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Utilization of Photobacterium Phosphoreum as an Indicator of Eutrophication of Seawater

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Abstract -Recently eutrophication has become a serious environmental concern in aquatic ecosystems. Because it is too technical and time-consuming to detect symptoms of eutrophication before the 'red tide', we considered the utilization of the luminous bacteria Photobacterium phosphoreum in detecting eutrophication at an early stage by incorporating the bacteria's bioluminescence and sensitivity to the surrounding environment. We tested the sensitivity of the bacteria by applying various toxin chemicals on it and confirmed its chemotaxis behavior. Hypothesizing that Photobacterium phosphoreum would show detectable changes in response to sensitive changes of concentrations of phosphate and nitrates, we conducted several experiments. By placing Photobacterium phosphoreum in varying conditions of nitrates, which are the main components of eutrophication conditions, we were able to observe reduction in its growth in increased level of phosphate and nitrate, confirming its viability. In addition, the change in luminosity of the bacteria in response to the different levels of phosphate and nitrate was also a clear indicator of changing environments close to levels of eutrophication. Thus, we were suggest Photobacterium phosphoreum can be an effective method in detecting eutrophication with much less technicality, which will allow us to resolve eutrophication issues at an earlier stage.

Keywords : Photobacterium phosphoreum (P.phosphoreum), eutrophication, chemotaxis, bioluminescence

I. INTRODUCTION

In the past few decades, eutrophication, a response of ecosystem to an addition of natural or artificial substances such as nitrate and phosphate, has risen to a serious environmental concern all around the world, causing much damage to fish population [1]. Although eutrophication is easy to detect after it has begun due to the "red tide," method to detect it before it proceeds have been too technical and time-consuming. Thus, it is necessary to develop a method that can reduce the time and technicality problem of existing methods.In this study, we consider the utilization of the luminous bacteria *Photobacterium phosphoreum* as a method of detecting eutrophication at early stage. *Photobacterium phosphoreum* has bioluminescence that is directly affected by the growth and survival of bacteria [2]. Thus, *Photobacterium phosphoreum* growth can easily be detected by the decrease in the luminosity that the bacteria produces. Additionally, *Photobacterium phosphoreum* is known to be very sensitive to the external environment [2]. Previous researches show that growth of *Photobacterium phosphoreum* is drastically changed by addition of toxic chemicals [3]. Similar bacteria, *Vibrio fischeri* has been shown to have positive chemotaxis to different concentrations of sugars and amino acids [4]. Thus, we hypothesized that *Photobacterium phosphoreum* will also show sensitive changes to concentrations of phosphate and nitrate, major chemicals that cause eutrophication. This sensitive change can then be visualized by the change in bioluminescence that the bacteria produce. To confirm this hypothesis, this study first confirms sensitivity of *Photobacterium phosphoreum* to the surrounding environment by showing its positive and negative chemotaxis. Then, we test the growth and luminosity change of *Photobacterium* to different concentrations of phosphate and nitrate. Thus, through this study we provide an effective method of detecting eutrophication through visible evidence utilizing *Photobacterium phosphoreum*, with much less technicality.

II. MATERIALS AND METHODS

The bacteria used for this research, *Photobacterium phosphoreum*, was acquired from Korean Collection of Type Cultures (KCTC). The bacteria was cultured in the LB broth (tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L). The LB liquid broth was made by dissolving LB broth powder in distilled water, used after cooling it for 15 minutes after sterilization in 120℃ 1.5atm. The LB-agar broth was also made by dissolving LB agar powder in distilled water and sterilizing it in the same condition as the LB liquid broth was sterilized, cooled to about 60℃ and poured on the cultivation plate, solidifying it before use. The same process was used for culturing bacteria in NB broth (0.5% Peptone, 0.3% beef extract/yeast extract, 0.5% NaCl) and R2A broth (Proteose peptone 0.5g/L, Casamino acids 0.5g/L, Dextrose 0.5g/L, Solube starch 0.5g/L, Dipotassium phosphate 0.3g/L, Magnesium sulfate 7H2O 0.05g/L, Sodium pyruvate, 0.3g/L).

