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Determine the Essential Micronutrients Intake by *Vibrio cholerae* in Association with Algae in Different Waters

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ABSTRACT

Phosphate, nitrite, nitrate are the micronutrients for *Vibrio cholerae* and algal growth. Current study intended to determine the utilization of these micronutrients by *V. cholerae* in association with algae in four different water (Distilled water, River water, Aritificial sea water and BOD dilution water). Amount of these micronutrients were estimated from stock water, control water and algal water by developing standard curve method. Among these four stock water, orthophosphate was the highest in amount (10.44µg/L) in BOD dilution stock water and significant positive changes (0.06 µg/L) from stock found in the algal water in River water microcosm. In stock sample , the highest amount of nitrite was found in both River and BOD dilution water (6.486µg/L) and the most significant increase (388.014µg/L) from stock was found in the control water of BOD dilution water. River stock water contained the maximum nitrate (1865.204µg/L) among four water types and the highest increase was found in the control water of River. In brief, the amount of phosphate, nitrite and nitrate varied with the types of water. Most cases amount of nitrite- nitrate increased by the decay of *V. cholerae* which used by algae for their growth and overloaded algae did not use phosphate from the surrounding water.

Keywords: Water quality, *Vibrio cholerae* O139, Essential micronutrient intake, and Aquatic environments.

INTRODUCTION:

The gram-negative bacterium *Vibrio cholerae* naturally inhabits aquatic ecosystems and is a part of the normal flora of brackish water. While the vast majority of environmental isolates are nonpathogenic, some strains are able to cause cholera in humans (Maurice Bilung *et al.*, 2019). Cholera toxin (CT) is a major virulence factor involved in the species' enter pathogenicity. Cholera is an acute diarrhoeal disease caused by *V. cholerae*, which affects millions of people and is responsible for the death of an estimated 95,000 people each year in at least 47 countries across the world (Sela *et al.*, 2023; Walton *et al.*, 2023). Though cholera-like illnesses have been reported in the various forms for millennia, there have only been seven pandemics of the disease recorded in the last 200 years. Despite a wealth of information on *V. cholerae*, the disease is still endemic in many countries and produces periodic outbreaks elsewhere (Walton *et al.*, 2023). Researchers suggested that favorable conditions for *V. cholerae* in water characterized by moderate salinity, high nutrient content, warm temperature, neutral or slightly alkaline p^H and the presence of aquatic macrophage , phytoplankton, zooplankton, fish, mollusks and crustaceans, all of these conditions are typical of estuaries and coastal swamps (Borroto 1997; Maurice Bilung *et al.*,

2019; Sela *et al.*, 2023; Walton *et al.*, 2023). Nitrates, nitrites, phosphates, carbon, oxygen play role in the growth of *V. cholerae*. Over the last few decades, the role of certain nutrients and micronutrients in the ecological survival, host colonization, and pathogenicity of the *V. cholerae* has become increasingly apparent (Epstein, 1993; McDonald *et al.*, 2023). The presence of nutrients such as phosphorus in water causes a tremendous development of algae or free-floating plants. An excessive quantity of those nutrients creates algal bloom (Dey *et al.*, 2021). It has been found that nitrates and phosphates in sewage and fertilizers cause eutrophication and scientists report an increase in intensity, duration and shifts in the biodiversity of algal blooms in many coastal, marine and fresh water worlds wide (Epstein, 1993; Dey *et al.*, 2021). In Bangladesh, cultivable *V. cholerae* O1 and O139 strains from aquatic sources can be isolated only during cholera epidemics (pre-monsoon, April - May, and post-monsoon, October - December) when numerous freshwater ponds or lakes are teemed with plankton blooms (Neogi *et al.*, 2012). The Cholera has been long associated with the seasonality of coastal algal bloom of Bangladesh (Epstein, 1993; Alam *et al.*, 2006; Neogi *et al.*, 2012). Scientists groups found correlation between outbreaks of *V. cholerae* and phytoplankton blooms (King *et al.*, 2022). Expression of many *V. cholerae* genes responded to nutrient conditions, including genes regulating nutrient uptake and metabolism, protein synthesis, cell growth and stress responses, environmental sensor systems, & flagellar struture & function (Borroto 1997). The aim of this paper was to determine the orthophosphate, nitrite, nitrate intake by *V. cholerae* in four different water (Distilled water, river water, artificial sea water, BOD dilution water) in association with *Anabaena* sp. in laboratory.

