# Interaction between *Rhodobacter sphaeroides* and Harmful Algal Bloom causing dinoflagellate *Amphidinium carterae*

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**Abstract**

The present study hopes to develop a further understanding regarding algal-bacteria interactions as an option for bioremediation. After the use of chemicals against the events of red tide proved to be impractical due to its detrimental effects on the aquatic ecosystem, recent studies focused on bioremediation. To examine the algal-bacteria interactions, an *in vitro* co-culture system consisting of marine bacteria *Rhodobacter sphaeroides* and HAB-causing dinoflagellate *Amphidinium carterae* were used as the model organisms. The population count of each species in the co-culture were monitored for 14 days as well as positive (pure *A. carterae*) control and negative (pure *Rb. sphaeroides*) control. The co-culture system used for investigating the interactions was established based on the interdependence of the two organisms and later the bacteria would prevail over the dinoflagellate. The results of the population count shows an inverse progression on the growth between the two organisms. Furthermore, the analysis of the population trend suggests that the bacteria growth was suppressed due to allelopathic interactions by the dinoflagellate specifically the production of toxins and chemical compounds detrimental to the growth of the bacteria proving that the bacteria was ineffective to mitigate the growth of the dinoflagellate.

***Keywords:*** *Bioremediation, mitigate, co-culture, dinoflagellate, harmful algal blooms*

**Introduction** In the Philippines, numerous algal blooming activities have been reported in areas around Manila Bay starting from the year 1988 until 2000 [1] and today, several coastal waters around the Philippines are infected by harmful algal blooms. The Bureau of Fisheries and Aquatic resources collects water samples and offers timely reports on the occurrence of these blooms. Harmful algal blooms (HABs) is the term used for the proliferation of algae, dinoflagellates, diatoms etc. that has adverse effects in the environment [2].

The occurrence of HABs has been causing fish kills and intoxicating water sources that could lead to health problems in humans and animals that could come in contact. To date, there are recent research focusing on the nature of dinoflagellates and how to control these organisms. However, these organisms are known for their unpredictable nature and their ability to adapt to their environment [3].

Researchers have formulated solutions that could kill red-tide causing dinoflagellates using various chemical compounds. One method used is by dispersing yellow clay which flocculates the dinoflagellate cells causing them to sink in the bottom [4]. Another method is the use of strong oxidant such as ozone. In the study conducted by Senco MR, Ozone was incorporated in the dinoflagellate culture and killed all of the dinoflagellate cells after a 10-minute exposure [5]. Although proven to be effective, these compounds threaten organisms inhabiting the same environment. Because of its lack of practicality, researchers proposed studies on bioremediation as a control mechanism against HAB [6].

The use of microorganisms such as marine bacteria proved to be successful in mitigating the growth of red-tide causing dinoflagellate. In a study conducted by Wagner et.al, the researchers co-cultivated a marine bacteria, *Dinoroseobacter shibae* and HAB-causing dinoflagellate, *Prorocentrum minimum* and observed the interactions between the two species. It was observed that the two species entered from a mutualistic to a parasitic relationship. The *D. shibae* became an endosymbiont to the P minimum and produces vitamin $B_{12}$ for the growth of the dinoflagellate and in return the *P. minimum* produces compounds and minerals for the bacteria. After 24 days the dinoflagellate population starts depleting while the bacteria population continues to grow [7].

This shows that during the first phase of the experiment, the bacteria and dinoflagellate degrades compounds such as DMSP and PHA as well as the production of vitamins and minerals that are beneficial for one another, however as soon as the bacteria cells multiplied exponentially compared to the dinoflagellate, it later caused the breakdown of the dinoflagellate cells from the inside, thus cell lysis [7].

