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# Attachment of V. cholerae with plankton biomass in two different ecological zones of Bangladesh

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## ABSTRACT

Seasonal variation and planktonic abundance has profound effects on the attachment of *V. cholerae* the devastating agent for causing cholera. As crustacean plankton is the primitive carrier for the cholera bacterium, which stage is the most efficient for the attachment to *V. cholerae* however is a most curious interrogation to resolve. Here in this experiment, laboratory microcosms were prepared with estuarine Mathbaria water, saline Paikgacca water and fresh water of lake. These three sets were with their twins among which one was with algal supplementation to feed the plankton and another was without any algae. After two months of rearing, it was found that, Mathbaria water and lake water supported the bacterial growth to enhance along with the nauplii production. On the other hand, among the three contaminated water sources (site-2, site-8 and site-11) in Mathbaria nauplii biomass showed highest peak during the infection period (March-May and September-November) in 2013 and 2014. In Chhatak also nauplii biomass showed highest peak at three contaminated ponds (site-1, site-10 and site-12) during the peak season in the studied year. During the current study nitrogen and phosphorus amount was higher in the contaminated ponds of Mathbaria when there was peak season of cholera. So, micronutrients as well as larval stages of crustacean plankton are dominant biological factor for causing cholera in these two regions of Bangladesh. This experiment will play as a role model to observe the intensity of the attachment of *V. cholerae* to copepods at their different stages as well as the interrelation of micronutrients in coastal region with the availability of cholera during peak season.

**Contribution/Originality:** In this experiment, microecosystem study is the nobility work where larval stages of copepods are subjected to this with which *V. cholerae* are more likely associated with them than the adult copepods. On the other hand, some micronutrients in the water body may influence the cholera during infectious period.

## 1. INTRODUCTION

Attachment of *V. cholerae* to various aquatic organisms has been well documented. The bacterium is strongly associated with plankton forming commensal and symbiotic relationships, mainly with copepods Islam, et al. [1]; Colwell and Huq [2] and Shukla, et al. [3]. The copepod exoskeleton has been shown to support large populations of vibrios, including the pathogenic species *V. cholerae* Tamplin and Colwell [4]; Colwell, et al. [5]; Colwell [6] and Huq, et al. [7]. Adherence to the roots of water hyacinth, common duckweeds, other freshwater plants and certain blue and blue-green algae has also been shown [1, 8]. The highly diverse zooplankton community *V.*

*cholerae* serogroup O1 has been reported to attach only to certain groups, notably copepods, cladocerans and rotifers [9]. *Vibrio spp.* produce an extracellular chitinase that aids their adhesion to the integument of planktonic crustaceans [10] explaining the widespread association of these bacteria with these arthropods.

The biomass is the mass of living biological organisms in a given area or ecosystem at given time. Biomass can refer to species biomass, which is the mass of one or more species, or to community biomass which is the mass of all species in the community. It can include microorganisms, plants or animals. The mass can be expressed as the average mass per unit area, or as the total mass in the community [11]. The impact of an environmental variable on population dynamics is typically largest when it is highly variable and affects population growth with a steep and monotonic functional response [12]. Temperature, salinity, stratification and nutrients are key environmental variables for plankton population dynamics, and these variables are also influenced by anthropogenic pressures such as eutrophication and climate change Suikkanen, et al. [13]; and Andersson, et al. [14].

Annual mean temperatures in temperate regions are generally a few degrees below the predicted evolutionary stable conditions for the local phytoplankton taxa [15]. Salinity stress can vary seasonally and influences growth performance of phytoplankton [16] and determines the species composition. Stratification and ice conditions can have complex effects on plankton by indirectly influencing the onset of the spring bloom and thus interactions between species [17, 18]. A number of studies demonstrated that in the subtropics, zooplankton biomass is low in comparison with temperate lakes of similar trophic state (e.g., [19, 20]) and that body size of zooplankton also is reduced [21]. Further, there appears to be less top-down control of phytoplankton by zooplankton grazers in subtropical lakes Havens and East [22] and Jeppesen, et al. [23].

Baumann, et al. [24] studied that all pathogenic vibrio species elaborate an extracellular chitinase and also investigated the association between these pathogenic vibrios and the chitin-containing zooplankton in the water column. The viability of *Vibrio cholerae* ecological habitat related to its survival and pathogenecity. Huq, et al. [7]; Akselman, et al. [25] and Shikuma and Hadfield [26] observed that *V. cholerae* attaches to abiotic and biotic surfaces (chitinous as well as gelatinous zoo and phytoplankton) as biofilms.

