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Population Dynamics of Vibrios in Biotic Biofilm in the Aquatic Environment of Bangladesh

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Abstract: The *Vibrio* sp. forming biofilm on biotic surface especially chitin and algae was investigated using artificial chitin and *Anabaena variabilis* from pure culture of laboratory and glued to plexiglass disc. The presence of culturable *Vibrio* spp. were investigated using cultural technique for TCBS agar medium after homogenization and physicochemical parameters were measured by standard techniques. The Pearson correlation coefficient applied by SPSS software. The results indicated that out of 13 sampling period, only *V. cholerae* O1 was isolated 7.7% sample while 30.8% samples were positive for *V. cholerae* non-O1, *V. proteolyticus* and *V. mimicus* from canal site. From pond ecosystem, all the chitin samples were negative for *V. cholerae* O1 but 15.4% were positive for *V. cholerae* non-O1 and *V. proteolyticus* and 30.8% samples were positive for *V. mimicus*. The biofilm formation is significantly correlated with the pH, DO and CO_2 concentration present of the corresponding water. This study indicates that biotic surface like chitin and algae could function to form biofilm and the water physicochemical parameters have the relationship with the *Vibrio* community present in the samples.

Key words: Algae, biofilm, chitin, physicochemical parameters, *vibrio*

INTRODUCTION

The World Health Organization (WHO) estimates that about 1.1 billion people globally drink unsafe water and (88%) diarrheal diseases in worldwide caused by using unsafe water, sanitation and hygiene (WHO, 2003). *Vibrios* are gram-negative bacteria, which often cause disease in humans. Infections by *V. cholerae* (Finkelstein, 1973), *V. mimicus* (Davis *et al*.,1981) and *V. proteolyticus* (Muniesa-Pe´Rez *et al*., 1996) are acquired through consumption of contaminated water and lead to excessive watery diarrhea (*V. cholerae* and *V. mimicus*) and septicemia (*V. vulnificus*). *Vibrio* species are ubiquitous in aquatic ecosystems. Although many *Vibrio* species are free living, a small group can form pathogenic or symbiotic interactions with eukaryotic hosts. Indeed, these *Vibrio* species alternate between growth within their hosts and prolonged survival in aquatic ecosystems. When *V. cholerae* is not wreaking havoc in the human intestine, it may be found in diverse aquatic environment such as estuaries, rivers, ponds etc (Colwell and Spira, 1992; Islam *et al*., 1995). Observational studies in Bangladesh epidemiologically link seasonal phytoplankton and zooplankton blooms with cholera outbreaks (Islam *et al*., 1993). In the aquatic habitats, *V. cholerae* is found as a free living bacterium or survive either as free-living planktonic organisms association with aquatic life forms, such as zooplankton, phytoplankton, crustaceans, algae, insects and plants (Islam *et al*., 1994; Heidelberg *et al*., 2002; Huq *et al*., 1983, 1986).

Bacteria attach to surfaces of this kind as widely-separated individuals, small colonial aggregates or confluent biofilm communities characterized by interactions between community members and a three dimensional architecture that provides channels through which nutrients and metabolic by-products circulate. Biofilms that form in multi-species habitats can be composed either of a single strain or of multiple strains or species (Davey and O'Toole, 2000). Numerous studies have investigated biofilm formation in *Vibrio* species. Adhesion to biotic and abiotic surfaces is likely an impotant adaptive strategy for V. cholerae survival in the environment. *V. cholerae* O1, non-O139, non-O139 and