*Rhodobacter sphaeroides*, a gram negative prokaryote that is also photosynthetic, which means it can convert light energy into chemical energy; these

bacteria are commonly found in open waters such as seas and oceans as well as aquatic sediments like ponds and lakes. This photosynthetic bacteria is also adaptive to its environment able to switch from fresh water source and salt water source. The bacteria is also a supplementary source of nutrients and proteins able to enhance its own biomass production as well as able to boost its cell growth and [14] *Amphidinium carterae* is one of the most frequent harmful algae that can cause large damages to coastal zones. It is known to be one of the toxin-producing species of the *Amphidinium* family. It is commonly found in Southeast Asian countries such as china, japan and Philippines. *A. carterae* is known for its bioactive compounds and substances such as cytotoxic macrolides, amphidinols, and amphidinolides, these compounds are relatively harm to the environment and has toxic effects on the organism that ingest the dinoflagellate [16].

Similarly this research also suggest in co-culture a marine bacteria, *Rhodobacter sphaeroides,* and the common HAB-causing dinoflagellate species *Amphidinium carterae.* Characterized as an adaptable bacteria, past studies stated that *Rb. sphaeroides* is very efficient and requires minimal conditions for growth [15] is used as a bacterial symbiont to check if *Rb. sphaeroides* can be used as a biological control against *Amphidinium carterae.* By monitoring the population counts of *Rb .sphaeroides* and *A.carterae.*

The objective of this study is to determine the population dynamics between the pure *Rhodobacter sphaeroides* and pure *Amphidinium carterae* cultures, and the co-culture of both organisms. It specifically aims to:

(i) Count the population (cell/ml) in every 48 hours for two weeks of:
*a). Amphidinium carterae* in vitro
*b). Rhodobacter sphaeroides* in vitro
*c). Amphidinium carterae* co-cultured with *Rhodobacter sphaeroides* in vitro

(ii) Compute the growth rate for two weeks of:
*a). Amphidinium carterae* in vitro
*b). Rhodobacter sphaeroides* in vitro
*c). Amphidinium carterae* co-cultured with *Rhodobacter sphaeroides* in vitro

(iii) Compare the growth for two weeks of
*a). Amphidinium carterae* in vitro
*b). Rhodobacter sphaeroides* in vitro
*c). Amphidinium carterae* co-cultured with *Rhodobacter sphaeroides* in vitro

(iv) Determine the relationship between *Amphidinium carterae* and *Rhodobacter sphaeroides.*

**Methods** The study took place in an in vitro setting and would only serve as a model and not the environment itself. Specifically, the study took place in the phycology laboratory of SEAFDEC/AQD, Tigbauan, Iloilo lasted for 14 days. In order to determine the interaction between marine bacteria, *Rhodobacter sphaeroides* co-cultured with HAB-causing dinoflagellate *Amphidinium carterae,* the relationship between the two organisms was determined by analyzing the population trend of the different setups. The population of each setup was counted every 48 hours for 2 weeks and the growth trend was established. The population trend will determine the interactions occurring between the two organisms and the nature of their relationship.

*Algal culture.* The *Amphidinium carterae* samples were acquired in the stock culture of the phycology department in SEAFDEC, Tigbauan, Iloilo City. For optimal growth and propagation, the *Ampidnium carterae* was pre cultured in the F-medium that used by SEAFDEC as their media for algal cultures [9]. The F-medium is composed of 500 ml of ozonated seawater, major nutrients such as $NaNO_3$, $NaH_2PO_4$, $Na_2SiO_3$and trace elements such as $NaNO_3$, $Na_2$ EDTA, Vitamin Stock, and Trace Metal. The medium was mixed and put inside 500 ml dextrose bottles. After acquiring a count of $1.20x10^6$ cells/ml, 4.17 ml was transferred into three dextrose bottles in order to obtain a final count of $1.00x10^4$ cells/ml. For illumination, the cultures were kept in shelves of laboratory where light was produced by a cool-white daylight fluorescent tubes.