## 2. MATERIALS AND METHODS

### *2.1. Micro-Ecosystem Study (Microcosms) of Copepods in Different Ecological Habitats*

#### *2.1.1. Preparation of Microcosms of Copepoda*

Three microcosms were set up with different sources of water collecting from Mathabaria (Cholera infected area), Paikgacca (saline water) and Dhanmondi Lake (Fresh water). Each microcosm was with two different subsets. The microcosms were designated as Mathbaria water microcosm (MW), Mathbaria water microcosm with algal feed (MW+AF), Paikgacca water microcosm (PW), Paikgacca water microcosm with algal feed (PW+AF), Lake water microcosm (LW) and Lake water microcosm with algal feed (LW+AF). Copepods were collected with plankton net of 64µm mesh size from Dhanmondi Lake. Salinity of the microcosms was 0.3 ppt, 3.6 ppt and 0 ppt for Mathbaria water, Paikgacca water and lake water respectively. They were then released into the microcosms after counting. All sets of microcosms were kept at room temperature (27°C).

### *2.2. Inoculation of Vibrio Cholerae*

*V. cholerae* O1 biotype El*Tor* N-16961 cells isolated from a pond of Mathbaria. Bacteria was grown in Luria-Bertani (LB) broth at 37°C for 18 h. After collection bacterial colony was washed with Phosphate Buffer Saline (PBS). The cells were then inoculated into following combinations, Mathbaria water (MW), Mathbaria water with alagal feed (MW+AF), Paikgacca water (PW), Paikgacca water with algal feed (PW+AF), Lake water (LW) and Lake water with algal feed (LW+AF) to a final concentration of $10^7$cfu/ml. Continuous aeration was provided the copepods at the room temperature. Sub samples from the beakers were taken to conduct plate culture, Direct Flouroscent Antibody (DFA) and multiplex Polymerase chain Reaction (mPCR).

### *2.3. Plate Count of Vibrio Cholerae O1*

Samples were diluted 10 fold serially in PBS and 100 µl of diluted samples were spread on the surface of TTGA (Taurocholate-Tellurite-Gelatine Agar) plates. Inoculated plates were incubated at 37° C for 24 h. After incubation, probable *V. cholerae* O1 colonies on plates were confirmed by slide agglutination test using polyvalent anti-O1 serum [27]. The confirmed colonies represented the total viable and culturable count of *V. cholerae*.

### *2.4. Multiplex Polymerase Chain Reaction (mPCR)*

The colonies confirmed as *V. cholerae* O1 by slide agglutination test (antigen-antibody reaction) were subjected to M-PCR for detection of O1serotype specific *rfb*O1genes encoding O-antigen and *ctxA* encoding subunit A of cholera toxin (CT) were amplified using M-PCR, details of which are provided elsewhere [28].

## 3. RESULTS AND DISCUSSIONS

### *3.1. Ex-Situ Experiments of Vibrio Cholerae Growth with Zooplankton and Chitin Extraction*

#### *3.1.1. Growth of Vibrio Cholerae in Mathbaria Water Micro-Ecosystem (Microcosm)*

In Mathbaria water two sets of microcosms i.e., microcosm supplemented with feed and without any supplemented feed was set. 150 copepods were released into the microcosms. Count of *V. cholerae* that was inoculated from a pure culture into the microcosm was ($1.9X10^6$) at day 0. Number of bacteria decreased with the decreased number of copepods such as after seven days of inoculation number of adult copepods was minimum. At the same time bacterial count was poor onto the counting plates. Nauplii emerged in the microcosm after eight days of rearing the plankton. Bacterial count again increased in number with the increased number of nauplii. At the end of the experiment, no. of bacteria totally depleted with the declining of nauplii and adult copepods (Figure 1 and Figure 2).

