



# Cell-specific detection of Alkaline Phosphatase in the Heterotrophic Bacteria *Vibrio alginolyticus*



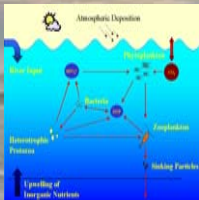
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## Abstract

In order to study the effects of inorganic phosphorus on the microbial ocean community, we focused on the production of the enzyme alkaline phosphatase in heterotrophic bacteria. We specifically examined *Vibrio alginolyticus* and its response to phosphorus depletion in the environment. It is observed that *V. alginolyticus* fluoresces under the ELF stain, which detects AP activity; however, the number of cells fluorescing is much smaller than anticipated compared to the total number of cells.

## Introduction

Phosphorus is a vital nutrient for marine life. Phosphorus cycling includes bacterial uptake of orthophosphates and phytoplankton assimilate it under suitable lighting conditions. Then the bacteria and phytoplankton are consumed by filter feeding organisms, and the food chain continues. There has been an ongoing debate on which element limits primary production in aquatic ecosystems. New studies have found that phosphorus could sometimes be the limiting nutrient. In the past decades, some work (Lomas, M.W et al.2004; Dyrhman, S.T et al.2002) has shown that primary production in marine systems can be limited by inorganic phosphorus. Studies of phosphorus have taken place to find out exactly its role is in limiting primary producers.



One way scientists have developed to study phosphorus activity within plankton is the study of cell surface enzymes, also known as "ectoenzymes", which are essential catalysts in the break down of dissolved organic phosphorus and the surfaces of particulate organic matter. Alkaline phosphatase is an ectoenzyme that is induced by a genetic response to phosphate deficiency.

It is responsible for dephosphorylation, or the removal of phosphate groups with attached phosphate esters. Although its regulation is not entirely understood, it is hypothesized that it is a means for the bacteria or plankton to produce free phosphate groups for uptake and use. One way to observe the activity of this enzyme is by using a specific method of staining known as Enzyme Labeled Fluorescence (ELF). It is an enzyme substrate that is used to label the alkaline phosphatase (AP) activity of a phosphorus stressed cell. It has been demonstrated that it can fluorescently label individual phytoplankton cells expressing AP activity and distinguish single cell AP activity (Carlsson, P. et al. 2001). It is clearly regulated by phosphate, it is apparent in -P cells and not in +P cells.

The ELF stain has been previously used for alkaline phosphatase testing on cyanobacteria and phytoplankton, but has rarely been used on heterotrophic marine bacteria (Carlsson, Per et al. 2001; Lomas, M.W et al.2004). Heterotrophic bacteria have been shown to have a high phosphate requirement for their overall growth (Cotner, J.B et al. 1997). This study aims to find if the heterotrophic bacteria, *Vibrio alginolyticus*, produces the enzyme and the regulation AP activity can be observed as well using the ELF substrate.



*Vibrio alginolyticus*

Since this assay has rarely been used with heterotrophic marine bacteria, the results could vary greatly. In past studies, there has been large variability in the level of ELF fluorescence due to differences in cellular AP expression or specific activity of the individual enzyme complexes (e.g. Lomas, M.W et al.2004).

## Materials and Methods

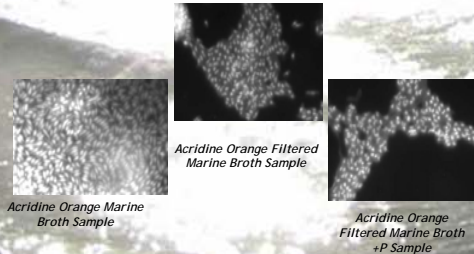


Aliquots of each sample were placed in eppendorf tubes

Observations were done by adding nutrients to a media which where there is little or no phosphorus to observe Alkaline Phosphatase or (AP) activity. The species *Vibrio alginolyticus* was grown from a culture for this study. The bacteria were grown in three different medias, standard Marine Broth, filtered Marine Broth, and filtered Marine Broth with inorganic phosphate (KH<sub>2</sub>PO<sub>4</sub>) added 22 hours after inoculation.

The bacterial cells were stained using the staining kit the ELF 97 Endogenous Phosphatase Detection Kit (Molecular Probes, E6601). The ELF 97 alcohol precipitate shows a fluorescence emission that is 480-500nm. To assess the effect of phosphorus stress, the standard ELF staining procedure was used. First, the ELF stain was prepared by using the standard protocol, 1:20 ratio. An aliquot of 500 µL of the three cultures were prepared with 25µL of ELF reagent. Another aliquot of each sample was also taken and 10% neutral buffered formalin, and placed in a refrigerator.

