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Interaction between *Chlorella vulgaris* and bacteria: interference and resource competition

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Abstract
Research of interaction mechanism between *Chlorella vulgaris* and two bacterial strains (Z-QD08 and Z-QS01) were conducted under laboratory conditions. Growth rates of bacteria and *C. vulgaris* were tested under co-culture conditions to evaluate the effects of concentrations of *C. vulgaris* and bacteria on their interactions. To test whether the availability of inorganic nutrients, vitamins and trace metals affects the interactions between *C. vulgaris* and bacteria, experiments were performed with or without the culture medium filtrate of *C. vulgaris* or bacteria. The results showed that the growth of *C. vulgaris* was promoted at low concentrations of bacteria (5×10^6 cells/ml), and expressed a positive correlation with the bacteria density, whereas opposite trend was observed for treatments with high bacteria density (10×10^6 cells/ml and 20×10^6 cells/ml). The growth rate of bacteria decreased with the increasing concentrations of *C. vulgaris*. The growth of bacteria Z-QD08 was inhibited by *C. vulgaris* through interference competition, while the mechanism for interaction between bacteria Z-QS01 and *C. vulgaris* was resource competition. The influence of cell density on the interaction between microalgae and bacteria was also discussed. These experiments confirm some elements of published theory on interactions between heterotrophic bacteria and microalgae and suggest that heterotrophic bacteria play an important role in the development of blooms in natural waters.

Key words: *Chlorella vulgaris*, bacteria Z-QD08, bacteria Z-QS01, interaction mechanism

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1 Introduction

Microalgae are considered as a major food source for animals in aquaculture because of their high protein and carbohydrate contents. The rapid development of aquaculture over the last few years has a significant impact on aquatic ecosystems, leading to the shortage of living microalgae as well as the eutrophication. Microalgae and associated bacteria are commonly used as artificial food to cultivate aquatic organisms based on their beneficial trophic relationships. Extracellular enzymes secreted by bacteria can convert large molecular substances, e.g., starch lipid protein nucleic acid, into smaller ones and mineralize organic matters to carbonate, nitrate, phosphate, and sulfate, while microalgae gain energy and matter and provide oxygen for microbes through photosynthesis (Pinhassi et al., 2004; Rooney-Varga et al., 2005). It has been inferred from the stimulation effect of decomposed DON solutions on the growth of *M. aeruginosa* that the decomposing activities of bacteria contribute significantly to the fast growth of microalgae (Du et al., 2013). Although bacteria can effectively decompose organic matters, they can create favorable microenvironments to promote the growth of microalgae by reducing the photosynthetic oxygen tension (Mouget et al., 1995). However, substrates such as vitamin and phytohormones produced by bacteria may also significantly promote the growth of microalgae (Gonzalez and Bashan, 2000; Watanabe et al., 2005; Croft et al., 2005). Previous studies have shown that the growth-promoting bacteria adhere to the microalgae, and the adhesion may reduce the diffusion distance and result in an efficient exchange of nutrients (Park et al., 2008).

Negative relationships between bacteria and microalgae have also been reported. Variations in chemical composition of microalgae in the presence of a bacterial community implicated that bacteria could limit the growth of microalgae by switching the stoichiometry of microalgae (Daufresne and Loreau, 2001; Danger et al., 2007). Previous studies have shown that bacteria can compete with microalgae for inorganic nutrients (Grover,

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2000). Some bacteria can inhibit the growth of algae by producing variable algicidal compounds (Nakashima et al., 2006; Sakata et al., 2011). In other studies, the ability of bacteria to degrade cell wall through cell to cell attachment has been considered as the potential cause of microalgal cells lyses (Furusawa et al., 2003; Chen et al., 2012). However, these effects have been shown to be species-specific inhibition of microalgae by bacteria (Fukami et al., 1997; Mayali and Azam, 2004). Although bacteria were implicated as inhibitory factors to microalgal growth in these studies, antibacterial metabolites or extracellular product were also found to be secreted by microalgae and could inhibit the growth of bacteria (Regunathan and Wesley, 2004; Ribalet et al., 2008).