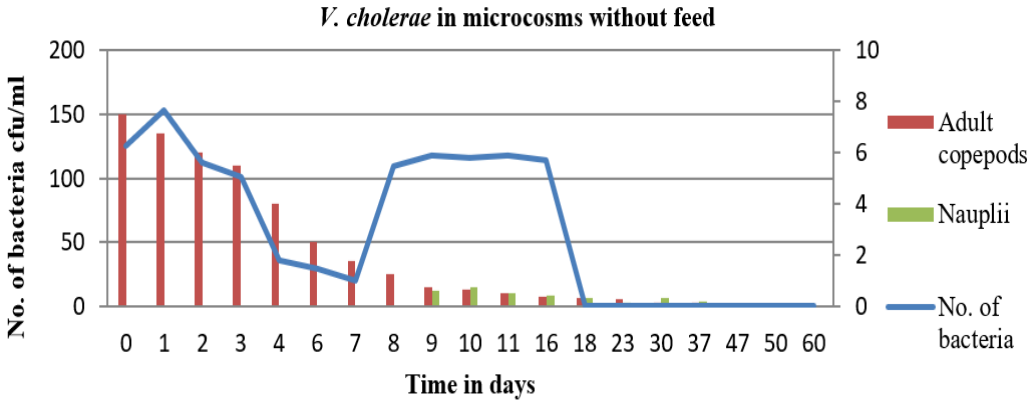


**Figure 1.** Growth of *V. cholerae* in Mathbaria water micrococsm (Without feed).

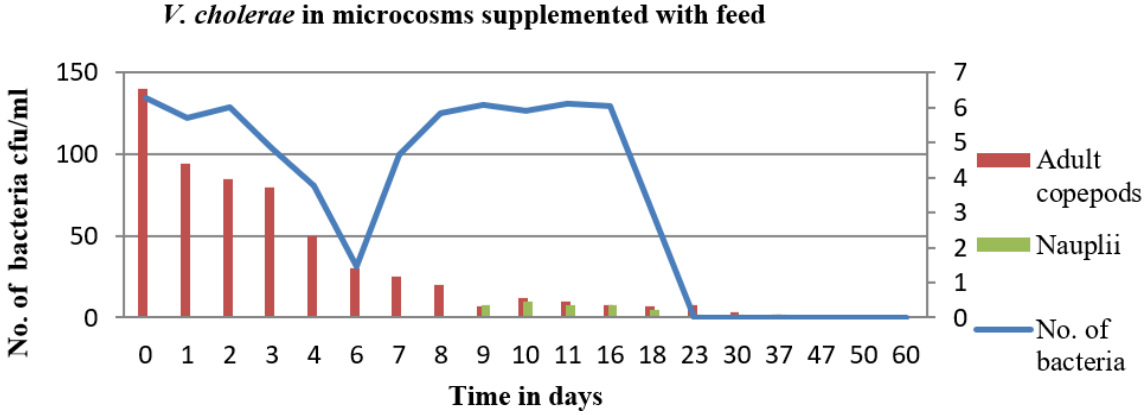


**Figure 2.** Growth of *V. cholerae* in Mathbaria water micrococsm (With feed).

### *3.2. Growth o Vibrio Cholerae in Paikgacha Water Micro-Ecosystem (Microcosm)*

Paikgacca pond water in comparison to Mathbaria had higher water salinity. Number of copepods declined quickly in both set of microcosms (PW and PW+F). Number of bacterial cells at first increased with time and then decreased. After seven days of rearing bacterial count on TTGA plate was ($4X10^5$ cfu/ml) and ($3X10^5$cfu/ml) (Figure 3 and Figure 4).

