# Communications

## Allelopathic interactions between the red-tide causative dinoflagellate *Prorocentrum donghaiense* and the diatom *Phaeodactylum tricornutum**

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**Abstract**

The interactions between the red-tide causing dinoflagellate *Prorocentrum donghaiense* and the marine diatom *Phaeodactylum tricornutum* were investigated using a co-culture experiment and an enriched culture filtrate experiment. The results showed that when the two microalgae were cultured together with different initial cell densities, the growth of one species was basically suppressed by the other one. In addition, the enriched culture filtrates of one species had generally inhibitory effects on the other one. Our result inferred that *P. donghaiense* and *P. tricornutum* would interfere with each other mainly by releasing allelochemicals into the culture medium, and that the degree of allelopathic effects was dependent on the initial cell densities and growth phases. The allelopathic interactions between microalgal species may contribute to the formation and succession of red tides.

Marine microalgae act as the basis of the marine food chain and reduce carbon dioxide levels in the atmosphere during photosynthesis. Sometimes, however, some species of marine microalgae proliferate and assemble so quickly that they may lead to red tide events, which have been reported to increase in frequency, intensity and geographic distribution during recent decades and have brought about many negative environmental and economic consequences (Kremp et al. 2012). Hence, there is an urgent need to understand the mechanisms underlying the outbreak of red tides, which is significant in developing effective management strategies to control them.

However, the outbreak mechanisms of red tides are very complex and are not fully understood (Cai et al. 2013). In particular, no satisfactory explanations have been provided to explain why some microalgal species are replaced in a phytoplankton community. Recent studies have shown that the forming of red tides can be dependent on multiple physical, chemical, meteorological and biological factors, such as wind, water current, disturbance, temperature, salinity, nutrient availability, predation of zooplankton, and so on (Smayda 1997, Laanaia et al. 2013, Persson et al. 2013).

Allelopathy, a widely existing natural phenomenon, refers to any direct or indirect, inhibitory and stimulatory effects of plants or microorganisms on others, by producing chemical compounds that are released into the environment (Rice 1984, Meiners et al. 2012). It is believed to be a competitive strategy to adapt to the environment (Cummings et al. 2012). Allelopathy is not a contributory factor towards the formation of harmful algal blooms, but may be important in the maintenance of these blooms (Jonsson et al. 2009). For a long time, research on allelopathy concentrated on terrestrial higher plants (Feng et al. 2010, Khan et al. 2012). The existing published research work on allelopathy in marine microalgae is somewhat limited (Addisie & Medellin 2012). Understanding the allelopathic interactions in marine

microalgae can provide deeper insight into successions in natural algal communities and outbreak mechanisms of harmful algal blooms (Legrand et al. 2003, Żak et al. 2012).

The dinoflagellate *Prorocentrum donghaiense* is an ecosystem-harmful algal bloom species that frequently occurs in Chinese coastal waters (Hu et al. 2012). For instance, in May 2002 a large-scale *P. donghaiense* causative bloom formed in the East China Sea. It lasted for about one month, and the affected area was larger than 1000 $km^2$; it had significant negative impacts on the aquatic environment, marine fisheries and even public health (Lu et al. 2005). Meanwhile, the phytoplankton organism *Phaeodactylum tricornutum* is a marine diatom. Under certain environmental conditions, it can also over-proliferate in coastal waters, with the potential to destroy the natural marine ecosystems in the vicinity, and hence to cause great losses to the economy (Cai et al. 2009). However, until now no report has been available on the allelopathic interactions between the disruptive *P. donghaiense* and *P. tricornutum*, not to mention the roles of initial cell density and growth stage as related to allelopathy.

Therefore, in this study we used axenic strains of *P. donghaiense* and *P. tricornutum* to assess their allelopathic interactions under controlled laboratory conditions. We first investigated their mutual interactions in a laboratory-designed co-culture experiment with several combinations of initial cell densities. Then, we further tested the allelopathic effects of the cell-free filtrates of one species on the growth of the other one by growing the microalgal cells in the presence of enriched culture filtrates.