Photobacterium phosphoreum was cultured in the shaking incubator when the bacteria was cultured in the liquid broth, and was cultured in the incubator when the bacteria was cultivated in the agar-solid broth, both in temperatures 25-28C. Luminosity of *Photobacterium phosphoreum* was confirmed after positioning *Photobacterium phosphoreum* made by streaking on agar broth in a dark space.

A. Chemotaxis Test

Two types of assay was used to test the chemotaxis of *Photobacterium phosphoreum*: soft agar method for positive chemotaxis and capillary method for negative chemotaxis. The methods are described below.

1) Soft Agar Method

TB-SW (1% tryptone, 0.88% NaCl, 0.62% MgSO4, 0.072% CaCl2, 0.038% KCl) or TN broths (1% tryptone, 0.5% NaCl) with 0.2% agar was melted with a microwave, poured on a dish/plate, and cooled. When making the culture dish, culture medium was heated with a microwave and cooled, and the substance was added before it was fully solidified. This medium was poured into a petri dish or a 6 - well plate. We poured 25ml in the dish, and 4ml in each plate of the 6well plate. Later, we inoculated 20µl of the bacteria the middle of the agar and compared how much it diffused after 8 hours [5].

2) Capillary Method

Photobacterium phosphoreum was inoculated on a TB-SW broth and cultured inside a shaking incubator. After scorching and closing one end of a radius 0.2mm capillary with the flames of an alcohol lamp and heating it slightly again with the flames, the other open end of the capillary was placed in the solutions with the dissolved substances, enabling the solutions to be sucked inside the capillary. *Photobacterium phosphoreum* broth solution was centrifuged and the remaining *Photobacterium phosphoreum* cells was added in buffer solution. 300 µl of this solution was placed in each 1.5ml tubes. Capillaries with different substances in them was inoculated in each of the filled tubes. After an hour, capillary was taken out and cleansed with sterilized distilled water, smashed open the closed end of it, and put in the solution inside in the capillary in 1.5ml tubes with 1ml of distilled water already present. 50 µl of bacteria solution in the capillary that was diluted in 1ml distilled water was inoculated on the LB agar broth, and the number of colonies was compared after 24 hours [5].

B. Luminosity and Growth Test

To test the change in growth and luminosity of *Photobacterium phosphoreum* due to phosphate and nitrate the following method was used [6].

1) General Procedure

a) Mix NaCl and MgCl¬2 to make 35‰ saline solution
b) Inoculate *Photobacterium phosphoreum* in 5ml liquid NB and culture for 12 hours at 25℃
c) After culture is done, centrifuge (3000rpm) 1ml of *Photobacterium phosphoreum* broth solution and leave only the bacteria
d) Add solution from a) in *Photobacterium phosphoreum* from c) and mix well
e) Add designated concentration of phosphate and nitrate in solution from d) and place at 25℃for1 hour. Culture each solution in solid NA broth and photograph the result in a dark room.

The designated concentrations of phosphate and nitrates were varied in half-way conditions having the reference concentrations as that of the aquatic environment when eutrophication actually occurs in the water. (Phosphate 0.02mg/L, nitrates 0.2mg/L.)

III. EXPERIMENT RESULTS

A. Chemotaxis Experiment

1) Positive Chemotaxis (Soft Agar)

We first made two kinds of soft agar cultivation dishes; one with nutrients added to the TB-SW broth and another without the nutrients added to the TB-SW broth. The cultivation dishes were inoculated with *Photobacterium phosphoreum* and afterwards, resulting motions were examined.

Serine(2mM), an amino acid, and glucose(1%) was added in the broth and the bacteria was inoculated at the center of the agar dish, we were able to observe that the radius of diffusion was smaller than that of the cultivation dish without serine or glucose (figure x). This is because the bacteria consumes the serine or glucose, the serine or glucose in the broth diffuses toward the center where the bacteria is located, and the bacteria itself moves inwards. Thus, we can say that in *Photobacterium phosphoreum*, Positive Chemotaxis is observed toward serine and glucose. It was originally known that serine and glucose are attractants to both E.Coli and Vibrio Fischeri, and we were able to suggest that *Photobacterium phosphoreum* might share similar qualities in this aspect.