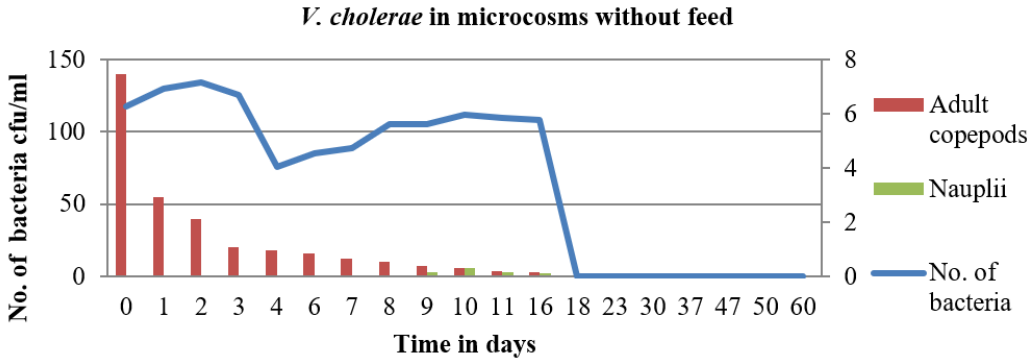


**Figure 3.** Growth of *V. cholerae* in Paikgacca water micrococsm (Without feed).

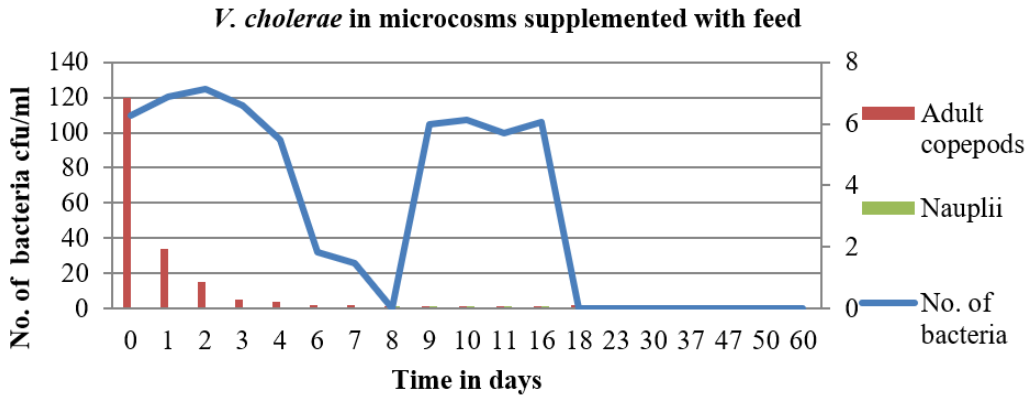


**Figure 4.** Growth of *V. cholerae* in Paikgacca water micrococsm (With feed).

### *3.3. Growth of Vibrio Cholerae in Lake Water Micro-Ecosystem (Microcosm)*

Microcosms prepared with Dhanmondi lake water had more or less similar results with that of Mathbaria water microcosms. Number of copepods and bacterial count declined after seven days of experiment. Then the bacterial cells increased with emerging number of nauplii. This condition continued upto 16$^{th}$day and then decreased (Figure 5 and Figure 6).

