (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and the General Time Reversible model (Nei and Kumar, 2000). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5618)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 25.10% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Gene sequences have been deposited in the NCBI GenBank with accession numbers MN173411-MN173417.

2.3. Effect of oil-degrading bacterial isolate on the growth of dinoflagellates

2.3.1. Identification of growth-promoting bacterial isolates

A single colony of each bacterial isolate was transferred into a10 mL plastic tube containing 5 mL of Marine Broth (BD Difco, NJ, USA) media and placed on a shaking incubator at 37 °C, 120 rpm for 48 h. Then, 1.5 mL of cultures were harvested by centrifugation (3200 x *g* 30 min), pellets were washed twice with fresh algal media (F/2), and these pellets were inoculated into 75 mL plastic tissue culture flasks (Corning, NY, USA) containing 50 mL of log growth phase axenic dinoflagellate cultures (*A. carterae* and *Pe. sociale*) with a final bacterial concentration of ca. 10^6 cells mL^{-1}. Subsamples were taken from duplicate flasks at 6-7 day intervals prior to the onset of the declining growth phase of *A. carterae* and *Pe. sociale*. To measure the cell density of these

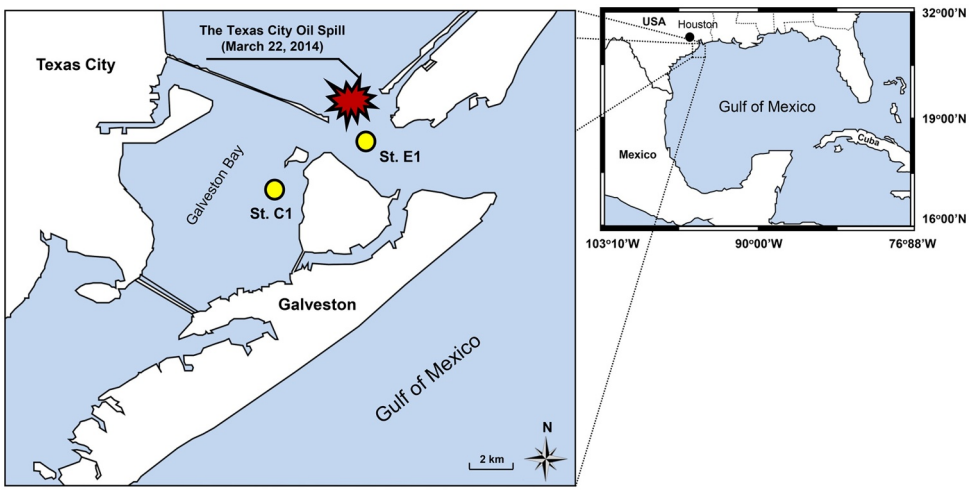

Fig. 1. Map of sampling sites in Galveston Bay. Sampling site coordinates: C1, 29.325°N 94.832°W and E1, 29.348°N 94.788°W.

dinoflagellates, samples were fixed with Lugol's solution at a final concentration of 1% and stored at 4 °C until analysis. Lugol's preserved samples were transferred into Sedgewick-Rafter chambers, and dinoflagellate cells were counted at 100 × magnification using a light microscope (Olympus, Tokyo, Japan). The growth effect was calculated using the following equation: growth effect (%) = ($A_{TREATMENT}$- $A_{CONTROL}$)/$A_{CONTROL}$ x 100, where $A_{CONTROL}$ and $A_{TREATMENT}$ are the number of algal cells in control and treatment, respectively. In these preliminary tests, the values were calculated based on cell numbers of each dinoflagellate in stationary growth phase and compared maximum densities between control and bacterial treatments in order to evaluate variation in final growth yield (Table S1). For further analysis, we selected bacterial isolates that showed high final growth yield of dinoflagellates in stationary growth phase; two bacterial isolates (C1-T3 and E1-Gal-T2) were chosen.

2.3.2. Changes in algal growth in co-culture with bacterial isolates

The two chosen bacterial isolates were inoculated into xenic cultures of four different dinoflagellates at a final concentration of ca. 10^6 cells mL^{-1}. Subsamples were taken from duplicate flasks at 6-7 day intervals prior to the onset of the declining growth phase of each dinoflagellate culture, and growth effect of each bacterial isolate on those dinoflagellates in stationary growth phase was calculated using the above equation.

To better understand the effect of the bacterial isolates on dinoflagellate growth, the two bacterial isolates were inoculated into two axenic dinoflagellate cultures at three different bacterial densities. For this experiment, incubation and harvesting of bacterial cells was performed under the same conditions as mentioned above, and then they were inoculated into cultures of *A. carterae* or *Pe. sociale* in log-growth stage at final bacterial densities of 10^5, 10^6, and 10^7 cells mL^{-1}. For enumeration of changes in dinoflagellate and bacterial densities, subsamples were taken from duplicate flasks at day 0 (after bacterial inoculation), 1, 3, 5, 7, 10, 13, 16, and 20. In this experiment, we investigated variation in growth of dinoflagellates depending on bacterial treatment through calculation of growth effect (%) in treatments and comparison of the overall growth rate during the period of exponential growth phase. All subsamples were fixed with 1% Lugol's solution (for algal counts) and 2% glutaraldehyde (for bacterial counts), and stored at 4 °C until analysis. Growth effect in bacterial treatments was calculated by the same method as above, and bacterial cells were enumerated using an Accuri C6 flow cytometer and BD CFlow Plus Software (BD Biosciences, CA, USA) after staining with SYBR Green II (Lonza, NJ, USA) as previously described in Liu et al. (2013).