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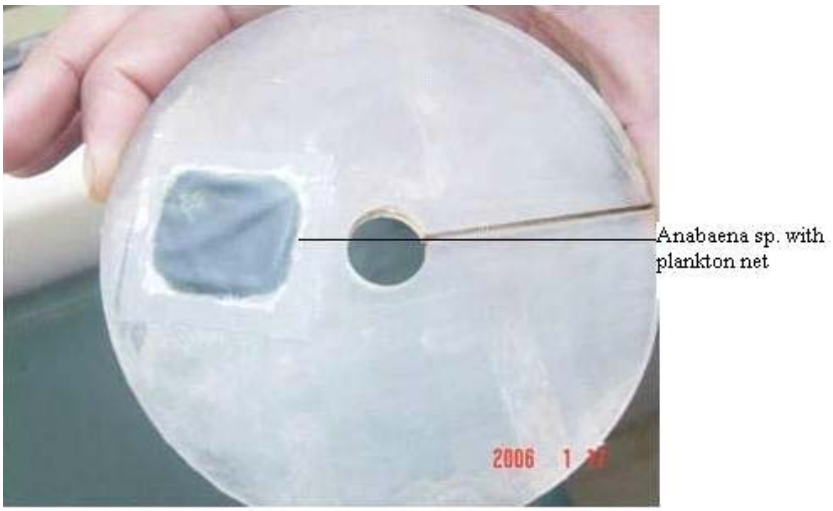

Fig. 1: Plexiglass disc glued with anabaena and then plankton net is overlaid on *Anabaena* sp.

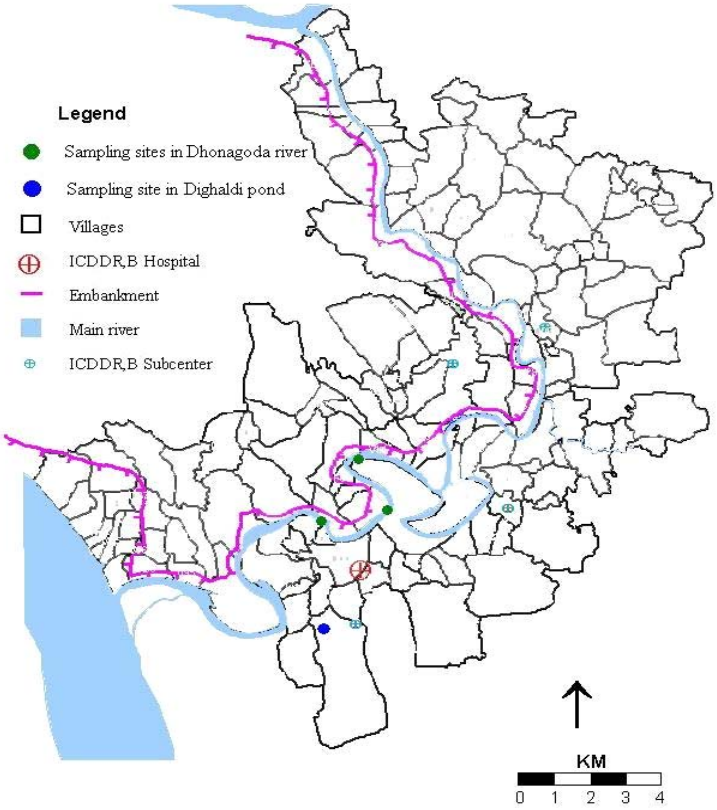

Fig. 2: The map Chandpur district showing the sampling area

other *Vibrio* produce chitinases and are capable of utilizing chitin as acarbon and nitrogen source (Garay *et al*., 1985). *V. cholerae* adsorbed onto chitin skeletons may persist for long amounts of time and be transported to more one geographical location by currents and tides (Colwell, 1996). Not only the chitin but also the close contact between the algal and the heterotrophic community in attached biofilms favors the use of algal material by microorganisms within the biofilm (Haack and McFeters, 1982; Nakano, 1996). Algal accumulation

and activity enhance the heterotrophic community's use of organic matter by increasing the amount of substrate available for bacteria (Espeland *et al*., 2001; Romani and Sabater, 1999). *V. cholerae* attached to biotic surfaces, such as mucilaginous sheath of algae and chitinous flora and can derive nutrients from digestion of the surface. By contrast *V. cholerae* attached to an abiotic (non-nutritive) surface can obtain nutrients only from the water column (or adsorbed from the water column onto the abiotic surface). The concentrations of such nutrients (in the water column or adsorbed from it onto the surface) are typically low. However this situation is dramatically altered if the abiotic surface also contains other species which are nutrient producers or supplier. These primary food producing organisms include various kinds of phytoplankton and the nutrient supplier is the chitin.