Meanwhile, we found out that with fructose and sugar, the experimental results were not much different with that of the control group. Such results of *Photobacterium phosphoreum* were blatantly different with the features of *Vibrio Fischeri*, which showed chemotaxis with fructose and

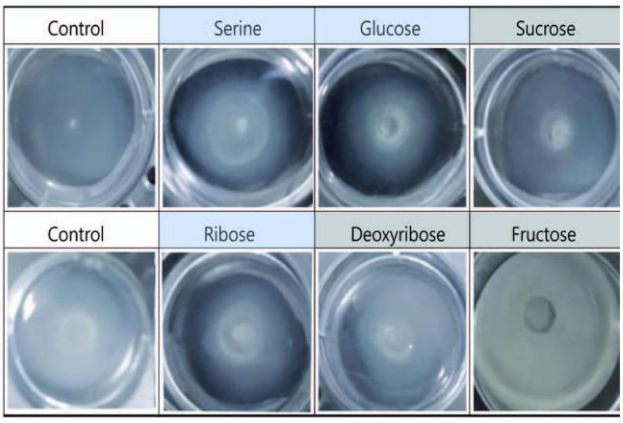

Figure 1. Positive chemotaxis shown in six-well plate in soft agar method *(3.1.1.)*

sugar, as well as serine and glucose. *Photobacterium phosphoreum* also showed chemotaxis to ribose and did not show any sort of taxis with deoxyribose, which was quite common with chemotaxis features of *Vibrio Fischeri.*

2) Negative Chemotaxis (Capillary)

Soft agar diffusion method can be only used to substances that the bacteria can consume. For other substances such as metal ions, there needs to be an alternative method of testing chemotaxis and one that is generally used is the capillary test. The capillary test is performed by placing the substance inside a very thin capillary and placing the capillary into the bacterial solution itself. If the bacteria shows positive chemotaxis toward the substance then the bacteria will move into the capillary over time and if the bacteria shows negative chemotaxis toward the substance it will move away from the capillary. Chemotaxis is confirmed by observing how many colonies of bacteria are present inside the capillary.

In this study, we examined chemotaxis of *Photobacterium phosphoreum* toward metals that prevents the illumination of *Photobacterium phosphoreum.* Selenium and Chrome are two substances that suppress the illumination of the bacteria, and this mechanism is actually utilized in real life to monitor content of Selenium or Chrome in toxic substances. We initially estimated that *Photobacterium phosphoreum* will show negative chemotaxis toward these toxic chemicals, placed 10mM of SeO2, K2CrO4 inside each capillaries and examined the movement of *Photobacterium phosphoreum*. Positioning the capillaries inside the bacterial solution for an hour and letting the bacteria enter the capillary, we diluted the bacteria inside the capillary and cultivated them on the LB broth. As a result, in comparison with the reference case (chemotaxis buffer solution), bacteria extracted from capillaries with Selenium and Chrome showed less number of colonies, suggesting a negative chemo-attraction.

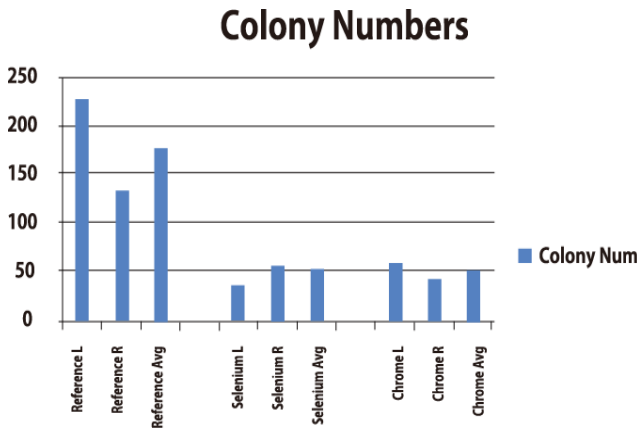

Figure 2. Colony number in plate where each of toxic chemical was added (3.1.2.)