2.4. Growth promoting mechanism of the two oil-degrading bacterial isolates

2.4.1. Verification of dissolved growth promoting substance

To determine whether or not excreted substances from the two bacterial isolates (C1-T3 and E1-Gal-T2) are capable of enhancing the growth of dinoflagellates, 10 mL of bacterial culture were harvested by centrifugation at 1400 g for 30 m after incubation in Marine broth 2216 (Difco) at 37 °C for 48 h and washed three times with 5 mL of fresh F/2 media. Then, these bacterial cells were inoculated into 50 mL of fresh F/2 media and incubated for 7 days at the same condition as the algal cultures: 20 °C under cool-white fluorescent lamps (photon flux of 60 μE m^{-2} s^{-1}) on a 12-h light:12-h dark photoperiod. The bacteria were filtered from the conditioned medium using a 0.2 μm pore-size syringe filter, and then the filtrates were inoculated to axenic *A. carterae* and *Pe. sociale* cultures with 10% (v/v) concentration. Additionally, to set-up the control, the same cell number of *A. carterae* and *Pe. sociale* were inoculated into same volume of fresh F/2 media. Lastly, subsamples were taken at 7 day intervals (day 0, 7, 14, 21, and 28), fixed with Lugol's solution at a final concentration of 1%; cell numbers of the two dinoflagellates were measured as above.

2.4.2. Effect of bacterial isolates on dinoflagellate growth under nutrient-limited conditions

To confirm if the dissolved growth promoting substances from these bacterial isolates might be nutrients, nutrient-limited media (nitrogen, phosphorus, trace metal, and vitamins) were used in this experiment. Each limited medium was made of 100-times lower concentration of the respective nutrient, compared to F/2 medium. Then, incubation and harvesting of bacterial isolates were conducted as mentioned above. However, those isolates were washed with the nutrient-limited media, and inoculated into the respective nutrient-limited media, containing *A. carterae* and *Pe. sociale* at a final bacterial concentration of about 10^6 cell mL^{-1}. Subsamples were taken from duplicate flasks prior to the onset of the declining growth phase of each dinoflagellate at 7 day intervals (sample of day 21 from *A. carterae* was omitted), and the growth changes in dinoflagellates following addition of bacteria was measured using microscopic cell counts as described above.

2.5. Effects of bacterial communities exposed to crude oil on the growth of Prorocentrum texanum

To elucidate whether the BCs altered by crude oil exposure could assist the formation of the *Prorocentrum* bloom after the Texas City "Y" oil spill in 2014, we exposed free-living (FL) bacteria in *P. texanum* culture to crude oil and investigated the changes in this dinoflagellate's growth in response to co-culture with oil-exposed bacteria. All experiments were carried out in triplicate.

2.5.1. Changes in bacterial community composition by exposure to crude oil

The FL bacteria were obtained from *P. texanum* cultures in stationary growth phase, by removal of dinoflagellate cells using a 3.0 μm pore-size membrane filter (Isopore, Germany), and amended with the water accommodated fraction (WAF) of 100 ppm concentration of Louisiana Light Sweet (LLS) crude oil. The WAF was prepared following the protocol of Singer et al. (2000) with modifications. To minimize bacterial contamination from crude oil, the LLS crude oil was filtered with a 0.22 μm pore-size syringe membrane filter (Isopore, Germany) before addition to L1 media for WAF preparation. After addition of the WAF into FL bacteria, they were incubated at 20 °C for 38 days under cool-white fluorescent lamps (photon flux of 60 μE m^{-2} s^{-1}) on a 16-h light:8-h dark photoperiod.

To characterize changes in bacterial community structure in response to crude oil exposure, FL bacteria before and after treatment were harvested by centrifugation (3200 x *g*, 30 min); (*i*) samples for before oil treatment were obtained from FL bacteria right after removal of *P. texanum*, (*ii*) samples for after oil treatment were obtained from WAF-exposed bacteria right before bacterial inoculation into culture of *P. texanum*. DNA was extracted using DNeasy Blood and Tissue kit following the manufacture's protocol (Qiagen, Hilden, Germany), and DNA samples were submitted to the Research and Testing Laboratory Genomics (RTL Genomics, Lubbock, TX, USA) for Illumina MiSeq sequencing of the bacterial 16S rRNA genes. For this study, it was chosen to amplify the V1–V2 region using the 28f (5′-GAG TTT GAT CNT GGC TCA G-3′) (Handl et al., 2011) and the 388r (5′-TGC TGC CTC CCG TAG GAG T-3′) (Francés et al., 2004) primers based on available assays at RTL Genomics, and on their advice based on their annotated internal database to classify sequences. Briefly, amplifications were performed in 25 μL reactions with Qiagen HotStar Taq master mix (Qiagen Inc., Valencia, CA, USA), 1 μL of each 5 μM primer and 1 μL of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA, USA) using the following thermal profile: 95 °C for 5 min, then 25 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and a 4 °C hold. A second PCR was also performed and the amplification products visualized with eGels (Life Technologies, Grand Island, New York). Generated sequences were processed and quality checked by the RTL Genomics data analysis pipeline. The sequence reads were then sorted by

length from the longest to the shortest and clustered into operational taxonomic units (OTUs) at a 3% divergence using the USEARCH clustering algorithms (Edgar, 2010) to prefix dereplication. OTU selection was performed using the UPARSE OTU selection algorithm (clustering method in USEARCH) (Edgar, 2013) to classify the large number of clusters into OTUs, and chimera checking was performed using the UCHIME chimera detection software (Edgar et al., 2011). Taxonomic identifications were made by comparing the OTU sequences against a database of high-quality sequences derived from the NCBI database using the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007). The term 'unknown' was assigned when the algorithm was not able to make a confident determination of the taxonomic classification at a certain level (number of matching taxonomic levels/number of total taxonomic levels >51%). The data were analyzed by RTL Genomics using R software (3.0.1). Generation of a rarefaction curve plot of the number of OTUs vs the number of sequences was performed. All sequences have been deposited in the NCBI GenBank with accession number PRJNA554405.

