THE IMPACT OF BIOFILM FORMATION ON THE SURVIVAL OF *Vibrio cholerae* IN AQUATIC ENVIRONMENT AND ITS ROLE IN PATHOGENICITY

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Abstract

Vibrio cholerae, the causative agent of epidemic cholera, has been established as an autochthonous flora of the aquatic environment, persisting predominately as non-culturable cells within clusters of biofilms and in association with plankton. Although biofilm has been proposed to serve as an important reservoir for *V. cholerae*, very little is known about the factors responsible for biofilm formation in the natural aquatic ecosystem, including its role in the active growth and survival, especially how the bacterium turns actively growing to initiate the seasonal outbreaks of cholera. Laboratory microcosm prepared with water collected from estuarine ecosystem of Mathbaria, Bangladesh showed slower biofilm formation and extended culturability for 68 days at 4°C, as compared to microcosms that were maintained at higher temperatures (30°C, 37°C, and 45°C), suggesting biofilm formation to be temperature dependent and negatively linked to loss of culturability. Mathbaria water (MW) microcosm supported the active growth of *V. cholerae* O1 for an extended period up to 54 days under higher pH (8.0-9.0), whereas microcosms having pH 6 and pH 7 supported the active growth for only 26 days and 40 days, respectively. Instant ocean (IO; salinity 1%) microcosms maintained at room temperature supported the active growth of *V. cholerae* for 54 days, while the IO microcosms with 0%, 2%, and 4% salinity supported the active growth for only 26 days. The biofilm formation was robust in Luria Bertani (LB) broth with 2% salinity and pH range 7.0-9.0 in presence of calcium chloride (10mM) and magnesium chloride (40mM). MW microcosm supplemented with dehydrated shrimp chitin chips (MW-CC) as the single source of nutrient supported both active growth of toxigenic *V. cholerae* O1 for up to six months and biofilm formation by the bacteria. *V. cholerae* O1 cells in chitin-associated biofilms remained metabolically active even in a high acidic environment without losing either viability or virulence. Biofilm-bound coccoid, nonculturable *V. cholerae* O1 cells maintained in MW microcosm for 495 days were able to be culturable upon passage through rabbit ileal loop (RIL). Genetic screening by polymerase chain reaction (PCR) of *V. cholerae* cells in MW microcosm revealed loss of several virulence and related genes including *ctxA, ace, zot*, *rstR, tcpA, acf, toxT* and two of four *mshA* genes. The mutant strains lacking *mshA* genes produced significantly less amount of biofilm in the air-water interface of the borosilicate glass tubes when compared with the parent strain. Pulsed-field gel electrophoresis (PFGE) analysis of the genomic DNA (*Not*I-digested) of the *mshA*-mutant daughter strains revealed them to be clonal, as they had identical PFGE pattern, but they differ from the parent strain. Results obtained from the field-based investigations in estuarine ecosystem of Mathbaria, Bangladesh have shown the presence of non-culturable *V. cholerae* cells as a component of the bacterial community of which only a negligible proportion could be enumerated when conventional culture methods were employed. In conclusion, *V. cholerae* biofilms formed *in situ* in response to physicochemical, and related biotic and abiotic factors of aquatic environment thus serve as an important reservoir where the bacterium can take refuge to, and persist as dormant cell until the favorable season returns for them to be actively growing again to increase the probability of infecting human and spreading cholera epidemics.

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Abbreviations

(-) ve	Negative
(+) ve	Positive
µg	Microgram
ATCC	American Type Culture Collection
cfu	Colony Forming Unit
et al	and others
etc	et cetra
Fig.	Figure
g	Gram
h	Hour
EPS	Exopolysacharide
kbp	Kilo base pair
L	Liter
M	Molar
mg	Milligram
ml	Milliliter
min	Minute
mm	Millimeter
µl	Microliter
N	Normal
ng	Nanogram
nm	Nanometer
^{0}C	Degree Centigrade
OD	Optical Density
pH	Negative logarithm of hydrogen ion concentration
rpm	Rotation per minute
sec	Second
sp.	Species
spp.	Species
v/v	volume/volume
w/v	weight/volume
UTI	Urinary Tract Infection
VP	Voges-Proskauer
VSP	Vibrio Pathogenicity Island
PFGE	Pulsed Field Gel Electrophoresis
UPGMA	Unweighted Pair Group Method with Arithmatic
CT	Cholera Toxin
TCP	Toxin Coregulated Pilus
MSHA	Mannose-Sensitive Hemolysin Agglutination Pilin
Φ	Phage

1.1 . INTRODUCTION

Vibrio cholerae O1 is the causative agent of epidemic cholera, a severe dehydrating diarrhoeal disease that kills thousands of people each year and remains a serious health threat globally. Cholera is endemic in the Ganges delta of Bay of Bengal, occurring twice yearly in epidemic form (Glass *et al.,* 1982; Alam *et al.*, 2006b). *V. cholerae* is an autochthonous bacterium of estuarine waters (Colwell and Spira**,** 1992**;** Lipp *et al*., 2002). Despite *V. cholerae* has been established as a native flora of the aquatic ecosystem (Colwell and Spira**,** 1992**;** Lipp *et al*., 2002), yet the bacterium is isolated only infrequently from surface water by culture methods during epidemic periods (Islam *et al*., 1994b) and very rarely between the epidemics (Huq *et al*., 1990). Fluorescent antibody-based studies show that *V. cholerae* O1 can be present in aquatic environments throughout the year (Xu *et al.*, 1982) mostly in viable but non-culturable (VBNC) state. Although non-culturability has long been proposed to be a survival strategy for cholera bacteria in the aquatic environment, the reservoir and mechanism by which these non-culturable cells regain culturability to initiate seasonal cholera are not fully understood. Despite the rate of isolation of *V. cholerae* O1 from fresh water being very low (Huq *et al*., 2005), recent epidemiological and ecological surveys carried out in Bangladesh provide firm evidence for an estuarine niche for *V. cholerae* causing endemic cholera in the estuarine villages of Mathbaria (Colwell, 1996a; Alam *et al*., 2006a).

Bacteria in the natural ecosystem can be found as actively growing (culturable) or as dormant and nonculturable cells (Roszak and Colwell, 1987). Nonculturable bacteria represent the part of a bacterial population unable to be cultured on bacteriological media by traditional methods, yet retaining metabolic activity detectable by various methods (Besnard *et al.*, 2000). Bacterial cells may enter a dormant state in response to stresses such as unfavourable temperature beyond the range required for their growth, elevated or declining osmotic concentrations (tidal flow of seawater), suboptimal oxygen concentration, or exposure to light (Oliver, 2000; Oliver, 2005). Many studies have shown that *V. cholerae* O1 can become non-culturable when environmental conditions are not conducive to active growth (Xu *et al.*, 1982; Colwell and Huq, 1994a). The non-culturable bacterium however can retain the metabolic activity (Xu *et al.*, 1982; Rollins and Colwell, 1986; Nilsson *et al.*, 1991) suggesting they are viable but not culturable.

Although viability (Kogure *et al*., 1979) and infective potential (Colwell *et al.,* 1985) of nonculturable cells of some species of bacteria have been documented (Rahman *et al.,* 1996), it is not well understood whether and how non-culturable cells revert to active culturable state.

In the aquatic environment, nonculturable bacteria can be found as coccoid cells (Xu *et al.*, 1982; Colwell and Huq, 1994a) either attached to substrates, including sediment particles, marine snow, fecal and detritus particles in the water column, zooplankton and phytoplankton, or as free-living single or aggregated cells within biofilms (Velji and Albright, 1993). Attachment of vibrios to aquatic surfaces is believed to be highly adaptive, and many studies have demonstrated their attachment to environmental surfaces such as plants, filamentous green algae, zooplankton, crustaceans and insects (Huq *et al*., 1986; Shukla *et al*., 1995; Tamplin *et al*., 1990). Copepods, in general, *Acanthamoeba castellanii* (Huq *et al*., 1984; Abd *et al*., 2007), *Acartia tonsa*, and *Eurytemora affinis* (Rawlings *et al*., 2007), all contain chitin and are colonized by *V. cholerae.* Copepods, in particular, occur in the natural aquatic environment in seasonal blooms (Colwell and Huq, 1994b). Chitinous substrates influence the population dynamics of *V. cholerae* in a number of ways, including food availability, adaptation to environmental nutrient gradients, tolerance to stress, and protection from predators (Pruzzo *et al*., 2008). In the aquatic environment, chitin is heavily colonized by chitinolytic bacteria that are responsible for mineralization of this insoluble polysaccharide (Gooday *et at*., 1991). *V. cholerae* associated with crustaceans have been found to occur predominantly in a nonculturable state (Roszak and Colwell, 1987), mostly as the aggregates or cluster of biofilms (Alam *et al*., 2006a).

Biofilm is defined as an assemblage of bacterial cells trapped and enclosed in a matrix primarily of exopolysaccharide material secreted by them which helps the individual cells to remain attached together forming a consortia either free floating or in association with a solid surface (Donlan, 2002). Such assemblages can comprise a population that develops from a single species or a community derived from multiple microbial species. Biofilm can protect its colonizers from the effect of antibiotics, pollutants, heavy metals, and changes in pH and temperature (Parsek and Singh, 2003). Microbial biofilm formation is initiated by attachment of the microbes to a surface, followed by cell-cell interactions to form multiple layers, and construction of three-dimensional units

including water channels through which nutrients can diffuse in and waste products can diffuse out (Costerton *et al.*, 1995, Watnick *et al.*, 1999). Bacterial biofilms, have been a subject of intense interest in recent years due to the predominance of biofilm-associated bacteria in natural environments, the complex developmental pathway that bacteria follow in forming a biofilm, and the role of biofilm formation in antibiotic-resistant bacterial infections (Davey and O'Toole, 2000; Mah and O'Toole, 2001).

There are many reports of accelerated rates of conjugation in bacterial biofilms (Angles *et al.*, 1993; Hausner and Wuertz, 1999) which suggests that evolution by horizontal transfer of genetic material may occur rapidly in biofilm, making it the perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors, and environmental survival compabilities. For example, acquisition of resistance gene has been reported in *Streptoccus* biofilm when *Bacillus subtilis* strain harbouring conjugative transposon which confers resistance to tetracycline was introduced in a microcosm containing dental plaque (Roberts *et al.,* 1999). Although, Alam *et al*. (2006a) reported that toxigenic *V. cholerae* cells exist predominantly as aggregates of structured biofilms, how these biofilms bound *V. cholerae* O1 cells persist and respond to environmental stimuli is not well understood. Furthermore, question remains as to what factor(s) influences the bioflim-bound coccoid *V. cholerae* cells to turn actively growing? The study presented here was designed to understand the natural forcings that influence the biofilm formation, and under what condition(s) *V. cholerae* comes out of the biofilms and becomes actively growing as part of their annual life cycle in the estuarine environment of Bangladesh.

1.2. LITERATURE REVIEW

1.2.1. CHOLERA

Cholera is an acute intestinal infection caused by toxigenic *V. cholerae* characterized in severe form by massive watery diarrhoea and recurrent vomiting resulting in rapidly progressive dehydration and death if not promptly and adequately treated. Two distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemics that progressively affect many countries in multiple continents over the course of many years (Kaper *et al.,* 1995).

1.2.2. CLASSIFICATION OF *V. cholerae*

The Genus *Vibrio* belongs to the Family Vibrionaceae (Krauss *et al*., 2003; Bronze and Greenfield, 2005). Some of the members are pathogenic to man and animal while others are non-pathogenic. The well known and rather frightening member that is responsible for epidemic and pandemic cholera is *V. cholerae.* This species (*V. cholerae*) is differentiated into >200 serogroups (serovars) on the basis of it's 'O' antigen of outer membrane (Shimada and Sakazaki, 1977). Among them two serogroups are mainly toxigenic, i.e. *V. cholerae* O1 and O139. *V. cholerae* O1 has been subtyped into two biotypes: Classical and El Tor based on some biochemical properties and susceptibility to bacteriophages (Sakajaki, 1970). *V. cholerae* O139 has no biotype. Both *V. cholerae* O1 Classical and El Tor have three serotypes: Ogawa, Inaba and Hikozima. *V. cholerae* that don't agglutinate with the antisera raised against either *V. cholerae* O1 or O139 are called non-agglutinating vibrios (NAG vibrios) or *V. cholerae* non-O1/non-O139 (Kaper *et al.,* 1995).

1.2.3. MORPHOLOGY AND CULTURAL CHARACTERISTICS OF *V. cholerae*

V. cholerae is a gram negative, non-spore forming, curved rod shaped bacterium that is oxidase positive (Krauss *et al*., 2003; Ryan and Ray, 2004; Bronze and Greenfield, 2005). It is very motile and has a single polar flagellum (Krauss *et al*., 2003). They are non-acid-fast and non-spore forming bacteria. They are facultatively anaerobic and can grow on ordinary media at optimum temperature of 37°C and optimum pH of 8 (Pollitzer, 1955). They grow as yellow colonies on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar and black-centered colonies with characteristics hallows around them on Taurocholate tellurite gelatin agar (TTGA) (Pfeffer and Oliver, 2003).

1.2.4. TRANSMISSION OF CHOLERA

Cholera is typically spread by consumption of water that is contaminated with infectious feces (Krauss *et al*., 2003; Ryan and Ray, 2004). Epidemic caused by infectious raw fish and seafood have been reported (Krauss *et al*., 2003). Humans, water birds, shellfish, fish, and herbivores have been found to contain the infectious agent (Krauss *et al*., 2003). The infectious dose ranges between 10^6 and 10^{11} ingested vibrios (Krauss *et al*., 2003). The infectious dose depends on gastric acidity (lower acidity levels reduces the number of vibrios required for infection (Krauss *et al*., 2003). The incubation period can range from a few hours to 5 days after infection (Krauss *et al*., 2003).

1.2.5. SEASONALITY OF CHOLERA

Once endemicity is established in an area, cholera tends to settle into a clear seasonal pattern. In Bangladesh, for example, the major cholera season begins after the monsoons, in August or September, with the number of cases reaching a peak in the winter period 1 to 3 months later and then rapidly declining. The beginning of the cholera season generally coincide with the warmest mean temperature, a fall in the level of river water, and the cessation of rainfall; it ends with the dry, cold weather (Gangarosa *et al.,* 1974; Glass *et al.,* 1982). For reasons that are not well defined, cases caused by classical biotypes tend to occur somewhat later, with onset in November or December (Glass *et al.,*

1982). Seasonal patterns may also differ by geographic area, with peak cholera season in Calcutta, India (less than 200 miles away), occurring in April, May, and June (Gangarosa and Moseley, 1974). Studies in Bangladesh indicate that when cholera season starts, illnesses occur simultaneously in multiple locations and are attributable to multiple strains (i.e., there is not a discernible pattern of spread of a single strain from one community to another) (Glass *et al.*, 1982). However, the underlying mechanisms of the seasonal increase in cholera cases are still not well understood. It is possible that an (as yet undefined) environmental cue triggers a rapid increase in counts of *V. cholerae* in the environment; as human cases start to occur, they, in turn, amplify the number of organisms present, leading to even more cases. Colwell and Spira (1992) have hypothesized that zooplankton blooms play a key role in this process, as noted above, *V. cholerae* colonize copepods and other zooplankton, and zooplankton blooms are known to occur in Bangladesh after the summer monsoon, when cholera season starts.

1.2.6. ECOLOGY OF *V. cholerae*

Previously, it was assumed that infected people spread cholera to other susceptible individuals through faecal contamination of water and food and global movement of populations accounted for the global movement of the disease. Recent studies of the aquatic environment, however, have shown that *V cholerae*, including strains of O1 and O139, are normal inhabitants of surface waters, particularly brackish waters, and survive and multiply in association with zooplankton and phytoplankton quite independently of infected human beings (Colwell *et al*., 1977; Nair *et al*., 1988; Huq *et al*., 1983; Islam *et al*., 1990). Because global climate changes may affect the estuarine environment including growth of plankton, the populations of the vibrios associated with water and plankton could also be modified. The continuing presence of cholera in the Indian subcontinent and the re-emergence of cholera in other continents may be highly dependent on environmental factors such as temperature, pH, salinity, dissolve oxygen etc. (Pascual *et al*., 2000; Colwell, 1996b).

The life cycle of *V cholerae* consists of two distinct phases: one while remaining inside the host and another while persisting in the aquatic environment. *V. cholerae* can be found as free swimming cells, attached to surfaces provided by plants, filamentous green

algae, copepods, crustaceans, insects (Colwell, 1996b; Islam *et al*., 1994c) and egg masses of chironomids (Broza and Halpern, 2001). Biofilm formation (Watnick *et. al*., 2001) and entry into a viable but non-culturable state in response to nutrient deprivation (Colwell, 2000) are thought to be important in facilitating the persistence of *V. cholerae* within natural aquatic habitats during periods between epidemics (Reidl and Klose, 2002). Neither the genetic events that help the organism to lead a life in association with plankton nor the biofilm ecology of vibrios on abiotic surfaces are completely understood. Although *V cholerae* is part of the normal estuarine flora, toxigenic strains are mostly isolated from the environment in areas probably contaminated by infected individuals. Environmental isolates from areas that are distant from regions of infection do not generally harbor the cholera toxin genes (Faruque *el al*., 1998a). Two large DNA regions, the *Vibrio* pathogenicity island (VPI)-1 and the CTX prophage, encode the major virulence factors in *V. cholerae* O1/O139 (Faruque *et al.,* 1998a), i.e. the CTX element, which is encoded by the genome of a filamentous bacteriophage (Waldor and Mekalanos*,* 1994), and the toxin-corregulated pillus (TCP), and accessory colonization factor (ACF) gene cluster, referred to as the TCP pathogenicity island (Kovach *et al.,* 1996). The structures of the TCP pathogenicity island and the CTX genetic element are suggestive of horizontal transfer of these gene clusters as a possible mechanism for the origination of new pathogenic clones of *V. cholerae* (Faruque *et al*., 1998a). Two crucial sequential steps in the evolution of a pathogenic *V cholerae* have been hypothesized: firstly, strains have to acquire the VPI (which most environmental strains do not have); and secondly, having acquired the CTXф receptor, the TCP positive strains need to be infected with and lysogenised by CTXф (Waldor and Mekalanos, 1996; Faruque *et al*., 1998b; Mekalanos *et al*., 1997).

Experiments in animals indicate that the intestinal milieu may be an site where *V. cholerae* strains can acquire these mobile elements efficiently (Waldor and Mekalanos, 1996; Lazar and Waldor, 1998). Thus, *V. cholerae* can be visualized as an autochthonous marine bacterium that colonizes and thrives in the human gut during phases of infection while the pathogenic bacteria usually spend most of the time between epidemics in its“original” habitat, i.e. aquatic environment, particularly estuary.

1.2.7. BIOFILM FORMATION: A SURVIVAL MECHANISM OF BACTERIA IN ENVIRONMENT

Biofilms are surface attached communities composed of microorganisms and extra-polymeric substances they produce (Costerton *et al*., 1995). The biofilm structure appears to be largely determined by the production of slime-like matrix of extracellular polymeric substances (EPS), which provides the structural support, for the biofilm (Flemming *et al*., 2000). Biofilms can play a significant role of in environmnental, industrial and clinical settings (Davey and O'Toole, 2000; Donlan, 2002; Parsek and Singh**,** 2003). The biofilm mode of growth is probably the preferred lifestyle in the microbial world as it enhances survival in natural settings (Yildiz and Kolter, 2008). Because surfaces adsorb and concentrate nutrients that are usually scarce, growth on surfaces is favoured (Yildiz and Kolter, 2008). In addition, biofilms may provide the constituent microbes protection from predators, such as protozoa and viruses, and from toxic compounds, such as antimicrobial agents (Davey and O'Toole, 2000; Donlan, 2002).