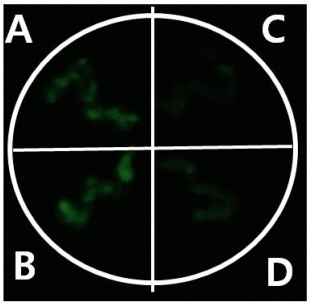

Figure 3. *Photobacterium phosphoreum* culture in types of seawater

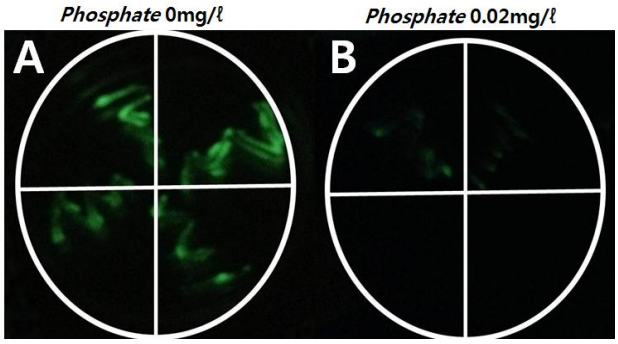

Figure 4. Effect of phosphate on Photobacterium phosphoreum culture (3.2.2.)

B. Luminosity and Growth Test

1) Confirmation of survival of *Photobacterium phosphoreum* culture in various types of seawater.

In this experiment, the bacteria was cultured in four different fresh seawater acquired from sushi restaurants that day (A, B, C, and D). By inoculating *Photobacterium phosphoreum* in the 4 kinds of acquired seawater in A, B, C, and D, we were able to confirm that *Photobacterium phosphoreum* grows well in seawater from A, B, and D, but not in seawater from C.

2) The effect of phosphate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

Photobacterium phosphoreum in the *Photobacterium phosphoreum* culture without phosphate showed normal growth, indicated by the clear luminosity (A). However, *Photobacterium phosphoreum* in the culture with 0.02mg/ℓof phosphate, condition of eutrophication, did not show survival of *Photobacterium phosphoreum* (B). This result shows that *Photobacterium phosphoreum* is unable to grow in phosphate level that indicates condition for eutrophication (0.02mg/ℓ).

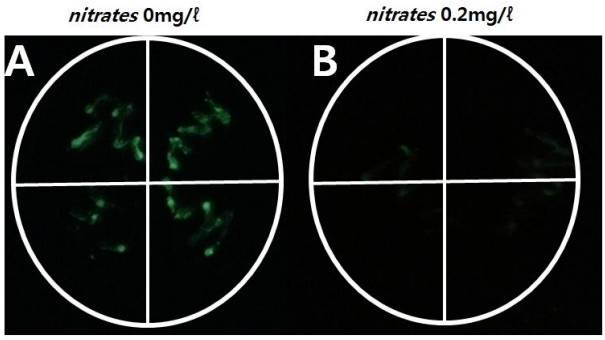

Figure 5. Effect of nitrate on *Photobacterium phosphoreum* culture.(3.2.3)

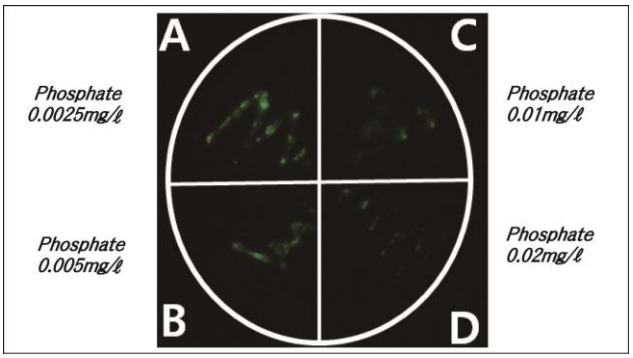

Figure 6. Effect of different concentrations of phosphate on Photobacterium phosphoreum culture (3.2.4.)