Though biofilm is very common phenomena in aquatic environment, but most of the studies of *Vibrio* cholerae with only biofilm, but the present study was undertaken to find out the role of chitin and algae as biotic surface in biofilm of *Vibrio* sp. and the relationship with the physicochemical parameters of water.

MATERIALS AND METHODS

Biofilm sampling device: The Biofilm device developed by the Maryland Sea Grant program, USA was used for the present study. It consists of plexiglass discs strung on nonfilament line at predetermined depths, one end of the line anchored to the bottom, the other and suspended from a float. Each biofilm sampling device had three pelxi-glass sampling discs, 1st one located 12 cm depth from the surface water (within the photic zone); 3rd one 12 cm from the bottom (near the Bentic zone); and the second at mid depth. Besides, another sampling device was also studied, each of which contained two pelxi discs. One of which contained 1 g of autoclaved artificial chitin chips and the other one's with 1 g of pure Anabaena variabilis a filamentous blue green algae. Chitin and A. variabilis were trapped by 20 mm phytoplankton net whose boarders were sealed by special type of glue that protect any unwanted entrance of environmental phytoplankton and zooplankton (Fig. 1). Then the Pelxi-glass discs with *A. variabilis* was placed 12 cm depth from the surface (within the photic zone), and the chitin containing plexi-disc was placed 5 cm below from *A. variabilis* containing plexi-disc. Adjustment of discs sampling positions were undertaken as needed by changes in the depth of the water sources.

Sampling period and sites: Two locations within the Matlab study site were selected. One of them was a canal and another was a pond. A canal of the Megan River, adjacent to the Matlab laboratory and hospital; and a pond located close to the river embankment (not in direct communication with the river delta system) were selected for sample collection (Fig. 2) and the samples were collected within every two weeks intervals for six months. The study was conducted from August 2005 to January 2006.

Sampling and processing: The discs of the biofilm device were taken out of the water. The biofilm samples were taken by scraping with the edge of a razor and preserved in 3.0 mL phosphate Buffered Saline (PBS) at <10ºC during transport to the laboratory. All the samples were transported to the laboratory inside a cooler box and processed in the Environmental Microbiology Laboratory of ICDDR,B within 24 h of collection. 1.0 mL-uncrushed sample was kept was kept with 3.5 mL PBS (Phosphate Buffered Saline, pH 7.4) and 500 μL formalin for phytoplankton and zooplankton enumeration. 2.0 mL of sample was homogenized with 1 mL of PBS using a stedfast stirrer (Model 300, Fisher Scientific, USA). 1.0 mL of homogenate was enriched in 10 mL 1% APW (Alkaline Peptone Water; pH 9.0) and incubated 4-6 h at 37ºC. 450 μL of biofilm samples was enriched with 6.25 μL yeast extract and 5 μL nalidixic acid and incubated at room temperature overnight in the dark after which the samples were fixed with formalin (40% formaldehyde solution).