1.2.7.1. Advantages of bacterial survival in biofilm

Many studies have indicated the advantages of forming a biofilm in comparison to survival as individual cells in many bacterial species. The advantages of bacterial biofilm formation are summarized below under different headings.

1.2.7.1.1. Protection from adverse environment

Bacteria can experience a certain degree of shelter and homeostasis when residing within a biofilm, which is surrounded by an extrapolymeric substance matrix. This matrix is composed of a mixture of components, such as exopolysaccharide (EPS), protein, nucleic acids, and other substances. The best studied of these components is EPS. Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular EPS excretions into the surrounding environment. EPS plays various roles in the structure and function of different biofilm communities. Biofilm-associated cells can be more resistant to many toxic substances such as antibiotics, chlorine, and detergents (Costerton *et al*., 1987). The reason for this resistance may be due to the decreased

diffusion into the biofilm (De Beer *et al*., 1994; Suci *et al*., 1994), decreased bacterial growth rate in a biofilm (Evans *et al*., 1990), biofilm-specific substances such as exopolysaccharide (Yildiz and Schoolnik, 1999), and the bacterial cell to cell communication termed as quorum-sensing specific effects (Davies *et al*., 1998; Hassett *et al*., 1999). EPS has also been reported to provide protection from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation (Flemming, 1993). In some cases, EPS has also shown to sequester metals, cations, and toxins (Decho, 1990; Flemming, 1993).

1.2.7.1.2. Nutrient availability and metabolic cooperativity

Attachment to surfaces and formation of a biofilm may provide an adaptive advantage for nutrient acquisition in aquatic organisms. For example, less soluble and less easily metabolizable large organic compounds (i.e. humic acids) which are found adsorbed to aquatic surfaces may provide nutrients to attached bacteria (Mills and Powelson, 1996). The highly permeable water channels interspersed throughout the biofilm in the areas surrounding the microcolonies have been compared to a primitive circulatory system (Davey and O'Toole, 2000). The water channels inside the biofilm most likely provide an effective means of exchanging nutrients and metabolites with the bulk aqueous phase, enhancing nutrient availability as well as removal of potentially toxic metabolites (Costerton *et al*, 1995).

The metabolic characteristics of bacteria within a biofilm community are most often distinct from those of their planktonic counterparts (Davey and O'Toole, 2000). The elaborate architecture of biofilm can provide the opportunity for metabolic cooperation, and niches are formed within these spatially well-organized systems (Davey and O'Toole, 2000). Consequently, the bacteria are exposed to an array of distinct environmental signals within a biofilm. For instance, cells situated near the center of a microcolony are more likely to experience low oxygen tensions (Davey and O'Toole, 2000). Biofilms may provide an ideal environment for the establishment of syntrophic relationships (Davey and O'Toole, 2000). Syntrophism is a special case of symbiosis in which two metabolically distinct types of bacteria depend on each other to utilize certain substrates, typically for energy production (Davey and O'Toole, 2000). Other factors, such as chitinous exoskeletons of crustaceans can also provide nutrition directly to the attached

bacteria (Huq *et al*., 2008). Ca^{2+}, which is abundant in marine and brackish water ecosystems, is shown to play an important role in the formation of biofilms by directly stabilizing intercellular interactions, as has been shown for *P. aeruginosa* and *Streptococcus downei*, presumably by forming intercellular salt bridges (Huq *et al*., 2008).

1.2.7.1.3. Acquisition of New Genetic Traits

Horizontal gene transfer is important for the evolution and genetic diversity of natural microbial communities. The importance of studying gene transfer in natural environments has recently been emphasized due to the emergence of multidrug-resistant bacteria (Davies, 1994), the extensive use of antibiotics to promote growth in domestic animals (Witte, 1998), and the use of genetically engineered microorganisms in industrial processes (Jansson and Prosser, 1997, Sayler and Ripp, 2000). The prevalence of plasmids in bacteria from diverse habitats is well established, and gene transfer by conjugation is one of the best understood mechanisms for dissemination of genetic information. Conjugation is a likely mechanism by which bacteria in biofilms transfer genes within or between populations. Although there may be fewer incidences of mating events within a biofilm, the "fixed" close quarters within biofilms are likely to favor conjugation. A study reported isolation of novel plasmids containing mercury resistance genes from bacteria residing in biofilms by combining nutrient deprived recipient cells (a strain of *Pseudomonas putida*) in marine environments (Dahlberg *et al*, 1997). Another study examined transfer of conjugative transposon conferring resistance to tetracycline in *Streptococcus* sp. while surviving with *Bacillus subtilis* (Roberts *et al*., 1999).

1.2.7.2. Steps involved in biofilm formation

Several steps are involved in biofilm development by bacteria. These are:

i. Initial attachment
ii. Formation of microcolonies

iii. Maturation of microcolonies into an EPS-encased mature biofilm

iv. Dispersal of cells from biofilm structures

The basic steps leading to the formation of a multi-species biofilm are outlined in Fig. 1.1 (Watnick and Kolter, 2000).

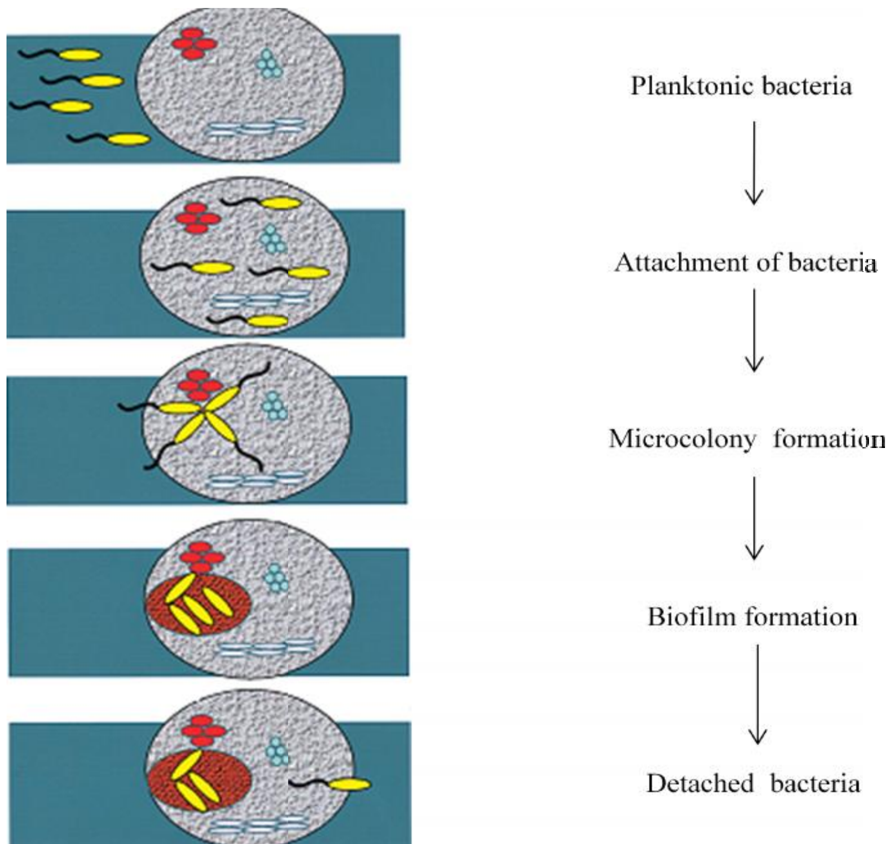

Fig. 1.1. A schematic representation of the steps a new bacterial species takes in forming a biofilm on a rock previously colonized with multiple species of bacteria. The yellow bacteria represent an aquatic species that swims towards the rock using polar flagella, forms random loose attachments to the rock, migrates over the surface to form a microcolony, and finally produces exopolysaccharide to form a three-dimensional biofilm. When environmental conditions become unfavorable, some of the bacteria may detach and swim away to find a surface in a more favorable environment.

1.2.7.2.1. Initial attachment

First, the bacterium generally approaches the surface so closely that motility is slowed (Watnick and Kolter, 2000). The process of initial attachment is believed to begin when bacteria sense certain environmental parameters that trigger the transition from planktonic growth to life on a surface (Fletcher and Pringle, 1986; Nyvad and Kilian, 1990; O'Toole

and Kolter, 1998b; O'Toole *et al*., 2000a; Poulsen *et al*., 1993; Stanley, 1983; Wang *et al*., 1996). The environmental cues that control this transition can vary greatly among organisms. *P. aeruginosa* can form biofilms under most conditions that allow growth (O'Toole and Kolter, 1998a), but some strains of *E. coli* K-12 will may not form biofilms in minimal medium unless supplemented with amino acids (Pratt and Kolter, 1998), and *E. coli* O157:H7 has been reported to make a biofilm only under low-nutrient conditions (Dewanti and Wong, 1995).

V. cholerae also appears to utilize different pathways for initial attachment step of biofilm formation depending on the surface to which the organism attaches. For example, *in vivo* experiment has shown that the Tcp pilus is required for colonization of the intestine (Herrington *et al*., 1988). However, Tcp appears to play no role in attachment to abiotic surfaces in aquatic environment. Here, it is the pilus encoded by the *msh* locus having no role in pathogenesis, that is required for attachment to abiotic surfaces (Thelin and Taylor, 1996). Abiotic surfaces can be further subdivided into nonnutritive (plastic, glass, metal, etc.) and nutritive (e.g., chitin). The MshA pilus also appears to speed the attachment of bacteria to the surface (Davey and O'Toole, 2000).

1.2.7.2.2. Formation of microcolonies

After initial attachment the biofilm forming bacterium generally develop a transient association with the surface and/or other microbes previously attached to the surface. This transient association probably allows it to search for a place to settle down and forms a stable association as a member of a microcolony (Watnick and Kolter, 2000). Flagellum-mediated motility is required for parallel movement to the surface in addition to bringing the bacteria into proximity to the surface (Davey and O'Toole, 2000). Flagella are important for bringing bacteria into close proximity with a surface and for bacterial spread across the surface (Davey and O'Toole, 2000).

1.2.7.2.3. Maturation of microcolonies into an EPS-encased mature biofilm

With time, microcolonies usually develop into a mature biofilm that is often associated with the production of EPS. As cells adjust to an immobile life on a surface, they enrally lose their flagella and increase the production of EPS. Exopolysaccharide expression and

biofilm elaboration are markedly enhanced in certain bacteria, including the pseudomonads, *V. cholerae*, and *E. coli*, the staphylococci and the streptococci, when glucose or another readily utilizable carbon source is abundant (Ammendolia *et al*., 1999; Burne *et al*., 2003; Jefferson *et al*., 2004; O'Toole *et al*., 2000a). Alginate has been implicated as a likely EPS in biofilm development in *P. aeruginosa*. In *P. aeruginosa*, the transcription of *algC*, a key gene involved in the biosynthesis of alginate, is induced soon after the bacteria attach to the surface (Davies and Geesey, 1995). The downregulation of flagellum synthesis has been shown to be linked with the upregulation of alginate synthesis (Garrett *et al*., 1999). The formation of a biofilm is a developmental process in which a quorum sensing signal molecule, an auto-inducer, functions to induce the secretion of EPS and leads to the formation of a characteristic three-dimensional biofilm architecture (Nadell *e. al.,* 2008*).* Davies *et al*., (1998) have shown that an acylated homoserine lactone fuctions as a developmental signal in the formation of the characteristic three-dimensional biofilm architecture in *P. aeruginosa*. The observation that a mutant of *P. aeruginosa* unable to synthesize the major quorum-sensing molecules acylhomoserine lactones (acyl-HSLs) was radically altered in biofilm architecture clearly demonstrated that these molecules may regulate the formation of biofilm structures in this organism. The typical biofilm architecture and resistance to SDS could be restored by the addition of exogenous acyl-HSLs (Davies *et al*., 1998). These data strongly suggest that cell-cell communication is essential for this bacterium to establish a well-ordered surface community.

1.2.7.2.4. Dispersal of cells from biofilm structures

Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, as a result of changes in nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects. The mechanisms underlying the process of shedding by actively growing cells in a biofilm are not well understood. Gilbert *et al.* (1993) showed that surface hydrophobicity characteristics of newly divided daughter cells spontaneously dispersed from either *E. coli* or *P. aeruginosa* biofilms differ substantially from those of either chemostat-intact biofilms or resuspended biofilm cells. These researchers suggested that these differences might explain newly divided daughter cells' detachment.

Hydrophobicity was lowest for the newly dispersed and steadily increases upon continued incubation and growth. Boyd and Chakrabarty (1994) showed that enhancing alginate lyase expression gene substantially decreased the amount of alginate produced as the major component of the EPS of *P. aeruginosa*, which corresponded with a significant increase in the number of detached cells. Brading *et al.* (1995) have emphasized the importance of physical forces in detachment, stating that the three main processes for detachment are erosion or shearing (continuous removal of small portions of the biofilm), sloughing (rapid and massive removal), and abrasion (detachment due to collision of particles from the bulk fluid with the biofilm).

Characklis (1990) noted that the rate of erosion from the biofilm increases with increase in biofilm thickness and fluid shear at the biofilm-bulk liquid interface. With increase in flow velocity, the hydrodynamic boundary layer can be decreased, resulting in mixing and turbulence closer to the biofilm surface. Sloughing is more random than erosion and is thought to result from nutrient or oxygen depletion within the biofilm structure (Brading *et al*., 1995). Sloughing is more commonly observed with thicker biofilms that have developed in nutrient-rich environments (Characklis, 1990). The mode of dispersal apparently affects the phenotypic characteristics of the organisms. Eroded or sloughed aggregates from the biofilm are likely to retain certain biofilm characteristics, such as antimicrobial resistance properties, whereas cells that have been shed as a result of growth may revert quickly to the planktonic phenotype (Donlan, 2002).

1.2.8. Mechanism of biofilm formation

Environmental signals that may influence initial attachment during the process of bacterial biofilm formation are osmolarity, pH, iron availability, oxygen tension, and temperature (Fletcher, 1996; Nyvad and Kilian, 1990; O'Toole *et al*., 2000b; O'Toole and Kolter, 1998b; Pratt and Kolter, 1998). Although the details of the environmental signals triggering biofilm development may vary from organism to organism, it is evident that environmental parameters have a profound impact on the transition between planktonic and biofilm growth (Davey and O'Toole, 2000). Three distinct signaling pathways, namely quorum-sensing pathway, flagellum-dependent pathway and phase variation pathway are known for EPS production in *V. cholerae* and each may operate via discrete

signals and/or microenvironments. In the quorum-sensing pathway, the absence of the transcriptional regulator HapR results in enhanced EPS synthesis and biofilm formation (Hammer *et al*., 2003; Jobling *et al*., 1997; Vance *et al*., 2003; Zhu *et al*., 2003; Heithoff *et al*., 2004); in the flagellum-dependent pathway the non-flagellated cells induce the expression of EPS synthesis and biofilm formation (Lauriano *et al*., 2004; Watnick *et al*., 2001; Heithoff *et al*., 2004).; whereas in thephase variation pathway, two distinct morphological variants termed smooth and rugose [wrinkled] are yielded and the rugose variant is associated with an enhanced capacity to produce EPS (Yildiz *et al*., 2001; Yildiz *et al*., 1999**;** Heithoff *et al*., 2004). However, recent evidence suggests that two main systems are responsible for biofilm formation and regulation in *V. cholerae*, namely, the matrix exopolysaccharide and quorum sensing **(**Huq *et al.,* 2008). QS is a cell-cell communication process that involves the production, secretion, and detection of chemical signal molecules known as autoinducers (AIs) allowing bacteria to synchronize the behavior of the population. *V. cholerae* produces two AIs termed as cholera autoinducer 1 (CAI-1, (*S*)-3-hydroxytridecan-4-one, and autoinducer 2 (AI-2, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate) and responds to them by using parallel phosphorelay signaling systems (Chen *et al*., 2002; Miller *et al*., 2002.). The two AI molecules are expressed based on the cell density, in the low cell density state the response regulator, LuxO activates the expression of genes to destabilize the mRNA encoding HapR, a major regulator of QS (Jobling *et al*., 1997; Lenz *et al*., 2004). This relay culminates in the expression of low-cell density specific genes, including genes required for biofilm formation and virulence factor production (Hammer *et al*., 2003; Miller *et al*., 2002; Zhu *et al*., 2003; Zhu *et al*., 2002). When the cell density increases, the AIs accumulate and their phosphatase activity leads to dephosphorylation of LuxO and expression of HapR that in turn repress genes involved in biofilm formation as well as virulence (Lenz *et al*., 2004; Miller *et al*., 2002Hammer *et al*., 2003; Miller *et al*., 2002; Zhu *et al*., 2003).

1.3. AIMS AND OBJECTIVES

Although *V. cholerae* cells exist mostly as nonculturable cells within thick clusters of biofilms in the estuarine environment of Bangladesh, how these biofilm bound nonculturable cells behave in the aquatic environment at different seasons of the year driven by environmental changes is not clearly known. Therefore, the present study was carried out in both laboratory microcosms and estuarine environment of Bangladesh to

understand the role of different environmental factors on biofilm formation as well as survival and growth of *V. cholerae* cells.

The major aims of the present study were as below:

1. To understand the impacts of various physicochemical conditions on the survival and biofilm formation of *V. cholerae.*
2. To examine the role of aquatic chitin on the survival and biofilm formation of *V. cholerae*.
3. To understand whether non-culturable *V. cholerae* cells residing within the clusters of biofilms can be reverted to cultivable state.
4. To examine whether any genetic change occurs in *V. cholerae* cells while surviving inside the biofilms.
5. To observe the role of bacterial biofilms in the annual life cycle of toxigenic *V. cholerae* in the estuarine ecosystem of Bangladesh.

2. MATERIALS AND METHODS

As *V. cholerae* is an estuarine bacteriam, the present study was designed with the intention to understand the influence of the physicochemical and related abiotic factors on biofilm formation as well as on growth and survival of *V. cholerae* in both microcosm and estuarine aquatic ecosystem of Bangladesh

2.1. DESCRIPTION OF STUDY AREA

2.1.1. MATHBARIA

Mathbaria is located adjacent to the Bay of Bengal, approximately 400 km southwest of Dhaka (Fig. 2.1). Mathbaria is also an administrative unit under the district of Pirojpur, with a police station and a Thana Health Complex (THC). The THC in Mathbaria is Government run at the community level and is a rural health care facility containing hospital beds. In this study, samples were collected from patients attending the THC of Mathbaria and three man-made ponds that are heavily used by Mathbaria people for drinking and other domestic purposes. The major river, Baleshwar, flows along the western boundary of Mathbaria, on the other side of which is located a tropical mangrove forest of the Sundarbans, the temporary island system of that part of the Bay of Bengal.