Both the axenic strains of the dinoflagellate *Prorocentrum donghaiense* Lu and the marine diatom *Phaeodactylum tricornutum* (Bacillariophyta) were obtained from the Institute of Hydrobiology, Jinan University, Guangzhou, China, and were routinely cultivated under standardised conditions at constant irradiance (70 $\mu$mol $m^{-2}$ $s^{-1}$) and temperature (23°C) in a 12 h/12 h (light/dark) photoperiod cycle. The artificial seawater was passed through a 0.45 $\mu$m filter prior to being used for culture medium preparation, and an f/2 nutrient solution was used in the experiments (Guillard 1973). The salinity of the artificial seawater was 30 PSU and the initial pH of the culture was approximately 7.0.

The microalgal cells were cultivated to the exponential growth phase for use. They were inoculated into 250-mL Erlenmeyer flasks containing fresh f/2 seawater medium; the total experimental volume was 100 mL. The initial cell densities were set at $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$ for the two microalgae respectively. Hence, the resulting combinations of initial cell densities of *P. donghaiense* and *P. tricornutum* were respectively (1) $1.0 \times 10^4$ cells $mL^{-1}$ each; (2) $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$; (3)

$1.0 \times 10^5$ and $1.0 \times 10^4$ cells mL$^{-1}$; and (4) $1.0 \times 10^5$ cells mL$^{-1}$ each. As controls, both microalgae species were cultured individually at initial cell densities of $1.0 \times 10^4$ and $1.0 \times 10^5$ cells mL$^{-1}$. During the maintenance of the experimental stages, the glass flasks containing algal cells were shaken three times every day by hand at the set time, and they were randomly rearranged to minimise the effects of light or temperature gradients in the plant growth chamber. The growth conditions were the same as stated above, and all experiments were carried out in triplicate. Based on the cell growth characteristics of these microalgae, culture samples were collected in the beginning growth stage (BGS), lag growth stage (LGS), exponential growth stage (EGS) and stationary growth stage (SGS), basically on Day 1, Day 4, Day 7 and Day 10 respectively. Thereafter, an 0.5 mL volume of solution was sampled, and microalgal cell densities were counted using a haemocytometer under an optical microscope after the cells were preserved (Cai et al. 2013).

In order to verify the effects of allelopathic compounds of one microalga on the growth of the other, the culture filtrates of *P. donghaiense* and *P. tricornutum* in the exponential growth phase were collected separately. The culture media were first filtered through 0.45 $\mu$m, and then through 0.22 $\mu$m pore-size Millipore membrane filters to prepare sterilised cell-free filtrates. 100 mL of each filtrate were adjusted to the same concentrations as the f/2 medium by the addition of nutrients including nitrate and phosphate, trace metals and vitamins. The culture filtrates of *P. donghaiense* were used to cultivate *P. tricornutum*; those of *P. tricornutum* were used to cultivate *P. donghaiense*. The initial densities of the two microalgae cultivated in the filtrates were also set at $1.0 \times 10^4$ and $1.0 \times 10^5$ cells mL$^{-1}$. The cells cultured in 100 mL fresh f/2 enriched seawater were used as controls. The growth conditions were kept the same as described above, and the cell densities were assessed with reference to the above methods. Moreover, the specific growth rate ($\mu$, divisions d$^{-1}$) was calculated to monitor the growth of cells using the following equation: $\mu_{n+1} = (\ln X_{n+1} - \ln X_n) / (t_{n+1} - t_n)$, where $X_{n+1}$ and $X_n$ [cells mL$^{-1}$] are the respective cell densities at times $t_{n+1}$ and $t_n$ (d). Statistical tests were conducted using Microsoft Excel 2003 (Microsoft Company, USA) and SAS (SAS Institute Inc., Cary, NC, USA). Statistical significances were determined by repeated ANOVA, and the $t$-test was also used to analyse the data on the same sampling day when necessary. The probability level of 0.05 was used as the threshold for statistical significances. All the data from this study were expressed as means with standard errors (mean$\pm$ SE).

