Prorocentrum micans promote and Skeletonema tropicum disfavours persistence of the pathogenic bacteria Vibrio parahaemolyticus

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The genus Vibrio comprises a diverse group of species, of which most are halophilic and many pathogenic. Vibrio cholerae, the causative agent of cholera epidemics worldwide, is well known for its contribution to hundreds of thousands of events of death every year1. Vibrio parahaemolyticus and V. vulnificus are the most common human pathogenic vibrios in the marine environment, and are coupled to food poisoning with sporadic fatal results2. Infections of V. parahaemolyticus have been reported in increased numbers around the world3. In tropical and warm-temperate waters, cases of Vibrio-induced diseases are common4, while in cold-temperate waters reported cases occur but are rare5,6. According to one study, ten percent of all gastroenteritis in India are related to V. parahaemolyticus7. High abundances of V. vulnificus and V. parahaemolyticus are found in oysters and other filtering shellfish, and infections are frequently associated to consumption of those, if eaten raw or poorly cooked3. It is estimated that 1-2% of all V. parahaemolyticus isolates are pathogenic7.

The growth of Vibrio is known to be affected by changes in water temperature, salinity, presence of zooplankton, and sometimes also by phytoplankton biomass2,8. In general, Vibrio spp. tolerates a wide range of salinities and tends to be more common in warmer water2,9. In tropical waters, where the temperature and salinity are more stable throughout the year, the variation in phytoplankton biomass seems to be an important determinant of Vibrio growth10. In a mesocosm study conducted in India, the community structure of phytoplankton was found to affect the Vibrio abundance. For instance, Coscinodiscus spp. had a positive effect on Vibrio growth, whereas Skeletonema tropic was negatively correlated with Vibrio abundances11. Furthermore, in northern temperate coastal waters, Eiler et al.12 has shown that abundances of Vibrio tend to be higher in more saline waters, are affected by different nutrient levels, and increase with higher concentrations of dinoflagellates such as Prorocentrum. Dinoflagellates are important contributors to primary production globally. They appear in high abundances during different time of the year and blooms can develop rapidly and reach high densities with up to thousands of cells per mL13. Thus, increased knowledge of how different phytoplankton groups and species, affect the growth of Vibrio can therefore provide better and safer management of fisheries and aquacultural products.

The aim of this study was to experimentally examine how the presence of two common phytoplankton species in the Arabian Sea14,15, a dinoflagellate Prorocentrum micans, and a diatom...
**Skeletonema tropicum**, affect the persistence and potential growth of an environmental strain of *V. parahaemolyticus*.

The study was conducted in Mangalore on the southwest coast of India (December 17-23, 2009). During the experiment the temperature was set to 25°C, light-intensity was 50 µE m⁻² s⁻¹ at a photoperiod of 12 h light:dark. Prior to the experiment start, a growth curve of *P. micans* (CCMP1589) and *S. tropicum* (isolated from Arabian Sea in Dec 2008) was constructed to identify the start and duration of log- and lag-phases. Forty mL of f/2 medium and 1 mL of algal culture were added to each of 3 flasks (NUNC 50 mL). One mL was subsampled from each flask every morning during 7 days, and fixed in 12 µL Lugol’s Iodine. The number of cells per ml in the samples was counted in a Sedgwick Rafter 1 cm³ chamber (Wildlife supply company, USA) and plotted over time. By fitting exponential trend lines to the curves, it was possible to predict the growth rate of the cultures. *Vibrio parahaemolyticus* (environmental isolate 24ST belonging to serotype O4:K37, identified at College of Fisheries, Mangalore) was prior to the addition in the experiment flasks cultured in luria-broth (LB) media (3.5% NaCl) to late exponential phase and then centrifuged and the bacterial pellet was washed and then diluted in sterile seawater. The exponential phase of *V. parahaemolyticus* growth in the LB-media, in the same conditions, had earlier been identified spectrophotometrically by optical density (600 nm) measurements.