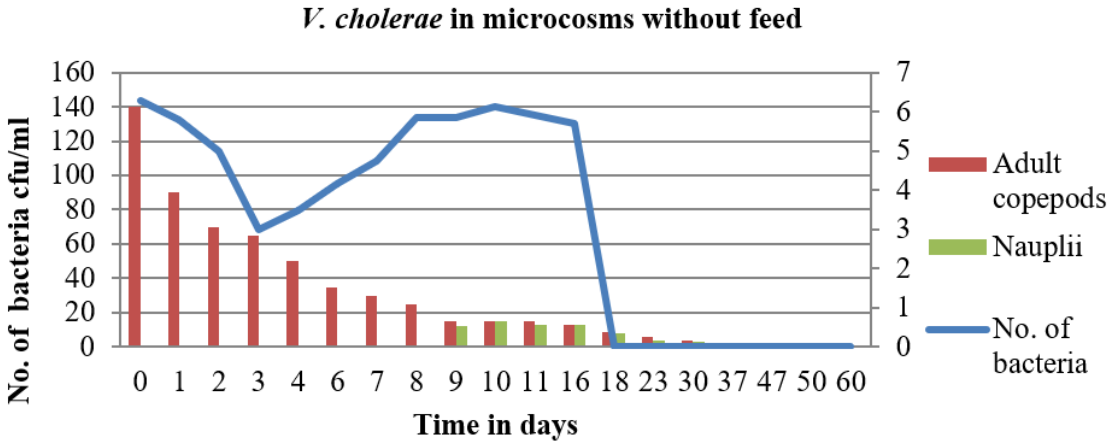


**Figure 5.** Growth of *V. cholerae* in Lake water micrococsm (Without feed).

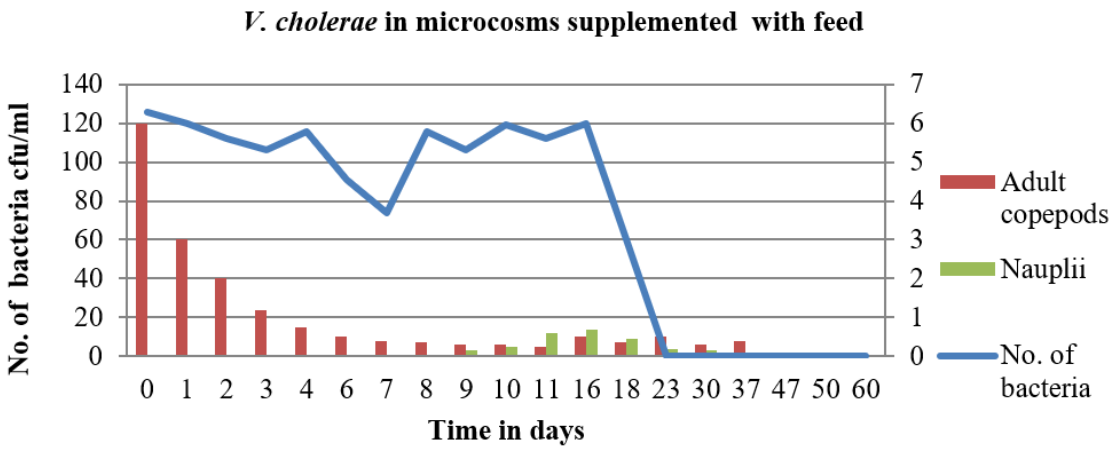


**Figure 6.** Growth of *V. cholerae* in Lake water micrococsm (With feed).

### *3.4. Relationships with Nauplii Biomass in Ponds During Infection Periods*

In Mathbaria, site-2 (Jotishkanti Bepari's Pond), site-8 (Mathbaria Canal) and site-11 (Commissioner Bari Pond) were identified as contaminated water sources for *Vibrio cholerae* infection. They are shown in solid lines in the Figure 7 and 8 for sampling year 2013 and 2014. Except Mathbaria canal (Site 8) in 2013 the nauplii were available in highest in biomass in the contaminated and non-contaminated water bodies.

In 2014, the biomass of Nauplii showed the similar trends for abundance. The contaminated and non-contaminated ponds water sources were evitable at peak for spreading the bacteria. Thus, the occurrence of nauplii biomass can initiate the infection of *Vibrio* in the ecosystem along with other factors. As the other water bodies (in dotted lines) were found to be rich in nauplii biomass, the contamination could aid in spreading the *V. cholarae* infection in the area. Except Mathbaria canal in October 2013, minimum biomass was available at 94.3 g per cubic meterof nauplii in contaminated and non-contaminated ponds. There may be some other reasons in lowering the nauplii in the canal (site 8) in Mathbaria.

In Chhatak, site-1(Govt. Pond near Thana Health Complex), site 10 (Surma River Ghat-2: Cement factory ghat) and site-12 (Mondolibhog Girl's High School Pond) were identified as contaminated water sources for *Vibrio cholerae* infection. They are shown in solid lines in the Figure 9 and 10 for sampling year for 2013 and 2014. All contaminated and non-contaminated ponds showed single highest peak of biomass in the seasons.

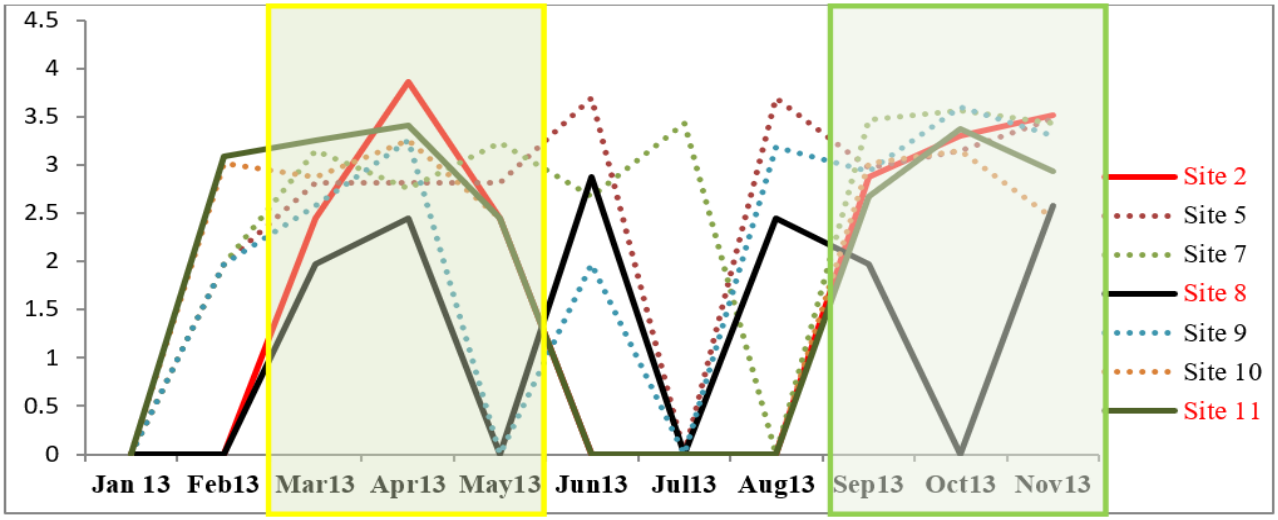


**Figure 7.** Mathbaria nauplii data, showing the monthly weight (log) distributions and their relationships with two infection seasons and the ponds (solid line sites 2, 8 and 11 were the infected ponds) in 2013. The non-contaminated pond data were shown as in dotted line. March to May and September to November were the infected season of Mathbaria. The December sampling was missing due to strike in communication. The highlighted zone is the disease outbreak period of the sampling sites.