Enumeration of total *Vibrio* sp. and identification of *Vibrio* sp.: Enrichment for *Vibrio* species and viable bacterial counting were performed by procedure describes elsewhere (Islam *et al*., 2007). In brief, chitin, Anabaena and other biofilm samples was homogenized using a steadfast stirrer (Model 300, Fisher Scientific, USA). One ml of homogenate was enriched in Alkaline Peptone Water (APW) and incubated for 6 h at 37ºC. The other portion of homogenate were directly placed on thiosulfate-citrate-bile salts- sucrose (TCBS, Himedia) agar plates and incubated at 37ºC for 16-18 h. The number of viable *Vibrio* isolates was estimated as CFU/g of biofilm samples. The total counts were found after adding all the counts from chitin, algae and plexiglass disc. The primary and secondary enrichments into APW for *Vibrio* detection were performed as described previously (Baumann *et al*., 1984; Kelly *et al*., 1992). From the enrichment samples in APW, 2 loopful were taken and inoculated onto TCBS and TTGA plates and incubated at 37ºC for 18-24 h. Suspected *Vibrios* colonies were further characterized following the procedures described earlier (Islam *et al*., 1995). In brief, strains were identified as *V. cholerae* if they fulfilled the following criteria: Gram negative, oxidase positive, produced acid from sucrose but not inositol and decarboxylated lysine and ornithine but not arginine. All the Strains were serotyped and biotyped following the procedures

Table 1: Presence of *V. cholerae* O1 and *V. cholerae* O1, O139, *Vibrio proteolyticus*, *Vibrio mimicus* in 13 samples each from chitin, surface, middle, bottom, *Anabaena* discs and water from canal site of Matlab, Bangladesh

Isolates	Device	1	2	3	4	5	6	7	8	9	10	11	12	13	Total (%)
Vibrio	Chitin	01	0	0	0	0	0	0	0	0	0	0	0	0	1(7.7)
cholerae O1	Surface	01	0	0	0	0	0	0	0	0	0	0	0	0	1 (7.7)
	Middle	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0)
	Bottom	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0)
	Anabaena sp.	01	0	0	0	0	0	0	0	0	0	0	0	0	1(7.7)
Vibrio	Chitin	0	01	0	0	01	01	01	0	0	0	0	0	0	4 (30.8)
cholerae	Surface	01	0	0	01	0	0	0	0	0	0	0	0	0	2 (15.4)
non-O1/	Middle	0	0	01	01	0	0	0	0	0	0	0	0	0	2 (15.4)
non-O139	Bottom	0	0	01	0	0	0	0	0	01	0	0	0	0	2 (15.4)
	Anabaena sp.	0	01	0	01	0	0	0	01	0	01	0	0	0	4 (30.8)
Vibrio	Chitin	0	0	0	0	0	0	0	01	0	0	0	0	0	1(7.7)
proteolyticus	Surface	0	0	0	0	0	01	0	0	0	0	0	0	0	1(7.7)
	Middle	0	0	0	0	0	01	0	0	0	0	0	0	0	1(7.7)
	Bottom	0	01	0	0	0	0	01	0	0	0	01	0	0	3 (23.1)
	Anabaena sp.	0	0	0	0	0	0	0	0	0	0	01	0	0	1 (7.7)
Vibrio	Chitin	0	0	0	0	0	0	0	01	0	01	01	01	0	4 (30.8)
mimicus	Surface	0	0	0	0	0	0	0	0	0	0	0	01	0	1(7.7)
	Middle	0	0	0	0	0	0	0	0	0	01	0	01	0	2 (15.4)
	Bottom	0	0	0	0	0	0	0	0	0	0	0	01	0	1 (7.7)
	Anabaena sp.	0	0	0	0	0	0	0	0	01	0	0	01	01	3 (23.1)
Total *Vibrios*		04	03	02	03	01	03	02	03	01	03	03	05	01	35

Table 2: Presence of *V. cholerae* O1 and *V. cholerae* non-O1, non-O139, *Vibrio proteolyticus*, *Vibrio mimicus* in 13 sampleseach from chitin, surface, middle, bottom, *Anabaena* discs and water from pond site of Matlab Bangladesh