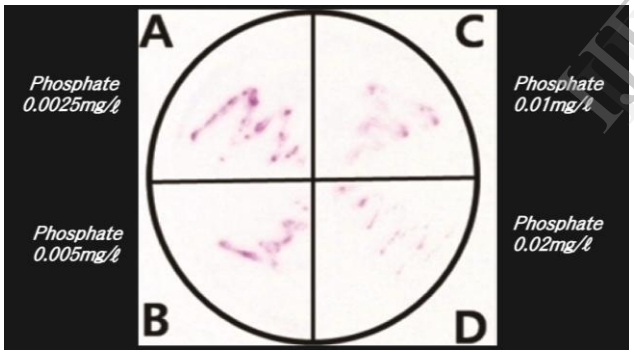

Figure 7. Contrasted image of Figure 6. (3.2.4.)

3) The effect of nitrate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

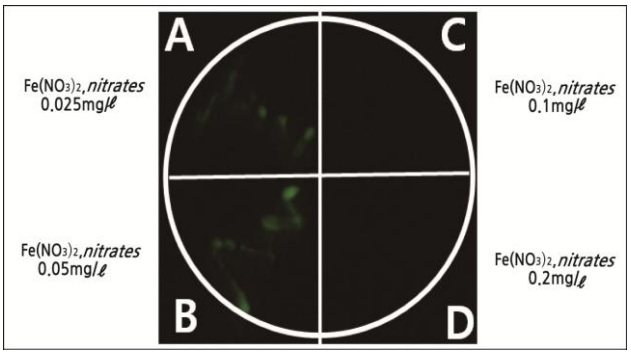

Figure 8. Effect of different concentrations of $Fe(NO_3)_2$ on *Photobacterium phosphoreum* culture (3.2.5.)

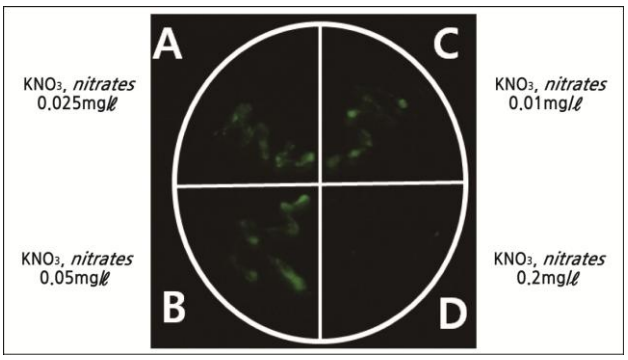

Figure 9. Effect of KNO_3 on *Photobacterium phosphoreum* culture (3.2.5)

In this experiment, nitrate was not added in one culture of *Photobacterium phosphoreum* (A), and in the other culture (B), 0.2mg/ℓ of nitrate, which is a condition of eutrophication, was added.

Photobacterium phosphoreum in the *Photobacterium phosphoreum* culture without nitrate showed normal growth, indicated by the clear luminosity (A). However, *Photobacterium phosphoreum* in the culture with 0.2mg/ℓof nitrate, condition of eutrophication, did not show survival of *Photobacterium phosphoreum* (B). This result shows that *Photobacterium phosphoreum* is unable to grow in nitrate level that indicates condition for eutrophication (0.2mg/ℓ).

Experiment 1 and 2 showed that *Photobacterium phosphoreum* did not survive in the concentration of nitrate and phosphate during eutrophication; however, since conditions used in these experiments were conditions after eutrophication had already happened, a test of response of *Photobacterium phosphoreum* to lower concentrations of phosphate and nitrate is necessary to use the bacteria as an indicator of eutrophication.

4) The effect of different concentrations of phosphate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

Phosphate was added in concentrations of 0.0025mg/ℓ (A), 0.005mg/ℓ (B), 0.01mg/ℓ (C), and 0.02mg/ℓ (D) in each culture of *Photobacterium phosphoreum*.

Photobacterium phosphoreum did not survive in 0.02mg/ℓof phosphate, condition of eutrophication, but at the lowest level of 0.0025mg/ℓ bacteria survived and only showed reduction.