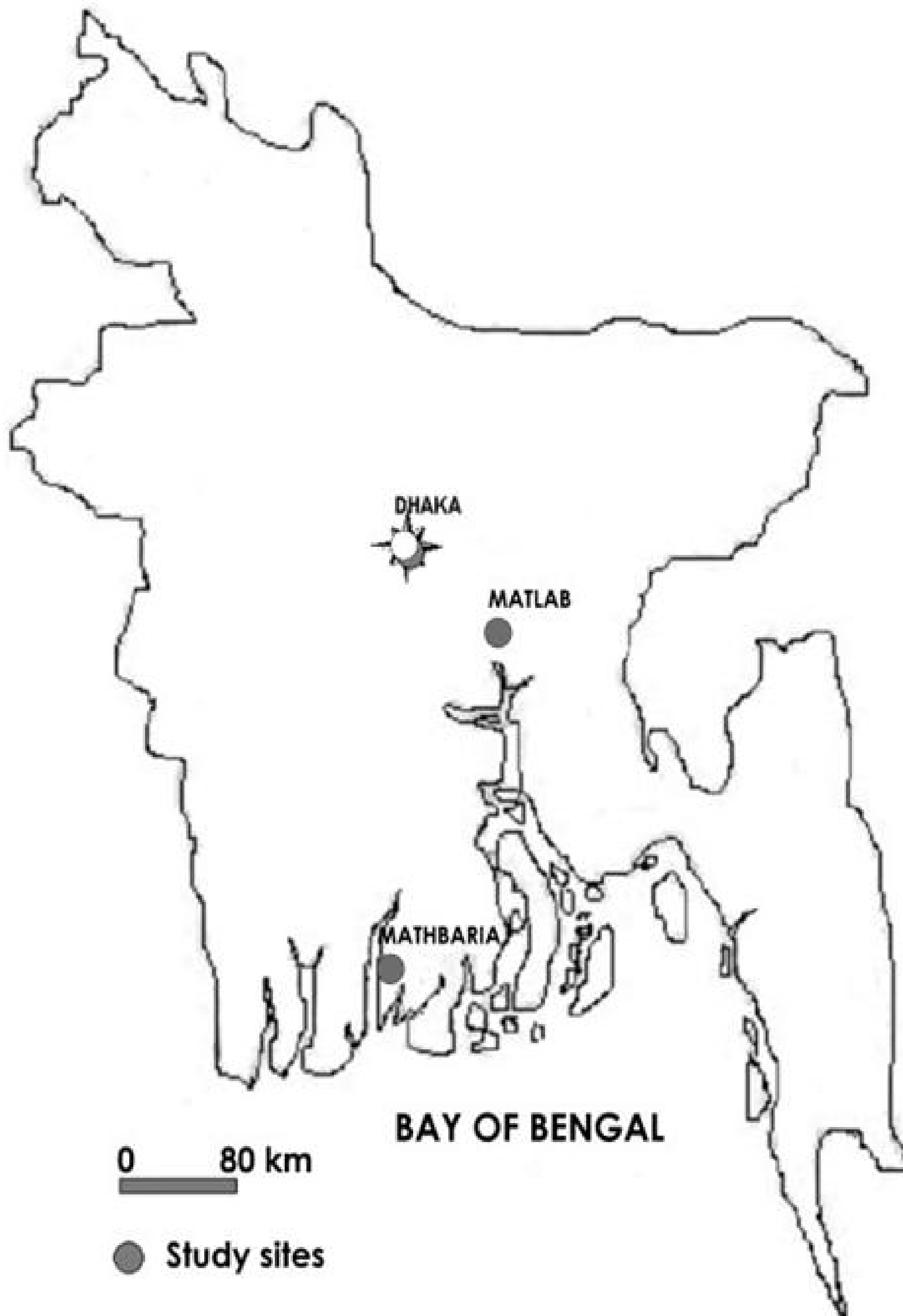

Fig. 2.1. The sample collection area as shown in the map

Fig. 2.2. Kachichira pond of Mathbaria (one of three study sites)

2.2. COLLECTION OF ENVIRONMENTAL SAMPLES AND PROCESSING

Water and plankton samples were collected every 2 weeks between January and December 2007 from three ponds (natural bodies of water used by resident people for washing utensils and drinking) located in Mathbaria (Fig. 2.2). All samples were collected by using aseptic techniques in sterile dark Nalgene bottles (Nalgene Nunc International, St. Louis, Mo.), placed in an insulated plastic box, and transported at ambient air temperature from the site of collection to the central laboratory of the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b), in Dhaka. All samples were processed the following day, with approximately 20 h elapsing between sample collections in the field and processing in the laboratory. For sample collection, 100 liters of water was filtered successively through 64- and 20-µm-pore-size nylon nets (Millipore Corp., Bedford, Mass.) (with a 64-µm-pore-size [64-µm] net placed sequentially in front of the 20-µm nylon net, with each having a collecting bucket at the base), and 50-ml portions of the concentrates were collected initially to determine crude estimations of the zooplankton and phytoplankton, respectively. During this process of filtration, 200 ml of filtrate water from the 20-µm mesh net was collected as

representative of water, to be analyzed for planktonic (unattached, free-living) bacteria. Both 64- and 20-µm plankton samples were further concentrated in laboratory using respective size plankton nets (specially devised, netted plastic beakers) to a final volume of 5 ml each. For bacteriological analysis, the plankton samples were crushed by using a glass homogenizer (Elberbach Corp., Ann Arbor, Mich.) to release attached bacteria. Water samples were concentrated by filtration through a 0.22-µm-pore-size bacteriological membrane filter (Millipore), and the retained contents on the membrane filter were washed into phosphate-buffered saline, pH 7.0 (PBS). Both concentrated water and homogenates were used for direct plating analyses, as well as for enrichment of *V. cholerae* in alkaline peptone water (APW; Difco Laboratories, Detroit, Mich.), Acridine Orange Direct counting (AODC), Direct Fluorescent Antibody (DFA) assay, and Multiplex-Polymerase Chain Reaction (M-PCR).

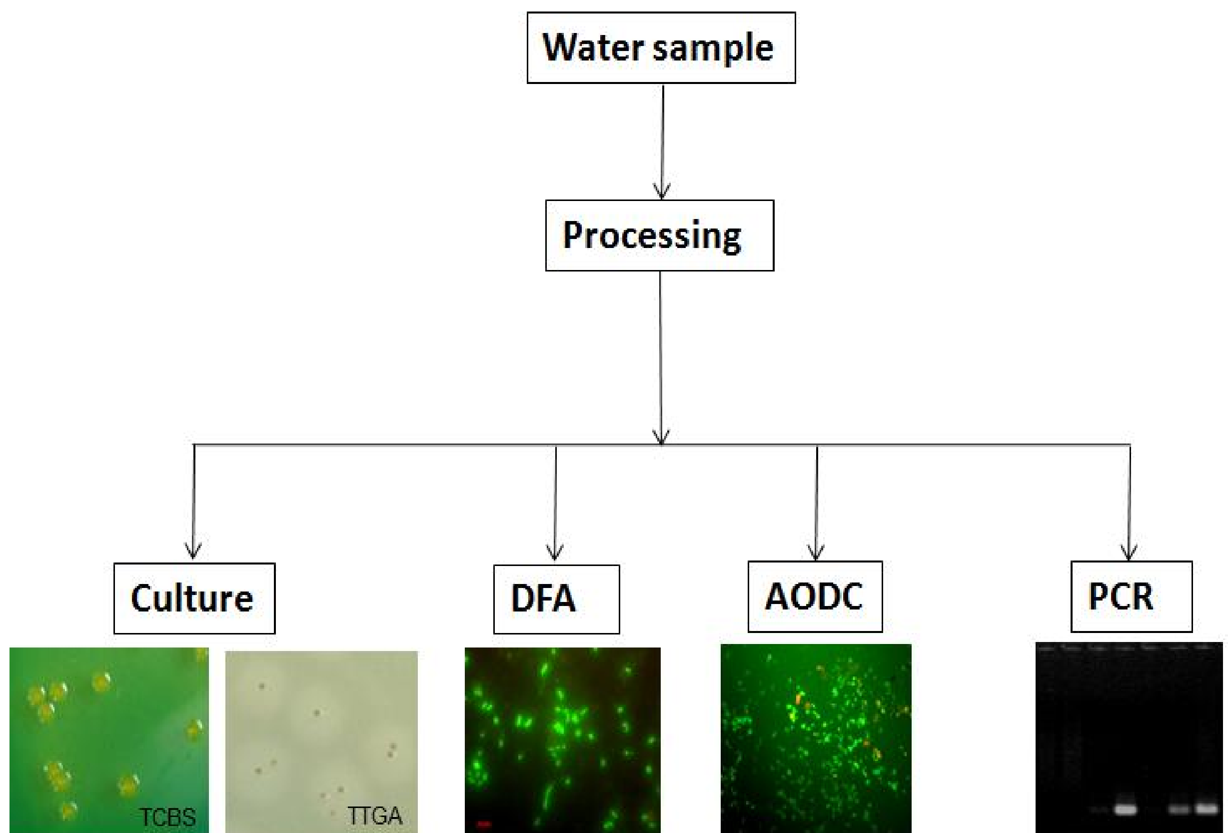

Fig. 2.3. Schematic representation of processing and analysis of environmental samples

2.3. PREPARATIONS OF MICROCOSMS

2.3.1. Estuarine microcosms

Microcosms (Fig. 2.4) were prepared with water collected from a pond in Mathbaria that serves as a drinking water source for villagers and had been tested positive for *V. cholerae* O1 by culture, DFA, and PCR (Alam *et al*., 2006a; 2006b). Water for the microcosms (600 ml in each 1 liter conical flask) was filtered using 0.22 µm membrane filtration to eliminate particulate matter (both biotic and abiotic) and autoclaved. *V. cholerae* O1 biotype El Tor (EM-226) cells isolated from a pond of Mathbaria were grown in Luria–Bertani (LB) broth at 37°C for 18 hrs, harvested, washed with PBS, and inoculated to a final concentration of 10^7 cfu/ml into microcosm flasks. To examine biofilm formation, and survival and growth of *V. cholerae* cells in estuarine environment microcosms were incubated at room temperature and 4^0C and designated as MW-RT and MW-4C respectively. In order to observe effect of temperature on survival of *V. cholerae,* microcosms were incubated at different temperature (4, 30, 37 and 42ºC) and designated as MW-45C, MW-4C, MW-30C and MW-37C. To observe effect of pH on survival of *V. cholerae*, microcosms were prepared with Mathbaria water having different pH (6.0, 7.0, 8.0 and 9.0) and designated as MW-pH6, MW-pH7, MW-pH8 and MW-pH9. In order to observe influence of salinity on survibility of *V. cholerae*, microcosms were prepared with instant ocean having different concentration of salinity (0, 1.0, 2.0 and 4.0%) and designated as MW-0%, MW-1%, MW-2% and MW-4%. Samples from all of the microcosms were collected aseptically at selected time intervals and examined by plating on thiosulfate citrate bile-salts sucrose (TCBS), Taurocholate Tellurite Gelatin agar (TTGA) and Luria–Bertani agar (LBA) media; serogroup was confirmed using polyvalent and monoclonal antiserum specific for *V. cholerae* O1 (Nandi *et al*., 2000) . Aliquots of the samples were also examined by direct fluorescent monoclonal antibody (DFA) staining (Brayton and Colwell, 1987; Hasan *et al*., 1994) and multiplex-polymerase chain reaction (M-PCR) to detect toxigenic *V. cholerae* O1, following methods described elsewhere (Hoshino *et al*., 1998).

2.3.2. MW microcosms supplemented with shrimp chitin chips

Chitin was extracted from the carapaces of a large crustacean, the 'golda' shrimp (*Macrobrachium rosenbergii*), collected from an estuary geographically adjacent to the coastal village of Mathbaria, Bangladesh following procedures described elsewhere (Sen, 2005). The chitin shells were washed, autoclaved and dried at 60°C overnight and cut aseptically into small pieces. A microcosm was constructed using 600 ml filtered (0.22µm membrane) and autoclaved Mathbaria water in 1 liter sterile conical flask, as described above. *V. cholerae* O1 biotype El Tor (EM-226) cells in exponential phase, collected after growth in LB broth at 37°C and washed with PBS, were inoculated to a

Fig. 2.4. Microcosm constructed with sterile Mathbaria water and *V. cholerae* O1

final concentration of 10^7 cfu/ml into MW microcosms supplemented with shrimp chitin chips (CC 0.3%; w/v) as sole source of nutrient. The microcosm amended with chitin chips, designated MW-CC, was sealed aseptically and incubated at room temperature, as above. Samples were collected from the microcosms at selected time intervals and plated on TCBS and LBA. Simple staining, DFA staining, and M-PCR were also performed to

detect and enumerate *V. cholerae* O1. When culturable cell counts in the aqueous phase of the microcosm had declined to <10 on all culture media, the chitin residue at the bottom of the flask was treated with concentrated HCl (pH 1.6 - 1.8) for 30 min to kill loosely attached bacteria, alkalinized with NaOH (pH 7.0 - 9.0) for 30 min, and the residue collected as a pellet by centrifugation. The pellet was washed with PBS several times and homogenized in PBS using a sterilized glass homogenizer (Elberbach Corp., Ann Arbor, Mich.) to dislodge firmly attached bacteria. This homogenate was enriched in alkaline peptone water (APW) at 37^0C for 24h, as described previously (Huq *et al*., 1990), plated on TCBS and LBA, and analyzed by DFA and M-PCR to detect and enumerate *V. cholerae* O1 (Alam *et al*., 2006a; Brayton and Colwell, 1987; Hasan *et al*., 1994; Hoshino *et al*., 1998; Nandi *et al*., 2000).

2.3.3.Culture

Samples from all the microcosms and environment were first diluted in PBS. An amount of 100 µl of diluted samples from different dilutions were plated on TCBS, TTGA, LBA for culturable *V. cholerae* and Heterotropic plate (HP) for total culturable bacterial count, and incubated at 37°C for 24h. After incubation, characteristic colonies of *V. cholerae* O1 were confirmed by serological tests and counted.

2.3.4. Enrichment and plating

Samples were enriched in APW at 37°C for 6 to 8h before plating as described previously (Huq *et al*., 1990; Islam *et al*., 1994a). Approximately 5 µl of enriched APW broth was streaked by using an inoculating loop on both TCBS and TTGA, and incubated at 37°C for 18 to 24h. Colonies with the characteristic appearance of *V. cholerae* were confirmed by serological tests using polyvalent and monoclonal antibodies specific for *V. cholerae* O1 and finally, by molecular methods (Nandi *et al*., 2000).

2.3.5. Simple staining

Water samples and Chitin chips from the microcosms were aseptically collected and placed on clean glass slides, air-dried, stained with 0.1% crystal violet (Sigma, St. Louis, Mo.), washed, and visualized using a light microscope (Axioskop 40; Carl Zeiss AG, Gottingen, Germany). Images were recorded with a digital camera attachment (AxioCam MRc; Carl Zeiss AG, Gottingen, Germany).

2.3.6. AODC

AODC was performed as described by Hobbie *et al*., (1977). Briefly, samples were preincubated overnight in the dark, with 0.025% yeast extract (Difco Laboratories, Detroit, Mich.) and 0.002% nalidixic acid (Sigma). After incubation, samples were fixed with 4% formaldehyde. The samples were diluted 10-fold in a series and stained with acridine orange (Sigma) at a 0.1% (w/v) final concentration for 2 min. Samples were filtered through polycarbonate filters (Millipore, pore size 0.2 µm, dia. 25 mm) pre-stained with Irgalan black dye. The stained bacterial population on the membrane filter was counted using an epifluorescent microscope (Carl Zeiss; Axioskop 40). The total direct bacterial count (including viable and VBNC bacteria) was averaged from the bacterial numbers obtained by counting 10 microscopic fields. Photographic records of the bacterial cells and their biofilms were captured using a digital camera (AxioCam MRc) connected to the epifluorescent microscope.

2.3.7. DFA

DFA was done according to a method described elsewhere (Brayton and Colwell, 1987). Samples were preincubated overnight, in the dark, with 0.025% yeast extract (Difco) and 0.002% nalidixic acid (Sigma). After incubation, samples were fixed with 4% formaldehyde.

Procedure of DFA:

1. A thin smear of the processed sample was made by adding 5 µl on a well and then spreading the contents to cover the well.
2. Similar thin smears of both positive and negative control were also prepared following the above mention procedure.
3. The slide containing sample, positive and negative controls were air dried at room temperature.
4. 5 µl of absolute ethanol or methanol was added to each control or sample to fix the smear and then air dried.
5. 10 µl of Cholera DFA reagent (specific for *Vibrio cholerae* O1) obtained from New Horizon Diagnostic Corp. (Columbia, Md.) was added to each well.
6. The slide was placed in a covered, moist chamber, and incubated at 37^0C for 30 minutes in the dark.
7. The slides were washed thoroughly with PBS protecting from light.
8. The slides were then air dried in the dark.
9. A drop of Fluorescent Mounting Medium was added on the slide and covered with a cover slip.
10. The stained samples were observed under the fluorescence microscope (Axioskop 40) connected to a digital camera (AxioCam MRc) at a magnification of 1000x with oil immersion.

Photographic images of the *V. cholerae* cells and their biofilms were captured using a digital camera connected to the epifluorescent microscope.

2.3.8. PCR

A single-primer-pair PCR for the amplification of *V. cholerae* species specific gene *ompW*, encoding outer membrane protein OmpW, was carried out as described elsewhere (Nandi *et al*., 2000). The genes responsible for O-antigen biosynthesis and for generation of serotype-specific determinants are located in the *rfb* region on the *V. cholerae* chromosome. The *wbe* gene specific for *V. cholerae* O1, the *ctxA* gene encoding subunit A of cholera toxin and *rstR*2 encoding transcriptional repressor were amplified by using M-PCR. PCR was also carried out for the genes listed in the table 2.1.

2.3.8.1. Extraction and Purification of Chromosomal DNA

Chromosomal DNA was isolated by using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. The method is described briefly here:

1. 1ml of an overnight culture was taken in a 1.5ml micro centrifuge tube.
2. The cells were centrifuge at 13,000 rpm for 5 minutes. The supernatant was removed and cells were collected as pellet.
3. 600µl of nucleic acid lysis solution was added to the cell pellet by pipetting until the cells were resuspended.
4. The cells were incubated at 80°C for 5 minutes to lyse the cells and then allowed to cool at room temperature.
5. 3µl of RNase Solution was added to the cell lysate and the tube was inverted for 2–5 times to mix the content properly.
6. The content was incubated at 37°C for 15–60 minutes and cooled to room temperature.
7. 200µl of protein precipitation solution was added to the RNase-treated cell lysate and mixed vigorously in a vortex machine.
8. The sample was cooled in ice for 5 minutes.
9. The sample was centrifuged at 13,000 rpm for 3 minutes.
10. The supernatant containing the DNA was Transfered to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.
11. The content was mixed gently by inverting the microcentrifuge tube until the thread-like strands of DNA form a visible mass.
12. The DNA was centrifuged at 13,000 rpm for 2 minutes.
13. The supernatant was carefully poured off and the tube was drained on clean absorbent paper.

Table 2.1 PCR primers used in this study

Serial	Target gene	Primer sequence	Amplicon	Reference
1	*mdh-1*	5'-ATG AAA GTC GCT GTT ATT-3'	892	O'Shea et al. 2004
	mdh-2	5'-GTA TCT AAC ATG CCA TCC-3'		
2	*groEL1A*	5'-GAT CCA TAT GGC TGC TAA AGA CGT ACG-3'	1,600	O'Shea et al. 2004
	groEL1B	5'-CTA GGT CGA CTT ACA TCA TGC GGC CCA TGC-3'		
3	*msha398*F	5'-GGA ACG TGG CAC AAA TG-3'	3,000	O'Shea et al. 2004
	*msha398*R	5'-TGA CGT AAG TGA GCC GC-3'		
4	*msha400*F	5'-AAG ATG AAA TCG GGT TG-3'	2,212	O'Shea et al. 2004
	*msha400*R	5'-TAT CTG GCG ACG CTT GC-3'		
5	*msha403*F	5'-GAA CCG ATT TAT CTT TAG GAG-3'	3,874	O'Shea et al. 2004
	*msha403*R	5'-TGA CCG CCA TTA TCT GAT AC-3'		
6	*msha406*F	5'-CGA GTA TTA AGG TAC TGA AGG-3'	596	O'Shea et al. 2004
	*msha406*R	5'-ATC GGT CAG CTT GAT CG-3'		
7	*tcpA* El Tor R	5'-CGA AAG CAC CTT CTT TCA CAC GTT G-3'	453	Rivera et al., 2001
	tcpA F	5'-CAC GAT AAG AAA ACC GGT CAA GAG-3'		
8	*tcpA* Class R	5'-TTA CCA AAT GCA ACG CCG AATG-3'	620	Rivera et al., 2001
	tcpA F	5'-CAC GAT AAG AAA ACC GGT CAA GAG-3'		
9	*acfB*1	5'-GAT GAA AGA ACA GGA GAG A-3'	1,180	O'Shea et al. 2004
	acfB 2	5'-CAG CAA CCA CAG CAA AAC C-3'		
10	*rstR2* F	5'-CCA TGA TTT AAG ATG CTC-3'	500	Mwansa et al., 2006
	rstR3A R	5'-TCG AGT TGT AAT TCA TCA AGA GTG-3'		
11	*ctxA* F	5'-ACA GAG TGA GTA CTT TGA CC-3'	308	Hoshino et al.1998
	ctxA R	5'-ATA CCA TCC ATA TAT TTG GGA G-3'		
12	*rfbO1* F	5'-GTT TCA CTG AAC AGA TGG G-3'	192	Hoshino et al.1998
	rfbO1 R	5'-GGT CAT CTG TAA GTA CAA C-3'		
13	*zot* F	5'-TCG CTT AAC GAT GGC GCG TTT T-3'	947	Rivera et al., 2001
	zot R	5'-AAC CCC GTT TCA CTT CTA CCC A-3'		
14	*ace* F	5'-TAA GGA TGT GCT TAT GAT GGA CAC CC-3'	316	Trucksis et al., 1993
	ace R	5'-CGT GAT GAA TAA AGA TAC TCA TAG G-3'		
15	*toxR* F	5'-CCT TCG ATC CCC TAA GCA ATA C-3'	779	Rivera et al., 2001
	toxR R	5'-AGG GTT AGC AAC GAT GCG TAA G-3'		

14. 600µl of room temperature 70% ethanol was added and the tube was inverted gently for several times to wash the DNA pellet.
15. The DNA was centrifuged at 13,000 rpm for 2 minutes and the ethanol was aspirated carefully.
16. The tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 10–15 minutes.
17. 100µl of DNA rehydration solution was added to the tube and the DNA was re-hydrated incubating the solution overnight at room temperature.
18. The DNA was stored at –20°C.