2.5.2. Change in Prorocentrum texanum growth during co-culture with bacterial communities exposed to crude oil

A 25 mL subsample of WAF-exposed bacterial community was centrifuged (3200 x *g*, 30 min) and washed two times with fresh L1 media to remove crude oil. Harvested bacteria were then inoculated into 75 mL plastic tissue culture flasks (Corning, NY, USA) containing 50 mL of log phase *P. texanum* culture, and the growth change in *P. texanum* after addition of these FL bacteria was investigated. To enumerate algal and bacterial cells, subsamples were taken at day 0 (after bacterial inoculation), 1, 3, 5, 7, 9, 11, 15, and 18, and preserved with Lugol's solution or glutaraldehyde at a final concentration of 1% and 2%, respectively. Enumeration of algal- and bacterial cells and calculation of growth effect were conducted same as mentioned above.

In addition, to set up a positive control, the same volume (25 mL) of *P. texanum* culture under stationary growth phase was filtered through a 3.0 μm pore-size membrane filter, and these filtrates were centrifuged (3200 x *g*, 30 min) for bacterial harvest. Then, the harvested bacterial cells were washed with fresh L1 media twice, and transferred to 75 mL plastic tissue culture flasks (Corning, NY, USA) containing 50 mL of log phase *P. texanum* culture. Enumeration of dinoflagellate and bacterial cells were carried out as described above.

2.6. Statistical analyses

All statistical analyses were performed using SPSS software ver. 21 (IBM Inc., IL, USA). The Student's *t*-test were used for testing differences in cell number of dinoflagellates between control and treatment. A two-way analysis of variance (ANOVA) test was conducted to determine statistically significant differences in dinoflagellates densities among control and each bacterial treatment (different inoculation densities of the two bacterial isolates); the two independent variables were set as bacterial inoculation density (control and each treatment) and sampling day. All pairwise comparisons using post hoc tests (Duncan and Scheffé) were carried out to confirm the absence of significant differences between all possible pairs of averages.

3. Results

3.1. Co-culture of dinoflagellates and oil-degrading bacteria

3.1.1. Xenic cultures of dinoflagellates

Final growth yield of dinoflagellates was higher after co-culture with the two oil-degrading isolates (C1-T3 and E1-Gal-T2) (Table 1). The maximum dinoflagellate cell densities in bacterial treatments were significantly higher (Student's *t*-test, $p < 0.05$), compared to controls for xenic *K. brevis* (43.8% − 50.7% increase), *P. gracile* (33.1% − 49.6%), *P. minimum* (30.6% − 46.3%), and *P. texanum* (37.9% − 38.9%) cultures.

Table 1
Variation in final growth yield of four xenic dinoflagellate cultures due to co-culture with two bacterial isolates (C1-T3 and E1-Gal-T2). The values were the averages of growth effects of each bacterial treatment in stationary growth phase and standard deviations (*K. brevis*, $n = 12$; *P. gracile*, $n = 6$; *P. minimum*, $n = 8$; *P. texanum*, $n = 6$). The asterisks represent the statistical significance of the difference (Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$) in dinoflagellate cell abundance between control and bacterial treatments.

Species	Strain No.	Growth effect (%)	
		C1-T3	E1-Gal-T2
Karenia brevis	SP3TOX	50.7 ± 39.4	43.8 ± 38.7
Prorocentrum gracile	PATX-3	33.1 ± 19.3	49.6 ± 18.2
Prorocentrum minimum	PATX-1	46.3 ± 10.9	30.6 ± 19.9
Prorocentrum texanum	CCMP3349	37.9 ± 8.5	38.9 ± 14.6

3.1.2. Axenic cultures of dinoflagellates

To better understand the impact of oil-degrading bacterial isolates on dinoflagellate growth, two axenic (bacteria-free) strains of dinoflagellates were inoculated with the two bacterial isolates, each at three different bacterial densities (from 10^5 to 10^7 cells mL^{-1}). Additionally, during the period of this experiment, axenic culture conditions in control were consistently checked through measuring bacterial cells by a flow cytometer. A growth promoting effect on *A. carterae* was shown from exponential growth phase (day 3 or 5) until the death phase (day 10) in both bacterial treatments (Fig. 2a and c); the overall growth rate during the period of the exponential growth phase in control were 0.16 ± 0.01 (mean ± standard deviation, $n = 2$), whereas the range of this growth rate in treatments was 0.19–0.26. In addition, the level of growth enhancement was significantly different depending on inoculation densities of each bacterial isolate (two-way ANOVA, $n = 12$, $p < 0.05$, F: 6.949–12.220, DF: 3). In *Pe. sociale* culture, the growth enhancement in E1-Gal-T2 treatment was evident from day 1 and was consistent across the growth curve (Fig. 2b and d); the overall growth rate during the period of the exponential growth phase in control were 0.07 ± 0.00, whereas it was ranged from 0.08 to 0.17 in treatments. Whereas, growth enhancement of *Pe. sociale* in C1-T3 treatment was variable even though a clear growth promoting effect was observed in the highest bacterial inoculation treatment (~10^7 cells mL^{-1}). The level of growth enhancement of *Pe. sociale* was significantly different in accordance with inoculation densities of the respective bacterial isolate (two-way ANOVA, $n = 20$, $p < 0.05$, F: 4.406–20.246, DF: 3).

