

Survival of *Vibrio cholerae* O139 in Association with *Anabaena variabilis* in Four Different Microcosms

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Abstract: Investigation was carried out to see the association of *Vibrio cholerae* O139 with *Anabaena variabilis*, in four different types of water. Conventional media were used to count culturable bacteria while epi fluorescence microscopy to count the viable but non culturable (VBNC) *V. cholerae* O139. It was found that the culturable form of *V. cholerae* O139 found 9 days in the control artificial sea water and 4 days in river water and BOD dilution water. However, in association with *A. variabilis*, culturable form was found for 6 days in artificial sea water, 4 days in distilled water, 1 day in BOD dilution water, 4 days in river water. The bacterial count of surrounding water decreased inversely with the bacterial count in association with algae. Among these four microcosm water, associations of *V. cholerae* O139 with algae was higher in artificial sea water. In control water of four microcosms, direct viable count (DVC) gradually decreased with time. In association with algae the DVC increased after some time then reached a peak in 4-8 days depending on the types of water. The epi fluorescence microscopy indicated that *V. cholerae* O139 survived for more than one month in association with algae. Present study demonstrated that survival of *V. cholerae* O139 appears to get advantages in association with *A. variabilis* in different types of water.

Key words: Water type, *Vibrio cholerae*, microcosm

INTRODUCTION

Cholera which is a major public-health problem in developing countries, caused by infection of the intestine with toxigenic *Vibrio cholerae*^[1]. In health children, HIV patients are at vulnerable risk of death if infected by this disease. Even this disease can kill a healthy adult within hours. A global cholera update by World Health Organization (WHO) indicates a grand total of about 236-896 cases of cholera from 52 countries with about 6311 deaths which is overall 79% higher than the number of cases notified in 2005^[2]. Bangladesh and other South Asian countries is endemic zone for cholera. Cholera is endemic in Bangladesh and maintains a regular seasonal pattern^[3]. In this country, cholera epidemics occur twice every year, the highest peak during post monsoon (September-January) and second smaller peak during pre monsoon (March-May). During interepidemic period *V. cholerae* cannot be cultured from the surface water whereas in epidemic season it can be isolated from the patient body as well as from surface water^[4].

V. cholerae O139 have been identified as an etiological agent of cholera since 1993^[5]. Out of 150 sero groups, only *V. cholerae* O1 and O139 are related to the epidemics of cholera^[5,6,7]. The rest of the non O1 and non O139 serogroups of *V. cholerae* do not have

epidemic and pandemic potential^[8]. They are known to cause sporadic or small outbreaks of diarrhoea and extra intestinal infections^[9]. When *V. cholerae* is not wreaking havoc in the human intestine, it may be found in diverse aquatic environments such as estuaries, rivers, ponds etc.^[7,10]. In the aquatic environment *V. cholerae* can survive either as free living planktonic organisms in the water column or associated with phytoplankton and zooplankton^[11,12]. In natural habitat *V. cholerae* remains attached to *Anabaena* sp and *V. cholerae* O1 was found to be persistent in the mucilaginous sheath of a blue green alga *A. variabilis* in the artificial aquatic environment^[13].

Some investigators studied viability of *V. cholerae* O1 in different aquatic environment. Pruzzo *et al.*,^[14] found that in artificial sea water *V. cholerae* O1 remain viable for 40 days without losing their adhesive properties in presence of copepods and chitin. Feachem^[15] demonstrated that *V. cholerae* survived upto one month at 4°C and 2-14 days at 20-30°C in clean water.

The occurrence of culturable *V. cholerae* O139 in various components of the aquatic environment in Bangladesh has been published in 1996^[16]. However, a few studies have been done in regard to the association of *V. cholerae* O139 with blue green algae with respect to changes in physicochemical features of water.

During the present study, attempts were made to compare the growth pattern (both in culturable and non-culturable state) of *V. cholerae* O139 in association with *A. variabilis* in four different types of water VIZ. artificial sea water, distilled water, river water and BOD dilution water. If this virulent agent of cholera has got broad spectrum survival, it would have a greater potency of availability in nature; this would help in public health safety.