In 2014, the biomass of Nauplii showed the same trends for abundance in all water bodies. The contaminated and non-contaminated ponds water sources were evitable to be at peak in nauplii biomass in September to November, the cholera spreading season. However, the peak was again varied throughout the year in the water bodies in the Chhatak area. In 2013, two earlier peak were observed in March and June, while in 2014 were in April and onwards. The occurrence of nauplii biomass can only be maintained in September to November to initiate the infection of *Vibrio* in the ecosystem along with other factors. As the ecosystem was fresh water, the occurance of infection may take other factors to initiate to the process in the vicinity. Other water bodies (in dotted lines) were also found to be rich in nauplii biomass, any contamination in the non-infectious pond ecosystem could aid in spreading the *V. cholarae* infection in the area. In Chhatak, minimum biomass of nauplii was available at 94.3 g per cubic meterin contaminated and non-contaminated ponds.

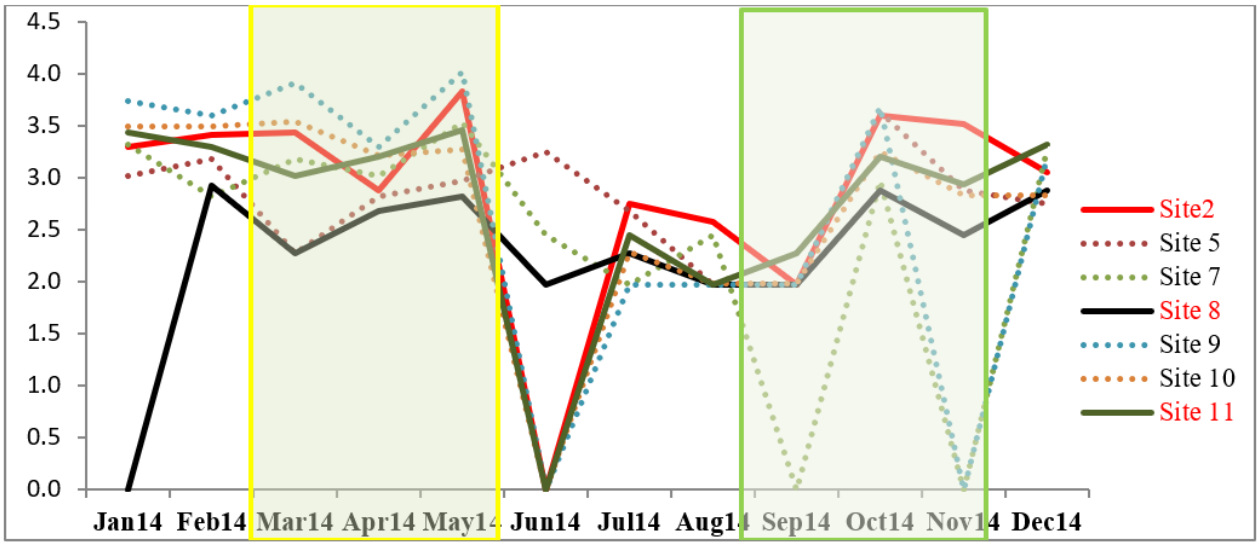


**Figure 8.** Mathbaria nauplii data, showing the monthly weight (log) distributions and their relationships with two infection seasons and the ponds (solid line sites 2,8 and 11 were the contaminated ponds) in 2014. The non-contaminated pond data were shown as in dotted line. March to May and September to November were the infected season of Mathbaria. The highlighted zone is the disease outbreak period of the sampling sites.