We conducted a co-culture experiment using different initial cell densities of *P. tricornutum* and *P. donghaiense* (Figure 1). When the initial cell den-

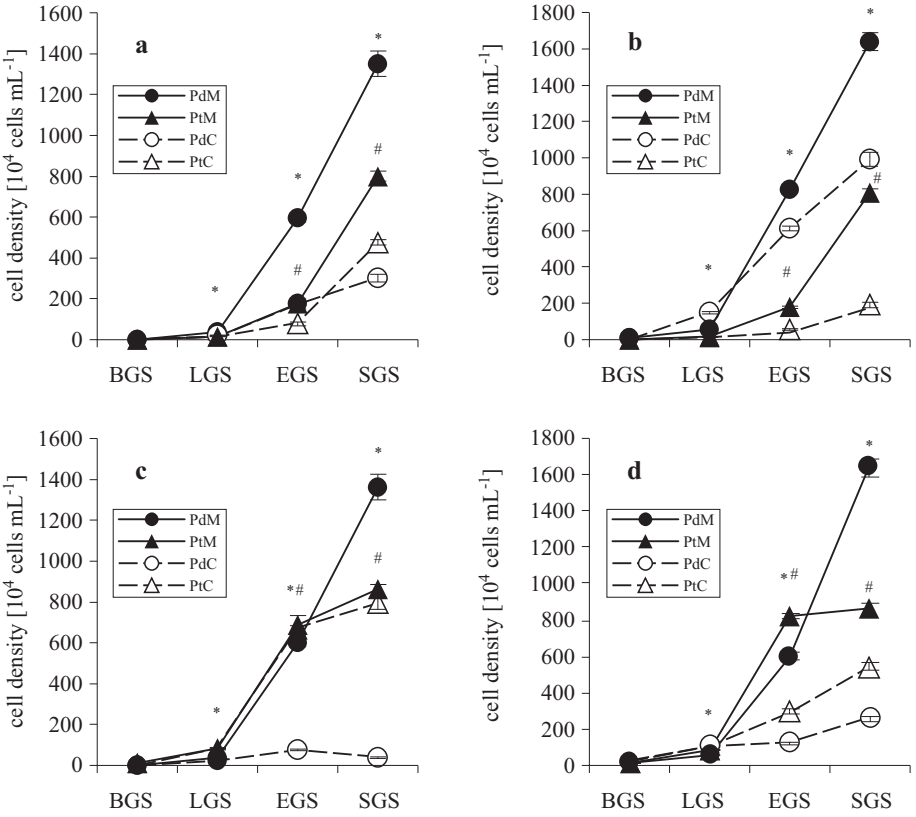


**Figure 1.** Growth interactions between *Prorocentrum donghaiense* and *Phaeodactylum tricornutum* showing growth of *P. donghaiense* when cultured alone (filled circles) or co-cultured with *P. tricornutum* (empty circles), and of *P. tricornutum* when cultured alone (filled triangles) or co-cultured with *P. donghaiense* (empty triangles). PdM – *P. donghaiense* in the monoculture; PdC – *P. donghaiense* in the co-culture; PtM – *P. tricornutum* in the monoculture; PtC – *P. tricornutum* in the co-culture. BGS – beginning growth stage; LGS – lag growth stage; EGS – exponential growth stage; SGS – stationary growth stage. '*' represents a significant difference between PdM and PdC in each sample period; '#' represents a significant difference between PtM and PtC in each sample period. a) Initial cell densities: *P. donghaiense* – $1.0 \times 10^4$ cells mL$^{-1}$, *P. tricornutum* – $1.0 \times 10^4$ cells mL$^{-1}$, b) initial cell densities: *P. donghaiense* – $1.0 \times 10^5$ cells mL$^{-1}$, *P. tricornutum* – $1.0 \times 10^4$ cells mL$^{-1}$, c) initial cell densities: *P. donghaiense* – $1.0 \times 10^4$ cells mL$^{-1}$, *P. tricornutum* – $1.0 \times 10^5$ cells mL$^{-1}$, d) initial cell densities: *P. donghaiense* – $1.0 \times 10^5$ cells mL$^{-1}$, *P. tricornutum* – $1.0 \times 10^5$ cells mL$^{-1}$

sities of *P. tricornutum* and *P. donghaiense* were set at $1.0 \times 10^4$ cells mL$^{-1}$, the growth of *P. tricornutum* in the co-culture was significantly inhibited from LGS onwards, and its cell densities at EGS and SGS were only about 45% and 60% of those in the monoculture ($P < 0.0001$). The growth of

*P. donghaiense* was also noticeably suppressed in the co-culture, with the cell densities at EGS and SGS being approximately 30% and 20% of those in the monoculture ($P < 0.0001$) (Figure 1a).