3.2. Potential mechanism of bacterial isolates to enhance dinoflagellate growth

To determine whether the oil-degrading bacterial isolates can release a growth promoting substance, algal media (F/2) containing exudates from each bacterial isolate was added to axenic algal cultures. The growth of both *A. carterae* and *Pe. sociale* were clearly stimulated in these treatments (Fig. 3); the overall growth rates during the period of the exponential growth phase in control were 0.21 ± 0.01 (*A. carterae*, mean ± standard deviation, $n = 2$) and 0.12 ± 0.01 (*Pe. sociale*), whereas these growth rates in treatments were 0.24 - 0.25 (*A. carterae*) and 0.14 - 0.15 (*Pe. sociale*). This growth stimulation was initiated from the exponential phase (day 7) and was consistent through the death phase. The maximum densities of both dinoflagellates were also higher in bacterial treatments, compared to in control.

In addition, to determine whether the released growth promoting substances may be providing nutrients, the respective nutrient-limited (nitrogen, phosphorus, trace metal, and vitamins) media were used. In co-culture with C1-T3 and E1-Gal-T2, growth of the two dinoflagellates was clearly enhanced in every nutrient limited condition, and the maximum cell densities in these bacterial treatments were similar to those in normal F/2 media (Fig. 4).

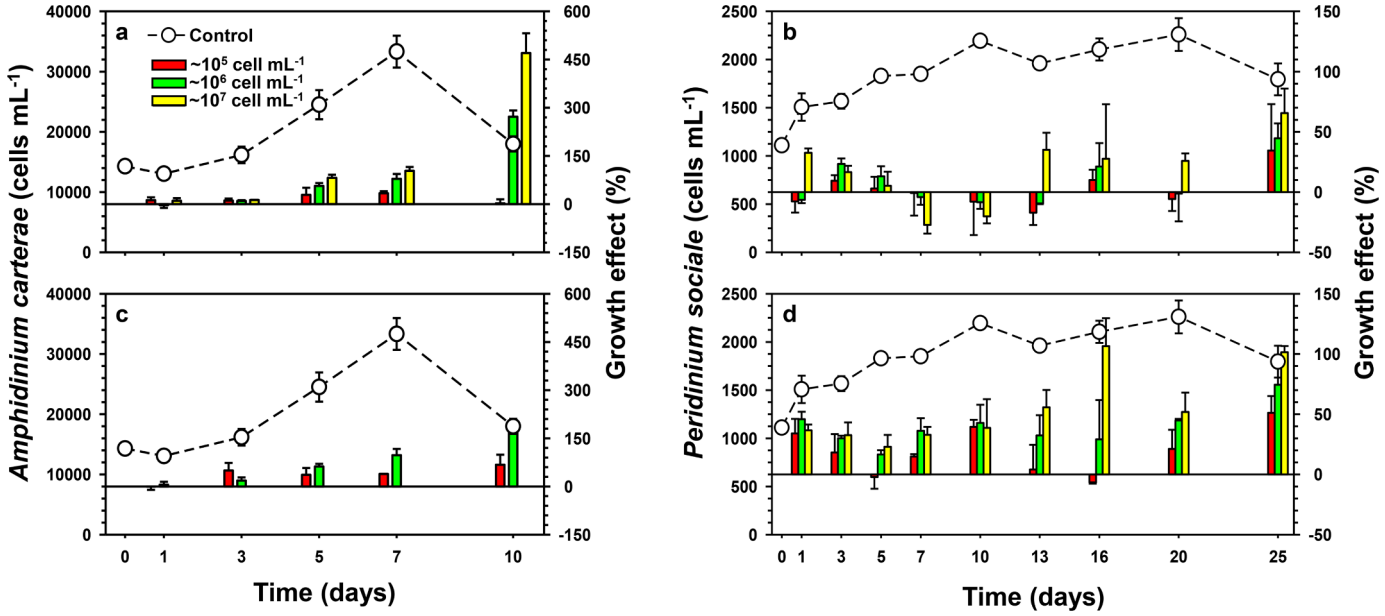

Fig. 2. Change in the growth of axenic *Amphidinium carterae* (a and c) and *Peridinium sociale* (b and d) after co-culturing with three different inoculation density of the two bacterial isolates, C1-T3 (a and b) and E1-Gal-T2 (c and d). Line plot indicate abundance of each dinoflagellate in control (non-treatment), and bar charts show growth effect on each dinoflagellate in treatments to control, respectively. Error bars indicate the standard deviation of mean value (n = 2).