2.3.9. Biofilm assay

Quantification of biofilm was performed according to the method described by O'Toole and Kolter (1998b) and Watnick *et al*. (1999). Borosilicate glass tubes were filled with 1 ml LB broth and inoculated with *V. cholerae* O1 (EM-226) El Tor cells grown in Luria–Bertani (LB) broth at 37°C for 2 h. After inoculation, tubes were incubated at different temperature (4, 30, 37 and 45°C) for 24 h to observe effect of temperature on biofilm formation. To observe effect of pH on biofilm formation, tubes containing LB broth with different pH (3, 4, 5, 6, 7, 8 and 9) were inculated and kept at 37°C for 24 h. To observe influence of salinity, calcium and magnesium on biofilm formation, tubes containing LB broth with different salinity (0, 1, 2, and 4%), tubes containing LB with different concentration of calcium (0, 1.25, 2.5, 5, 10 and 20mM) but no sodium chloride and tubes containing LB with different concentration of magnesium (0, 10, 20, 40 and 80mM) but no sodium chloride were inculated and kept at 37°C for 24h. Broth cultures from all the tubes were poured off, tubes were then rinsed vigorously with distilled water to remove non adherent cells, filled with 1.2ml of a 0.1% crystal violet solution (Sigma), allowed to incubate for 30 min, and again rinsed vigorously with distilled water. Biofilm formation was quantitated by measuring the optical density at 600nm (OD_{600}) of a solution produced by extracting cell associated dye with 1.5ml of dimethyl sulfoxide (DMSO). All experiments were performed at least three independent times and samples were performed in triplicate. Data shown are a representative experiment and error bars represent standard error of mean.

2.3.10. Animal passage of microcosm water harvouiring nonculturable V. cholerae cells

Cells of nonculturable *V. cholerae* of MW-RT and MW-4C microcosms were collected separately by filtering 10 ml water sample through a 0.22 μm membrane and the cells were suspended into 2 ml PBS aseptically. Bacteria washed from the surface of the membrane filters were aseptically transferred to sterile eppendorf tubes in duplicate (1.0 ml each) of which 1.0 ml was enriched in 9 ml APW in sterile 50 ml flask for 6–8 h at 37°C to determine that no culturable cells were present. The remaining 1 ml sample from each microcosm was injected into Rabbit Ileal Loop (RIL) following methods described elsewhere (Oliver, 2005). The filtrate (1 ml) containing "free cells" from each microcosm

was also inoculated into the RILs. Among seven inoculated RILs, the 1st (N16961, positive control) and 2nd (EM-226, parent strain) loops showed highest amount (4^+) of fluid accumulation whereas the 3rd (negative control) and 4th, 5th, 6th and 7th loops containing nonculturable cells showed no fluid accumulation. The rabbit was killed after 18–20 h and entire loop of the RILs containing nonculturable cells were collected aseptically and enriched in APW at 37°C for 6–8 h before plating (Huq *et al*., 1990). A loopfull of enriched APW was streaked onto TCBS agar and TTGA plates, and incubated at 37°C for 18–24 h. Presumptive *V. cholerae* colonies were confirmed by serology. After that two representative colonies were subjected to genetic screening, biofilm assay and molecular typing.

2.3.11. Molecular typing of V. cholerae by Pulsed Field Gel Electrophoresis (PFGE)

2.3.11.1. Preparation of PFGE Agarose Plugs from Cell Suspensions

Cell suspensions were made in 2 ml Cell Suspension Buffer from 14-16 h Gelatinase Agar (GA) plates. The concentration of the cell suspensions were adjusted with the Dade Micro scan Turbidity Meter as described in the following steps:

The digital output on the instrument was tested by inserting CSB “blank” tube in the left position (position 1) of the turbidity meter and one of the other tubes of CSB in sample position (right) (position 2) of the turbidity meter. The reading should be 0.00±0.01.

A cotton swab was used to remove growth from the agar plates into the appropriate Falcon tube that contains 2 ml of CSB. The cells were suspended by rubbing against the wall the tube gently so that the cells are evenly dispensed and formation of aerosol is minimized.

The tubes were inserted into the sample position to check the reading (desired range is 0.48-0.52).

If the reading was greater than 0.60, additional CSB was mixed and the reading was checked again until it is within the desired range.

The cell suspensions were kept in ice-bath. 98 µl of each cell suspensions were transferred to labeled micro centrifuge tubes using 100 µl pipette and tip.

The micro centrifuge tubes were placed in floating rack and incubated at 37ºC water bath for 5 minutes.

The cell suspensions were removed in 1.5 ml tubes from water bath and 4 µl Proteinase K was added to each 98 µl cell suspension and mixed by closing tubes and tapping side of tubes.

98 µl melted 1% Seakem Gold agarose that was kept in 56ºC water bath was added to one of the 98 µl cell suspensions and mixed gently by pipetting.

Immediately part of mixture was dispensed into previously labeled appropriate wells in plug molds. The plugs were allowed to solidify for 10-15 min at room temperature.

2.3.11.2. Lysis of Cells in Agarose Plugs

25 µl Proteinase K Stock solution (20mg/ml) was added per 5 ml of cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine) just before use. 5 ml of cell lysis buffer was dispensed to each of the 50 ml tubes. The plugs were disposed in the cell lysis buffer. The tubes were placed in a rack in 54ºC shaking water bath and incubated for 1 hour with constant agitation.

2.3.11.3. Washing of Agarose Plugs after Cell Lysis

Tubes were removed from shaking water bath and the lysis buffer was poured off. 15 ml pre-warmed (50ºC) sterile Type 1 water was added to each tube and returned to shaking water (50ºC). Tubes were shaken for 15 minutes. Water was poured off and these wash steps were repeated. After that, the water was poured off and 15 ml pre-warmed (50ºC) sterile TE was added and the tubes were shaken in shaking water bath (50ºC) for 15

minutes. This steps were repeated for four times and TE was poured off each time and finally 1 ml TE was added and the plugs were stored at 4ºC for subsequent step(s) in 1.5 ml tubes.

2.3.11.4. Restriction Digestion of DNA in agarose plugs with *Xba*I and *Not* I

***2.3.11.4.1. Requirements*:**

1. Filtered deionized water
2. 10X H buffer
3. *Xba* I enzyme (10 U/ml)
4. *Not* I enzyme (10 U/ml)

Plugs were removed from tubes containing TE with wide end of spatula and were placed in a sterile disposable petri dish and were cut at a 2 mm wide slice from test samples and transferred to the labeled 1.5ml micro centrifuge tubes containing 200 µl diluted H buffer (1:10 dilution). The rest of plugs were replaced in original tubes that contained 1 ml TE Buffer. Two 2 mm wide slices of *Salmonella* ser. Braenderup standard plugs were cut and transferred to tubes of diluted H buffer. The tubes were incubated in room temperature for 10-15 min. After incubation of plug slices H buffer were removed. Then each of the plugs was immersed in 200 µl of reaction mixer, which was prepared according to the following calculations:

Table 2.2 Reaction mixture for Marker

Reagent	Quantity
10x *Xba* I restriction enzyme buffer	20 µl
Xba I restriction enzyme (10U/ml)	2 µl
`BSA (20U/ml)	2 µl
Filtered deionized water	176 µl
Total	**200 µl**

Table 2.3: Reaction mixture for *V. cholerae* O1

Reagent	Quantity

10x *Not* I restriction enzyme buffer	20 µl
Not I restriction enzyme (10U/ml)	1 µl
BSA (20U/ml)	2 µl
Filtered deionized water	177 µl
Total	**200 µl**

The samples and the control tubes were incubated at 37ºC water bath for at least 18 h.

2.3.11.4.2. Casting Agarose Gel and Loading Restriction Plug Slices on the Comb

1 g of Seakem Gold (SKG) Agarose (Bio-Rad) was added to 100 ml of 0.5X TBE buffer in a 500 ml Erlenmeyer flask. The slurry was heated in the microwave oven until the agarose was dissolved completely. The temperature of the slurry was equilibrated to 54-58^0 C in a water bath. The casting apparatus of the PFGE was assembled according to the instruction manual (Bio-Rad). The comb was put onbench top and the plugs were loaded on the bottom of the comb teeth; The *Salmonella* ser. Braenderup standard plug slices were put on teeth, 1, and 15 and the samples were loaded on the remaining teeth. Using a Pasteur pipette, the edges of the casting platform were sealed with a small quantity of the agarose solution and allowed to set. Then the remainder of the warm agarose solution was poured into the casting stand for a thickness of approximately 5-6 mm. The gel was allowed to solidify for 30 minutes at room temperature.

2.3.11.5. Electrophoresis

PFGE was performed with the Contour Clamped Homogenous Electric Field (CHEF-DRII) apparatus from the Bio-Rad laboratories (Richmond, CA, USA). The electrophoresis chamber was filled with approximately 2.5 liters of running buffer (0.5 X TBE). Preparations of the electrophoresis apparatus were performed according to the instruction manual (Bio-Rad). The gel and the platform assembly were placed into the frame. It was insured that the gel was covered by about 2 mm of buffer. The temperature of the running buffer was adjusted to 14^0 C and the flow rate of the buffer through the electrophoresis cell was maintained approximately at 0.75 liter per minute. Electrophoresis was done at 6 volts for 18 h (initial switch time 2.2s; final switch time

63.8s). After the end of run, the gel was stained with ethidium bromide (0.5 μg/ml) solution for 30 minutes at room temperature and then de-stained in sufficient distilled water for 1 h. The gel was visualized on the UV transilluminator and photographs were taken as with a digital camera connected the UV transilluminator. The DNA size standards used was the *Salmonella* serotype *Braenderup* (H9812) ranging from 20.5 to 1135 kb.

2.3.11.6. Dendrogram

The test fingerprint image was normalized according to the standard, and the molecular weights of the DNA fragments were determined. The gel image analyzed included two reference lanes of classical and El Tor biotypes. The reference strains of classical *V. cholerae* O1 and of El Tor *V. cholerae* O1 were O395 and N16961, which are used routinely in our laboratory.

The molecular weight of the DNA fragments was determined by using Quantity One software (version 4.4.1; Bio-Rad). The degree of genetic diversity between the new variants, and the classical and El Tor strains was determined by using Diversity Database software (version 2.2; Bio-Rad). The similarity among the strains was determined using the Dice coefficient, and the cluster analysis was carried out using the unweighted-pair group method using average linkages (UPGMA).

3. RESULTS

3.1. BIOFILM FORMATION AND SURVIVAL OF *V. cholerae* O1 IN MICROCOSM

To understand whether *V. cholerae* can form biofilm for their survival in microcosm the laboratory, a *V. cholerae* O1 strain (EM-226) was initially tested. Two sets of microcosms prepared with 600 ml Mathbaria coastal pond water (MW) in 1 L conical flasks and inoculated with approximately similar numbers of the *V. cholerae* O1 strain. However, the microcosms were incubated at different temperatures, one set at room temperature (25°C) and another at 4C. Microcosm kept at room temperature yielded initial counts at day 1/0h of ~10^7 cfu/ml of *V. cholerae* O1 when plated on TTGA and LBA plates, whereas counts on TCBS agar were ~10^5 cfu/ml in both microcosms i.e. at RT and 4°C (Table 3.1). On the other hand, culture independent DFA counts of *V. cholerae* O1 at day 1, were ~8.0 x 10^8 cells/ml in both microcosms incubated at different temperature. M-PCR with template DNA prepared from the two microcosms on day 1, amplified the genes for *wbe* (specific for *V. cholerae* O1)*, ctx*A (encode subunit A of cholera toxin) and *rstR* (encode transcriptional repressor) (Table 3.1). Plate counts of MW microcosms maintained at room temperature (MW-RT) varied significantly from those maintained at 4°C (MW-4C). Both microcosms showed a gradual reduction in plate counts on TTGA and LA with passage of time. However, an even faster decline in plate counts on TCBS agar was observed, with no counts after day 15. Conversely, counts for both MW-RT and MW-4C microcosms varied significantly on TTGA and LA at day 15, and subsequently, with respect to time of incubation, continued to decline; eventually no *V. cholerae* counts were obtained after 40 and 68 days, respectively (Table 3.1). As the monitoring for culturable *V. cholerae* O1 in the microcosms continued for several weeks gradual decline in the numbers of culturable cells occurred, as was observed by the fewer colonies appearing on TTGA and LBA, notably at 40 days, the bacterial colonies were tiny (<1.0 mm), grew very slowly, and did not grow on TCBS.

Interestingly, DNA templates obtained from both microcosms supported amplification of *wbe* and *ctxA* genes by M-PCR up to 54 days (Table 3.1). At day 68, when non culturable count was observed in MW-RT, samples from MW-4C yielded only a few colonies on TTGA and LBA, and the DNA template from the MW-RT did not amplify

all the target genes after PCR but MW-4C microcosm sample amplified genes for *wbe* but not *ctx*A and *rstR*2 (Table 3.1). At this stage of extended incubation, representative tiny colonies on TTGA and LBA were confirmed as *V. cholerae* O1. However, the tiny colonies did not appear on TTGA and LBA after plating of MW-4C sample at day 85 and thereafter, even when concentrates of larger volumes of samples (10 ml) from microcosms were enriched and plated on TTGA and LBA. Furthermore, template DNA from the samples of day 85 and afterwards did not amplify the genes *wbe*, *ctxA* and *rstR2*.

Table 3.1. Impact of biofilm formation on the growth and survival of *V. cholerae* O1 cells

Day	Incubation condition	Plate count (cfu/ml)			Agglutinate	DFA	Multiplex PCR		
		TCBS	TTGA	LA		Cells/mL	*wbe*	*ctx*A	*rts*R
1	*V. cholerae* O1 in microcosm MW-RT	6.2×10^5	1.1×10^7	1.2×10^7	+	8.0×10^8	+	+	+
	V. cholerae O1 in microcosm MW-4C	6.2×10^5	1.2×10^7	1.2×10^7	+	8.0×10^8	+	+	+
15	*V. cholerae* O1 in microcosm MW-RT	2.1×10^2	4.3×10^4	6.0×10^5	+	9.0×10^7	+	+	+
	V. cholerae O1 in microcosm MW-4C	1.2×10^2	3.2×10^6	3.7×10^6	+	5.0×10^8	+	+	+
26	*V. cholerae* O1 in microcosm MW-RT	<10	4.0×10^3	3.0×10^3	+	BF, coccoids & few rods	+	+	+
	V. cholerae O1 in microcosm MW-4C	<10	3.2×10^5	1.4×10^6	+	5.0×10^8	+	+	+
40	*V. cholerae* O1 in microcosm MW-RT	<10	3.1×10^2	2.9×10^2	+	**BF, coccoids & few rods**	+	+	+
	V. cholerae O1 in microcosm MW-4C	<10	1.6×10^4	2.9×10^4	+	**BF, coccoids & few rods**	+	+	+
54	*V. cholerae* O1 in microcosm MW-RT	<10	<10	<10	–	BF	+	+	+
	V. cholerae O1 in microcosm MW-4C	<10	3.6×10^3	1.1×10^4	+	BF, coccoids & few rods	+	+	+
68	*V. cholerae* O1 in microcosm MW-RT	<10	<10	<10	–	BF	–	–	–
	V. cholerae O1 in microcosm MW-4C	<10	8.0×10^1	6.2×10^2	+	BF, coccoids & few rods	+	–	–
85	*V. cholerae* O1 in microcosm MW-RT	**<10**	**<10**	**<10**	NG	BF	–	–	–
	V. cholerae O1 in microcosm MW-4C	**<10**	**<10**	**<10**	NG	BF	–	–	–
99	*V. cholerae* O1 in microcosm MW-RT	<10	<10	<10	NG	BF	ND	ND	ND
	V. cholerae O1 in microcosm MW-4C	<10	<10	<10	NG	BF	ND	ND	ND

MW-RT= Mathbaria pond water microcosm at room temperature; MW-4C= Mathbaria pond water microcosm at 4°C; NG=No growth; ND=Not done; BF=Biofilms

Unlike the plate counts, which declined sharply with time of incubation, the DFA counts did not change for both the MW-RT and MW-4C microcosms, until day 15. As shown in Table 3.1, the counts for MW-4C microcosm further remained unchanged

(~10^8 cells/ml) until day 26, whereas those for MW-RT were not countable from day 26 onwards because of the aggregation of cells within biofilm (Fig. 3.1).

Microscopic observations also revealed that *V. cholerae* O1 cells in microcosms changed their morphology along with time. The curved rod-shaped *V. cholerae* cells observed at day 1 gradually transformed into coccoid cells. *V. cholerae* cells appeared as single comma/rod shaped bacteria at day 1 (Fig. 3.1 A), however, these cells assembled in a coordinated pattern in microaggregates on day 2 (Fig. 3.1 B), in small clusters at day 7 (Fig. 3.1 C), and finally in large cluster of biofilms harboring mostly coccoid cells at day 45 (Fig. 3.1 D).

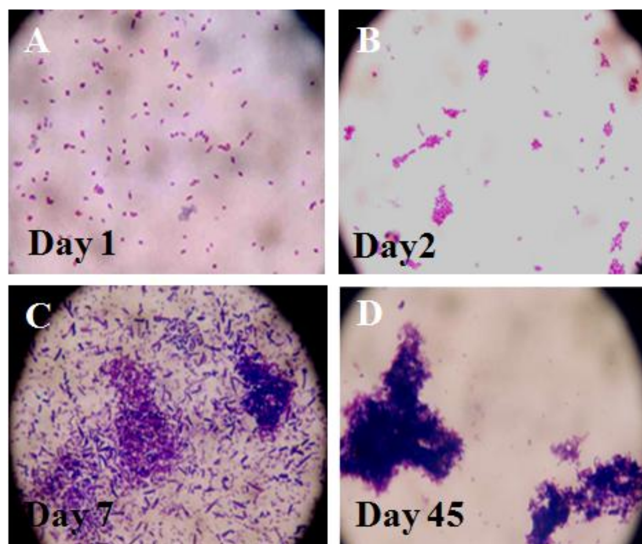

Fig. 3.1. Micrographs showing different stages of biofilm formation by *V. cholerae* cells in microcosm prepared with the pond water collected from Mathbaria, a cholera endemic area of Bangladesh. At day 1, cells appeared as single cells (A), microcolony formation at Day 2 (B), formation of large clusters of cells at day 7 (C) and formation of thick biofilm at day 45 (D). Samples were stained with crystal violet, observed under light microscope (Carl Zeiss; Axioskop 40) and images were captured using a digital camera (AxioCam MRc) attached to the microscope.