*Bacterial culture.* The bacteria *Rhodobacter sphaeroides* samples were acquired at UPV Miag-ao, College of Fisheries and Ocean Sciences through Prof. Sharon N. Nunal who has been working on with the species in her research.The bacterium *Rhodobacter sphaeroides* was pre-cultured in Nutrient agar ($Na^-$) and incubated for 48 hours in room temperature. 0.2 mg of the bacteria was transferred into a new plate with nutrient agar and incubated again for 48 hours and counted the population afterwards. After acquiring a count of $1.00x10^{12}$ cfu/ml, the method was repeated but the bacteria was diluted five times before spread-plating into the new plate in order to obtain a count of $1.00x10^7$ cfu/ml. The diluted bacteria was then transferred into three 500 ml dextrose bottles.

*Algae and Bacteria Co-culture.* eparate cultures were prepared for the co-culturing of these organisms. The same method was used in order to acquire the needed population for each species. As soon as the dinoflagellate reaches cells/mL 1.00x104 and bacteria reaches 1.00x107 cells/mL. This was determined through counting the population of each samples, dinoflagellate through hemocytometer [9] and spread plate method for bacteria, and diluting the media to achieve the desired population density. Both samples were co-cultured to one another and was transferred in a 500mL dextrose bottle with F - medium however the setup did not contain vitamine. Then will be placed in a well-lit area equipped with aerators.

*Sampling.* The set-ups were monitored every 48 hours in 2 weeks and the population for each species was counted during the duration.In counting the *Amphidnium carterae,* the researchers used a hemocytometer. 1 ml of the sample was acquired

from the control and co-cultured set-ups and placed in the hemacytometer to be counted [9]. As for the *Rhodobacter sphaeroides*, 1ml of the sample acquired from the controlled and co-cultured set-up was used to count the cells per day. After acquiring the 1 ml of sample, it will be diluted until six times. Then, using the spread-plate method in order to count the bacteria cells. The 1ml sample will be plated into the media composed of nutrient agar and after waiting another 72-96 hours for incubation, we counted the colonies formed in the media representing the population of the bacteria in CFU or colony forming units.

$$\text{Algal Population (cells/ml)} = \frac{\text{Total count/number of blocks counted}}{1.00 \times 10^4 \text{ cells/ml}}$$

*Growth Rate Computation.* The growth rate (log) of the *Amphidinium carterae* and *Rhodobacter sphaeroides* in pure and in co-culture was calculated using the following formula:

$$\mu = \frac{(\ln(\text{Final population count}) - \ln(\text{Initial count}))}{(\text{Time final}) - (\text{Time initial})}$$

*Safety Procedure.* The liquid content of the setup containing the dinoflagellate was poured into a container with 10mg chlorine. The same method was used for the disposal of the solid materials but in a separate chlorine container. Chlorine ensures the eradication of the dinoflagellate present in the setup. The solid materials were then rinsed with tap water and finally air-dried for a period of three to four days. After three to four days, the waste liquid was then disposed into the water drainage system of SEAFDEC/AQD as per their protocol.

**Results and Discussion.** The population trend of the different species was observed in each set-up (see Figure 1), in the *Amphidinium carterae* pure culture, it exhibited an overall increase in its population with no signs of death phase, as depicted from the consisted positive trend from day 1 until day 13. The *Rhodobacter sphaeroides* pure culture initial count started at 6.00 x $10^5$ cfu/mL and then directly entered death phase. As time progressed, there are no signs indicating growth and the population count steadily decreased, reaching a population count of 0 cfu/mL by day 11.

The *Amphidinium carterae* in the co-culture also display a similar trend in its growth likewise with the pure culture. However, after reaching its peak by day 7, the species later entered death phase, reaching a count of 0 by day 11. On the other hand, the *Rhodobacter sphaeroides co-culture* started with an initial count of approximately 5.00 x $10^3$ cfu/mL which also directly entered its death phase similar to the pure culture but by day 5, there is a slight increase in the population count until day 11 but then reached a count of 0 cells/mL by the end of the experiment.