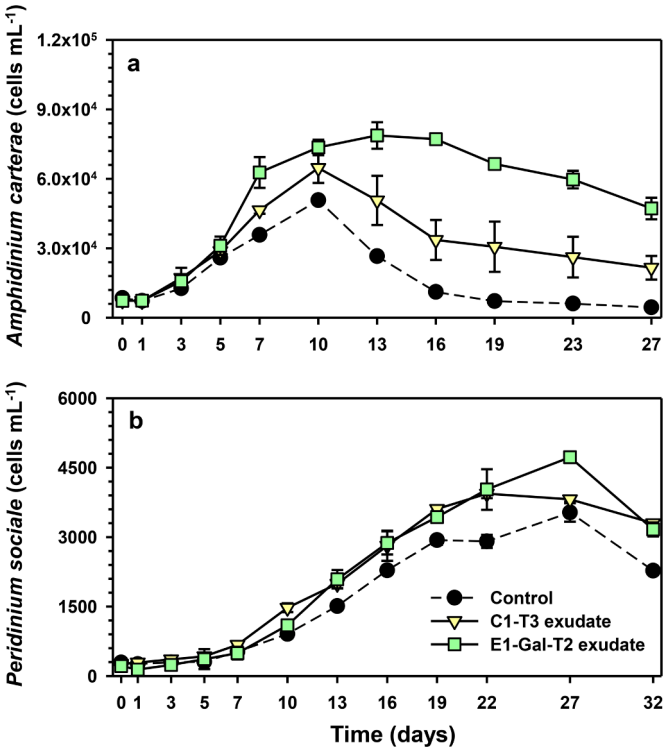

Fig. 3. Growth of *Amphidinium carterae* (a) and *Peridinium sociale* (b) in normal (control) and bacteria-conditioned (treatments) F/2 media. Error bars indicate the standard deviation of mean value (n = 2).

3.3. Effect of oil-exposed bacterial communities on the growth of *Prorocentrum texanum*

3.3.1. Change in bacterial communities according to crude oil exposure

Bacterial community structures were clearly different before and after crude oil treatments (Fig. 5). Before crude oil exposure, the classes Actinobacteria (5.1 ± 1.1%), Alphaproteobacteria (77.1 ± 0.6%) were dominant, and the genus *Roseobacter* (66.3 ± 3.0%) was predominant within the Alphaproteobacteria. After crude oil exposure, there were clear changes in bacteria community composition; Alphaproteobacteria (56.3 ± 0.6%) decreased, whereas the relative abundance of Beta- (12.4 ± 0.4%) and Gamma-proteobacteria (3.4 ± 0.2%) increased, becoming dominant. At the genus level, genera *Seohaeicola* (40.4 ± 0.6%) and *Limnobacter* (12.4 ± 0.4%) were greatly increased and became dominant taxa after the crude oil treatment. Interestingly, the genus *Roseobacter* was the dominant bacterial taxa both before (66.3 ± 3.0%) and after (10.4 ± 1.1%) crude oil exposure, but its proportion was decreased with increasing crude oil exposure.

3.3.2. Growth of Prorocentrum texanum in co-culture with oil-exposed bacterial communities

Growth of *P. texanum* were clearly stimulated when cultured with the oil-exposed BCs (Fig. 6a and c); the overall growth rates during the period of the exponential growth phase in control and treatment were 0.2 ± 0.01 (mean ± standard deviation, n = 3) and 0.23 ± 0.01, respectively. A significant (Student's t-test, $p < 0.05$) growth enhancement of *P. texanum* was observed from day 5, and it was consistent through the end of experiment (day 18, late stationary growth phase). Growth promoting effect on *P. texanum* in bacterial treatment ranged from 10.7 to 34.8% (Fig. 6c), and the highest value was observed at day 11 (the late exponential growth phase). However, inoculation of the resident BCs (without crude oil exposure; positive control) did not significantly affect the growth of *P. texanum* (Fig. 6b and d). Unlike previous experiments, there was no significant ($p > 0.05$) growth effect of bacterial inoculation on *P. texanum*, even though the cell density of this dinoflagellate in the positive control was slightly higher than the control during exponential growth phase (day 1, 5, and 7). Rather, growth of *P. texanum* was generally inhibited (from −30 to −2.72%) during the late exponential growth phase.

The initial bacterial densities in the treatments and positive controls were relatively higher (2.03–4.96 times) than the control due to bacterial inoculation (Fig. 6e and f). Both sources of bacteria showed similar growth patterns; bacterial cells reached the maximum density at day 3, and remained constant until the end of experiment.