MATERIALS AND METHODS:

Sources of bacteria and algae strains

A strain of *V. cholerae* O139 (1852), an environmental strain was isolated from fresh produce sample collected from the department of Botany, Jagannath University, Dhaka, Bangladesh. This strain was reconfirmed by culture, biochemical, and serological, physiological and molecular technique following standard procedure (Tison, 1999; Sarker *et al*., 2019). The *Anabaena variabilis* belonging to the phylum Cyanophyta, used in this study was initially collected and maintained from the department of Zoology, University of Dhaka, Bangladesh.

Preparation of inoculum

V. cholerae O139 strains were grown on taurocholate tellurite gelatine agar (TTGA) plates at 37^{o}C for 18- 24 hrs. A loopful of growth was mixed properly in 5 ml 0.1% NaCl (pH 7.0) saline water to prepare inoculum suspension. To make sure concentration of the prepared inoculum was log 107 cells/mL, was compared with 0.5 standards MacFarland solutions. The number of per millilitre was assessed by using drop plate method (Hoben & Samasegaran, 1982).

Preparation of microcosm water

In the study, four types of microcosm water was used to set four sets of microcosm. Artificial sea water, Distilled water, River water and BOD dilution water were used in four different microcosms. Artificial sea water and BOD dilution water were prepared in the laboratory using standard method. Artificial sea water was prepared by adding NaCl (23.926G/kg of solution), Na_2SO_4 (4.008G/kg of solution), $NaHCO_3$ (0.196/kg of solution), KCl (0.677G/kg of solution), KBr (0.098G /kg of solution), $MgCl_2$ (1.071g/mL), $CaCl_2$ (1.085 g/mL) in distilled water. BOD dilution water was prepared by the adding Phosphate buffer solution (8.5g KH_2PO_4 + 21.75g K_2HPO_4+ 33.4g Na_2HPO_4.7H2O+1.7g NH4Cl), Magnesium sulphate solution (22.5g $MgSO_4.7H_2O$), Calcium choloride (27.5g), Feric choloride (0.25g), in 1000mL distilled water. River water was collected from Dhonagoda river of Matlab, Bangladesh.

Nutrient intake experiment

The flasks were kept on racks close to windows so that the *Anabaena* sp. received direct sunlight for photosynthesis. Four 250mL experimental flasks, containing 100 mL of the Distilled water, River water , Artificial sea water and BOD dilution water respectively, were inoculated with defined number of *V. cholerae* and 1 gm wet (weight measured by an electronic balance) of *Anabaena* sp. from pure culture was added. Four control flasks were used for four experimental flasks. Four 250mL control flasks contained 100mL of water from these four stocks with

defined number bacterium (*V. cholerae*) but without algae. Another four 250 mL flaks were used where only stock solutions of these four types of water were kept. Sampling of water from each type of flasks started after 30 min which was considered as 0 hr reading.

Determination of orthophosphate in water sample

Sufficient volume of water sample (eg. 30mL) was filtered with 0.45µm pore sized filter paper. This separated the dissolved phosphorous from suspended forms. 50mL of filtrate water into conical flask was added. One drop phenolphthalein indicator was added. When red color was appeared it was dissolved with Sulphuric acid (5N). 8 mL of combined reagent (Sulphuric acid (5N) 50 mL, Potassium antimonyl tartrate solution 5 mL, Ammonium molybdate solution 15 mL, and Ascorbic acid (0.1 M) 30 mL were mixed for 100 mL combined reagents) was mixed and shacked to mix thoroughly. After 10 minutes OD at 880nm were measured using reagent blank (distilled water) as a reference solution. Individual calibration curve from series of standards by plotting absorbance (OD) vs. phosphate concentration was prepared. mg P/L = {(Calculated value from standard curve) X 1000}/ Volume of sample