When the initial cell densities of *P. tricornutum* and *P. donghaiense* were set at $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$ respectively, the growth of *P. tricornutum* in the co-culture was significantly inhibited from LGS onwards, and its cell densities at EGS and SGS were only about 30% and 24% of those in the monoculture ($P < 0.0001$). The growth of *P. donghaiense* in the co-culture was prompted in LGS ($P < 0.05$), but it was also conspicuously suppressed in the co-culture at EGS and SGS ($P < 0.0001$) (Figure 1b).

When the initial cell densities of *P. tricornutum* and *P. donghaiense* were set at $1.0 \times 10^5$ and $1.0 \times 10^4$ cells $mL^{-1}$ respectively, the growth of *P. tricornutum* was virtually the same in both the co-culture and the monoculture, with the cell densities approaching $660 \times 10^4$ and $790 \times 10^4$ cells $mL^{-1}$ in EGS and SGS ($P < 0.05$). The growth of *P. donghaiense* was suppressed very significantly in the co-culture from LGS onwards, and its cell densities at EGS and SGS were $75 \times 10^4$ and $36 \times 10^4$ cells $mL^{-1}$, only approximately 13% and 3% of those in monoculture($P < 0.0001$) (Figure 1c).

When the initial cell densities of both *P. tricornutum* and *P. donghaiense* were set at $1.0 \times 10^5$ cells $mL^{-1}$, the growths of both *P. tricornutum* and *P. donghaiense* in the co-culture were significantly inhibited, their cell densities being about 63% and 15% of those in the monoculture at SGS ($P < 0.0001$) (Figure d).

Several studies have hinted that the composition and dynamics of algal communities may be influenced by allelopathy among algal species (Legrand et al. 2003). The earlier studies on allelopathy among algae concentrated on field observations. For example, Keating (1977) observed the allelopathic influence of successors and their predecessors on the blue-green bloom sequence in Linsley Pond, a eutrophic lake, over a period of three years. In eutrophic lakes, diatom bloom populations varied inversely with the levels of the preceding blue-green algal populations, and blue-green algal dominance could be attributed to the allelopathic effects (Keating 1978). In the current study, we conducted laboratory experiments under controlled conditions to exclude the effects of environmental factor variation. Besides, in order to reveal the growth and interactive effects between *P. tricornutum* and *P. donghaiense*, we used their axenic strains. That is because previous bi-algal culture experiments indicated that bacteria were either directly or indirectly associated with algal toxin production, and that bacteria exerted some influence in the interaction between microalgal species (Tarutani et al. 2000). Nagasaki et al. (1994) showed that bacterial attack and viral

infection might play a role in the algal bloom initiation, succession or termination. Therefore, in our study, the allelopathic activity of microalgae might be the most likely explanation for the stimulatory or inhibitory effects of one species on the other co-cultured one.

Our laboratory results showed that the growth and interactive effects between *P. tricornutum* and *P. donghaiense* were dependent on the initial cell density of each species: a higher initial cell density generally resulted in stronger allelopathic effects between them. In addition, when the initial cell densities of *P. tricornutum* and *P. donghaiense* were set at $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$ respectively, growth promotion effects of *P. tricornutum* on *P. donghaiense* were detected at LGS, implying that allelopathic interactions were very complex and also time-dependent. We did not attempt to delve into the nature of this stimulation, but it could be assumed that some stimulatory compounds were excreted by *P. tricornutum* and that *P. donghaiense* was susceptible to such a concentration at that growth stage (Yamasaki et al. 2010). Our results were consistent with those of Gantar et al. (2008), who found that the interactive effects between strains of Cyanobacteria and green algae depended both on the concentration of allelopathic compounds and on the time of exposure.