**Table 1.** Specific Growth Rate of *Rhodobacter sphaeroides* and *Amphidinium carterae* in pure and co-cultures (log population/days)

| Setup | Specific Growth Rate |
|---|---|
| *Rhodobacter sphaeroides* in pure culture | -1.157 |
| *Rhodobacter sphaeroides* in co-culture | -1.276 |
| *Amphidinium carterae* in pure culture | 0.5008 |
| *Amphidinium carterae* in co-culture | 0.2723 |

The population counts of each sample of the dinoflagellate, *A. carterae,* in two different setups have different values but show a similar trend across the graph. The specific growth rate of was computed for each species during their log phase showing the nature of their trend during the peak of their growth. The *A. carterae* in the pure culture shows a greater increase in population size compared to the co-culture, as also observed in the graph. Each had an initial population of 1.00x$10^4$ cells/ml and gradually increased overtime until day 7 wherein the species in the co-culture started to decrease.

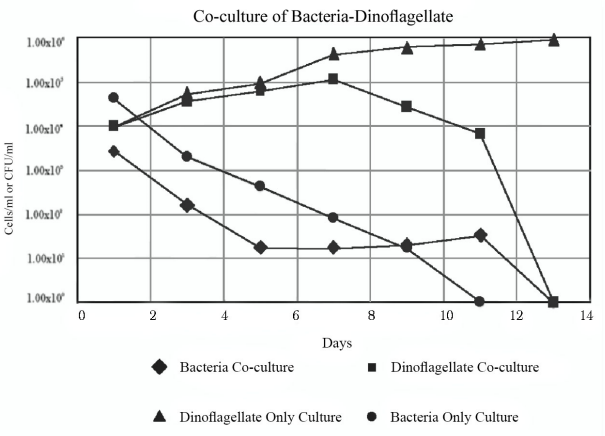


**Figure 1**. Population count of the different treatments in the experiment.

For the bacteria, it is shown that the *Rb. sphaeroides* in the co-culture shows a greater decrease in population compared to that of the pure culture, it is also observed in the graph that the bacteria exhibited a similar trend with each other. Both differ in the initial count however express a similar trend with one another. The bacteria in both setups gradually decreased in population, however in the co-culture the bacteria appeared to grow. Nevertheless, both arrived at a population count of 0 by the end of the duration.

The system used to analyze the interaction between the *Amphidnium carterae* and *Rhodobacter sphaeroides* co-culture was based on the relationship wherein the *Rb. sphaeroides* synthesizes vitamin $B_{12}$ as an essential nutrient for the growth of the *A. carterae* and in return the *A. carterae* produces vitamins that are beneficial for the bacteria [7]. A medium without vitamin $B_{12}$ was used in the co-culture to establish that the dinoflagellate relied on the bacteria for the vitamin $B_{12}$.

The pure culture of the *Amphidinium carterae* showed continuous increase in growth throughout the experiment. The pure culture of the dinoflagellate reached its exponential growth by day 5 to day 6 with the sudden increase in population thereafter. Without the presence of other organisms in the setup, the *A. carterae* population increases. This is due to the compatibility of the dinoflagellate to propagate in the F-medium.

As observed in the pure culture of the *Rb. sphaeroides*, the population count of the species started to drop from day 1. This setup was assigned as the negative control of the experiment to establish that the organism must rely on the presence of the dinoflagellate in its environment. The medium used for the experiment was only favorable for the growth of the *A. carterae*, thus expecting that the bacteria will continue to decline without the presence of the dinoflagellate.

In the first five days of the experiment, the *A. carterae* in the co-culture consistently grew, similar with the pure culture. Even with the absence of vitamin $B_{12}$ in the system, the *A. carterae* was able to grow due to the presence of the *Rb. sphaeroides*, the relationship between the two organisms is also similar with a previous study by Wagner-Dobler et.al [7]. However, in the present study, only the *A. carterae* benefits from the interaction between the species as the population of the *Rb. sphaeroides* decreases in time. An inverse progression is observed in the growth between the species suggesting that while the *A. carterae* benefits from the *Rb. sphaeroides*, the dinoflagellate kills the bacteria in the process.