Nine ventilated culture flasks (Nunc 600 ml) were prepared with f/2 media. Three of them were kept as controls, three with added *P. micans* and three with *S. tropicum*. Density of *P. micans* and *S. tropicum* added to the experimental flasks was based on natural bloom conditions. According to the pre-experimental growth curves, the algal cultures had reached exponential phase after four days. *P. micans* at a concentration of 150 cells ml⁻¹ and *S. tropicum* at 500 cells ml⁻¹ (Fig. 1). Subsequently, *V. parahaemolyticus* cells in exponential growth phase was added to the experimental flasks, in a concentration corresponding to *Vibrio* spp. concentration in a previous study from the Mangalore coast. Abundances of algal cells were estimated daily by microscope counting during the entire experiment. After two days the experiment was terminated and samples for *V. parahaemolyticus* quantification were collected from all flasks, they were processed as follows: Fifty ml was filtered from each flask in the experiment on membrane filters (0.2 µM, Pall Corporation). DNA was extracted by a phenol-chloroform based protocol according to Godhe et al. (2008) and diluted in 50 µl milli-Q water. Real-Time PCR for quantification of *V. parahaemolyticus* from the experiment were carried out following the protocol of Thompson et al. (2004). To each reaction well in a 96-well plate (Thermo-Fast detection plate: ABgene), 12.5 µL Sybr green PCR master mix (Applied Biosystems, Warrington, United Kingdom), 9.5 µL milli-Q water, 1 µL of each Primer (100 nM final conc., gvc 567/gvc 680R, Thompson et al.²³ and 1 µL template DNA was added, in triplicates. DNA extracted from a reference *V. parahaemolyticus* strain (Vp 43364, Culture Collection University of Gothenburg) with known number of copies (i.e. 11) of 16S rDNA was used as standard, in 10 time dilutions (ranging from 5 × 10⁻³–5 × 10⁶ cells µL⁻¹ DNA template). Numbers of cells per µL in the standard were calculated according to the protocol “Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR” (Applied Biosystems, appliedbiosystems.com). Furthermore, DNA from Vp43364 was added to the mastermix and used as an internal standard, in a concentration corresponding to approximately 10 cells per µL added to each reaction well, and used as an internal standard when running the real-Time PCR. Number of cells per ml was calculated from the standard curve where the estimated concentrations in the different dilutions were plotted against the Ct values from the real-Time PCR, after subtracting the amount of internal standard and correcting for the filtered volume of water and the dilution volume of the extracted DNA. To compare the concentration of *V. parahaemolyticus* in the controls and with the *P. micans* treatment, a t-tests were performed (*p < 0.05*) in SPSS 17.0.

At the initiation of the experiment, when *P. micans* had reached 150 cells mL⁻¹ and *S. tropicum* 500 cells mL⁻¹, *V. parahaemolyticus* was added (Fig. 1). At the end of the experiment, the recorded *V. parahaemolyticus* concentration was 4 × 10⁴ cells mL⁻¹ in the *P. micans* treatment, in the controls (without algae) the concentration was 8 × 10⁵ cells mL⁻¹, and in the *S. tropicum* treatment *V. parahaemolyticus* was undetectable (Fig. 2).

The concentration of *V. parahaemolyticus* differed significantly between the control flasks and the flasks where *P. micans* was added, (df=4, *p=0.019*). This suggests that *P. micans* facilitate the persistence of
Vibrio, and confirms indications from previous field studies\textsuperscript{12,18}, which were conducted in colder waters. Reason for the association is suggested to be due to the dinoflagellates release of bioavailable substances\textsuperscript{19}, which is utilized by the bacteria. Results also suggest that S. tropicum affects the bacteria negatively, since V. parahaemolyticus was below detectable level after 24 hour incubation. This result support what was previously found by Naviner et al.\textsuperscript{20}, that Skeletonema appears to have antibacterial activity. According to the study of Asplund et al.\textsuperscript{10}, fluctuations of phytoplankton biomass are of great importance in oligotrophic coastal water at lower latitudes. This contrast seasonal Vibrio dynamics from temperate coastal areas where temperature and salinity fluctuations are the most important factors for predicting Vibrio abundances\textsuperscript{2,21}. Thus, in countries like India, where the water is warm all year round the bacteria will probably be continuously present in the water and the fluctuations in their concentration will probably depend on other environmental factors, like accessibility of dissolved organic carbon. Thus, factors like phytoplankton abundance and taxonomic composition of the phytoplankton community are important when predicting Vibrio growth. Since phytoplankton sporadically appears in high densities, the results we present here are of great interest when it comes to facilitate or impede growth of pathogenic bacteria such as V. parahaemolyticus.

Filtrating animals, like mussels and oysters, assimilate phytoplankton and the bacteria that are associated with the algae will accumulate in the animals and can then pose a threat to human health. Future experiments should focus on how V. parahaemolyticus is affected by other mono cultures of phytoplankton or multispecies communities dominated by different phytoplankton groups. It would then be beneficial to combine the information on how different phytoplankton species or groups affect the number of V. parahemolyticus combined with other factors known to impact the

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{growth_curves.png}
\caption{Growth curves of a) P. micans and b) S. tropicum in the experiment, where V. parahaemolyticus was added at day 4 in the P. micans culture and at day 2 in the S. tropicum culture respectively.}
\end{figure}
survival, persistence and proliferation of \( V. \) \( parahaemolyticus \) such as salinity and temperature.

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