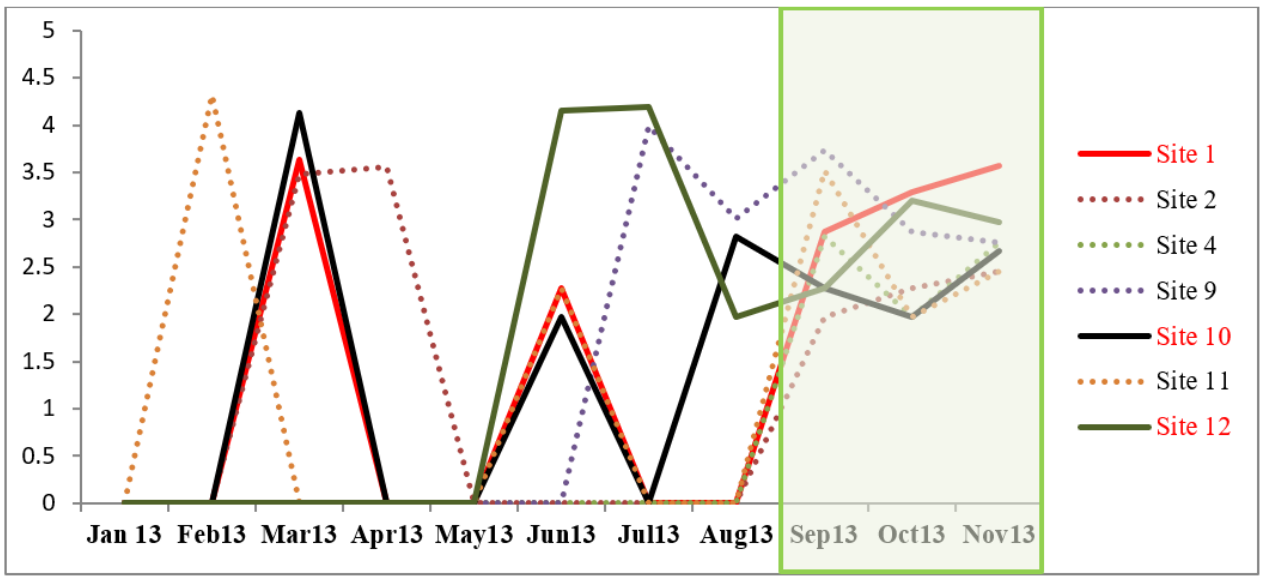


**Figure 9.** Chhatak nauplii data, showing the monthly weight (log) distributions and their relationships with single infection season and the ponds (solid line sites 1,10 and 12 were the contaminated ponds) in 2013. The non-contaminated pond data were shown as in dotted line. September to November were the infected season of Chhatak. December sampling was missing due to communication strike. The highlighted zone is the disease outbreak period of the sampling sites.

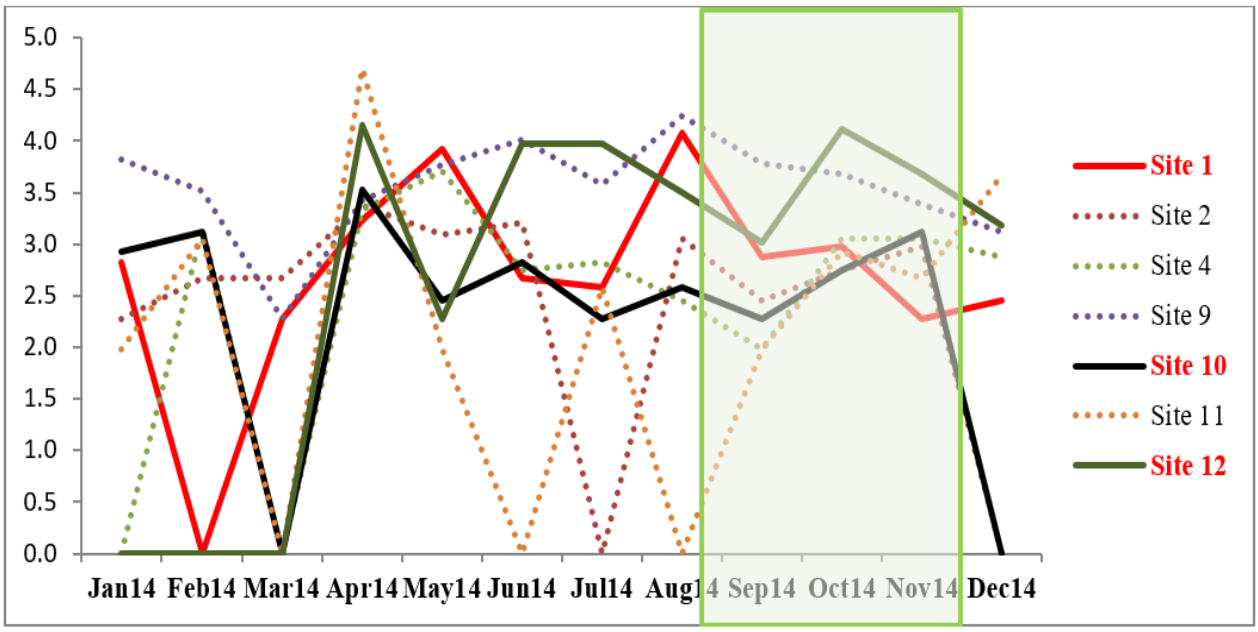


**Figure 10**. Chhatak nauplii data, showing the monthly weight (log) distributions and their relationships with single infection season and the ponds (solid line sites 1,10 and 12 were the contaminated ponds) in 2014. The non-infected pond data were shown as in dotted line. September to November were the infected season of Chhatak. The highlighted zone is the disease outbreak period of the sampling sites.