Isolates	Device	1	2	3	4	5	6	7	8	9	10	11	12	13	Total positive
Vibrio	Chitin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cholerae O1	Surface	0	0	0	0	01	0	0	0	0	0	0	0	0	1 (7.7)
	Middle	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Bottom	0	0	0	0	01	0	0	01	0	0	0	0	0	2 (15.4)
	Anabaena sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio	Chitin	0	0	0	0	0	0	01	0	0	0	0	01	0	2 (15.4)
cholerae	Surface	0	0	0	01	01	0	0	0	0	0	0	0	0	2 (15.4)
non-O1/non	Middle	0	0	0	0	0	0	0	0	0	01	0	01	0	2 (15.4)
O139	Bottom	0	01	0	01	0	0	0	0	01	01	0	01	0	5 (38.5)
	Anabaena sp.	0	01	0	0	0	0	0	0	0	0	0	0	0	1 (7.7)
Vibrio	Chitin	0	0	0	0	0	0	01	0	01	0	0	0	0	2 (15.4)
proteolyticus	Surface	0	0	0	0	0	0	0	0	0	01	0	0	0	1 (7.7)
	Middle	0	0	0	0	0	01	0	0	0	0	0	0	0	1 (7.7)
	Bottom	0	0	0	0	0	01	01	0	0	01	0	0	0	3 (23.1)
	Anabaena sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio	Chitin	0	0	0	0	0	0	01	01	01	01	0	0	0	4 (30.8)
mimicus	Surface	0	0	0	0	0	0	01	0	0	0	01	01	0	3 (23.1)
	Middle	0	0	0	0	0	0	01	01	0	01	0	0	01	4 (30.8)
	Bottom	0	0	0	0	0	0	0	01	01	0	0	0	0	2 (15.4)
	Anabaena sp.	0	0	0	0	0	0	01	0	01	0	0	0	0	2 (15.4)
Total *Vibrios*		0	02	0	02	03	02	07	04	05	06	01	04	01	37

described by Kelly *et al*. (1992). Other types of *Vibrios* were identified according to Kelly *et al*. (1992).

Measurement of chemical parameters: The temperature, total dissolved solids (TDS), dissolved oxygen (DO) and pH were measured using portable meters (HACH Conductivity Meter, Cat. No. 51800-18; HACH Portable Dissolved Oxygen Meter, Cat. No. 51850-18; Sension TM6, CO, USA and Orion Portable pH Meter, Cat. No. 210 A; Orion Research, MA, USA). The CO_2 was measured by titrimetric methods following standard procedures (APHA, 1998).

Identification of phytoplankton: The uncrushed formalin-preserved samples were shaken gently for proper mixing from which 1 ml sub-sample was drawn by pipette and transferred into a Sedgewick-Rafter counting cell (APHA, 1998). The samples were then observed under a compound binocular microscope for identification. The phytoplankton was identified following the procedures described by various authors (APHA, 1998; Islam and Khatun, 1966; Islam and Nahar, 1967; Islam *et al*., 1992; Prescott, 1984).

Statistical analysis: Pearson correlation analysis ($p<0.05$ = * and $p<0.01$ = **) was carried out with the statistical SPSS (version 15) programme for Windows in order to examine the relation between total cultural *Vibrios* from biofilm, physicochemical parameters of water and total phytoplankton counts from water.