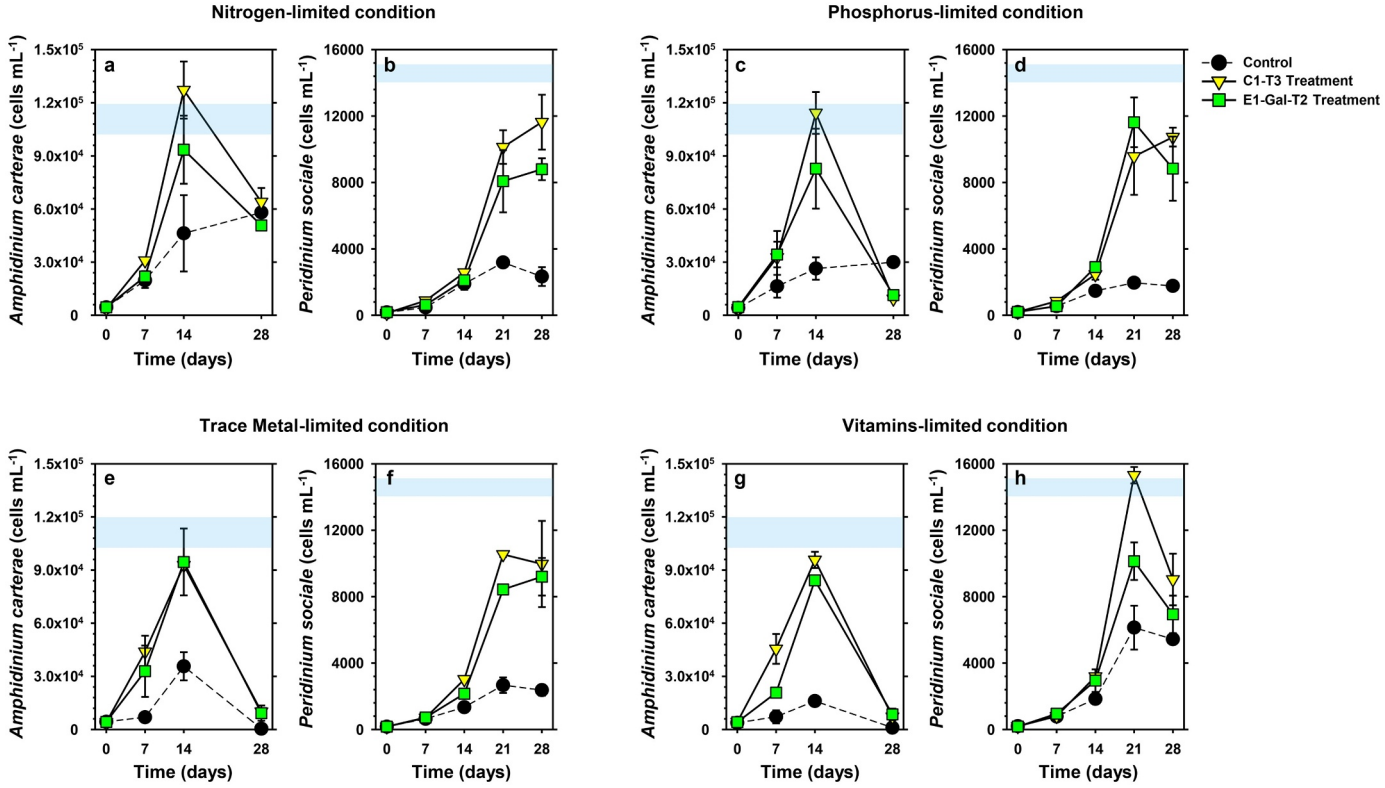

Fig. 4. Change in growth of *Amphidinium carterae* (a, c, e, and g) and *Peridinium sociale* (b, d, f, and h) in the respective nutrient-limited media after co-culturing with the two bacterial isolates (C1-T3 and E1-Gal-T2). This experiment was conducted under nitrogen- (a and b), phosphorus- (c and d), trace metal- (e and f), and vitamins- (g and h) limited conditions. Blue box indicates the maximum cell abundance of each dinoflagellate under normal F/2 media. Error bars indicate the standard deviation of mean value (n = 2).

4. Discussion

The association between oil spills and the formation of HABs has been observed, but there is lack of explanation on whether or not oil spills are able to lead to the formation of HABs. Recent findings show that BC exposed to crude oil and light clearly enhanced the growth of the harmful dinoflagellate *Karenia brevis* in laboratory experiments (Park and Buskey, 2020). Thus, we hypothesized that oil exposure

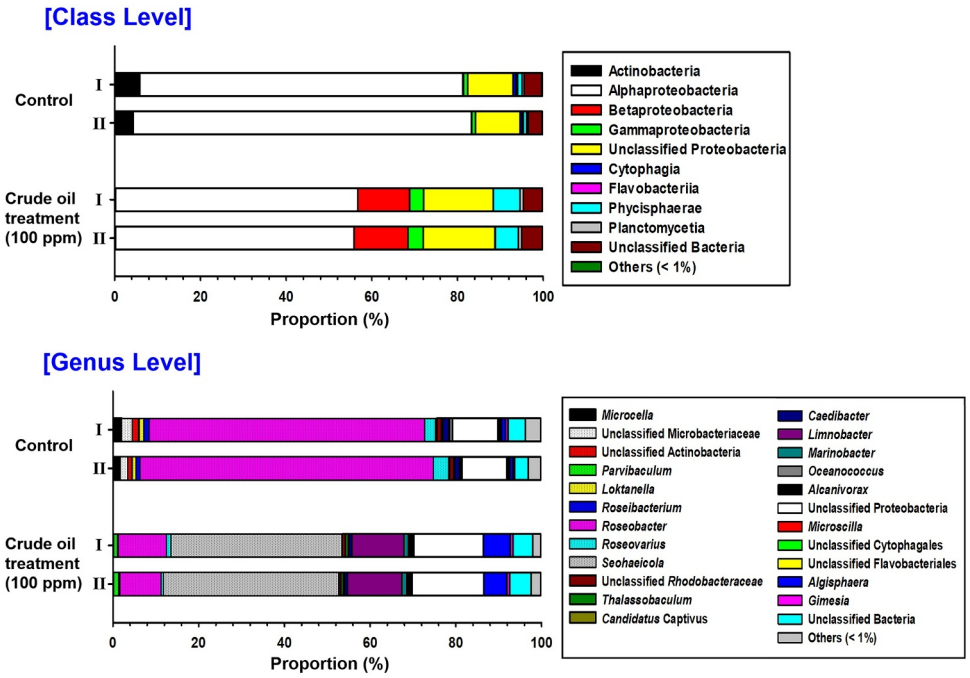

Fig. 5. Community compositions of free-living bacteria which were isolated from *Prorocentrum texanum* culture at class (top) and genus (bottom) levels before (control) and after (treatment) crude oil exposure.