The study of interactive mechanisms between bacteria and microalgae is expected to contribute to the enhancement of the production and quality of bait to feed, prevention of eutrophication and enable the selection of the optimal combinations of microalgae and bacteria for aquaculture. *Chlorella vulgaris* is widely used as food sources in aquaculture and has been detected as one of the dominant algal species during bloom (Spolaore et al., 2006). The major objective of this study is to examine the interaction between axenic *C. vulgaris* and commensal bacteria. We detected the effects of concentrations of bacteria (Z-QD08 and Z-QS01) and *C. vulgaris* on their interactions, identified their relationship and elucidated the mechanism involved in the interaction between *C. vulgaris* and its co-cultured bacteria.

2 Materials and methods

2.1 *Microalgal culture*

Axenic strains of *Chlorella vulgaris* Beijerink was obtained from Ocean University of China. The algae were grown in modified f/2 medium (Guillard, 1975) under 20°C with 70 μmol photons m^{-2} s^{-1} at 12 h: 12 h light: dark cycle in incubators. The initial pH and salinity of the culture medium were adjusted to be 8.0 and 30, respectively. All flasks containing the microalgae were shaken manually twice per day.

2.2 *Acquisition of bacteria*

Bacteria Z-QD08 and Z-QS01 belonged to *Pseudomonas* genera and *Bacillus*, respectively. Both strains were separated, identified, and preserved at ultra low temperature in laboratory.

2.3 *Effects of bacteria (Z-QD08 and Z-QS01) and Chlorella vulgaris concentrations on their interactions*

To test the effects of bacteria on *C. vulgaris*, 5×10^6, 10×10^6 and 20×10^6 cfu/ml of Z-QD08 or Z-QS01 suspension were added to the culture of *C. vulgaris* at the concentration of 10^6 cells/ml, respectively.

To test the effects of microalgae on bacteria, Z-QD08 or Z-QS01 suspension was added to bacteria-free cultured suspension of *C. vulgaris* with different initial concentrations, 0.5×10^6, 1×10^6 and 2×10^6 cells/ml. Triplicates were employed for each trial.

2.4 *Mechanism of the interactions between Chlorella vulgaris and co-cultured bacteria*

C. vulgaris was cultivated in f/2 medium. At the stationary growth phase, *C. vulgaris* was harvested by filtration using autoclaved membrane filters (0.22 μm). The effects of filtrates on bacteria growth were then tested. Single bacteria strain was incubated in the mixture of the filtrates and 2216E+ culture medium or 2216E medium alone. Triplicates were employed for each trial.

Filtrates from medium of bacteria cultured for 18 h were prepared by filtration on autoclaved membrane filters (0.22 μm). *C. vulgaris* was inoculated into the culture medium filtrate and f/2 + culture medium filtrate, respectively, with *Chlorella vulgaris* cultured in f/2 medium as controls. Triplicates were employed for each trial.

The initial cell density of *C. vulgaris* was set at 10^6 cells/ml.

2.5 *Counting of Chlorella vulgaris and bacteria*

Cell concentrations of *C. vulgaris* were counted by blood cell counting plate. The number of bacteria was counted by plate culture method. Relative growth rate was calculated using the method reported in previous studies (Zhou et al., 2011).

2.6 *Date statistics*

Statistical analysis (t-test) and all graphs were conducted using OriginPro7.0 software.

3 Results

3.1 *Interactions between Chlorella vulgaris and co-cultured bacteria*

The cell concentration of *C. vulgaris* increased during the culture period and reached the maximal value at Day 10. Cell concentrations of bacteria showed an increased trend at the first six days, but decreased over the rest period of the experiment (Fig. 1).

The growth of *C. vulgaris* was promoted when co-culturing with Z- QS01 (Fig. 2). The cell concentration reached the highest value at Day 8, and showed a decreased trend thereafter. The growth of bacteria Z-QS01 was suppressed strongly at the presence of *C. vulgaris*, showing a decreasing trend at the first eight days and started to grow after Day 8.

3.2 *Effects of initial concentrations on the interaction between Chlorella vulgaris and co-cultured bacteria*

Figure 3 shows the impact of bacteria initial concentrations on the growth of *C. vulgaris*. The growth of *C. vulgaris* was promoted at the bacteria initial concentration of 5×10^6 cfu/ml (y=22.733+0.582x, R=0.575). However, the growth of *C. vulgaris* was inhibited at treatments with initial bacteria densities of 10×10^6 cells/ml and 20×10^6 cfu/ml, showing negative correlations with bacteria density (10×10^6 cfu/ml: y=18.889–0.892x, R=–0.836; 20×10^6 cfu/ml: y=14.792–0.965x, R=–0. 912).