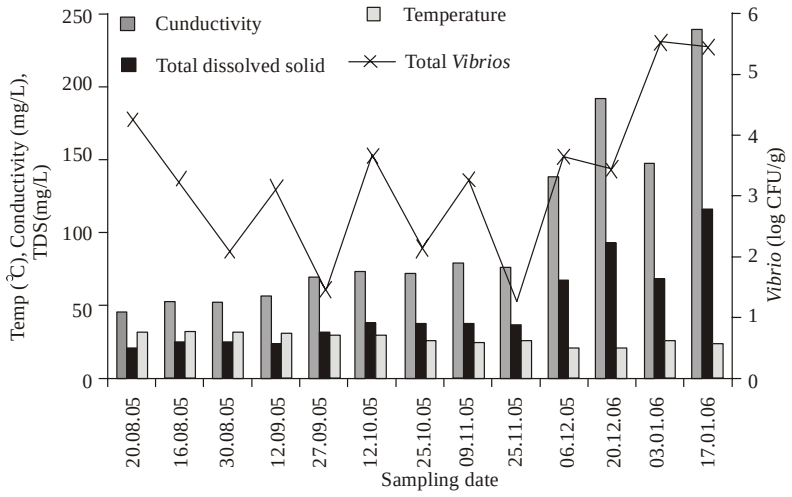

Fig. 3: Relationship of temperature, conductivity and TDS with total *Vibrio* sp. from canal site at Matlab in Bangladesh

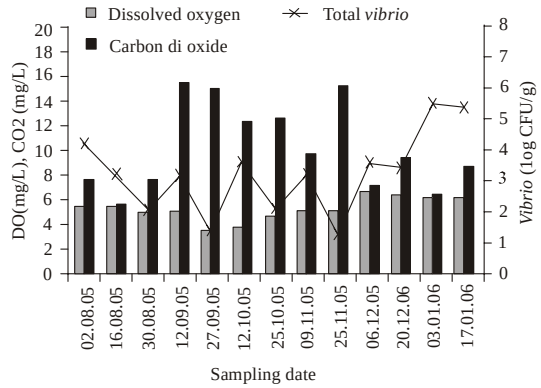

Fig. 4: Relationship of CO_2 and DO with total *Vibrio* sp. from canal site of Matlab in Bangladesh

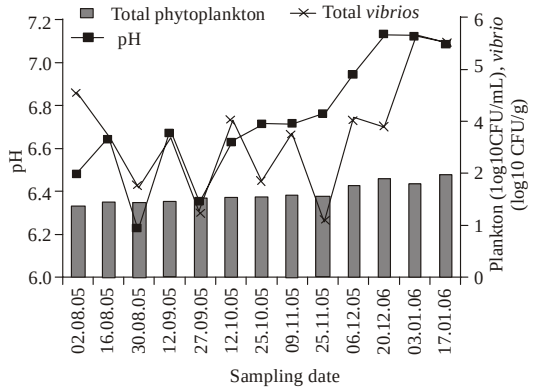

Fig. 5: Relationship of phytoplankton, pH with total *Vibrio* sp. from canal site of Matlab in Bangladesh

RESULTS

Abundance of culturable *Vibrios* in biofilm samples Table 1 and 2 show *V. cholerae* O1, *V. cholerae* non-O1, *V. proteolyticus* and *V. mimicus* isolates from canal and pond site during different months. Ever round were positive for *Vibrios* from canal but pond site were negative for round 1 and 3 (Table 1 and 2). The chitin yielded 7.7%(1/13) *Vibrio cholerae* O1 and 30.8% (4/13) *V. cholerae* non-O1, *V. proteolyticus* and *V. mimicus* from canal site. The phytoplankton yielded 7.7% (1/13), 30.8%, 7.7%(1/13), 23.1%(3/13) *V. cholerae* O1, *V. cholerae* non-O1, *V. proteolyticus* and *V. mimicus*, respectively. No *V. cholerae* O1 was found in culturable form from pond and the chitin yielded 15.4%(2/13), 15.4 and 30.8% (4/13) of *V. cholerae* non O1, *V. proteolyticus* and *V. mimicus*, respectively. The plankton contained 7.7%(1/13) and 15.4% *V. cholerae* non-O1 and *V. mimicus*, respectively. Culturable *V. cholerae* O1 was isolated from canal only in the month of August but *V. cholerae* non-O1, non-O139, *V. proteolyticus* and *V. mimicus* was isolated many months during the study period (Table 1). The chitin yielded 15.4%(2/13), 15.4%(2/13) and 30.8 %(4/13) of *V. cholerae* non-O1, *V. proteolyticus* and *V. mimicus*, respectively.