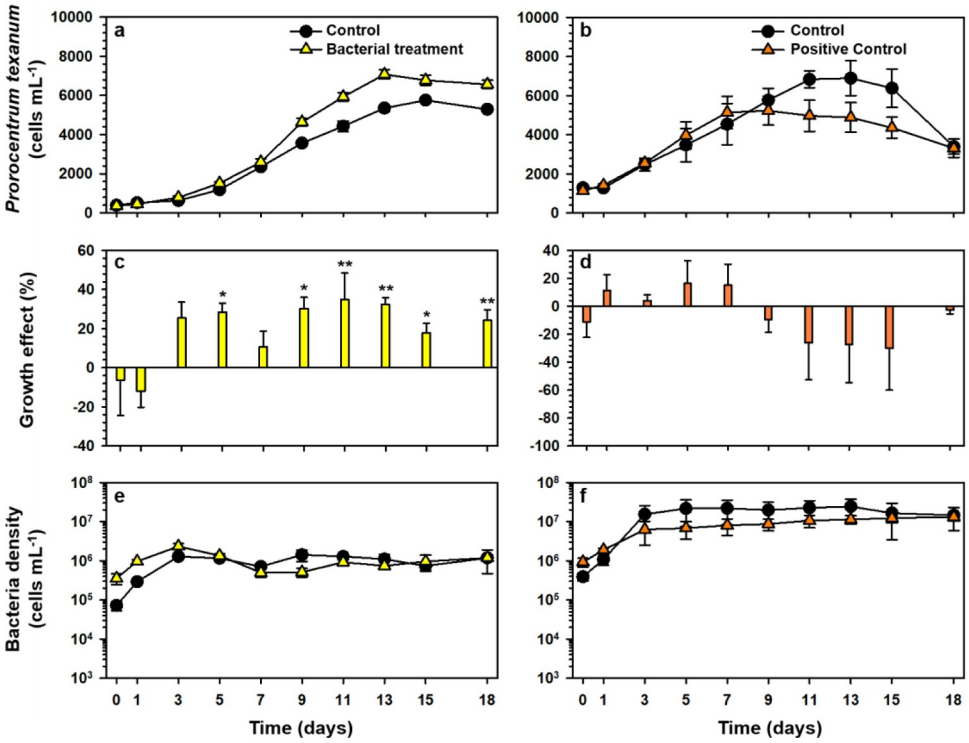

Fig. 6. Change in abundance of *Prorocentrum texanum* (a-d) and bacteria (e and f) after co-culture with altered bacterial communities exposed crude oil (a, c, and e) and bacterial communities in *P. texanum* culture as positive control (b, d, and f), respectively. Middle panels (c and d) show the relative abundance of *P. texanum* in non-control group, compared to control. The asterisks on the bar charts (c and d) represent the statistical significance of the difference (Student's *t*-test, $*p < 0.05$, $**p < 0.01$). Error bars indicate the standard deviation of mean value ($n = 3$).

changes the bacterial communities (BCs), and in turn these altered bacterial communities affect phytoplankton growth. To address this hypothesis, we exposed the resident BC of a *P. texanum* culture to oil WAF, then tested the ability of this altered BC to enhance dinoflagellate growth. While the altered BC enhanced *P. texanum* growth, this growth promoting effect could be caused by the addition of the oil-exposed BC or by an increase in overall bacteria number, and in particular any symbiotic taxa originally present in the BC. However, addition of the resident BC did not enhance growth, and in fact inhibited growth during stationary phase. Thus, the growth promoting effects on *P. texanum* most likely originate from BCs exposed to crude oil rather than from the addition of a symbiotic BC established in this dinoflagellate culture.

It is well known that hydrocarbon degrading bacteria are generally enhanced in crude oil-polluted environments (Cappello et al., 2007; Meng et al., 2016). In our results, there was clear variation in bacterial community structure depending on crude oil exposure; after crude oil exposure, the proportion of Roseobacter greatly decreased, but there were large increases in the proportions of known oil-degrading genera *Seohaeicola, Limnobacter* and *Algisphaera* (Kamalanathan et al., 2019; Mishamandani et al., 2016; Vedler et al., 2013). Based on previous findings in free-living bacteria (Park et al., 2018), the genus *Seohaeicola* was a dominant taxon that was closely associated with the growth of *P. minimum*. In addition, members of the genus *Limnobacter* have been reported as bacterial taxa associated with harmful dinoflagellates in laboratory and field studies (Hattenrath-Lehmann and Gobler, 2017; Danish-Daniel et al., 2016). Although there have been no reports on the association between the genus *Algisphaera* and dinoflagellates, many members of the class Phycisphaeraceae, including *Algisphaera* have been isolated from the phycosphere (Fukunaga et al., 2009; Yoon et al., 2014), suggesting that the members of this bacterial group are likely to symbiotically associate with phytoplankton. These findings suggest that increases in growth-associated bacteria after crude oil exposure may contribute to the growth enhancement of *P. texanum*. Indeed, a *Prorocentrum* bloom formed after the Texas City "Y" oil spill. Given our findings, change in the bacterial community structure due to the oil spill might affect the formation of a *Prorocentrum* bloom after the oil spill event.

Oil-degrading bacteria were directly isolated from sediment samples that were collected from oil-contaminated sites in Galveston Bay after the Texas City "Y" oil spill in this study. A total of seven bacteria were successfully established as cultures, and all were phylogenetically identical to *Bacillus megaterium* based on the sequence for the V1-V2 regions in the 16S rRNA gene (Table S1 and Fig. S1). Although this bacterial taxon has been widely known to be capable of degrading hydrocarbons, including polycyclic aromatic hydrocarbons (Lin and Cai, 2008; Adam et al., 2001; Carmichael and Wong, 2001; Jørgensen et al., 2000), the physiological characteristics can vary depending on species or strain. The oil degrading activity of the two bacterial isolates (C1-T3 and E1-Gal-T2) which showed relatively higher growth promoting effects on *A. carterae* and *Pe. sociale* in the preliminary test (Table S1) were checked through measurement of extracellular enzymatic activities as described by Hoppe (1993), prior to examining effect of those isolates on growth of dinoflagellates. Our results showed that they may be capable of degrading oil (Table S2).