5) The effect of different concentrations of nitrate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

Two types of nitrate, $Fe(NO_3)_2$ and KNO_3 was added

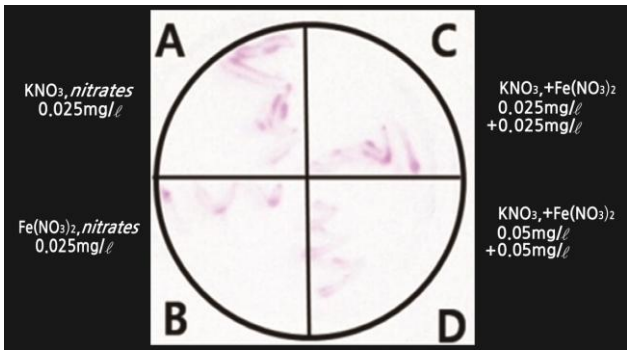

Figure 10. Contrasted image of Figure 9 (3.2.6.)

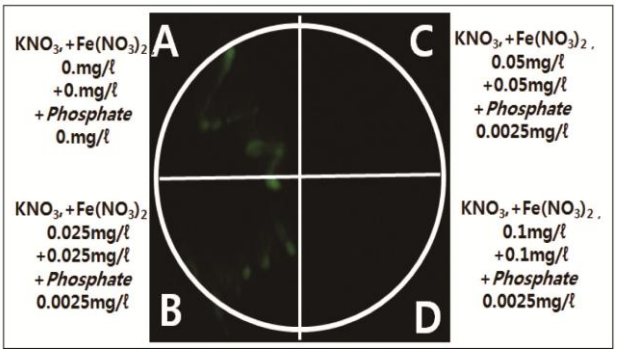

Figure 11. Effect of combination of nitrate and phosphate of different concentrations on *Photobacterium phosphoreum* culture. (3.2.7.)

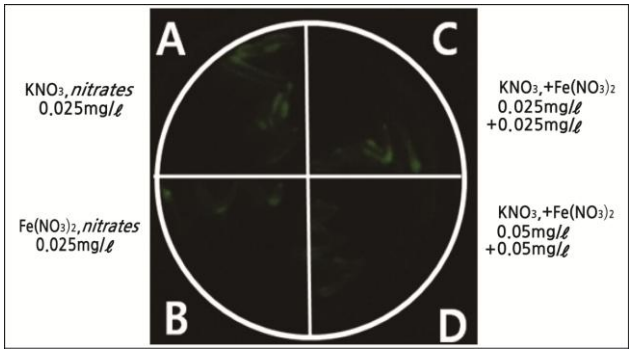

Figure 9. Effect of mixed concentrations of nitrates on *Photobacterium phosphoreum* culture (3.2.6.)

in concentrations of 0.0025mg/ℓ (A), 0.005mg/ℓ (B), 0.01mg/ℓ (C), and 0.02mg/ℓ (D) in each *Photobacterium phosphoreum* culture.

In different concentration of two types of nitrate, growth of *Photobacterium phosphoreum* decreased at 0.05mg/ℓ of both $Fe(NO_3)_2$ and KNO_3.

6) The effect of different concentrations of two types of nitrate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

The mixture of two types of nitrates in designated concentrations was treated on *Photobacterium phosphoreum*. *Photobacterium phosphoreum* did not survive in the mixture of each nitrate with 0.05mg/ℓ concentration and survived but drastically reduced in numbers in the mixture of each nitrate with 0.025mg/ℓ concentration.

7) The effect of different concentrations of phosphate and two types of nitrate, cause of eutrophication, in average salinity of seawater (35‰) on *Photobacterium phosphoreum* culture

Phosphate and nitrate was mixed in different combinations and added to *Photobacterium phosphoreum* culture. No

phosphate and nitrate was added to the first culture (A), and 0.025mg/ℓ of KNO_3 + 0.025mg/ℓ of $Fe(NO_3)_2$ + 0.0025mg/ℓ of phosphate (B), 0.05mg/ℓ of KNO_3 + 0.05mg/ℓ of $Fe(NO_3)_2$ + 0.0025mg/ℓ of phosphate (C), and 0.1mg/ℓ of KNO_3 + 0.1mg/ℓ of $Fe(NO_3)_2$ + 0.0025mg/ℓ of phosphate (D) was added to each culture.