3.2. BIOFILM FORRMATION OF *V. cholerae* O1 IN MICROCOSMS: INFLUENCE OF PHYSICO-CHEMICAL PARAMETERS

3.2.1. Influence of temperature on biofilm formation of V. cholerae

To understand whether temperature can influence the biofilm formation of *V. cholerae* kept in microcosm conditions, a specific number of the bacterium was inoculated in similar microcosms and kept at four different incubation temperature, i.e., 45°C, 37°C, 30°C and 4°C. All the microcosms inoculated with *V. cholerae* yielded initial counts of ~10^7 cfu/ml when plated on TTGA and LBA, whereas counts on TCBS agar were ~10^6 cfu/ml (Fig. 3.2). Cultivable *V. cholerae* numbers in all microcosms showed a gradual reduction in plate counts with passage of time. However, an even faster decline in plate counts on TCBS agar was observed with no counts at day 15 in MW-45C, and at day 26 in MW-4C, MW-30C and MW-37C. Conversely, counts for all microcosms varied significantly on TTGA and LBA at day 15, and subsequently, with respect to time of incubation, continued to decline. Eventually, no cultivable *V. cholerae* O1 counts were observed on TTGA and LBA plates from samples after day 26, 40, 40 and 68 from microcosms kept at 45°C, 30°C, 37°C, and 4°C, respectively (Fig.3.2).

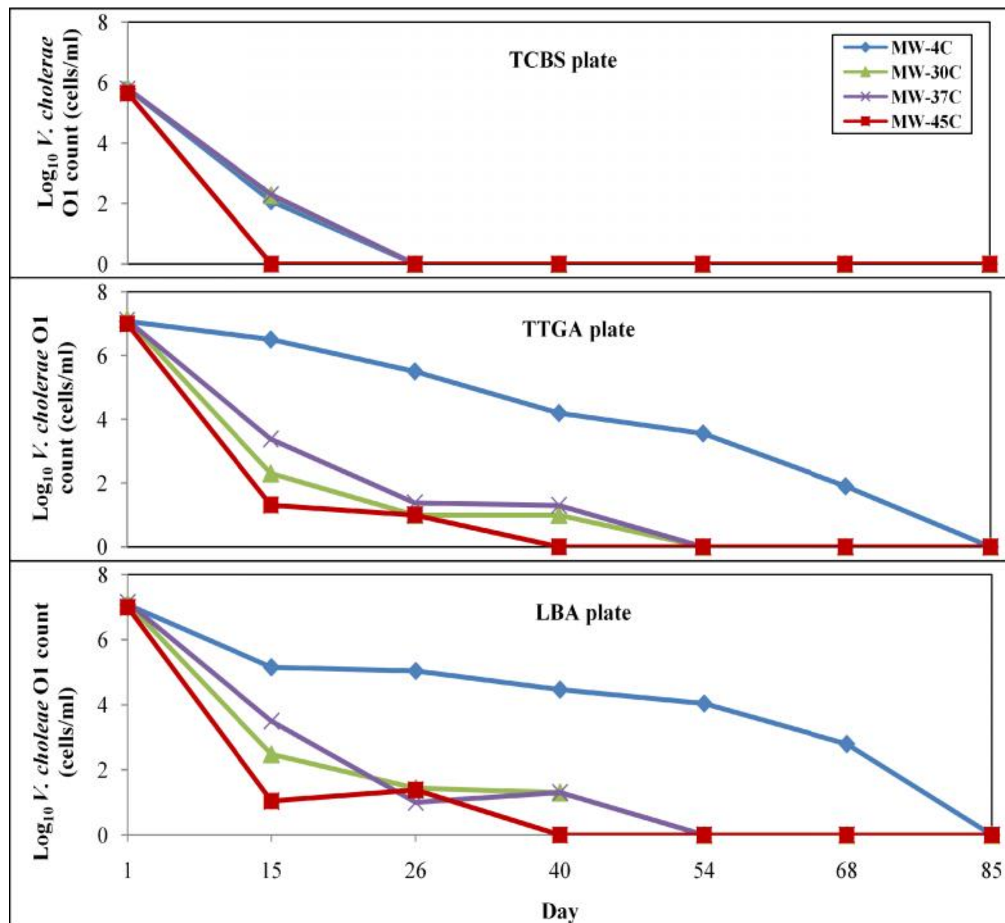

Fig. 3.2. Influence of temperature on the growth and survival of *V. cholerae* in microcosms prepared with pond water collected from Mathbaria, Bangladesh.

DFA micrographs showed that *V. cholerae* cells appearing as comma shaped bacteria at day 1, started to form microcolonies, which is the initial step of biofilm formation, at day 2 in all the microcosms incubated at different temperature. Eventually, the cells formed small to large aggregates of cells and finally thick clusters of biofilm. Interestingly, biofilm formation by *V. cholerae* cells in MW-4C was slower compared to that of other microcosms (Fig. 3.3). Matured biofilm was observed from day 40, 54, 54, and 85 onwards in microcosms kept at 45, 37, 30 and 4C, respectively.

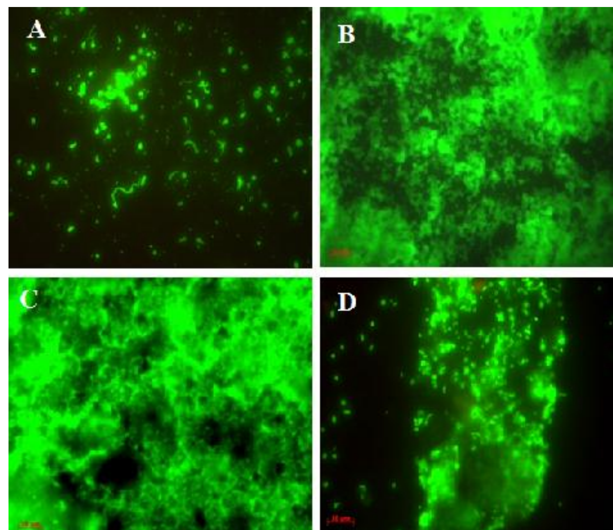

Fig. 3.3. DFA micrographs of biofilms formed by *V. cholerae* at day 54 in microcosms incubated at different temperature (A) 4^0C, (B) 30^0C, (C) 37^0C and (D) 45^0C. Samples were stained with fluorescent antibody specific for *V. cholerae* O1 and visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Microscopic images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm).

3.2.2. Influence of pH on biofilm formation of V. cholerae

To understand the effect of pH on biofilm formation capability of *V. cholerae*, the pH of Mathbaria water fractions used for microcosms were adjusted to 6.0, 7.0, 8.0 and 9.0. All the microcosms containing Mathbaria water with four different pH were inoculated with *V. cholerae* yielded initial counts of 10^6 cfu/ml when plated on TCBS agar plate whereas counts of 10^7 cfu/ml on TTGA and LBA plates (Fig. 3.4). *V. cholerae* count declined rapidly on TCBS agar plate, with no count at day 26 and onwards in all types of microcosms. A gradual reduction in the plate counts on TTGA and LBA was observed as time went on. Plate counts for *V. cholerae* cells in MW-pH6 and MW-pH7 continued up to 26 and 40 days, respectively. However, *V. cholerae* counts on TTGA and LBA in MW-pH8 and MW-pH9 continued for longer period of time (54 days).

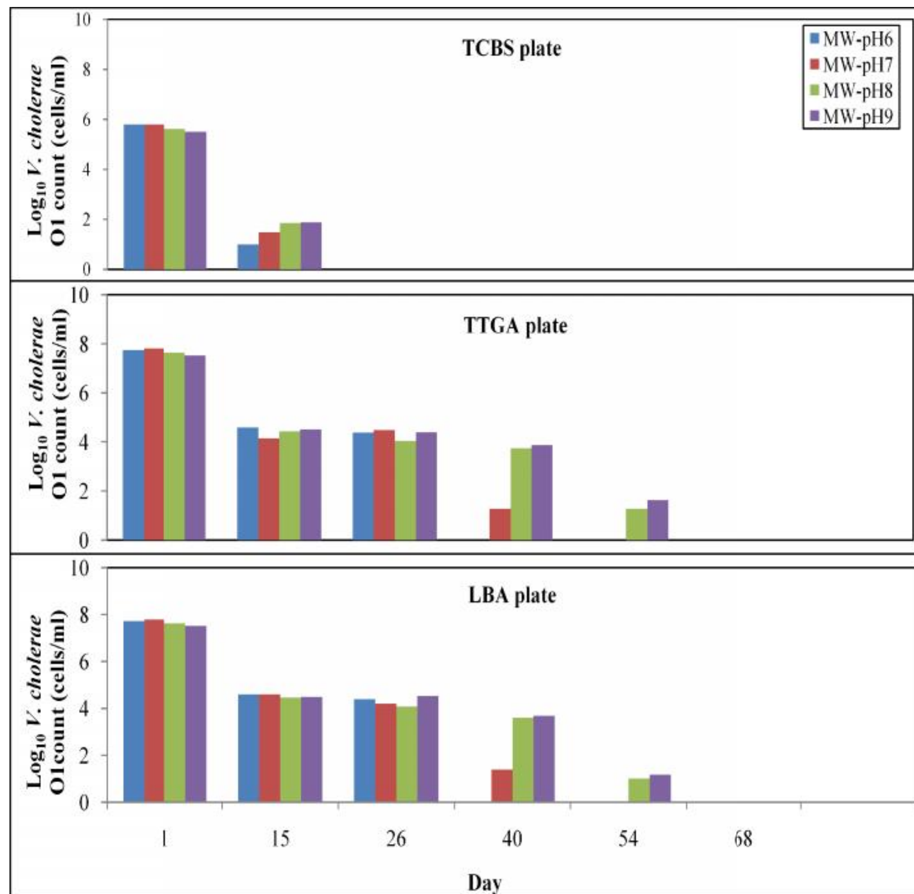

Fig. 3.4. Influence of pH on growth and survival of *V. cholerae* in microcosms constructed with water collected from a pond of Mathbaria.

Microscopic observations showed that *V. cholerae* cells formed microcolonies at day 2, and gradually, small clusters, large clusters and finally thick clusters of biofilms in all the microcosms at day 7, day 45 and day 54. Biofilm formation was higher in microcosms having pH 8.0-9.0 whereas lower in microcosms having pH 6.0-7.0 at day 54 (Fig. 3.5).

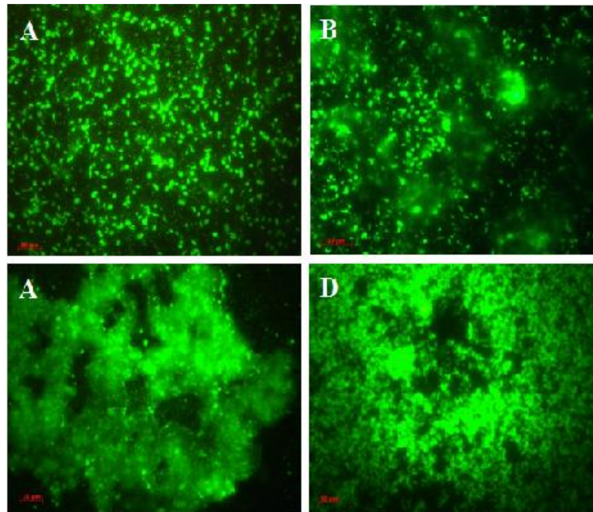

Fig. 3.5. Micrographs of biofilm formed by *V. cholerae* at day 54 in microcosms having different pH (A) 6.0 (B) 7.0 (C) 8.0 and (D) 9.0. Samples stained with fluorescent antibody specific for *V. cholerae* O1 were observed under epifluorescence microscope (Carl Zeiss; Axioskop 40). A digital camera (AxioCam MRc) attached to the microscope was used to capture the microscopic images. (Scale bars inserted in the figures were equal to 10 µm)

3.2.3. Influence of salinity on biofilm formation of V. cholerae

To understand the effect of salinity on biofilm formation capability, *V. cholerae* cells were inoculated into instant ocean microcosms having different concentration of salinity (0, 1.0, 2.0 and 4.0%). *V. cholerae* cells yielded initial counts of 10^6 cfu/ml when samples were plated from MW-0%, MW-1%, MW-2% and MW-4% on TCBS agar plate, whereas counts of 10^7 cfu/ml on TTGA and LBA plates (Fig. 3.6). A gradual decline in *V. cholerae* counts on TCBS agar plate was observed, with no counts at day 26 in MW-0%, MW-1%, MW-2% and MW-4% microcosms. *V. cholerae* counts on TTGA and LBA plates also showed gradual decline with passage of time and no cultivable counts were observed at day 40 in MW-2% and MW-4% microcosms, and at day 54 in MW-1% microcosm.

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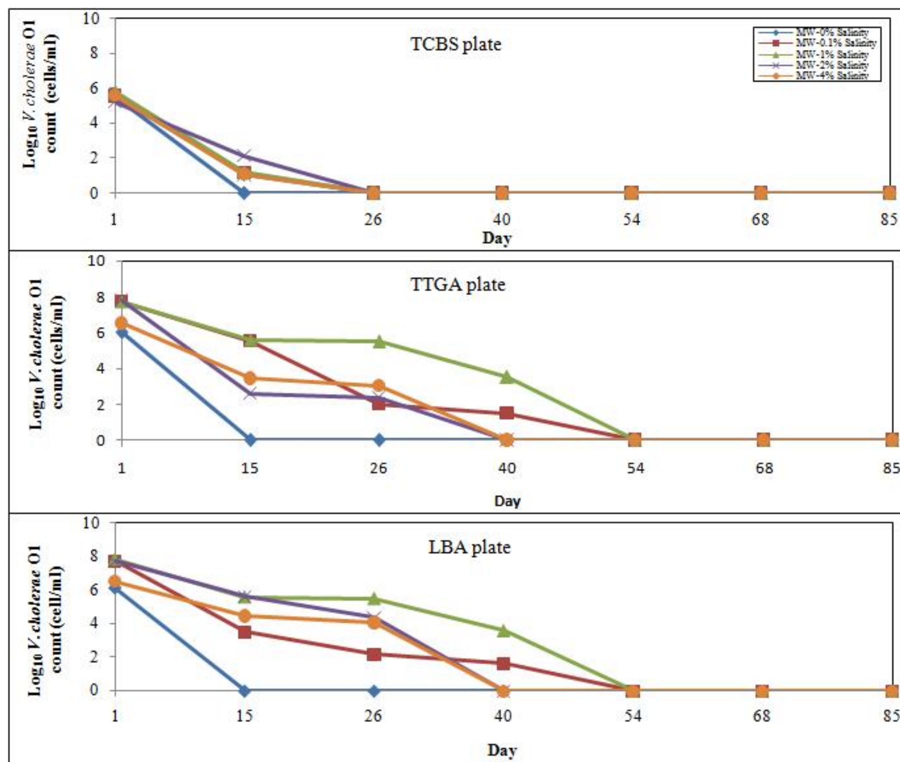

Fig. 3.6. Influence of salinity on growth and survival of *V. cholerae* in instant ocean having different concentration of salinity.

Under fluorescence microscope, *V. cholerae* cells were observed to form microcolonies, small clusters, large clusters and finally thick clusters of biofilms in all the microcosms at day 2, day 7 and day 45. DFA micrographs showed that biofilm formation by *V. cholerae* cells was higher in microcosms having salinity of 2 and 4% whereas lower in microcosms having 0-1% sainity when compared for the waters of the same day, e.g., day 54 (Fig. 3.7).

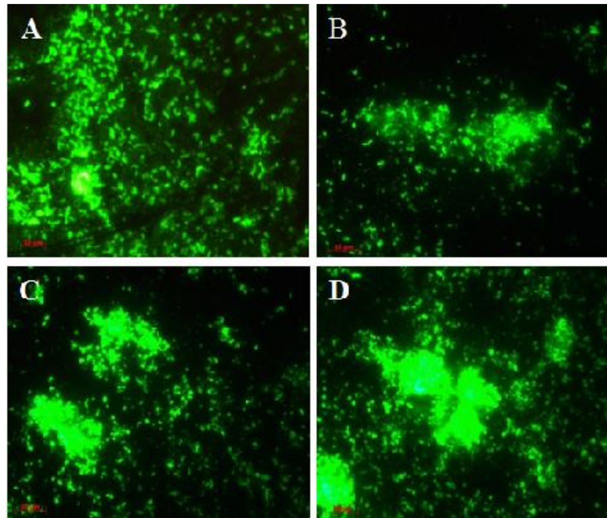

Fig. 3.7. DFA micrographs of biofilm formation by *V. cholerae* at day 54 in microcosms having different salinity (A) 0%, (B) 1%, (C) 2% and (D) 4%. Samples were stained with fluorescent antibody specific for *V. cholerae* O1 and visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

3.3. BIOFILM FORMATION OF *V. cholerae* O1 IN MEDIA: EFFECT OF PHYSICO-CHEMICAL PARAMETERS

3.3.1. Effect of temperature on biofilm formation of V. cholerae

To compare the biofilm formation capacity of *V. cholerae* at different temperatures, one representative strain was grown in 1 ml LB broth at 4, RT(25), 30, 37 and 45^0C temperature for 24h without shaking in 5 ml capacity borosilicate glass tubes. Differences in biofilm formation was measured by comparing the produced biofilms, which remained attached to the glass surface, by using crystal violet staining method. In Fig. 3.8, the biofilm formation of *V. cholerae* strain in LB broth was compared at different temperature. The results showed that this bacterium produced the highest amount of biofilm at 30°C whereas biofilm formation was lower at both lower (4°C) and higher temperature (45°C), e.g., at 4°C the *V. cholerae* strain produced biofilm which was equivalent to approx. 0.4 OD_{600} of cell density whereas at 30^0C approx. 0.95 OD_{600} of cell density.

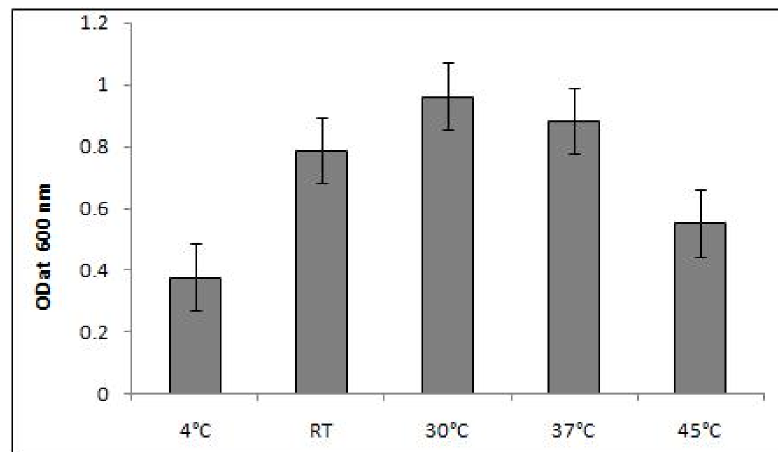

Fig. 3.8. Effect of temperature on biofilm formation of *V. cholerae* in LB broth after 24h of incubation. (Error bars mean standard error of mean).

3.3.2. Effect of pH on biofilm formation of V. cholerae

For analysis of biofilm formation capacity of *V. cholerae* at different pH, one representative strain was grown in 1 ml LB broth at 37^0C temperature for 24 h without shaking in 5 ml capacity borosilicate glass tubes. Biofilms produced by the cells on borosilicate glass tube were stained with crystal violet. The influence of pH on biofilm formation of *V. cholerae* was observed in LB broth after 24 h of incubation (Fig. 3.9). The amount of biofilm formation was different at different pH. Biofilm formation was low at lower pH (3.0-6.0) and high at higher pH (7.0-9.0) e.g., at pH 3.0-5.0 the *V. cholerae* strain produced biofilm which was equivalent to approx. 0.2-0.3 OD_{600} of cell density whereas at pH 7.0-9.0 approx. 0.45 OD_{600} of cell density.