Physicochemical variables: Figure 3, 4 and 5 demonstrate those physicochemical parameters of water and phytoplankton from canal sites and its relationship to form biofilm by *Vibrio* spp. It was observed that water temperature fluctuated between 24.1 and 33.5ºC, conductivity between 45.9 and 239.1µS/cm, total dissolved solids between 21.5 and 114.7 shown in the Fig. 3. DO between 3.5 and 6.7 mg/L (Fig. 4) and CO_2

Table 3: Relation of environmental parameters with the occurrence of *Vibrio* in the pond site of Matlab, Bangladesh

Date of sampling	Tem (ºC)	pH	DO (mg/L)	CO_2 (mg/L)	Conductivity (mg/L)	TDS (mg/L)	Plankton log_{10} cell/ml)	Total *Vibrio* (log_{10} CFU/g)
02.08.05	33.66667	6.63	5.05	6.10	198.0	70.5	4.67025	1.12
16.08.05	32.03333	6.65	4.53	8.32	135.0	62.0	5.40552	2.5
30.08.05	32.76667	7.46	7.90	5.95	127.7	60.7	5.05690	1.1
12.09.05	33.0000	6.76	5.35	14.08	114.8	54.6	5.24944	2.7
27.09.05	31.56667	7.20	6.73	10.84	141.9	67.7	4.74076	3.4
12.10.05	31.5000	7.16	6.54	11.32	154.5	73.8	4.19312	2.4
25.10.05	29.9000	7.20	6.31	9.73	156.9	74.6	4.01494	7.1
09.11.05	29.66667	6.98	5.72	8.95	205.1	109.5	3.91645	4.2
25.11.05	24.36667	7.04	5.78	8.62	205.6	105.2	4.32222	5.2
06.12.05	24.73333	6.80	7.16	7.70	209.5	116.7	3.93197	6.3
20.12.06	24.23333	6.75	3.52	10.6	201.0	96.3	4.62480	2.3
03.01.06	27.1000	6.74	4.34	8.86	204.0	95.8	4.88451	4.7
17.01.06	21.06667	6.71	3.16	8.56	223.0	106.6	3.78355	3.4

between 5.76 and 15.7. Total phytoplankton counts between log_{10} 3.69 and log_{10} 5.08 cell/mL and pH between 6.2 and 7.13 (Fig. 5). Total *Vibrio* were present the entire rounds and counts oscillated between log_{10}1.3 and log_{10} 5.6 CFU/g. The Table 3 shows the physicochemical parameters and phytoplankton of water from pond and total *Vibrio* sp. counts from biofilm samples. The pond water showed the temperature fluctuation between 21 to 33.6; pH changes between 6.63 and 7.46; DO between 3.16 and 7.16, CO_2 between 6.1 and 14.08 mg/L; Conductivity between 114.8 and 223 μS/cm; TDS between 54.6 and 106.6 mg/L; total phytoplankton log103.78 and 5.40 cell/mL (Table 3). Total *Vibrio* sp. found the entire rounds and vary between log_{10} 1.1 and log_{10} 6.3 CFU/g of biofilm (Table 3).

Pearson correlation: Analysis of the data from canal site with correlation coefficient (r) revealed that total culturable *Vibrio* sp. was positively correlated with pH (r = 0. 0.621), DO (r = 0.587), conductivity (r = 0.589), TDS (r = 0.58), total phytoplankton(r = 0.558) and negatively correlated with water temperature (r = - 0.187), water CO_2 (r = -0.605). A significant correlation was observed between pH (0.54*), DO (0.035*), CO_2 (0.028*), Conductivity (0.034*), TDS (0.037*) and total *Vibrio* sp. counts whereas water temperature and total phytoplankton were not strongly correlated. We also observed the relationship of total phytoplankton with other physicochemical parameters. It was found that total phytoplankton was positively correlated with pH (r = 0.129), DO (r = 0.595), conductivity (r = 0.253), TDS (r=0.245) and negatively correlated with water temperature (r = -0.0501) and CO_2 (r = -0.797). Only the DO (0.468*) and CO_2 (0.337*) showed significance correlation with plankton but not the others parameters. Pearson statistical analysis did not reveal any significant correlation between total *Vibrio* sp. counts and water physicochemical parameters and total phytoplankton. But the correlation coefficient showed that total *Vibrio* sp. counts from pond biofilm was positively correlated with pH (r = 0.0717), DO (r = 0.143), CO_2 (r = 0.085), Conductivity (r = 0.332), TDS (r = 0.535) and negatively correlated with water temperature (r = -0.448) and total phytoplankton (r = -0.552).