Results show that even when concentrations of nitrates are not at the level of eutrophication, if the phosphate and nitrate concentrations are mixed as in B, *Photobacterium phosphoreum*'s growth is reduced. This experiment showed that luminosity of *Photobacterium phosphoreum* decreased

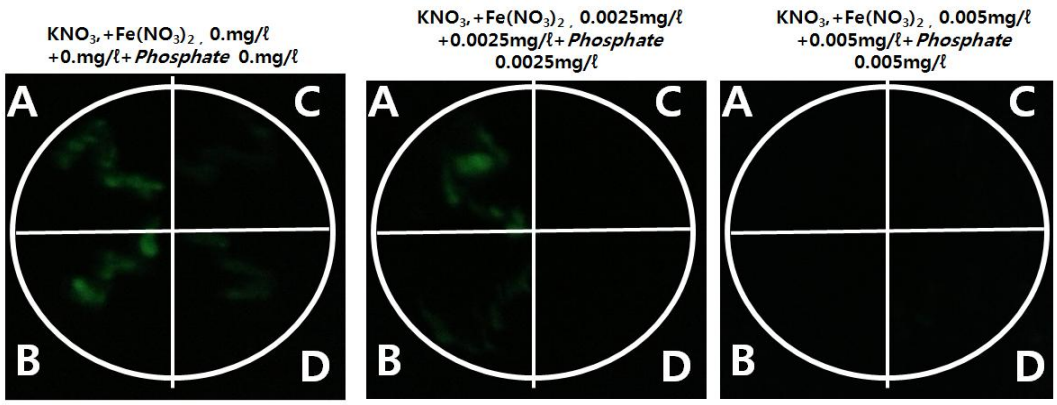

Figure 12. Effect of different concentrations of phosphate and two types of nitrate on *Photobacterium phosphoreum* in types of fresh seawater

before concentrations of nitrate and phosphate in seawater *Photobacterium phosphoreum* can be used as indicator organism for detecting red tide, result of eutrophication and for alerting the start of eutrophication in seawater.

8) The effect of different concentrations of phosphate and two types of nitrate, cause of eutrophication, in four different types of fresh seawater

In this experiment fresh seawater from the sushi restaurants were used instead of the saline water prepared in lab.

As a result of the experiment we were able to confirm that growth of *Photobacterium phosphoreum* was hampered when designated concentrations of phosphate and nitrates were injected into the prepared seawater.

IV. CONCLUSION

This study shows that *Photobacterium phosphoreum* can be utilized as a detector of eutrophication in seawater. The chemotaxis experiments confirmed the sensitivity of the bacteria. It was also confirmed that the bacteria successfully grows in seawater, as shown by the results of experiment 1. Results of test on *Photobacterium phosphoreum*'s growth in different concentrations of phosphate and nitrateshow that *Photobacterium phosphoreum* can detect eutrophication both after it has begun and before it has begun. As experiment 2 and 3 showed that *Photobacterium phosphoreum* did not survive when phosphate and nitrate was in the level of eutrophication, survival of *Photobacterium phosphoreum* in seawater can indicate a condition of eutrophication.

Furthermore, *Photobacterium phosphoreum* also showed reduction in its growth at lower level of phosphate and nitrate and can be utilized to detect eutrophication even at the beginning stage. Lastly, growth *Photobacterium phosphoreum* reduced at different mixture of phosphate and nitrate, practical condition of seawater at eutrophication. Additionally, similar test was confirmed using actual seawater, verifying the test results in practical manner. Decrease in luminosity following the reduction in *Photobacterium phosphoreum*'s growth is easily visible through photography, thus allowing for easy detection of eutrophication. This result presents us with more effective and visible method, as we can directly see the luminosity of the bacteria, rather than analyzing the chemicals in the sea water. In conclusion, utilization of *Photobacterium phosphoreum* can be an effective method in detection of eutrophication through changes in growth and luminosity.

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