We also observed the effects of enriched cell-free filtrates from one microalgal species on the growth of the other microalgal species at the initial cell densities $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$ (Figure 2). It was evident that the growth of *P. donghaiense* with initial cell densities of $1.0 \times 10^4$ cells $mL^{-1}$ was significantly inhibited by the filtrates from *P. tricornutum* cultures from LGS onwards ($P < 0.0001$). In contrast, when the initial cell density of *P. donghaiense* was $1.0 \times 10^5$ cells $mL^{-1}$, the enriched filtrates of *P. tricornutum* promoted the growth of *P. donghaiense* at LGS and EGS ($P < 0.05$), after which a significant inhibitory effect manifested itself at SGS ($P < 0.05$). Meanwhile, the growth of *P. tricornutum* at both $1.0 \times 10^4$ and $1.0 \times 10^5$ cells $mL^{-1}$ was inhibited in the presence of cell-free filtrates from *P. donghaiense* ($P < 0.0001$).

In the present study, besides the co-culture method, we also applied the cell-free filtrate method to assess the allelopathic interactions between *P. donghaiense* and the diatom *P. tricornutum*. These methods, as expected, produced some identical results. In general, growth inhibition of one species was recorded in both the co-culture experiment and the enriched filtrate experiment, indicating that the allelopathic effects of one species were acting on the other one. However, the extent of interference in the co-culture experiment was not quite identical with that in the enriched filtrate experiment. The degree of growth inhibition and promotion response of *P. donghaiense* and *P. tricornutum* cells was different in the co-