The two oil-degrading bacterial isolates (C1-T3 and E1-Gal-T2) showed a significant growth enhancement in the xenic dinoflagellate cultures (Table 1). However, since the xenic cultures contained their own BCs, it is hard to isolate the effects of inoculated bacteria on dinoflagellate growth. Thus, in this study, two axenic dinoflagellate cultures were not only used, but also the effect of bacterial density was explored. If these bacterial isolates are capable of stimulating dinoflagellate growth, higher bacterial inoculation density should show higher growth promoting activity. Growth promoting activities of the two isolates on two axenic dinoflagellates (*A. carterae* and *Pe. sociale*) were not only significant, but also those activities were significantly increased in accordance with increased bacterial inoculation density (two-way ANOVA test, $p<0.05$). These findings show that the two oil-degrading bacterial isolates from the oil-contaminated sites can enhance the growth of dinoflagellates, suggesting that oil-degrading bacteria may have played an important role in the formation of dinoflagellate blooms after the Texas City "Y" oil spill.

If this is the case, then it is important to elucidate the dinoflagellate-growth promoting mechanism of oil-degrading bacterial isolates.

Growth of the two dinoflagellate species were clearly stimulated when they were incubated in bacterial-conditioned F/2 media compared to dinoflagellates grown under normal F/2 media. This result indicates that those isolates are likely to release a dissolved growth promoting substance(s). To gain further insight, nutrient-limited media were used to determine whether these bacterial isolates are capable of enhancing dinoflagellate growth through releasing certain nutrients. In our results, growth of two axenic *A. carterae* and *Pe. sociale* cultures were inhibited under every nutrient-limited condition, but increased after co-culture with the two oil-degrading bacterial isolates, irrespective of which nutrient was deficient. It has been widely known that bacteria are able to re-mineralize nitrogen and phosphorous to support phytoplankton growth. For example, bacterial relatives of the two isolates used here are capable of solubilizing phosphorus and mineralizing organic phosphorus (Hu et al., 2013). There could be other types of substances, such as a phytohormones, to enhance growth of the dinoflagellates. Based on previous findings, various phytohormones (e.g., auxin, gibberellin, cytokinin, and abscisic acid etc.) can support growth of phytoplankton, including dinoflagellates, and *B. megaterium,* the same species as our bacterial isolates, is capable of secreting those phytohormones (Bentley-Mowat and Reid, 1969; Green et al., 2004; Karadeniz et al., 2006; Lee et al., 2019). The current results are, however, limited to clearly elucidate dinoflagellate-growth promoting mechanisms of the bacterial isolates, and further study will be necessary to determine whether the growth-promotion observed here is due to phytohormone production and/or some means of nutrient provision.

The potential role of oil-degrading bacteria in the formation of HABs after an oil spill is supported by the results of this study. The altered bacterial community composition due to crude oil exposure significantly enhanced the growth of *P. texanum*. In addition, the oil-degrading bacterial isolated from the oil-contaminated sites where a *Prorocentrum* bloom occurred after the Texas City “Y” oil spill showed a clear growth promoting activity on various dinoflagellates cultures, including three *Prorocentrum* species, through releasing certain dinoflagellate-growth promoting substances. Given these findings, an increase in oil-degrading bacteria after the Texas City “Y” oil spill may have positively contributed to the formation of the *Prorocentrum* bloom. It may not be remarkably surprising that oil-degrading bacteria could play an important role in the formation of HABs. It has been suggested that phytoplankton can adsorb, concentrate, and produce polycyclic aromatic hydrocarbons (PAHs), which are released into marine environments when oil spills happen, and hydrocarbon-degrading bacteria have been isolated from laboratory cultures of marine phytoplankton (Andelman and Suess, 1970; Gunnison and Alexander, 1975; Gol'man et al., 1973; Gutierrez et al., 2013; Zelibor et al., 1988; Kowalewska, 1999; Binark et al., 2000; Repeta et al., 2004; Zhang et al., 2018). Thus, Gutierrez et al. (2013) suggested that phytoplankton could have an ecological association with PAH-degrading bacteria through coevolution. Moreover, the bacterial taxa which are closely associated with HABs (e.g., Roseobacters and Flavobacteria) are known oil-degraders in marine environments (Rahman et al., 2002; Kim and Kwon, 2010; Buchan et al., 2014). There are still, however, a limited understanding of the association HABs and oil spills. For example, even though the oil-degrading bacterial isolates show growth promoting activity on dinoflagellates in this study, the proportion of phylum Firmicutes (including genus *Bacillus*) was low in the oil-contaminated sites after the Texas City “Y” oil spill (Gemmell et al., 2018). In addition, the effect of oil-degrading bacteria on the growth of other phytoplankton taxa, such as diatoms, has not been examined in this study, and it is unclear whether or not those bacteria are capable of enhancing the growth of dinoflagellates, selectively. Lastly, it is not certain whether oil-degrading activity of bacteria by itself may induce growth enhancement of phytoplankton in nature. Hence, to address these limitation, further extensive studies are needed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101802.

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