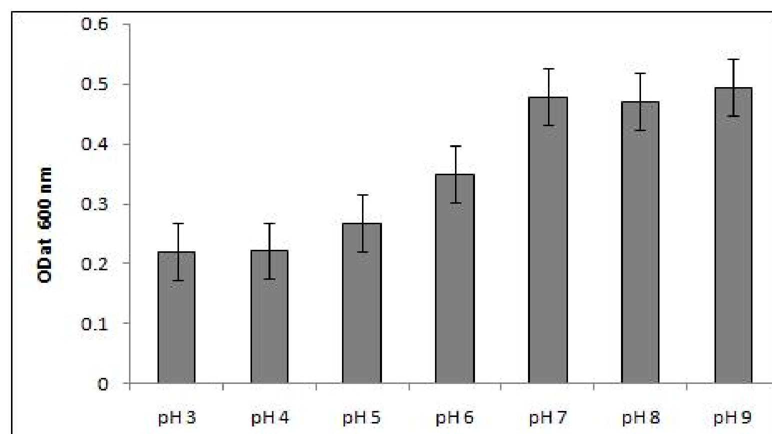

Fig. 3.9. Effect of pH on biofilm formation of *V. cholerae* in LB broth after 24h of incubation (Error bars mean standard error of mean).

3.3.3. Effect of salinity on biofilm formation of V. cholerae

To observe biofilm formation ability of *V. cholerae* at different salinity, one representative strain was grown in 1 ml LB broth having different concentration of salinity (0, 1, 2 and 4%) at 37^0C temperature for 24h without shaking in 5 ml capacity borosilicate glass tubes. Biofilms produced by the cells on borosilicate glass tube were stained using crystal violet staining method. The biofilm formation ability of *V. cholerae* in LB broth having different concentration of salinity was observed on borosilicate glass tube after 24h of incubation and compared (Fig. 3.10). *V. cholerae* cells produced highest amount of biofilm at 2% salinity and lowest amount at 0, 1 and 4% salinity. e.g., at 0% salinity, the *V. cholerae* strain produced biofilm which was equivalent to approx. 0.26 OD_{600} of cell density whereas at 2% salinity approx. 0.6 OD_{600} of cell density.

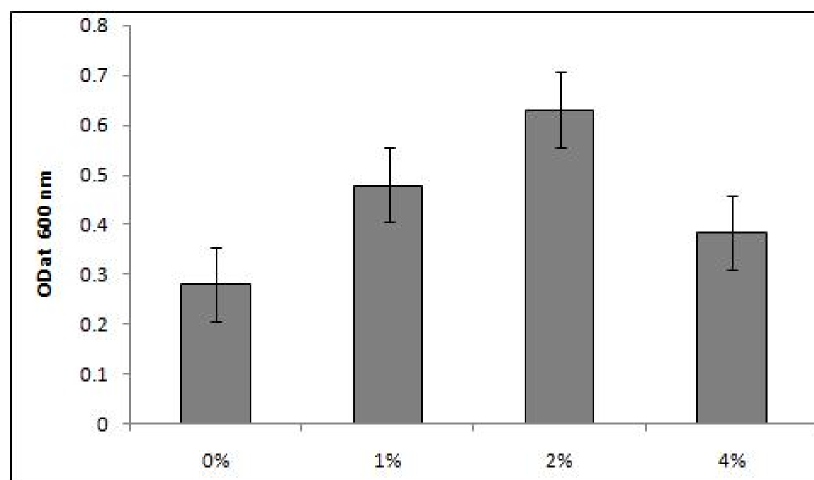

Fig. 3.10. Effect of salinity on biofilm formation of *V. cholerae* in LB broth after 24h of incubation (Error bars mean standard error of mean).

3.3.4. Effect of calcium on biofilm formation of V. cholerae

To examine biofilm formation capacity of *V. cholerae* at different concentration calcium, one representative strain was grown in 1 ml LB broth having different concentration of calcium chloride at 37^0C temperature for 24h without shaking in 5 ml capacity borosilicate glass tubes. Biofilms produced by the cells on borosilicate glass tube were stained using crystal violet staining method. In Fig. 3.11, the biofilm formation capacity of *V. cholerae* in LB broth having no salinity and different concentration of Calcium Chloride was compared. Amount of biofilm produced by *V.*

cholerae was highest (approx. 0.6 OD_{600} of cell density) in the medium containing 10mM Calcium Chloride.

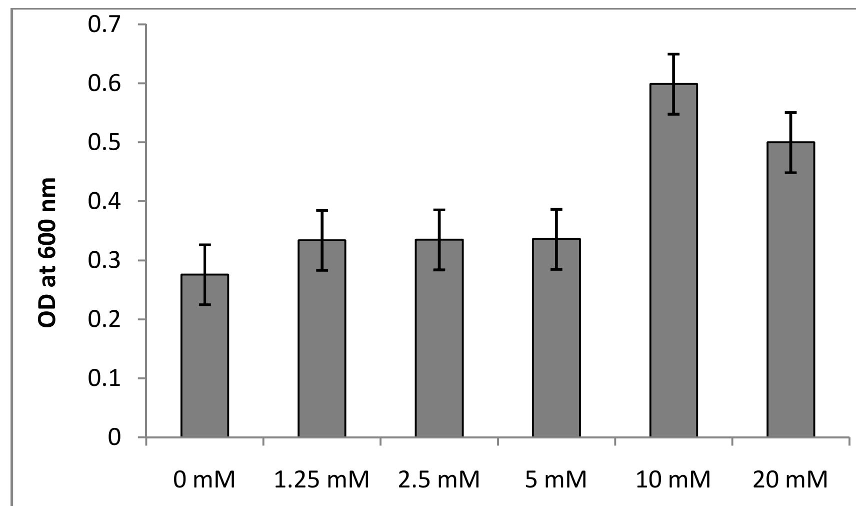

Fig. 3.11. Effect of calcium on biofilm formation of *V. cholerae* in LB broth after 24h of incubation (Error bars mean standard error of mean).

3.3.5. Effect of Magnesium on biofilm formation of V. cholerae

For analysis of biofilm formation capacity of *V. cholerae* at different concentration magnesium, one representative strain was grown in 1 ml LB broth having different concentration of magnesium chloride at 37^0C temperature for 24h without shaking in 5 ml capacity borosilicate glass tubes. Biofilms produced by the cells on borosilicate glass tube were stained with crystal violet. In Fig. 3.12, the biofilm formation ability of *V. cholerae* cells in LB broth having no Sodium Chloride and different concentration of Magnesium Chloride was compared. *V. cholerae* produced highest amount biofilm (approx. 0.63 OD_{600} of cell density) in the medium containing 40mM Magnesium Chloride.

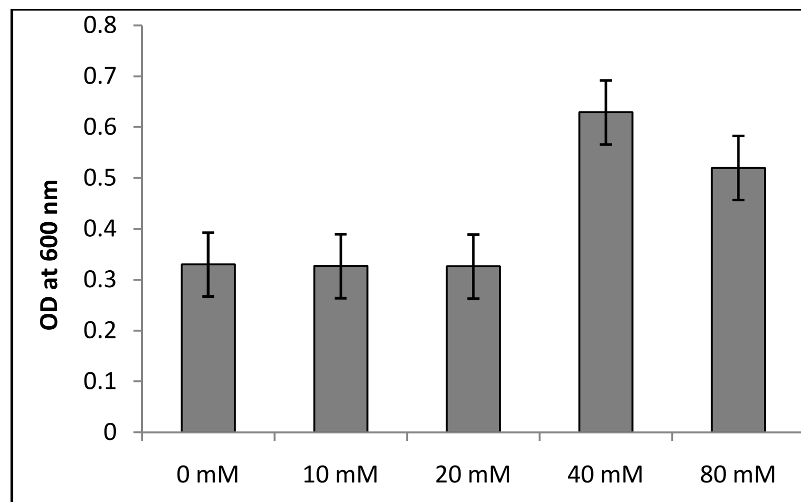

Fig. 3.12. Effect of Magnesium on biofilm formation of *V. cholerae* in LB broth after 24h of incubation. (Error bars mean standard error of mean)

3.4. IMPACT OF CHITIN ON BIOFILM FORMATION OF *V. cholerae*

In order to compare the effect of chitin on the biofilm formation of *V. cholerae,* one representative strain was inoculated in water without chitin (control, MW) as well as water with chitin (MW-CC) and incubated at room temperature (25°C) without shaking. The chitin was extracted from 'golda' shrimp (*Macrobrachium rosenbergii*). During different time intervals, a portion of water samples from the microcosms was collected and checked for *V. cholerae* counts following standard methods. The MW and MW-CC microcosms inoculated with *V. cholerae* O1 yielded initial counts of 10^7 cfu/ml on LBA plate and counts of 10^6 cfu/ml on TCBS agar plate (Table 3.2). At day 1, the initial DFA counts of *V. cholerae* for MW and MW-CC microcosms were 3.8 x 10^8 and 3.5 x 10^8 cells/ml, respectively. M-PCR employing template DNA prepared from the two microcosms at day 1 amplified primers for *V. cholerae* O1 specific *wbe* and *ctx*A (Table 3.2). Bacterial plate counts for both microcosms, MW and MW-CC, declined to 10^4 cfu/ml and 10^3 cfu/ml on LBA and TCBS agar plates, respectively, at day 7 and remained essentially stable until day 28. Subsequently, a gradual reduction in plate count occurred in both microcosms, although cell counts were higher by one log in the MW-CC microcosm, compared to the MW microcosm (Table 3.2). In MW microcosm, plate counts declined to <10 on TCBS agar after day 35 and on LBA plate after day 49 (Table 3.2). In contrast, the MW-CC microcosm, in which culturable cells were

detected for a longer period of time, cell counts declined to <10 on TCBS agar after day 76, whereas *V. cholerae* O1 produced colonies on LBA plate until day 174 (Table 3.2) but not longer, as the cell count declined to <10 at day 189.

To understand whether any genetic change has occured in *V. cholerae* cells while surviving in microcosm with chitin, samples collected from different times were subjected to screening for *wbe* and *ctxA* genes using PCR method. In the MW microcosm, the *wbe* and *ctxA* genes were amplified up to day 49 (Table 3.2), whereas DNA templates prepared from *V. cholerae* O1 cells inoculated and maintained in the MW-CC microcosm supported amplification of *wbe* and *ctxA* genes by M-PCR up to day 174, confirming presence of both of the structural as well as toxigenic genes of *V. cholerae* O1 (Table 3.2).

The physical appearance of the MW-CC microcosm, in which *V. cholerae* O1 was maintained in autoclaved estuarine water supplemented with shrimp chitin as sole nutrient, was essentially clear and the chitin chips were visible as intact pieces at day 1 (Fig. 3.13 A). Unlike the MW microcosm in which the aqueous phase was clear with the non-culturable cells settled to the bottom of the flask, the aqueous phase of the MW-CC microcosm became turbid with bacterial growth supported by chitin (Fig. 3.13 B). The process of chitin degradation by *V. cholerae* continued up to six months, after which degraded chitin and bacteria comprised dense sediment at the bottom of the flask.

Table 3.2. Comparision of *V. cholerae* counts in Mathbaria pond water (MW) microcosms with and without chitin[a,b]

Day	Microcosm without chitin						Microcosm with chitin					
	Plate count (cfu/ml)		Agglutinate[C]	DFA (Cells/mL)	Multiplex-PCR		Plate count (cfu/ml)		Agglutinate[C]	DFA (Cells/mL)	Multiplex-PCR	
	TCBS	LBA			O1 *wbe*	*ctx*A	TCBS	LBA			O1 *wbe*	*ctx*A
1	2.2×10^6	3.8×10^7	+	3.8×10^8	+	+	1.0×10^6	3.5×10^7	+	3.6×10^8	+	+
7	2.8×10^3	3.8×10^4	+	1.8×10^8	+	+	2.1×10^3	1.1×10^4	+	+[d]	+	+
14	1.9×10^3	1.7×10^4	+	1.8×10^8	+	+	1.4×10^3	1.9×10^4	+	BF[d]	+	+
21	1.6×10^3	2.8×10^4	+	BF	+	+	2.0×10^3	2.4×10^4	+	BF[d]	+	+
28	1.1×10^3	4.8×10^4	+	BF	+	+	1.9×10^3	1.3×10^4	+	BF[d]	+	+
35	1.7×10^1	2.4×10^2	+	BF	+	+	1.6×10^2	1.1×10^3	+	BF[d]	+	+
42	<10	1.0×10^2	+	BF	+	+	1.6×10^1	2.4×10^3	+	BF[d]	+	+
49	<10	6.8×10^1	+	BF	+	+	7.7×10^1	2.4×10^2	+	BF[d]	+	+
56	<10	<10	NG	BF	ND	ND	6.7×10^1	1.4×10^2	+	BF[d]	+	+
63	<10	<10	NG	BF	ND	ND	6.5×10^1	1.2×10^2	+	BF[d]	+	+
70	<10	<10	NG	BF	ND	ND	6.4×10^1	1.0×10^2	+	BF[d]	+	+
76	ND	ND	NG	ND	ND	ND	4.0×10^1	5.0×10^1	+	BF[d]	+	+
92	ND	ND	NG	ND	ND	ND	<10	1.1×10^1	+	ND	+	+
99	ND	ND	NG	ND	ND	ND	<10	2.5×10^1	+	ND	+	+
113	ND	ND	NG	ND	ND	ND	ND	2.1×10^1	+	ND	+	+
132	ND	ND	NG	ND	ND	ND	ND	2.0×10^1	+	ND	+	+
146	ND	ND	NG	ND	ND	ND	ND	2.1×10^1	+	ND	+	+
160	ND	ND	NG	ND	ND	ND	ND	2.1×10^1	+	ND	+	+
174	ND	ND	NG	ND	ND	ND	ND	1.0×10^1	+	ND	+	+
189	ND	ND	NG	ND	ND	ND	ND	<10	NG	BF[d]	+	+

a= Microcosm was constructed using Mathbaria pond water, a known reservoir of *V. cholerae* (Alam *et al*., 2006a).b= Microcosm was constructed using Mathbaria pond water, a known reservoir of *V. cholerae* O1 (Alam *et al*., 2006a) and chitin chips.c= *V. cholerae* O1 polyvalent and monoclonal antisera. d= Attachment of *V. cholerae* O1 cells on chitin chip. e= *V. cholerae* O1 cells from enrichment of HCl-treated homogenized chitin residues. MW= Mathbaria pond water microcosm, inoculated with *V. cholerae* O1 grown on Luria-Bertani broth and maintained at room temperature; MW-CC= Mathbaria pond water microcosm with chitin chips, inoculated with *V. cholerae* O1 grown on Luria-Bertani broth and maintained at room temperature; ND= Not done; NG= No growth; BF= Biofilms; CFU= Colony forming unit; DFA= Direct fluorescent antibody.

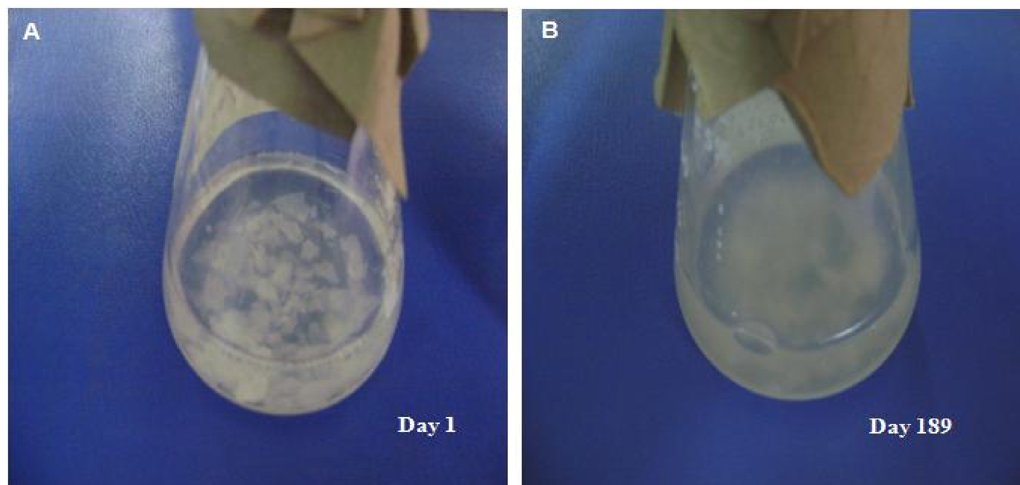

Fig. 3.13. External observation of the utilization of shrimp chitin in the MW-CC microcosm at room temperature (A) intact chitin chips at day 1 and (B) degraded chitin chips at day 189.

The shrimp chitin chips in the MW-CC microcosm had the morphology of complex hexagonal structural units, i.e., “building blocks” of chitin, when viewed under the compound (light) microscope (Fig. 3.14 A). Crystal violet staining of the shrimp chitin

chips collected from the MW-CC microcosm at day 1 showed no sign of bacterial colonization (Fig. 3.14 A). However, at day 7, the shrimp chitin chips clearly had been colonized by *V. cholerae* on the smooth surface of the chitin chips (Fig. 3.14 B). With passage of time, the chitin surface was further colonized by *V. cholerae,* with a larger number of cells appearing in clusters of biofilm, coupled with apparent signs of chitin degradation (Fig. 3.14 C). Finally, the hexagonal structures of chitin disappeared and a smaller, degraded residue was detected that was heavily colonized by *V. cholerae* O1 (Fig. 3.14 D). Although degradation of chitin continued, *V. cholerae* O1 cells were seen mostly as coccoid and embedded within biofilm clusters.

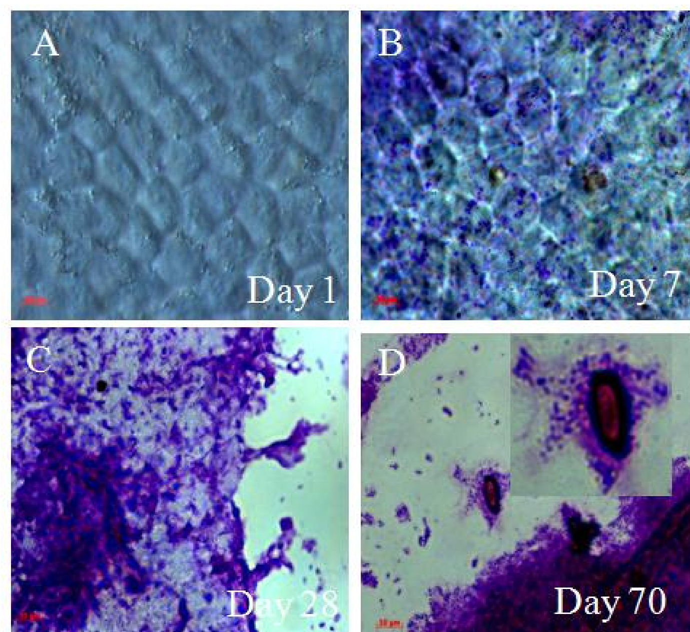

Fig. 3.14. Micrographs showing attachment and utilization of shrimp chitin by *V. cholerae* O1 in MW-CC microcosm. Samples were stained with 0.4% crystal violet. The stained samples were visualized using a light microscope (Carl Zeiss model Axioskop 40). Microscopic images were captured digitally (AxioCam MRc) and processed using Adobe Photoshop (version 5). (A) Hexagonal structure of intact chitin chip at day 1 (B) attachment and colonization of the chitin chip by *V. cholerae* cells at day 7 (C) aggregates of biofilm of *V. cholerae* cells on decaying chitin chips at day 28 and (D) thick biofilm and chitin residues colonized by *V. cholerae* cells showing utilization of chitin at day 70. (Scale bars inserted in the figures were equal to 10 µm).