As observed from another previous study conducted by Mandal et.al, the growth of the bacteria may be suppressed due to the production of toxins such as hemolysin by the *A. carterae* resulting to allelopathy [10]. The presence of bacteria triggered the *A. carterae* to produce hemolysin as a defense mechanism against foreign organisms [10][11]. Also, it has been observed in the study of Mandal et.al, that the *A. carterae* induced the growth of the bacteria by the production of Extracellular polymeric substances (EPS) but in some cases, the presence of bacteria triggers the dinoflagellate to release toxic compounds that are detrimental to the growth of the bacteria [10].

By day 7 the *A. carterae* population in the co-culture started to decrease continuously, reaching a population count of 0 cells/ml by day 13. This may be due to the depletion of nutrients in the media, because the water in the setup was not replaced throughout the duration of the experiment and the F - medium was only added once at the start of the experiment. The nutrients that are needed by the dinoflagellate are already used up and without the presence of the bacteria there will be no synthesis of vitamin $B_{12}$ that could support the growth of the dinoflagellate. Eventually, the population count of both species reached 0.

*Error analysis.* The study expected some error in the remaining duration of the experiment. A sampling error could have been a cause in some of the population counts that does not reflect a accurate count in the population of both species. Contamination was also present after 5 days of the experiment where a presence of Filimentos bluegreen was seen. It can be the cause of a single aeration airway present in the outdoor laboratory of Seafdec meaning that the aeration present in the sampling bottles were all connected to other sources that the Seafdec was also using causing the contamination. Another is the lack of sterile environment that could have also affected the experiment because of the location where there is a lack of quarantined surroundings and controlled area. Temperature in the outside laboratory may also have affected the growth of the cultures because there were no air condition present in the outdoor laboratory or any temperature controlling machine. The light source may also affect the outcome of the experiment due to the changing positions of the sun, sunlight may differ per day of the testing.

**Conclusion.** *Amphidinium carterae* inhibited the growth of the bacteria, *Rhodobacter sphaeroides,* along with the other factors that affected it in an *in vitro* setting. Because of the mechanism, an allelopathic interaction triggered by the dinoflagellate have caused the decrease in the population of the *Rb. sphaeroides*. As depicted in the graph, this follows an inverse relationship between the two organisms where the dinoflagellate is the more dominant species in the co-culture. Therefore, it could be concluded that in the present study, the interaction of the *A. carterae* towards the bacteria was antagonistic in nature while the *Rb. sphaeroides* was beneficial to the growth of the dinoflagellate. Furthermore, not only that the results of this study is contrary to the expected outcome, it also proved that the bacteria is unable to mitigate the growth of the dinoflagellate.

**Recommendations.** The present study would yield more accurate results if conducted again using more advanced equipment for counting the population in real time rather than using traditional methods, such as the use of spread plate method that may affect the results, in which takes time and might be affected by external factors when performing the said technique.

Also, it is advised that working in a more sterile environment be done to decrease contamination. Another is the re-supplementation of the bacteria culture to increase the survival rate of bacteria in an algal medium since it is found that the bacteria cannot survive in the medium used in the experiment due to the properties possessed by *Amphidinium carterae*.

To further determine the possible interactions of the bacteria with the dinoflagellate, it is recommended to use a similar mixture of media to all the cultures used in the study. Due to the removal of vitamin $B_{12}$ in the co-culture, it is advised that the removal of vitamin $B_{12}$ will also be applied to all cultures. This is advised in order to determine the behavior of the dinoflagellate only cultures and bacteria only cultures with the absence of vitamin $B_{12}$. The following results will then be compared in order to determine whether the bacteria has successfully supplemented vitamin $B_{12}$ to the dinoflagellate.

Furthermore, it is recommended to increase the sampling size so as to decrease sampling errors in the counting of bacteria cultures. Focus more on the interaction between *Rhodobacter sphaeroides* and *Amphidinium carterae* on a molecular level could give more data on proving the properties possessed by the dinoflagellate and add more detailed explanation of the activities found on the graph.

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