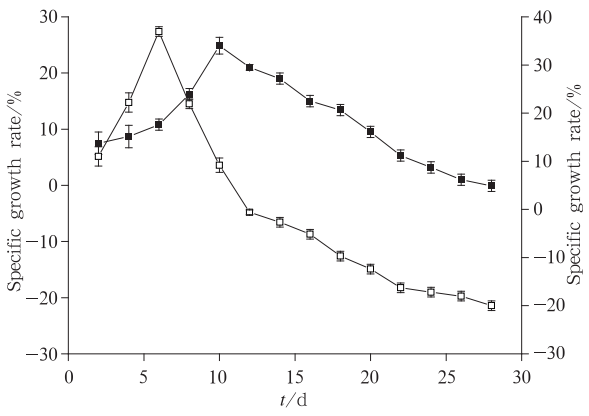

Fig.1. Variations of specific growth rate for *C.vulgaris* and co-cultured bacteria Z-QD08. ■ *C.vulgaris*, □ bacteria Z-QD08.

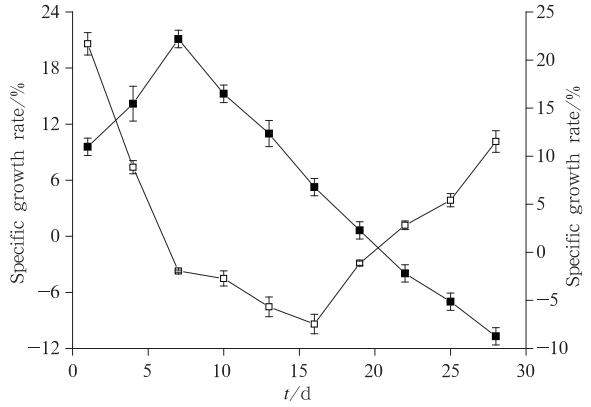

Fig.2. Variations of specific growth rate for *C.vulgaris* and co-cultured bacteria Z-QS01. ■ *C.vulgaris*, □ bacteria Z-QS01.

The growth of bacteria was suppressed with the increasing concentrations of *C. vulgaris* (Fig. 4). At the initial concentration of 0.5×10^6 cells/ml, the growth rate of bacteria decreased and reached the lowest value at Day 19. Similar trends were observed at initial inoculation concentrations of 1×10^6 cells/ml and 2×10^6 cells/ml. The growth rate decreased sharply and reached minimum on Day 12 and Day 10 respectively.

Regression analysis showed that the initial concentration of *C. vulgaris* and co-cultured bacteria had a significant relation, reflecting an underlying relationship between *C. vulgaris* and co-cultured bacteria.

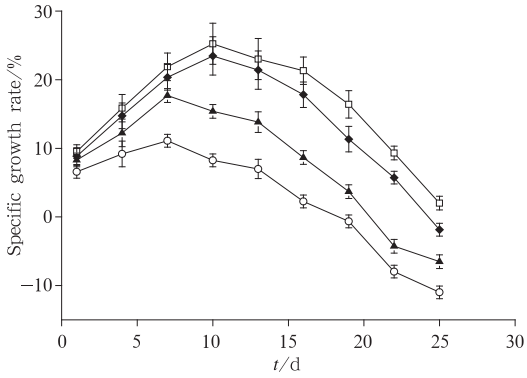

Fig.3. Effect of co-cultured bacteria on growth of *C.vulgaris* under different initial density conditions. ♦ control, □ 5×10^6, ▲ 10×10^6, ○ 10×10^6.