DISCUSSION

Vibrios are free-living in surfaces of freshwater, oceanic and estuarine environments. In aquatic environment it may persist in associations with chitinous exoskeleton of aquatic crustacean's copepods (Huq *et al*., 1983), crabs, prawns, shrimps and lobsters, mucilaginous sheath of algae (Islam *et al*., 1990, 1994) and planktonic biofilm communities. Biofilms have been the subject of intense interest in recent years due to predominance of biofilm-associated bacteria in natural environments and the increased antibiotic resistance of biofilm bacteria compared to the relative sensitivity of planktonic bacteria (Brooun *et al*., 2000). Understanding biofilm formation on natural chitinous surfaces may provide insight into both the ecology and adaptive survival mechanisms of an environmental bacterium and human pathogen. The whole genome analysis revealed that *Vibrios* contain chitinase enzymes (*chiS* genes) (Grimes *et al*., 2009; Meibom *et al*., 2004) and their findings reiterated that *Vibrio* sp. are able to utilize chitin an insoluble polymer of GlcNAc, found in chitinous exoskeleton of aquatic crustaceans copepods. The close contact between the algal and the heterotrophic community in attached biofilms favors the use of algal material by microorganisms within the biofilm (Haack and McFeters, 1982; Nakano, 1996). The main external components of zooplankton and phytoplankton (aquatic algae) are chitin and mucilaginous sheath, respectively. From this point of view the present research involved chitin and aquatic algae and the relationship of *Vibrio* spp. isolated by forming biofilm by them with physicochemical parameters was investigated. It has observed in laboratory experiment that most of the *Vibrio* spp. have pili, flagella and MshA, TCP, *chiS* usually regulate the formation of biofilm on chitin and *Anabaena*

act as nutrient and support sources, so both of it acts as biotic surface for the formation of biofilm. We have isolated most of the sampling time the V. cholerae non-O1, *V. proteolyticus* and *V. mimicus* but not the *V. cholerae* O1 might be reason of VBNC of cholera bacterium. The DFA counts showed that most of the time, *V. cholerae* O1 also present in the biofilm by both chitin and algae (unpublished data). The previous study from Bangladesh also suggested that both *V. cholerae* O1 and non-O1 were isolated from aquatic environment (Islam *et al*., 2007) but present study represented the biotic surface as chitin and *A. variabilis* in the environment. Islam *et al*. (2007) found that *V. cholerae* O1 and non-O1 were 4.2-8.3% and 16.7-33.3%, respectively whereas in the present study we isolated 7.7 and 30.8% from both chitin and *Anabaena*, respectively. The relationship of physicochemical parameters with *Vibrio* population was showed in the several studies (Sharma and Chaturvedi, 2007; Shar *et al*., 2010) and it was found some relationship with water temperature, pH but our study suggested strong positive correlation with pH, DO, conductivity and TDS. The previous study was related to free living *Vibrio* population but present study was conducted to find out the ecology of biofilm forming bacteria and how often it is controlled by physicochemical parameters of water. The counts of total phytoplankton are also influenced by DO and CO_2 which is vital in the water for plankton photosynthesis and survival.

The result of the present study revealed that *Vibrio* sp. could form biofilm using chitin and algae as biotic surface and the physicochemical parameters influence the formation of the biofilm community.

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