MATERIALS AND METHODS

Bacteria and algae strains and Preparation of inoculum: A strain of *V. cholerae* O139 (1852), an environmental strain was collected from the Environmental Microbiology Lab of ICDDR, B. This strain was reconfirmed by culture, biochemical and serological, physiological and molecular technique^[17]. The *A. variabilis* belonging to the phylum Cyanophyta, used in this study was initially collected from the department of Botany, Bangladesh, DU, Bangladesh and maintained as pure culture for long time at Environmental Microbiology Lab of ICDDR, B, Dhaka. For the current study algae was used from this stock culture and all experiments were conducted at International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B).

V. cholerae O139 strains were cultured on taurocholate tellurite gelatine agar (TTGA) plates at 37°C for 18- 24 hrs by following small modification of previously published method^[1]. In previous method, inoculum was prepared in 7 mL 0.1% NaCl solution (pH 7.0) but in this experiment prepared by mixing properly a loopful of culture in 5 mL 0.1% NaCl (pH 7.0) saline water. To make sure concentration of the prepared inoculum was \log_{10} 7 cells mL⁻¹, was compared with 0.5 standard MacFarland solution. A cell count per millilitre was determined by using drop plate method^[18].

Preparation of microcosm water: Four types of water VIZ. artificial sea water, distilled water, river water and BOD dilution water were used in four different microcosms in this study. Artificial sea water and BOD dilution water were prepared in the laboratory using method which was devised by Kester, Duedal, Cannors and Pytokowicz (1967)^[19] and standard method^[20] respectively. Artificial sea water was prepared by adding NaCl (23.926 G kg⁻¹ of solution), Na₂SO₄ (4.008 G kg⁻¹ of solution), Na HCO₃ (0.196 kg⁻¹ of solution), KCl (0.677 G kg⁻¹ of solution), KBr (0.098G kg⁻¹ of solution), MgCl₂ (1.071 g mL⁻¹), CaCl₂ (1.085 g

mL⁻¹) in 1000mL distilled water. BOD dilution water was prepared by adding Phosphate buffer solution (8.5g KH₂PO₄+21.75g K₂HPO₄+33.4g Na₂HPO₄.7H₂O+1.7g NH₄Cl), Magnesium sulphate solution (22.5g MgSO₄.7H₂O), Calcium chloride (27.5g), Feric chloride (0.25g), in 1000 mL distilled water. River water was collected from Dhonagoda river of Matlab, Bangladesh.

Survival experiment for culturable growth: Hundred milliliter of artificial sea water, distilled water, river water, BOD dilution water respectively was inoculated with defined number of *V. cholerae* and 1 gm wet *Anabaena* sp. was added from pure culture in 250 mL experimental flasks. One control flask (with defined number of *V. cholerae* but without algae) was used for every experimental flask. Sampling was performed from each experimental and control flask after 30 minutes was considered as 0 h reading. The samples were cultured in two TTGA media^[21] seperately. The steps of this experiment were followed by the procedures described by Islam *et al.*^[1] with few changes in amount. In brief, 0.05 gm wet weight of algae were collected with the help of a sterile spatula and then gently squeezed on the glass walls of the flasks to remove the excess water. This algae was homogenized manually in the glass made homogenizer with 1 mL 0.1% saline water. Then tenfold serial dilution was prepared and 25 µL proportions from each dilution were inoculated and spread on to the duplicated TTGA plates. The plates were incubated at 37°C for 24 h and the bacterial counts were derived from counts of individual colonies and expressed as CFU mL⁻¹ or gm (colony forming unit). Duplicated plates were used to average the bacterial count for each dilution.