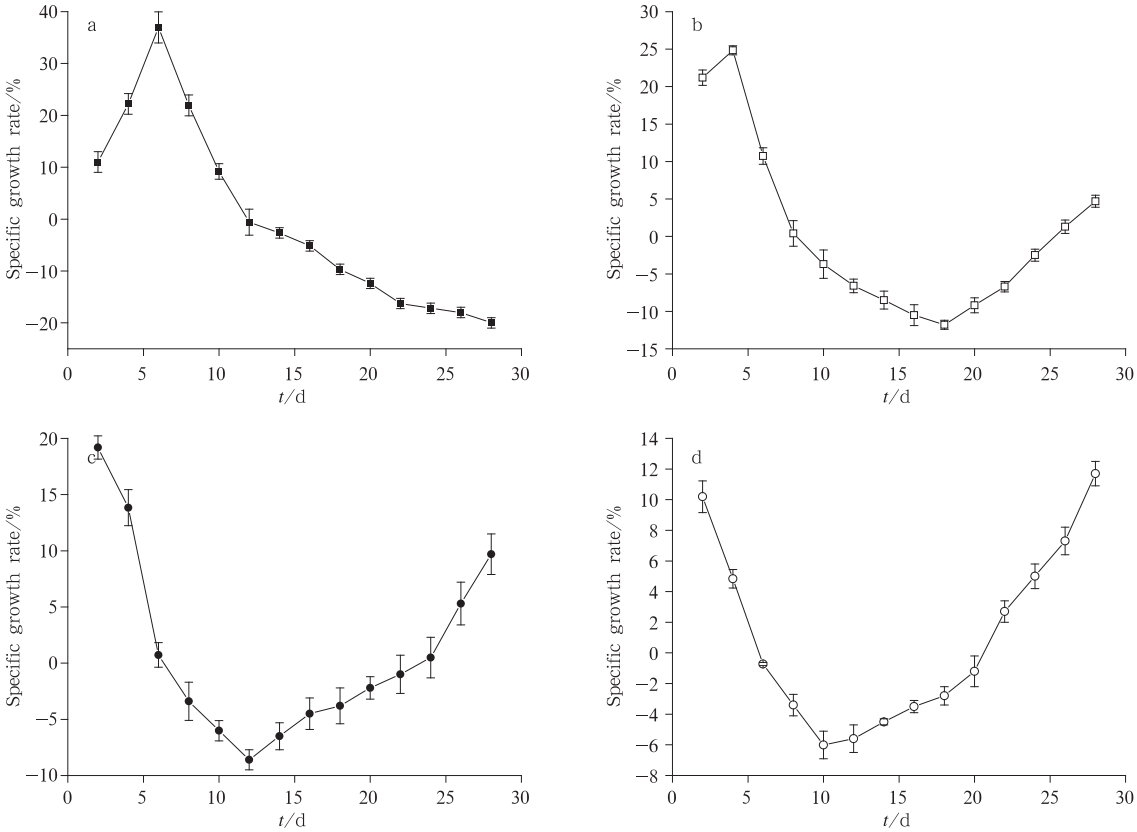

Fig.4. Effect of *C.vulgaris* on growth of co-cultured bacteria under different initial density conditions. ■ control, □ 0.5×10^6 cells/ml, • 1×10^6 cells/ml, ○ 2×10^6 cells/ml.

3.3 *Mechanism of the interaction of Chlorella vulgaris and co-cultured bacteria*

The growth of bacteria Z-QD08 was significantly inhibited with the addition of axenic culture medium from *C. vulgaris* (2216E+ filtrate of *C. vulgaris*) (Fig. 5). The lowest cell density of bacteria was observed at trials with the addition of bacteria free

culture medium of *C. vulgaris*.

The growth of bacteria Z-QS01 with the addition of 2216E and 2216E+ filtrate of *C. vulgaris* showed no significant difference, while significant suppressive effect was observed for bacteria with the addition of filtrate of *C. vulgaris*, implying that the shortage of nutrition rather than filtrate of *C. vulgaris* inhibited the growth of bacteria Z-QS01.

Significant suppression of *C. vulgaris* was observed in the presence of filtrate of bacteria Z-QD08, while no significant difference was observed at treatments of f/2 and f/2+ Z-QD08 filtrate (Fig. 7). Similar trend was observed for Z-QS01 (Fig. 8).

These results indicated that the interaction mechanism varied with different bacteria strains. The growth of bacteria Z-QD08 may be inhibited by *C. vulgaris* through interference competition, while the mechanism for interaction between bacteria Z-QS01 and *C. vulgaris* may be attributed to resource competition.