Determination of nitrite- nitrogen in water sample

Nitrite (NO_2) is determined through formation of a reddish purple azo dye produced at pH 2- 2.5 by the coupling diazotized Sulphanilamide with N- (1-napthyl1)- ethylene diamine dihydrochloride was added and mixed to dissolve and then diluted to 50 mL with distilled water . The sample pH was adjusted between 5 and 9 with the use of 1N HCl or NH_40H as required. 2 mL color reagent (5 mL 85% Phosphoric acid and 0.5 gm Sulphanilamide) was added to 40 mL water. After dissolving Sulphanilamide completely, 1 gm N-(l- napthyl)-ethylene diamine dihydrochloride was added and mixed to dissolve and then diluted to 50 mL with distilled water was added to each 30 mL sample solution. Color was developed within ten minutes or less. After 10 minutes of adding coloring reagent, the samples and the standards were measured at 543 nm of the absorbance. A standard curve was prepared by plotting absorbance of standards against NO_2-N concentrations. Then sample concentration was directly found from the standard (**Fig. 1**).

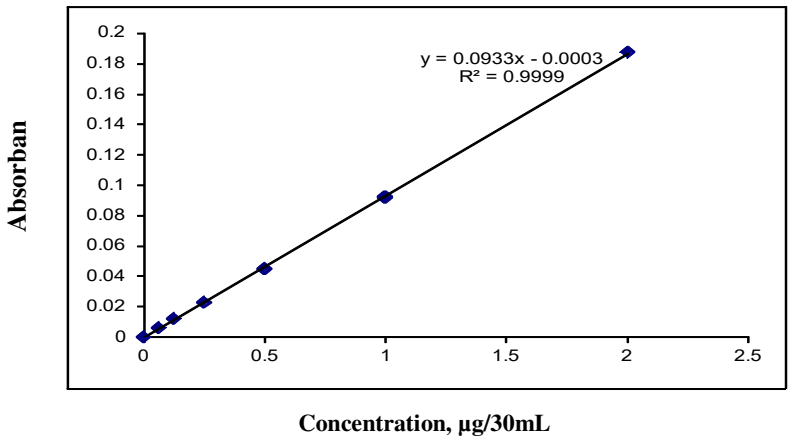

Fig. 1: Calibration curve for nitrite-nitrogen.

Determination of nitrate- nitrogen in water sample

Nitrate (NO_3) is determined in water sample through spectrophotometric method at (pH 2.0) (Ferree and Shannon, 2001). Standard KNO_3 solution was prepared by dissolving 0.0722gm KNO3 in deionized water. So, 1 mL KNO_3 solution = 100/µ KNO_3 -N and 100 microleter standard solution= 100/µ NO_3- N. Accor-ding to the above concentration the following standard solutions were prepared. The concentrations of the standard samples were 100µg/L, 80µg/L, 60 µg/L, 40 µg/L, 20µg/L and 10µg/L. The spectro-photometer was allowed to warm up for 30 min. For blank reading, the distilled water was acidified (pH 2.0) with concentrated Sulphuric acid. Readings were measured for standards and samples around at pH 2.0 and at weave length of 220 nm. Based on the standard curve the concentration of nitrate – nitrogen was cal-culated (**Fig. 2**).

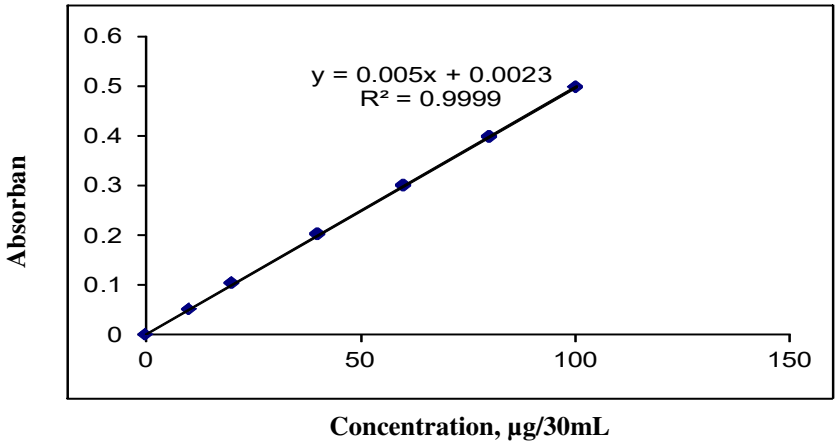

Fig. 2: Calibration curve for nitrate-nitrogen.