Fluorescent microscopy for viable but nonculturable growth: When the cells of *V. cholerae* O139 were failed to grow on TTGA media, they were thought to be enter the non-culturable state and samples were subjected to fluorescent antibody test according to the procedure described by Islam *et al.*^[22] to detect the non- culturable state and to detect direct viable count (DVC). Homogenates of algae and 1.0 mL of water from experimental flask and control flask of each set were taken into the eppendorf tubes containing yeast extract (0.025%) and nalidixic acid (0.002%). The tubes were incubated overnight in a dark place at room temperature. 5µL of sample from each eppendorf tube was placed on PTFE (polytetrafluoroethylene) coated well glass slides and were air dried and flame fixed. Then one drop (20 µL) of 1:10 *V.*

cholerae O139 polyclonal antiserum (made in ICDDR, B) was added to the wells and mixture was incubated at 37°C for 30 min in moist chamber. Slides were rinsed in sterile PBS, gently blotted and air dried. Fluorescein isothionate conjugated (FITC) antimouse Ig G (Sigma)(20 µL) diluted in PBS (1: 80) was added to the wells and incubated for 30 min at 37°C. The slides were then washed and mounted under coverslip with FA mounting oil. Finally the slides were examined under an epifluorescence Microscope (model 2 BH. 2, Olympus), culturable, *V.choleraei* O139 was used as a positive control.

RESULTS AND DISCUSSION

The Fig. 1 showed the survival pattern of *V. cholerae* O139 with *Anabaena* sp. in artificial sea water. In control flask water the culturable count increased 1.038 log₁₀ CFU mL⁻¹ from 0 h-24 h Then culturable count slightly decreased throughout 2nd day, 4th day and 6th day reading and reduced to 3.374 log at 9th day. After that no culturable *V. cholerae* O139 could be detected even after enrichment in APW. Incase of water where *Anabaena* sp. was floating (supernatant) the culturable count increased 1.113 log from 0-4 h and then the culturable count of *V. cholerae* O139 decreased to 2.225 log at 6th day. In association with algae the culturable count increased 0.961 log at 4th day of sampling.

In the control flask of distilled water (Fig. 2) the culturable count increased 0.873 log from 0 h to day 3. This count slightly decreased an amount of 0.382 log on day 9. In case of water where *Anabaena* sp. was floating the culturable count decreased an amount of 3.973 log from 0 h to day 3. In association with algae the culturable count increased 0.312 log from 0 h-4 h. This culturable count decreased an amount of 2.523 log at 3rd day reading.

In control flask of river water (Fig. 3) the culturable count increased 0.056 log from 0-6 h. Then culturable count gradually decreased 3.398 log at day 4. In case of water where *Anabaena* sp. was floating the culturable count gradually decreased 0.306 log from 0 h to day 2. In association with algae the culturable count gradually increased 0.286 log from 0 h to day 2. This culturable count deceased 1.136 log at day 4.

Figure 4 showed the survival and culturable pattern of *V. cholerae* O139 with *Anabaena* sp. in BOD dilution water. In contol flask water, the culturable count increased 0.325 log from 0 h to day 4. Incase of control water, *V. cholerae* O139 survived upto 4 days. In case of water where *Anabaena* sp. was floating the culturable count decreased to 1.19 log from 0 h of

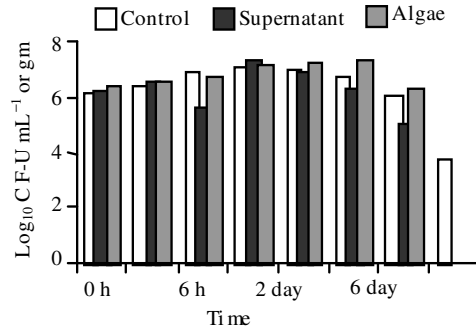


Fig. 1: Culurable count of *V. cholerae* O139 in artificial sea water microcosm (ph 8.0)

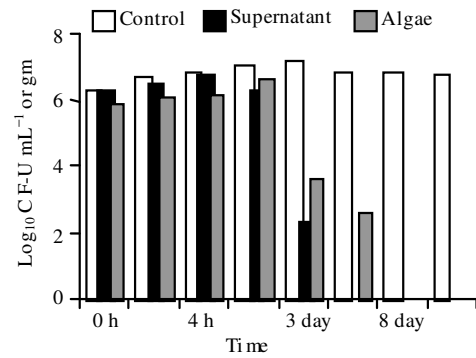


Fig. 2: Culurable count of *V. cholerae* O139 in distilled water microcosm (ph 8.0)