In *ex-situ* experiments of laboratory microcosms prepared with three different sources of water (Mathbaria, Paikgacca and Dhanmondi Lake water) *V. cholerae* was found to attach with the adult *Cyclops sp.* and their larval nauplii. Kogure, et al. [28] earlier reported that zooplankton promote the growth of *Vibrio* species. Huq, et al. [29] and Huq, et al. [30] showed that the survival of *V. cholerae* O1 is enhanced when it is grown with laboratory-grown planktonic copepods isolated from fresh and estuarine waters. Large numbers of *V. cholerae* was noted by those authors to be attached to plankton structures.

Microcosm study of *V. cholerae* O1 and their association with copepods in the present study revealed that they are influenced by the larval stages of the copepods than the adults. This activity was observed regarding the three different water made microcosms in the laboratory and the process continued upto four weeks. Huq, et al. [29] showed that *V. cholerae* associated with living copepods remained culturable at least 10 days or longer than *V. cholerae* associated with dead copepods.

### *3.5. Availability of Nutrients in Three Vibrio cholerae Inhabiting Ponds of Mathbaria*

*V. cholerae* the prime agent for causing horrible cholera needs some sort of nutrients for their survival and infectious activity. In Mathbaria, Nitrogen and Phosphorus amount as well as some other micronutrients were available in those infectious ponds of Mathbaria during peak season of cholera. During the study period, water sample of three heavily contaminated ponds were analyzed to observe the nutrients level for the production of primary producers (blue-green algae) which influence the growth and abundance of zooplankton to act as the reservoir of *V. cholerae*.

Total nitrogen content during the peak season of cholera was (0.812-0.504), (0.448-0.478) and (0.523-0.578) for site-2, site-8 and site-11 respectively. Phosphorus amount ranged between (28.8-34.4), (29.6-29.56) and (28.3-31.2) in the site-2, site-8 and site-11 sequentially. These amounts were higher than those measured in another peak season of cholera. On the other hand, zinc, iron and manganese were minimum or below detection limit during the seasonal abundance of cholera. Magnesium was higher ranging (8.96-11.13), (17.78-18.50) and (10.16-10.35) at site-2, site-8 and site-11 respectively.

**Table 1.** Amount of micronutrients analyzed of some contaminated ponds in Mathbaria during the peak season of cholera.

| Contaminated ponds | Cholera infection period | Total nitrogen (ppm) | Phosphorus (mg/100g) | Zinc (ppm) | Iron (ppm) | Magnesium (ppm) | Manganese (ppm) |
|---|---|---|---|---|---|---|---|
| Site-2 | April | 0.812 | 28.8 | <0.01 | 0.011 | 11.13 | <0.01 |
| | May | 0.504 | 34.4 | <0.01 | <0.01 | 8.96 | <0.01 |
| | October-1st week | 0.315 | 25.2 | 0.672 | 1.159 | 4.466 | 0.001 |
| | October-3rd week | 0.238 | 25.20 | 0.217 | 1.150 | 3.654 | 0.072 |
| Site-8 | April | 0.448 | 29.6 | <0.01 | 0.014 | 18.50 | <0.01 |
| | May | 0.478 | 29.56 | <0.01 | <0.01 | 17.78 | <0.01 |
| | October-1st week | 0.175 | 29.10 | 0.011 | 0.937 | 3.641 | 0.774 |
| | October-3rd week | 0.175 | 18.60 | BDL | 0.129 | 3.826 | 0.085 |
| Site-11 | April | 0.523 | 28.3 | <0.01 | 0.013 | 10.35 | <0.01 |
| | May | 0.578 | 31.2 | 0.089 | 0.0335 | 10.16 | <0.01 |
| | October-1st week | 0.280 | 21.00 | 0.0117 | 0.967 | 3.633 | 0.096 |
| | October-3rd week | 0.252 | 22.50 | 0.0117 | 0.397 | 3.612 | 0.023 |

*V. cholerae* the prime agent for causing horrible cholera needs some sort of nutrients for their survival and infectious activity. In Mathbaria, Nitrogen and Phosphorus amount as well as some other micronutrients were available in those contaminated ponds of Mathbaria during peak season of cholera. During the study period, water sample of three heavily contaminated ponds were analyzed to observe the nutrients level for the production of primary producers (blue-green algae) which influence the growth and abundance of zooplankton to act as the reservoir of *V. cholerae*.

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## 4. CONCLUSION

Micro ecosystem study of copepods in three water sources in laboratory condition inoculated with the pure culture of toxigenic *Vibrio cholerae* O1 revealed that the growth of bacteria increased with the increased production of nauplii.

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**Competing Interests:** The authors declare that they have no competing interests.
**Authors' Contributions:** All authors contributed equally to the conception and design of the study.

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