RESULTS AND DISCUSSION:

Orthophosphate intake

For all sets of the microcosm, the initial amount of orthophosphate was same in stock solution, in control water and in water of experimental flasks eg the amount of orthophosphate was 0.02µg/L in Distilled water, 0.03µg/L in River water, 0.01 µg/L in Artificial sea water and 10.44 µg/L in BOD Dilution water (**Table 1**). After one month time, no significant changes in the amount of orthophosphate of the control water, was found in Distilled water, River water and the Artificial sea water. This time, the estimated amount of orthophosphate was 0.015µg/L in Distilled water, 0.025µg/L in River water, >0.01µg/L in Artificial sea water (**Table 1**). In case of BOD dilution water, it's amount was increased up to 11.88 µg/L. In 30 days old River water, where alga were floating, the amount of orthophosphate was increased significantly (0.09µg/L though other samples did not show significant changes, eg. Distilled water (0.03µg/L), Artificial sea water (0.01µg/L) and BOD dilution water (10.56µg/L) (**Table 1**).

Table 1: The estimation of nutrient content (Orthophosphate) in µg/L in different water.

Types of water	Amount of Orthophosphate in stock water (µg/L)	Amount of Orthophosphate at O hr.		Amount of Orthophosphate after 1 month	
		In control water (µg/L)	In algal Water (µg/L)	In control Water (µg/L)	In algal Water (µg/L)
DW	0.02	0.02	0.02	0.01	0.03
RW	0.03	0.03	0.03	0.03	0.09
ASW	0.01	0.01	0.01	0.01	0.01
BOD dilution water	10.44	10.44	10.44	11.88	10.56

Footnote: DW= Distilled water, RW= River water, ASW= Artificial sea water.

Nitrite intake

At 0 hr, the amount of nitrate in stock water, was the lowest among three types of samples (stock water, control water, algal water), in Distilled water 4.731µg/ L, 6.486µg/L in River water, 2.976 µg/L in Artificial sea water, 6.486µg/ L for BOD dilution water (**Table 2**). Initial amount of nitrite was same to stock water in control flasks and experimental flasks in every sets of microcosm. Except the 30 days old control sample of River water, others contained the highest among three categories of samples. eg., 254.1 µg/L in the Distilled water, 359.4 µg/L in Artificial sea water,

394.5µg/L in BOD dilution water. The amount of nitrite in 30 days old algal water was 236.55 µg / L in Distilled water, 289.2 µg/L in Artificial sea water 254.1 µg/L in BOD dilution water (**Table 2**). In case of 30 days old River control water, the nitrite was detected 26.25 µg/L while in algal water, it was 131.25 µg/L (**Table 2**).

Table 2: The estimation of nutrient content (NO_2-N (µg/L)) in different water.

Types of water	Amount of nitrite in stock Water (µg/L)	Amount of nitrite at O hr.		Amount of nitrite after 1 month	
		In control Water (µg/L)	In algal Water (µg/L)	In control water (µg/L)	In algal Water (µg/L)
DW	4.731	4.731	4.731	254.1	236.55
RW	6.486	6.486	6.486	26.25	131.25
ASW	2.976	2.976	2.976	359.4	289.2
BOD dilution water	6.486	6.486	6.486	394.5	254.1

Nitrate intake

In Distilled water, the maximum amount of nitrate was detected in algal water (3063.35 µg/L) and the minimum amount found in control water (188.55 µg/L) after 30 days of the experiment (**Table 3**). At 0 hr, 219.381 µg/L nitrates were found in stock water, control water and in algal water. In River water its amount was 1865.204 µg/L in stock water at 0 hr which decreased to 188.55 µg/ L in water where algae were floating at day 30. The highest amount was found in the control River water (9531.65µg/L) after 30day. In case of the Artificial sea water 161.885µg/L, 3063. 35µg/L, 212.65.55µg/L were the amounts of nitrate (**Table 3**) present in stock water at 0 hr, in control water at day 30 and in algal water at day 30 respectively. In BOD dilution water, the content of nitrate was the lowest in stock water i. e. 18.14 µg/L at 0 hr while in the control water it increased to (3422.7 µg/L) and in water where algae were floating, it was 188.55 µg/L after 30 days (**Table 3**).