4 Discussion

Bacteria and microalgae exhibit mutual benefit relationship, in which bacteria benefit from *C. vulgaris* exudates, and microalgae growth is promoted by bacterial products such as inorganic substance, carbon dioxide and other growth factors. However, bacteria could become the parasites on algae, leading to death of algae, while algae can suppress growth of bacteria by secreting antibiotic compounds (Grossart and Simon, 2007). Moreover, competition for limiting nutrients such as phosphate can exist between bacteria and algae (Lekunberri et al., 2012).

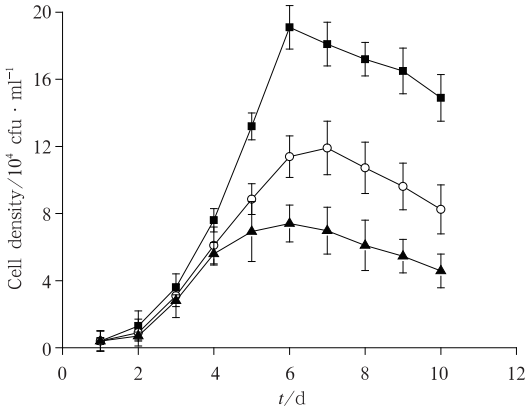

Fig.5. Effect of filtered fluid of *C.vulgaris* on growth of co-cultured bacteria Z-QD08. ■ 2216E, ○ 2216E+filtrate of *C.vulgaris*, ▲ filtrate of *C.vulgaris*.

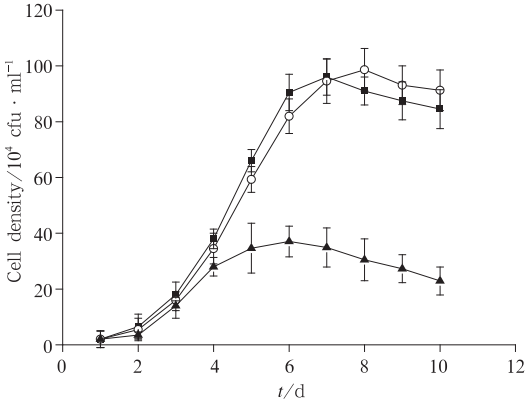

Fig.6. Effect of filtered fluid of C.vulgaris on growth of co-cultured bacteria Z-QS01. ■ 2216E, ○ 2216E+filtrate of *C.vulgaris*, ▲ filtrate of *C.vulgaris*.

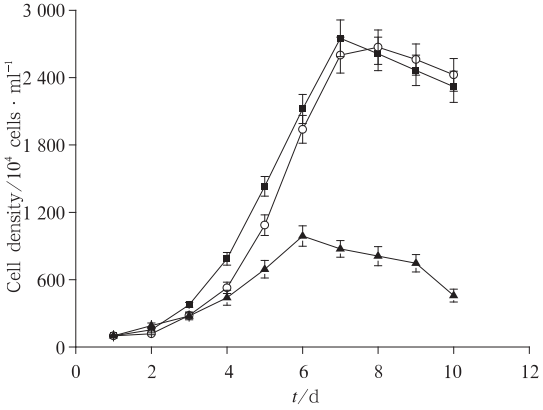

Fig.7. Effect of filtered fluid of bacteria Z-QD08 on growth of co-cultured *C.vulgaris*. ■ f/2, ○ f/2+filtrate of Z-QD08, ▲ filtrate of Z-QD08.

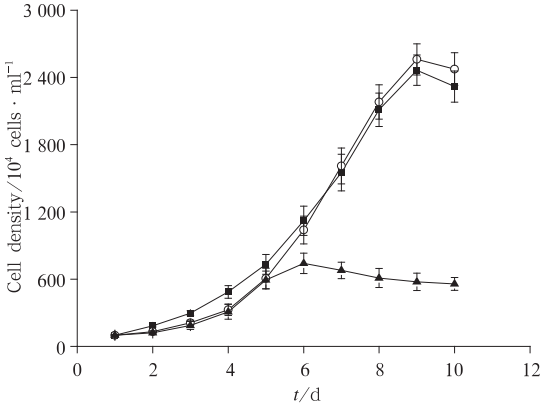

Fig.8. Effect of filtered fluid of bacteria Z-QS01 on growth of co-cultured *C.vulgaris*. ■ f/2, ○ f/2+filtrate of Z-QS01, ▲ filtrate of Z-QS01.