To understand the developmental stages of biofilm formation at different days of incubation, MW and MW-CC samples were examined using DFA technique. It was observed that the DFA counts of *V. cholerae* did not change in the MW microcosm until day 14 and cells in the MW microcosm could not be counted at day 21 because of transformation of *V. cholerae* cells into large biofilm harbouring mostly coccoid cells. Although initial DFA counts of *V. cholerae* in the MW-CC microcosm were much the

same as that of the MW microcosm, counting by DFA was not possible during later stage (Table 3.2) because of the clustering of cells and formation of micro-colonies that eventually turned into large biofilm clusters on the chitin (Fig. 3.15 A). At day 28, chitin degradation and biofilm formation had progressed (Fig. 3.15 B) simultaneously and the various sizes of degraded residues of the chitin were heavily colonized by *V. cholerae* O1 cells, mostly of coccoid morphology up to day 70 (Figs. 3.15 C).

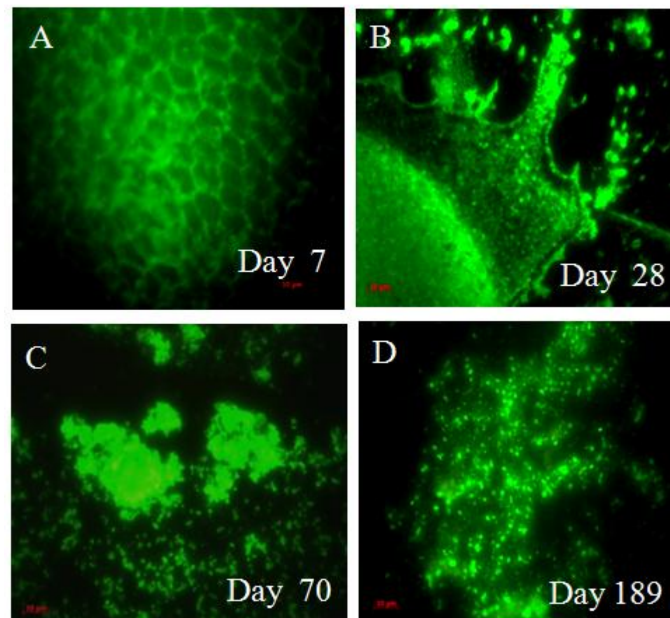

Fig. 3.15. Epifluorescent micrographs of attachment and utilization of shrimp chitin by *V. cholerae* O1 in the MW-CC microcosm. (A) Attachment and colonization of *V. cholerae* O1 on a chitin chip at day 7; (B) clusters of biofilm bound cells on a decaying chitin chip at day 28; (C) thick biofilm and small residues of chitin chips colonized by *V. cholerae* (utilization of chitin) at day 70; (D) typical cells, dividing cells, together with mostly coccoid cells of *V. cholerae* O1 in the homogenate of HCl-treated chitin residue at day 189. Samples were stained with fluorescent antibody specific for *V. cholerae* O1 and visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

In the MW-CC microcosm, *V. cholerae* O1 showed active growth up to day 174, after which the cells became completely non-culturable (<10 cfu) by day 189. Although microscopic observation of the MW-CC microcosm revealed large clusters of a biofilm that contained coccoid cells at day 189, a few rods were also detected. To examine whether the chitin biofilm serves as shelter for toxigenic *V. cholerae*, contents of the MW-CC microcosm were treated with concentrated HCl (pH 1.7) for 30 min, neutralized, and examined, revealing the HCl-treated homogenate of the chitin

biofilm preparation (Fig. 3.15 D) indeed contained 10^4 cfu/ml of culturable *V. cholerae* after enrichment in APW for 24 hours. This observation suggests that culturable *V. cholerae* cells persist within chitin-induced biofilms and withstand adverse environmental conditions, in this case high acidity. DNA templates prepared from these *V. cholerae* cells at day 189 amplified primers for *wbe* and *ctxA* by M-PCR (Table 3.2), providing evidence that toxigenic *V. cholerae* prevailed.

3.5. RESUSCITATION OF CULTURABLE CELLS FROM NONCULTURABLE STATE OF *V. cholerae* PRESENT IN BIOFILMS THROUGH ANIMAL PASSAGE

To understand whether nonculturable *V. cholerae* cells residing within clusters of biofilms can become cultivable upon availability of conducible environment nonculturable cells were subjected to rabbit ileal loop (RIL) assay that mimic the enriched environment of human intestine which may promote resuscitation of nonculturable *V. cholerae*. At day 450 and 500, water samples from microcosms MW-RT and MW-4C containing nonculturable cells were filtered through 0.22 µm membrane filters. DFA micrographs showed that the *V. cholerae* O1 cells retained on the 0.22 membranes were coccoid in morphology but occurred as clusters of cells within thick of biofilms (Fig. 3.16) and the cells observed in the filtrates were single

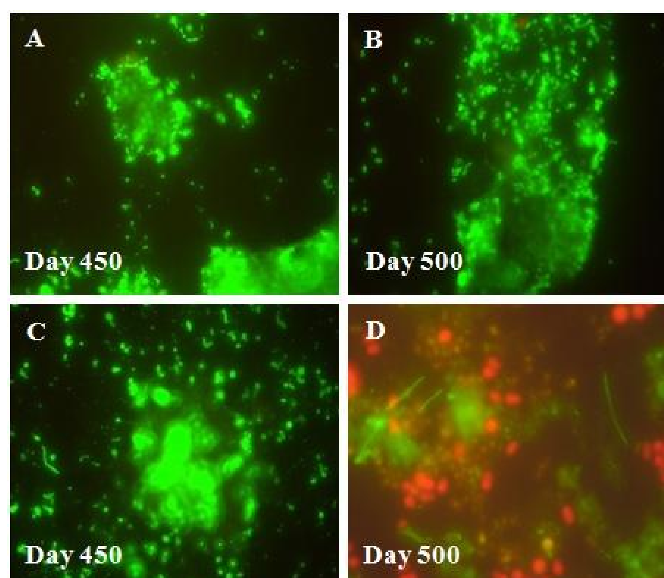

Fig. 3.16. Epifluorescence micrographs of nonculturable *V. cholerae* O1 cells within thick clusters of biofilms in microcosms MW-RT (A and B) and MW-4C (C and D). Samples were stained with fluorescent antibody specific for *V. cholerae* O1 and visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

and coccoid. Although in biofilm samples, cells were present predominantly as "biofilm", however, some cells were less than 0.2 um size which passed through the filter. These single and coccoid shaped cells were termed as "free cells". Among seven inoculated RILs, the 1st (N16961, positive control) and 2nd (EM-226, parent strain) loops showed highest amount (4^{+}) of fluid accumulation whereas the 3rd (negative control) and 4th, 5th, 6th and 7th loops containing nonculturable cells showed no fluid accumulation. Enrichment of animal passed "free cells", obtained from the filtrate of biofilm sample, did not yield culturable cells. In sharp contrast to the free cells, the clusters of nonculturable *V. cholerae* cells in biofilms collected only from MW-4C by filtration were able to be cultured after animal passage and enrichment of the entire loop in APW. These cells revived from nonculturable cells were then confirmed as *V. cholerae* O1 using polyvalent antiserum and subjected to molecular techniques following standard methods (see methodology section).

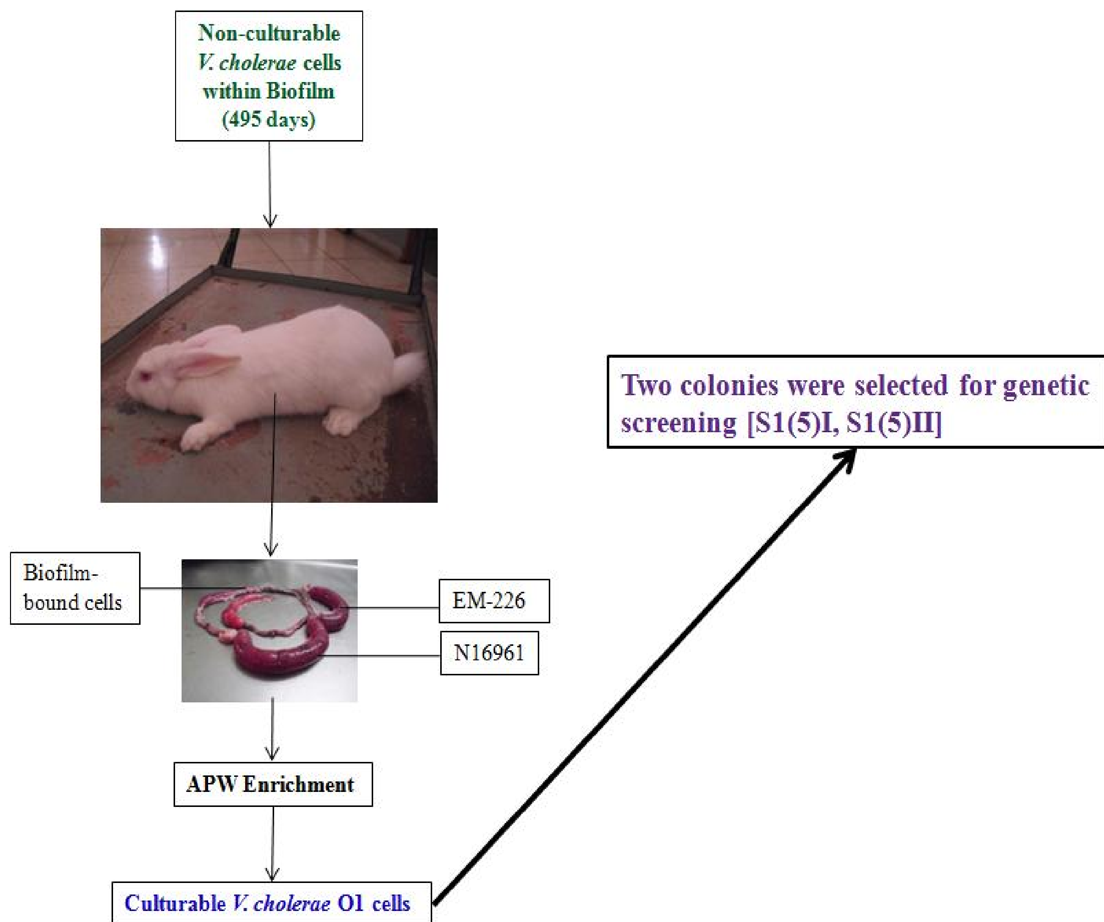

Fig. 3.17. Recovery of culturable *V. cholerae* O1 cells from biofilm-bound non-culturable cells through Rabbit Ileal Loop (RIL) assay.

3.5.1. Genetic screening of mutant strains cultured from biofilm-bound nonculturable cells

Two colonies of *V. cholerae* O1 recovered after animal passages were subjected to genetic screening to understand whether any genetic changes occur in strains recovered from biofilms. PCR results showed that both strains had lost the *ctxA, ace, zot* and *rstR2* genes of CTXф prophage. As *ctxA* and *rstR2* are located at two opposite termini of the CTXф genome, so it was assumed that the entire CTX prophage was lost from the bacterial genome. Both of the selected *V. cholerae* isolates revived after RIL enrichment had also lost the , Vibrio Pathogenicity Island (VPI) genes including toxin-corregulated pillus (*tcpA*) and accessory colonization factor (*acf*) and *toxT* (transcriptional regulator of ToxR regulon that activate *ctx* and *tcp* promoters). Moreover, PCR analysis showed that two of tested four mannose-sensitive

hemagglutinin (*mshA)* genes were also lost in the resuscitated *V. cholerae* isolates. *V. cholerae* cells use TcpA and AcfB for colonization of epithelium cells of the human intestine that is prerequisite for causing infection and MSHA for formation of three dimentional structures of biofilms.

Table 3.3. Prevalence of virulence-associated genes or gene clusters in *V. cholerae* O1 cells

Strain	House keeping genes			CTX prophage				VPI-I				MSHA			
	groEL	*mdh*	*rfb*O1	*rstR*	*ctxA*	*ace*	*zot*	*tcpA*	*toxT*	*acfB*	*toxR*	398	400	403	406
EM-226	+	+	+	E	+	+	+	E	+	+	+	+	+	+	+
S1(5)I	+	+	+	–	–	–	–	–	–	–	+	–	+	–	+
S1(5)II	+	+	+	–	–	–	–	–	–	–	+	–	+	–	+
N16961	+	+	+	E	+	+	+	E	+	+	+	+	+	+	+
O395	+	+	+	C	+	+	+	C	+	+	+	+	+	+	+

3.5.2. Measurement of biofilms formed by mutant strains of V. cholerae

Biofilm formation capability of *V. cholerae* O1 El Tor strain N16961, EM-226 and its respective MSHA mutant derivatives was compared qualitatively and quantitatively. Both mutant strains lacking two *mshA* genes were unable to produce biofilms on borosilicate glass tubes in air-water interface whereas parent strain (EM-226) and N16961 containing all the *mshA* genes were able to produce biofilms on borosilicate glass tubes (Fig. 3.18).

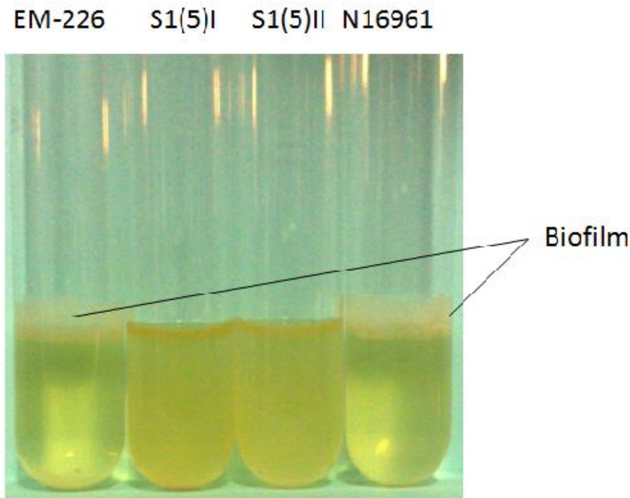

Fig. 3.18. Biofilm formation on borosilicate glass tubes by *V. choleae* incubated 24h at 37°C.

Crystal violet stained biofilms produced a purple colored ring on borosilicate glass tube as this strain has the capacity to bind biofilms. Parent strain (EM-226) and N16961 produced purple colored ring of biofilms on borosilicate glass tubes whereas mutant strains were unable to produce such ring (Fig. 3.19).

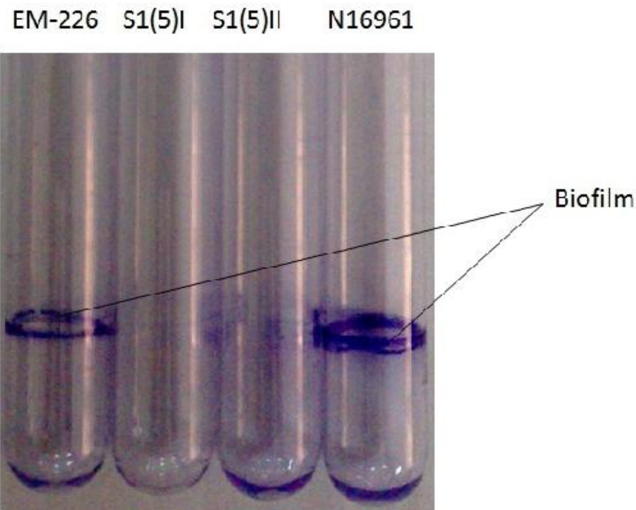

Fig. 3.19. Crystal violet stained biofilms of *V. cholerae* cells on borosilicate glass tubes after 24h of incubation

The biofilm produced by experimental strains on borosilicate glass tubes were stained with crystal violet. The quantification of biofilm revealed that mutants produced less amount of biofilm under the condition of this experiment as compared to its parent and reference strains (Fig. 3.20). So, it was inferred that deletion of *mshA* genes in mutant *V. cholerae* O1 strains might have caused the reduction of amount of biofilm formed.

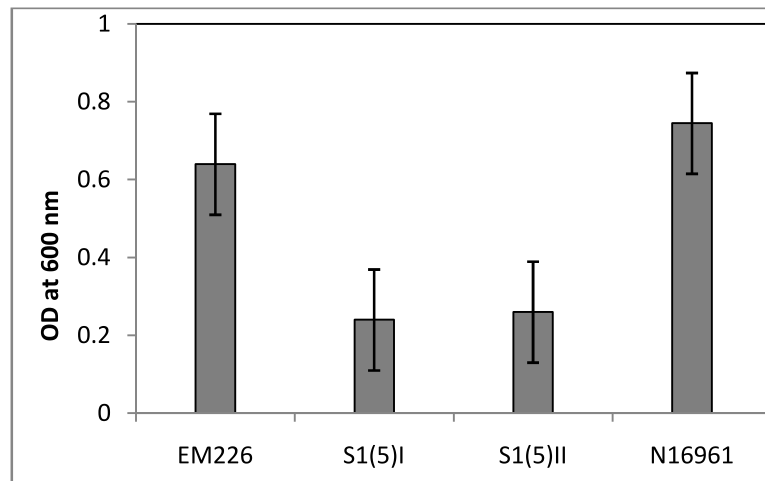

Fig. 3.20. Biofilm biomass formed by *V. cholerae* strains on borosilicate glass tubes after 24h of incubation. (Error bars mean standard error of mean)

3.5.3. Genomic fingerprinting of mutant strains

To understand the clonal relationship among the parent strain (EM-226) and its daughter (mutant) strains recovered from biofilms existing in the microcosm the strains were analyzed by PFGE. The *NotI* restriction digestion cut the chromosomal genome of parent strain into 24 fragments and of mutant strains into 22 fragments (Fig. 3.21) within the molecular size range from 6 to 350 kb. For cluster analysis, only fragments having molecular weight of 64 kb and more were considered.

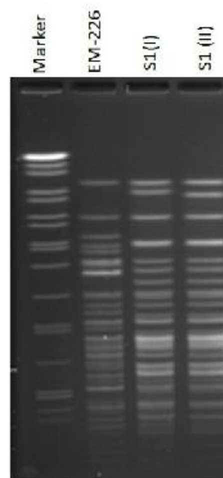

Fig. 3.21. PFGE patterns of *V. cholerae* O1

The level of similarity was defined using the Dice coefficient, and the similarity matrix was converted to a dendrogram by the use of UPGMA. It was observed that

both daughter strains were identical, although not 100% similar and had single band difference. However, the dendrogram analysis using UPGMA software showed that the daughter strains had only approx. 90% similarity with the parent strain (Fig. 3.22). Both of the daughter strains had differences in 7 bands when compared to the parent strain. Therefore, it can be concluded that substantial genetic rearrangements had occured in daughter strains while surviving within the thick clusters of biofilms.

Fig. 3.22. Dendrogram showing genomic fingerprint patterns of *V. cholerae* O1.

3.6. ROLE OF BIOFILMS IN THE ANNUAL LIFE CYCLE OF *V. cholerae* IN ESTUARINE AQUATIC ENVIRONMENT

In the natural estuarine water, culturability of bacteria varied from <1 to 2% of the total number of bacteria (Table 3.4), whereas culturability of *V. cholerae* O1 varied from 0 to 12% of total DFA counts of O1 cells at various seasons of the year (Table 3.5). Higher culturability of toxigenic V. *cholerae* together with bacterial population was recorded in spring (12% of DFA counts and 2% of AODC counts, respectively) and then in the fall/ autumn season (5% of DFA counts and 1% of AODC counts, respectively), while no culturability of *V. cholerae* O1 and lowest culturability of bacteria (<1% of AODC counts) was observed during winter and monsoon (Table 3.4; Table 3.5).