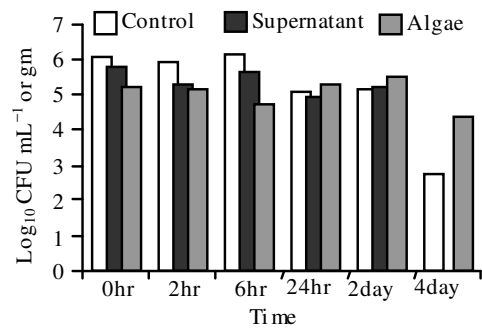


Fig. 3: Culurable count of *V. cholerae* in river water microcosm (pH 7.94)

sampling to 24 h. In association with algae the culturable count increased 0.488 log from 0-2 h of sampling but a decrease of 0.322 log was observed at 24 h reading.

In artificial sea water the multiplication of *V. cholerae* as VBNC state was observed in association with *Anabaena* sp. (Fig. 5). The initial count of VBNC cell was 6.68 log₁₀ cells/gm which increases to 7.28

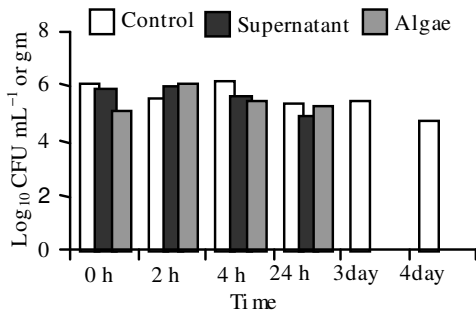


Fig. 4: Culturable count of *V. cholerae* in BOD dilution water (pH 7.70)

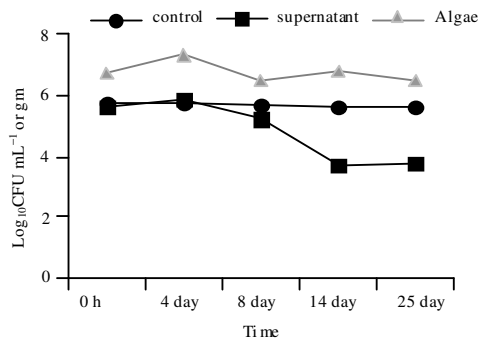


Fig. 5: Fluorescent antibody Direct Viable Count (DVC) of *V. cholerae* O139 in artificial sea water microcosm (pH 8.0)

log₁₀ cells/gm on day 4 and at the end of the experiment, the count was 6.431 log₁₀ cells/gm. The count in algal water at 0 h was 5.57 log₁₀ cells/mL, decrease to 3.69 log₁₀ cells/mL on days 14. At 30th day the count was 3.75 log₁₀ cells/mL. No significant increase or decrease of VBNC count was observed in control water.

In river water the VBNC count of *V. cholerae* in *Anabaena* sp. was increase to 7.1 log₁₀ cells/gm from the initial 6.11 log₁₀ cells/gm at 8 day while at 14th day, the count decrease to 4.41 log₁₀ cells/mL algal water (Fig. 6). Insignificant changes of VBNC count was found in control water.

In distilled water, the VBNC count of *V. cholerae* either inside or surface of the *Anabaena* sp. gradually increased at day 8 (from initial 6.15 log₁₀ cells/gm to 8.07 log₁₀ cells/gm) and then gradually decreased to 7.17 log₁₀ cells/gm at day 30 (Fig. 7). In *Anabaena* water VBNC of *V. cholerae* maintained a steady state with initial count and the end count (5.87 log₁₀ cells/mL at 0 h, 5.44 log₁₀ cells/mL at day 30). A decrease of 1.01 log₁₀ cells/mL was observed in control water from 0 h-day 30.