The growth rate of bacteria increased when co-culturing with *C. vulgaris* (Figs 2 and 4), which may be linked to the organic matter released from *C. vulgaris* and the nutrient composition of f/2 culture medium. Bacteria growth may be promoted due to the organic nutrients in seawater of culture medium. Quantity of organic matter varied over the growth of *C. vulgaris*. The content of organic matters was low at the logarithmic growth phase and increased quickly from the stationary phase to the decline phase, which may be attributed to the peak value of growth rate for bacteria. The inhibition effects of *C. vulgaris* on bacteria growth could be due to substance secreted by *C. vulgaris* at logarithmic phase, or the quick growth of *C. vulgaris* (DellaGreca et al., 2010).

The mechanisms of interactions between bacteria and algae varied in the previous studies. It has been shown that the inhibition of alga growth could be due to (1) production of antibiotic substances by the bacteria (Rooney-Varga et al., 2005), (2) synthesis of ectoenzymes (Zoppini et al., 2005), and (3) competition for inorganic matters (Trabelsia and Rassoulzadegana, 2011). Some substances released from bacteria could regulate

the growth of *C. vulgaris*. The growth of *C. vulgaris* was stimulated at low cell density of bacteria, but inhibited at a relative high density of bacteria. This mode of action is likely to be a result of growth hormone (GH) (Spoerner et al., 2012). However, such action varied among species. *Flavobacterium* sp. exhibited a strong inhibition on *Gymnodinium catenatum*, while the effects of inhibition on *Chattonella marina, Heterosigma akashiwo* and *Skeletonema acostatum* were not significant (Lovejoy et al., 1998). In the present study, the inhibition of bacteria Z-QD08 by *C. vulgaris* was caused by substances released from *C. vulgaris*. The growth of bacteria Z-QS01 was suppressed because of the interference of resource competition. The addition of bacteria Z-QS01 resulted in the growth inhibition of *C. vulgaris*, which may be attributed to the substances produced by bacteria. *Pseudomonas and Bacillus* can secrete some toxic substances into the culture medium, which can inhibit the growth of *C. vulgaris* by breaking respiratory chain or inhibiting cell wall synthesis (Vatsa et al., 2010; Zhao et al., 2012).

As shown in Figs 3 and 4, the growth rate of *C. vulgari* increased at relatively low initial densities of bacteria. The differences can be explained by the fact that different inoculation densities can form a special microenvironment of the algae and bacteria, within which substances released from species, such as carbohydrate, amino acid or toxic matters, influences the growth of species groups. In the present study, the high initial densities of bacteria led to the decrease of *C. vulgaris*, while opposite results were observed when the initial density of bacteria was low. A similar pattern was observed for growth of bacteria at treatments with different initial densities of *C. vulgaris*.

Table 1. Statistics results of effect of *C.vulgaris* on growth of co-cultured bacteria

Treatments with different concentration of *C.vulgaris*/cells·ml^{-1}	Regression equation	Coefficient of correlation (R^2)
5×10^6	Y=120.23+128.5$^{[-2\times(\frac{x-15.35}{41.14})^2]}$	–0.894
10×10^6	Y=71.6+83.02$^{[-2\times(\frac{x-17.92}{28.34})^2]}$	–0.932
20×10^6	Y=41.99+46.89$^{[-2\times(\frac{x-12.95}{31.67})^2]}$	–0.983

5 Conclusions

The growth of *C. vulgaris* was promoted at low concentrations of bacteria (5×10^6 cells/ml), showing a positive correlation with the bacteria density. The opposite trend was observed for treatments with high bacteria density (10×10^6 cells/ml and 20×10^6 cells/ml). The growth of bacteria decreased with the increasing concentrations of *C. vulgaris*.

The growth of bacteria Z-QD08 was inhibited by *C. vulgaris* through interference competition, while the mechanism for interaction between bacteria Z-QS01 and *C. vulgaris* was resource competition.

These results confirmed some elements of published theory on interactions between heterotrophic bacteria and microalgae, and suggest that heterotrophic bacteria play an important role in the development of blooms in natural waters.

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