These samples were diluted later to a 1:10 dilution with regular marine broth and stained with Acridine Orange to find the total cell counts. The aliquots were allowed to react 45 minutes in a dark room at room temperature. After the incubation, the samples were filtered into a 5-µm polycarbonate filter. The filters were then removed and incubated in 10% neutral buffered formalin for 45 minutes in a dark room at room temperature. These suspensions of colonies in formalin were then drawn onto another polycarbonate filter (black filters). The filters were mounted onto glass using the mounting media provided by the ELF kit. Samples are stable for several months when kept in a damp container at 4°C.



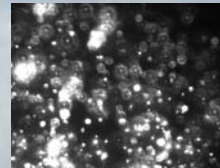
Acridine Orange Marine Broth Sample

Acridine Orange Filtered Marine Broth Sample

Acridine Orange Filtered Marine Broth +P Sample

## Results

Samples Viewed under UV Excitation



ELF fluorescence shown in a filtered marine broth sample



ELF fluorescence shown in a marine broth sample

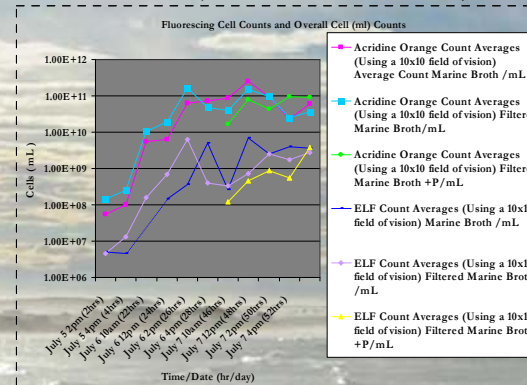


Chart A: The higher numbers are the Acridine Orange Counts, or overall counts of bacterial cells per mL sample, whereas the lesser counts are the ELF stain counts, or overall cells expressing AP activity per mL sample.

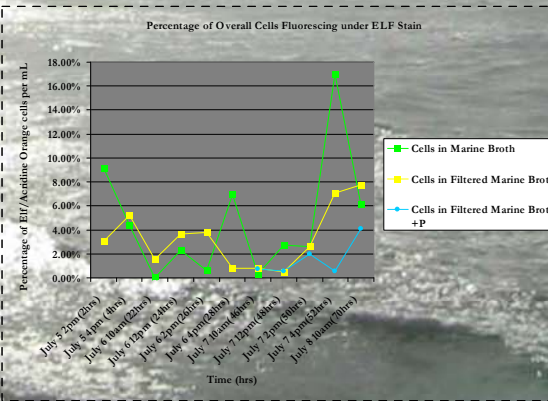


Chart B: The percentages of ELF stained cells over the overall number of cells per mL of each sample. The filtered marine broth with the added phosphorus percentage stayed lower than the other percentages, which is what one would assume. The marine broth percentage went from almost 0% to above 16%. However, the numbers of these percentages were inconsistent with the amount of phosphorus predicted in each media.

## Conclusion

Based on the graphs and calculations, alkaline phosphatase activity is expressed in the heterotrophic bacteria *Vibrio alginolyticus*, in phosphorus-limited environments. However, the collective percentages of ELF fluoresced cells based on the overall cell counts are extremely low, being all less than 14%. This is lower than anticipated since one of the medias used were phosphorus limited, the filtered Marine Broth.

The basis of the experiment was to observe if the bacteria expresses alkaline phosphatase activity, which was observed through the ELF, and if so, which media had the largest amount of cells fluorescing and the changes within each media. Now that this is known, one can try and see why the percentages were so low. There could have been many errors within the process limiting the number of fluorescing cells observed, such as the double filtering of the cells. The environments could have not been phosphorus limited enough, and regular phosphatase assays were not done to confirm the limitation in the medias. Since some phytoplankton species used in previous ELF experiments it did not work well with the dye, it is possible that the species used here also did not work as well. Lastly, the dilution used for the Acridine Orange slides was too little, making the slides difficult to count due to the dense and large numbers of cells per field of vision. Instead of a 1:10 dilution, a 1:100 dilution would make the slides easier to be viewed and quantification easier.

## Acknowledgements

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