Table 3: The estimation of nutrient content (NO_3-N (µg/L)) in different water.

Types of water	Amount of nitrate in stock water (µg/L)	Amount of nitrate at O hr.		Amount of nitrate after 1 month	
		In control water (µg/L)	In algal Water (µg/L)	In control water (µg/L)	In algal Water (µg/L)
DW	219.381	219.381	219.381	188.55	3063.35
RW	1865.204	1865.204	1865.204	9531.65	188.5
ASW	161.885	161.885	161.885	3063.35	212.65
BOD dilution water	18.14	18.14	18.14	3422.7	188.55

Phospahate, nitrite, nitrate are major and important micronutrients of bacteria. In case of orthophosphate concentration in the four microcosms containing different water, the initial amount of orthophosphate (mg/L) in stock water does not significantly differ with that of the 30 days old control water algal water. Overall presence of this micronutrient in three categories of samples was insignificant. Only in River

water where algae were floating, its amount (0.09 mg/L) significantly differed from other two samples (control (0.03 mg/L) and stock water (0.03 mg/L)). Normally during growth of algae (*Anabaena* sp.), it takes phosphate from water. So it can be explained by two possible phenomena: one is because of conversion of other forms of phosphorous into another phosphorous and other phenomenon is decaying of algae during this time. The river water environment enhanced this micronutrient in the experiment by either or both of these phenomena. Stwert and Gail, (1971) stated that excess phosphorus is stored in the vegetative cells of blue-green algae as polyphosphate bodies which may form within 60 min of adding phosphorus to phosphorus starved cells. It can assume that in our study, the used algae were sufficiently loaded by polyphosphate bodies and during the decomposition release phosphate in the form of orthophosphate.

In present study, amount (mg/L) of Nitrate (NO_3 -N) varied significantly in stock water (0 hr reading), control water (30 day reading) and in algal water (30 day reading) in each microcosm setup and also varied with water quality. Among these three types of water (Distilled water and Artificial sea water, BOD dilution water) total content of NO_3 - N increased in control water in comparison to the stock water while decreased in algal water. This condition can be explained in two manners: the degeneration of bacterial body can also release many nitrogenous products. It may increase the total amount of the nitrite in the control water and the intake of nitrate and nitrite by algae decrease the amount of NO_3 -N in algal water. It can be inferred that algae have utilized nitrate to produce different extracellular compounds like peptide, free nitrogen etc. Hence, the content of this compound decreased in water surrounded by algae. According to Islam *et al.* (1994a) blue-green algae produce a number of extracellular products which contain peptide, amine, and free nitrogen. This statement supports of loss of NO_3-NO_2 in experimental flasks. This trend is different in case of Distilled water. The amount of NO_3 did not differ significantly in stock water (0 hr) and in 30 days old control water but increased disproportionately in algal water. From this finding it can conclude that, the flask was either contaminated by other bacteria, which decayed and released nitrate or decomposition of algae due to the absence of food material in Distilled water. This can be supported by the statement that when the blooming algae die, they can produce lots of algae's toxin and nitrite concentration (Yang *et al.,* 2008).

CONCLUSION:

In aquatic environment, micronutrients play important role for bacteria and aquatic flora in a several ways such as their interaction, growth, colonization, ecological survival, pathogenecity etc. Although increasing the concentration of micronutrients can lead to eutrophication, it induce to produce the bloom of aquatic flora like algae. Aquatic flora can act as host, shelter and reservoirs for microorganisms. Several report has been revealed the association between *V. cholerae* and algal bloom. The present study result has exhibited that the amount of phosphate, nitrite and nitrate varied with the types of water. In 30 days old river water containing the floating alga, the amount of orthophosphate was increased significantly (0.09µg/L) while the amount (mg/L) of Nitrate (NO_3-N) was decreased significantly (188.55µg/L). In order to document nutrient removal from the aquatic ecosystem by phytoplankton accumulation, it was suggested that phytoplankton uptake was a key mechanism responsible for nutrient removal. Most cases amount of nitrite- nitrate increased by the decay of *V. cholerae* which used by algae for their growth and overloaded algae did not use phosphate from the surrounding water.

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CONFLICT OF INTERESTS:

The authors declare no conflicts of interest.

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