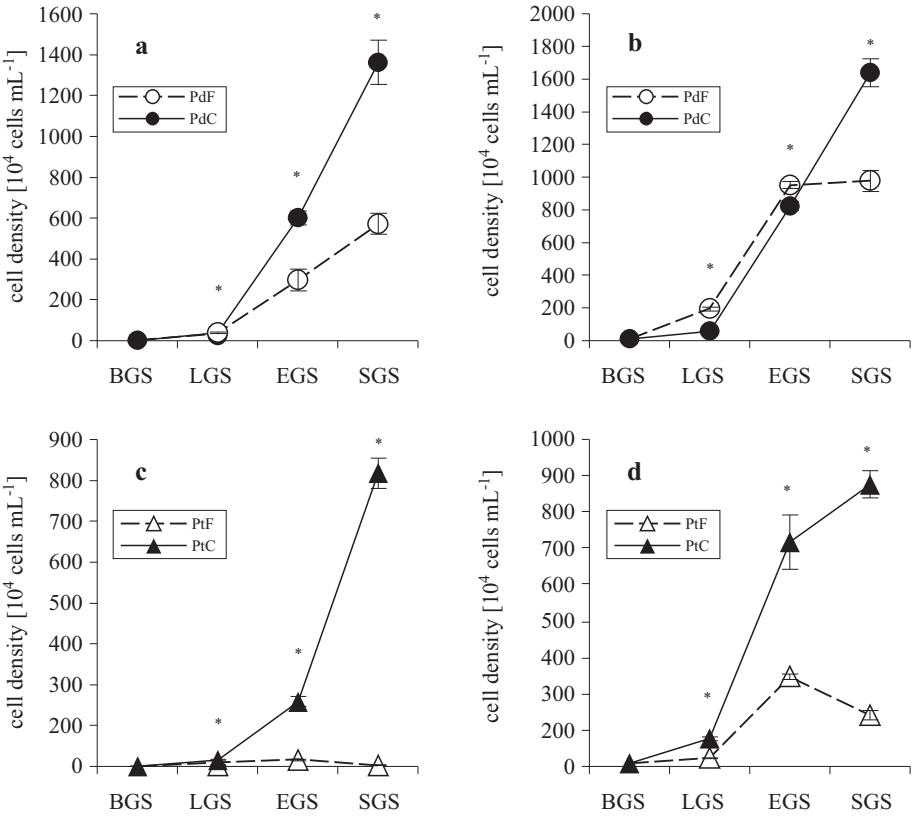


**Figure 2.** Growth of *Prorocentrum donghaiense* in the presence of cell-free filtrates from *Phaeodactylum tricornutum*, or of *P. tricornutum* in the presence of cell-free filtrates from *P. donghaiense*. PdF – *P. donghaiense* in the enriched cell-free filtrates from *P. tricornutum* (empty circles), and PdC – its control (filled circles); PtF – *P. tricornutum* in the enriched cell-free filtrates from *P. donghaiense* (empty triangles), and PtC – its control (filled triangles). BGS – beginning growth stage; LGS – lag growth stage; EGS – exponential growth stage; SGS – stationary growth stage. '*' – significant difference between the treatment group and the control group in the same sample period. a) Initial cell density of *P. donghaiense* – $1.0 \times 10^4$ cells $mL^{-1}$, b) initial cell density of *P. donghaiense* – $1.0 \times 10^5$ cells $mL^{-1}$, c) initial cell density of *P. tricornutum* – $1.0 \times 10^4$ cells $mL^{-1}$, d) initial cell density of *P. tricornutum* – $1.0 \times 10^5$ cells $mL^{-1}$

culture experiment and enriched filtrate experiment. This indicated that the allelopathic substances of *P. tricornutum* acting on *P. donghaiense* were probably different in their chemical nature or could have reacted antagonistically/synergistically in the co-culture (Yamasaki et al. 2007). An et al. (1996) assumed that the effect of the allelochemical pool of a plant might be characterised by two processes: the release and degradation of

the allelochemicals. Note that in our co-culture and filtrate experiments, the mode of allelopathy was different. Microalgal cells could rapidly and continuously release biologically-active allelochemicals into the culture medium in the co-culture, and this was also a result of the synergistic interaction of two or more compounds, some of which could have been degraded or lost in the filtrate experiment. Moreover, cell-to-cell contact in the co-culture was also responsible for the non-identical growth response of microalgal cells in the two methods. Nagasoe et al. (2006) found that the growth inhibition of *Gyrodinium instriatum* by *Skeletonema costatum* might require cell contact, but that *G. instriatum* did not affect *S. costatum*.

In recent years, mono-specific and multi-species blooms have been commonly observed in various coastal waters. Till now, however, no satisfactory explanations have been provided to explain why some microalgal species are able to dominate in a phytoplankton community, and it has been unclear how the succession of certain microalgal species forms. It has been increasingly clear that allelopathy is of special interest from an evolutionary perspective, since allelopathic substances can function as a defence (against microbes, viruses or competing plants) and represent adaptive characters that have been subjected to natural selection processes (Rengefors & Legrand 2001, Bertholdsson 2012). *P. tricornutum* and *P. donghaiense* can proliferate and assemble very quickly in coastal waters, which adversely affects the aquatic ecosystem (Cai et al. 2009). Therefore it is crucial to investigate the interaction between these two marine microalgae. In our study, we observed inhibitory and stimulatory interactions between the cell growth of *P. tricornutum* and *P. donghaiense*, and their allelopathic effects in the filtrates by investigating cell densities and specific growth rates (data not shown). This will be useful in elucidating the role of allelopathy in the succession of these two algae in the same ecosystem. Consequently, our findings have extended the field observations that in a natural ecosystem a monospecific bloom is replaced by another bloom, or that microalgal species form alternate blooms, such as *Skeletonema*, *Heterosigma* and *Prorocentrum* blooms.

In conclusion, our results from controlled laboratory experiments using axenic strains of *P. tricornutum* and *P. donghaiense* indicate that the growth of either species can be generally suppressed (or occasionally promoted) by the other, depending on initial cell densities and growth stages. The release of allelochemicals into the medium is an important way in which one species can affect another, which demonstrates that allelopathic interactions between/among sympatric microalgal species may involve a complex process in the formation and succession of harmful algal blooms. It needs to be pointed out that there are yet-to-be-defined mechanisms underlying

allelopathic interactions among phytoplankton. Work is in progress to clarify the factors responsible for the growth and interactions among phytoplankton, identify such allelopathic substances and assess the role of allelopathy in natural phytoplankton populations.

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