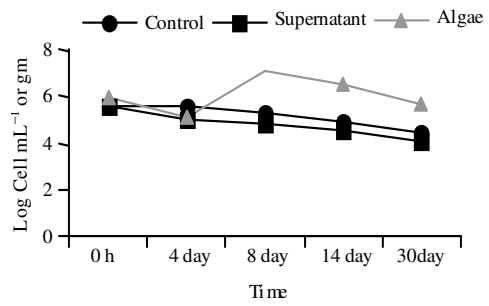


Fig. 6: Fluorescent antibody Direct Viable Count (DVC) of *V. cholerae* O139 in distilled water microcosm (pH 8.0)

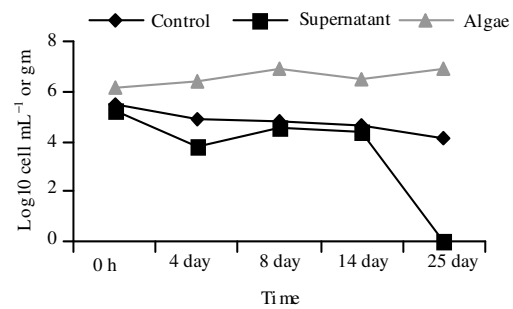


Fig. 7: Fluorescent Antibody Direct Viable Count (DVC) of *V. cholerae* O139 in River water microcosm (pH 7.94)

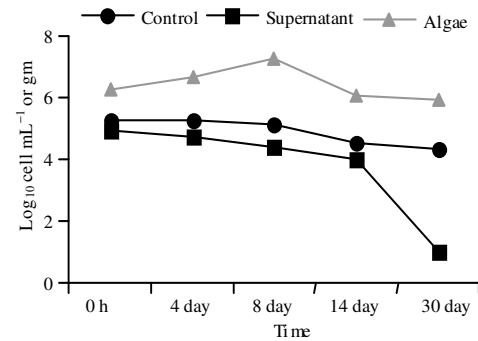


Fig. 8: Fluorescent antibody Direct Viable Count (DVC) of *V. cholerae* O139 in BOD dilution water microcosm (pH 7.70)

V. cholerae O139 of control water of BOD dilution water, did not show any significant changes in DVC count at 0 h for DVC was log₁₀ 5.252 cells/mL (Fig. 8). In association with *Anabaena* sp. the highest count of DVC was found at day 8 (log₁₀ 7.279 cells/mL). After day 8 the DVC counts gradually decreased to log₁₀ 5.960 cells/mL at day 30. In case of water where

Anabaena sp. was floating, DVC did not differ very much from 0 h to day 8 (\log_{10} 4.954 cells/mL, \log_{10} 5.176 cells/mL respectively). The DVC could be observed at day 14 sample and onwards.

Artificial sea water provided the best environment for the *V. cholerae* O139 for more attachment with algae in this experiment. On the other hand, BOD dilution water seemed less friendly for the growth of *V. cholerae* O139. This suggests that water quality has role in the survival of *V. cholerae* in association with *Anabaena* sp. This finding can be supported by previous studies. Water quality plays important role in bacteria-algal association and transmission of cholera^[23,24]. Here the culturable count in algal water was inversely proportionate to the count of bacteria-algal association. Higher count found in algal water at initial stage which gradually decreased within 2-4 days. This trend was completely opposite in bacteria-algal association. It inferred that *V. cholerae* O139 entered into the mucilaginous sheath of algae for shelter and nutrition. This finding is also supported by the study of Schneider and Parker^[25] where they found that *V. cholerae* can secrete an enzyme mucinase which degrade the mucin and mucin like substances present in the plant cells, helps to penetrate bacteria into algae. More studies have demonstrated that blue green algae, *Anabaena* sp. may act as a reservoir of *V. cholerae*^[26]. Islam^[27] also demonstrated that *V. cholerae* O1 can survive and attach in association with four marine algae longer period than water surrounding marine algae. This also infers that *V. cholerae* can make association with algae. Strain *V. cholerae* O139 remained in VBNC in blue-green algae in large number than in control water or in algal water. This result is concordance with another study by Munro^[28] where they found that in sea water VBNC *V. cholerae* O1 may survive for some times.

In brief this research leads to following findings: Survival rate of the bacteria with the algae depends on the quality of the water, *V. cholerae* O139 survives long time in control water because of the absence of nutrient intake by algae and interference of other bacteria and *A. variabilis* can act as a reservoir of *V. cholerae* O139 where they can hide for long time as VBNC form.

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