Table 3.4. Culturability and morphological variation of total bacteria during different seasons of the year in the coastal aquatic ecosystem of Bangladesh

Season	Month	Total bacterial count (cells/ml)	Culturable bacterial count (CFU/ml)	Culturability of bacteria (%)	Cell morphology
Winter	December, January and February	3.0×10^6	1.8×10^4	<1	Mostly coccoid, a few rods and short rods and thick biofilms
Spring	March, April and May	3.1×10^6	5.7×10^4	2	Mostly rods and dividing cells, a few short rods and coccoid cells, and biofilms
Monsoon	June, July and August	4.2×10^6	2.6×10^4	<1	Mostly coccoid, a few rods and short rods and thick biofilms
Fall	September, October and November	2.9×10^6	2.9×10^4	1	Mostly rods and dividing cells, a few short rods and coccoid cells, and biofilms

Under fluorescence microscope, acridine orange-stained micrographs showed the presence of bacterial cells mostly as green fluorescence for all the samples. The micrographs provided *in situ* evidence of how bacteria surviving in the natural aquatic ecosystem respond to stress by assembling, excreting exopolysaccharides, transforming into smaller and coccoid cells, and forming biofilm consortia during winter and monsoon (Fig. 3.23 A & C). Individual bacterial cells in the community, either free-living or within biofilm clusters varied significantly in size and morphology, suggesting multi-species consortia (Fig. 3.2). Although the presence of biofilm clusters was observed to be present in samples throughout the year, the appearance of mostly single rods, short rods, and coccoid-shaped bacteria, including dividing cells, was noted mainly during seasonal peaks of cholera (Table 3.4; Fig. 3.23 B & D). Cells that appeared as free-swimming and elongated rods to shorter rods during the spring and autumn had the morphology of short rods and coccoid cells and rarely as elongated rods during the winter months (December - January) when bacteria in the community appeared as clusters of cells within the biofilms (Table 3.4; Fig. 3.23 B & D).

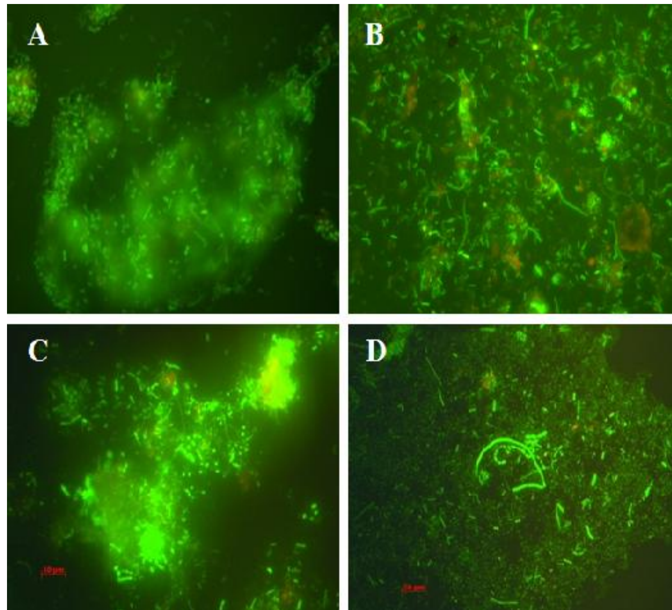

Fig. 3.23. Micrographs of stained bacterial communities occurring in the natural estuarine aquatic ecosystem of the mangrove forest, Sundarban, the Bay of Bengal, Bangladesh. The micrographs show bacterial biofilms in winter and monsoon (A and C) and free-living cells in spring and fall (B and D). Stained samples were visualized using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

Epifluorescent micrographs stained by DFA reagent showed the presence of *V. cholerae* O1 cells throughout the year (Fig. 3.24 A-D). Under fluorescent microscope, brightly fluorescing *V. cholerae* O1 cells appeared as individual free-swimming curved rods in spring and fall (Table 3.5; Fig. 3.24 B & D) and as clusters of cells during monsoon and winter (Table 3.5; Fig. 3.24 A & C). However, during the actively growing state, *V. cholerae* O1 cells appeared as curved rods with obvious cell division in spring and fall of the year (Table 3.5; Fig. 3.24 B & D). *V. cholerae* O1 cells within thick clusters of biofilms appeared to be very small, especially during the winter months, when biofilm formation intensified (Table 3.5; Fig. 3.24 A & C).

Table 3.5. Culturability and morphological variation of *V. cholerae* O1 at various seasons

Season	Month	*V. cholerae* O1 count by DFA (cells/ml)	*V. cholerae* O1 count by culture (CFU/ml)	Culturability of *V. cholerae* O1 (%)	Cell morphology
Winter	December, January and February	7.9×10^3	0	0	Mostly coccoid, few rods & thick biofilms
Spring	March, April and May	5.8×10^4	6.7×10^3	12	Mostly curved rods, few dividing rods & thin biofilms
Monsoon	June, July and August	2.3×10^3	0	0	Mostly coccoid, few rods & thick biofilms
Fall	September, October and November	2.1×10^4	1.0×10^3	5	Mostly curved rods, few dividing rods & thin biofilms

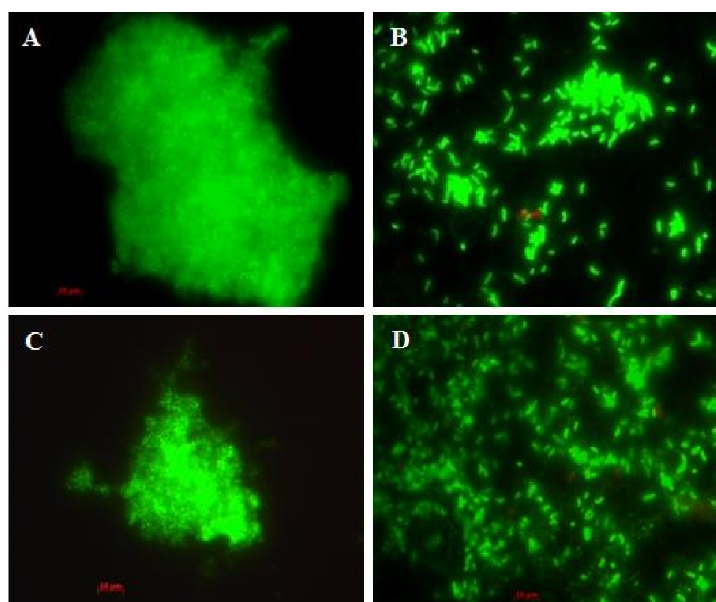

Fig. 3.24. Direct fluorescent monoclonal antibody (DFA) detection of naturally occurring toxigenic *V. cholerae* in the estuarine aquatic ecosystem. The micrographs show *V. cholerae* O1 biofilms in winter and monsoon (A and C) and free-living cells in spring and fall (B and D). Stained samples were visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

Primary clustering appeared to occur amongst cells of a given species such as *V. cholerae* (Fig. 3.25). The clusters become compact, with smaller, coccoid cells

dominant during inter-epidemic periods i.e. in winter and monsoon (Fig. 3.26). In estuarine environment, most of the bacteria were observed under the microscope as assemblages of various sizes and shapes and only less commonly as free-swimming single cells (Fig. 3.26). It was observed that *V. cholerae* O1 cells occurring as single cells can multiply in the natural environment but respond positively to the seasonal rise in temperature, probably by assembling and forming biofilm consortia. In the spring, when the water and air temperature began to rise, many single cells were observed. Bacterial cell assemblages occurred mainly late in the fall (September-November), when both the water and air temperature declined, notably during the early days of winter (December-February) and again after the spring during monsoon (June-August) rains. In both cases, most of the free-swimming cells assembled together to form clusters, secreting the exocellular polysaccharides visible in the stained samples.

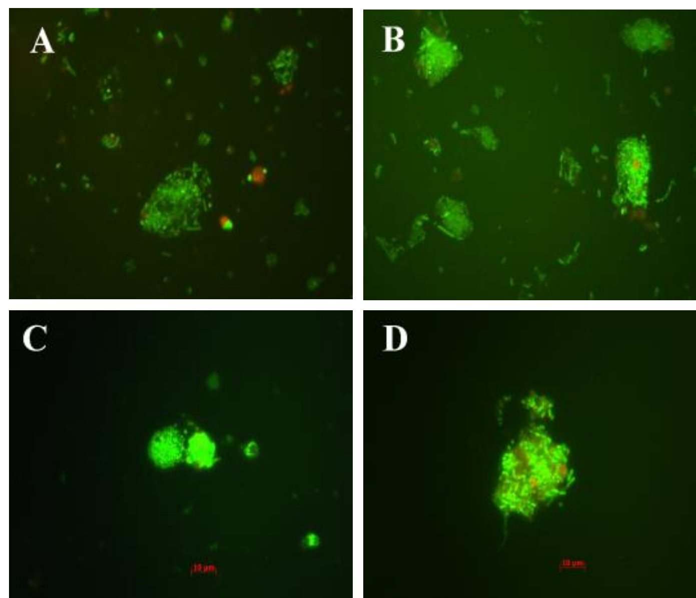

Fig. 3.25. Acridine Orange stained micrographs of bacterial communities forming microcolonies (A-F). These represents stages in bacterial biofilm formation, including cell assembly, secretion of exopolysaccharide to package into small consortia of a single population (D-F) or more than one population (A-C). Stained samples were visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 μm)

The assemblages of bacteria appeared either as a small microconsortium of a particular population (Fig. 3.25) or as a large assemblage comprising a diverse population (Fig.

3.26). There were clear signs of intricate coordination that could be detected by microscopic observations among the multiple species of populations as they assembled to form micro- or macro-colonies encapsulated by exogenous layers of polysaccharide (Fig. 3.25; Fig. 3.26).

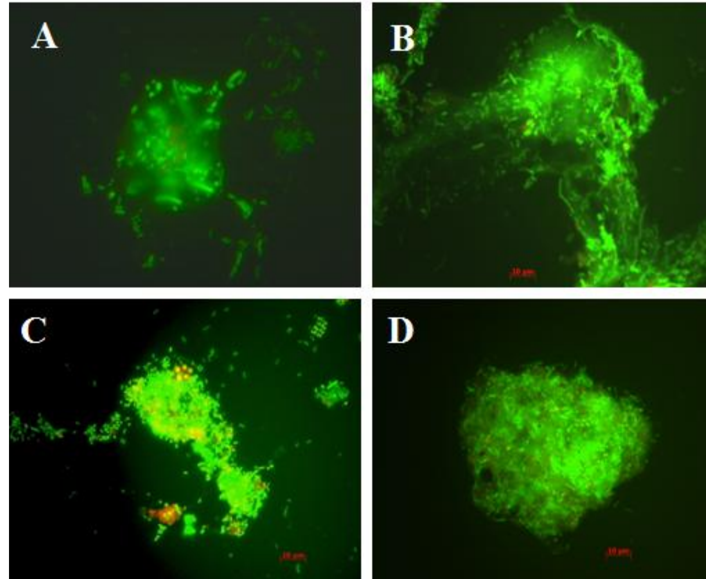

Fig. 3.26. *In situ* evidence of bacterial community as well as biofilm formation (A-F). These biofilm consortia were free-floating in the water. Stained samples were visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

According to the microscopic observation of the present study, bacterial populations formed biofilms on the surface of different zooplanktons (Fig. 3.27) or non-living particles (Fig. 3.28) in the aquatic environment.

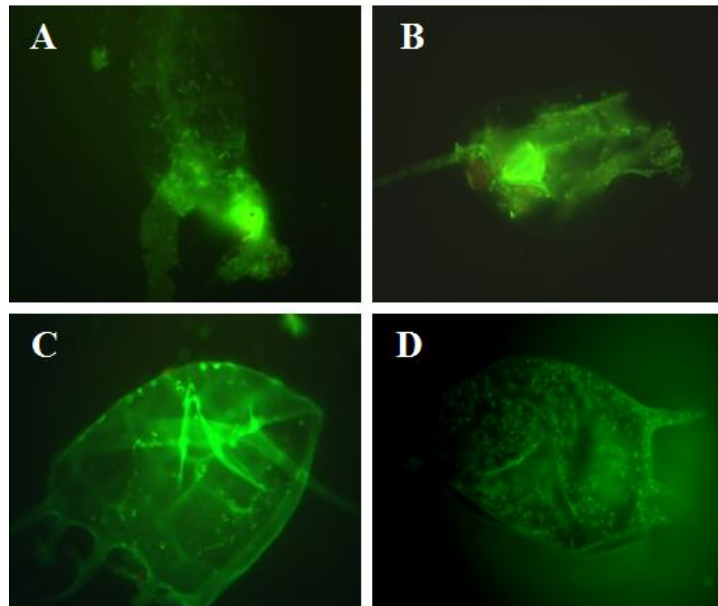

Fig. 3.27. Bacterial colonization of zooplankton in biofilms analogous to biofilms formed by free-floating bacteria. Bacterial populations reside within or on the chitinous structures of zooplankton, secreting exopolysaccharide to form the chitin-associated biofilm (A-F). Association and biofilm formation of Bacteria on Rotifer (Fig. D and E). Stained samples were visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

Biofilms attached to non-living particles also occurred free-floating in the water (Fig. 3.28). A noteworthy feature of particle-bound biofilms was the morphological diversity of the bacterial populations, with significant variation in size and shape, characteristic of multi-species consortia. Particle-bound biofilms were observed in all samples throughout the year. However, loosely adherent or active cells dispersed amongst biofilms, were rarely observed in samples collected during the winter, when active bacterial growth was minimal and the water temperature was low.

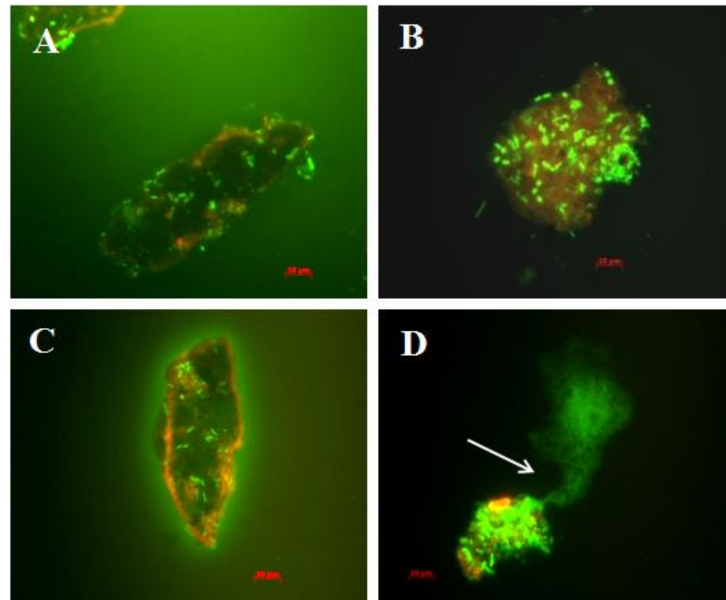

Fig. 3.28. Acridine orange stained micrographs of biofilm consortia are constructed by bacteria that produce the biofilm on living or non-living, free floating particulate matter. Arrow indicates semicircular cavities in the biofilm consortia. Stained samples were visualized by using an epifluorescence microscope (Carl Zeiss; Axioskop 40). Images were captured using a digital camera (AxioCam MRc) attached to the microscope. (Scale bars inserted in the figures were equal to 10 µm)

A notable feature of the particle-associated biofilms was that some cells were attached to the solid surfaces, with a few free-floating single cells (Fig. 3.28). These bioilms showed semicircular cavities which appeared hollow of various sizes within the same aggregation of biofilm consortia in given water samples (Fig. 3.28D).

Like other bacterial flora surviving in the aquatic environment, *V. cholerae* O1 cells were observed to form EPS- bound biofilms of various size and shapes during inter-epidemic periods (Fig. 3.29A, C). Those biofilms were free-floating, particle-bound, phytoplankton-associated and zooplankton-associated (Rotifer) (Fig. 3.29 A-D).

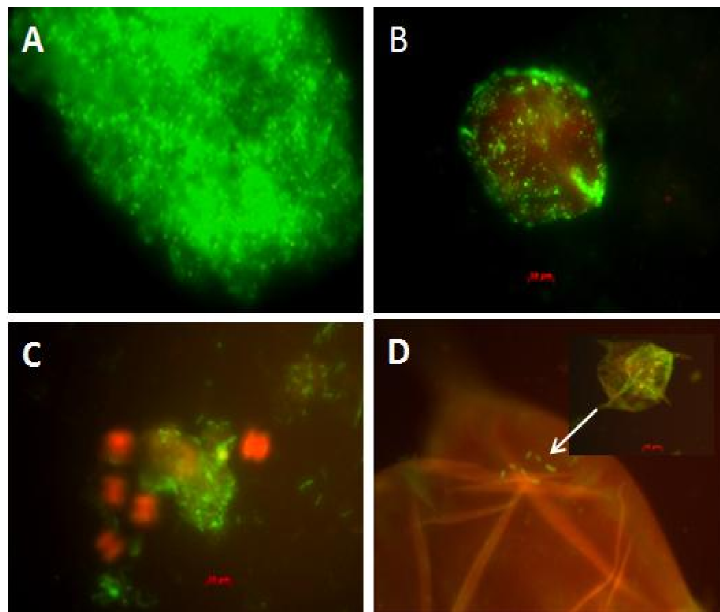

Fig. 3.29. Biofilms formation by *V. cholerae* O1 during inter-epidemic periods. The images show biofilms of *V. cholerae* in winter and monsoon: free-floating (A), particle-bound (B), phytoplankton-associated (C) and zooplankton-associated (Rotifer). Samples stained with fluorescent antibody specific for *V. cholerae* O1 were observed under epifluorescence microscope (Carl Zeiss; Axioskop 40). A digital camera (AxioCam MRc) attached to the microscope was used to capture the images. (Scale bars inserted in the figures were equal to 10 µm)

4. DISCUSSION

Vibrio cholerae is a resident flora of the aquatic ecosystem where the bacterium persists for most part of the year in a dormant (non-culturable) state, mostly in association with plankton (Brayton and Colwell, 1987; Huq *et al*., 1990). Although *V. cholerae* can respond to natural stimuli by changing shape from curved rod to small coccoid in appearance, which has been proposed to be a viable but non-culturable (VBNC) state (Brayton and Colwell, 1987; Huq *et al*., 1990), the bacterium can be found both as rod, short rod or coccoid within the biofilms occurring in natural aquatic ecosystem (Alam *et al*., 2006a). The biofilm-bound *V. cholerae* can be found as cell cluster either free-floating or in association with plankton or other substrates available in the aquatic ecosystem. Although *V. cholerae* can turn into actively growing, a time when they respond to culture method, before and during the seasonal epidemics of cholera (Colwell, 1996a), the factors involved in determining bacterial seasonal growth response and biofilm formation in the aquatic environment remain yet to be understood. In the present study, we present data showing how different physicochemical and related abiotic factors influence the seasonal growth response and biofilm formation of toxigenic *V. cholerae* O1 occurring in the natural estuarine aquatic ecosystem of Bangladesh.

V. cholerae and many related Gram-negative bacteria have been shown to become nonculturable under specific experimental conditions, although the time required for these cells to become nonculturable is variable (Rollins and Colwell, 1986; Nilsson *et al*., 1991; Islam *et al*., 2002; Gupte *et al*., 2003; Oliver, 2005). In this study, *V. cholerae* O1 cells in microcosms became nonculturable, as measured by TCBS agar, within 10–15 days, as has been reported by other investigators (Islam *et al*., 2002; Gupte *et al*., 2003; Oliver, 2005). However, samples from MW-RT and MW-4C microcosms showed extended survival of *V. cholerae* as culturable cells were found upto 40 and 68 days when the bacterium was plated on TTGA and LBA, , respectively. Initial plate counts on TCBS for all the microcosms were only 1% of the counts obtained on TTGA and LBA. Although TCBS is widely used medium for selective growth of vibrios, results obtained in the present study suggest this medium to be less supportive or inhibitory for *V.*

cholerae bacterium under stressed condition, as has been proposed previously by Pickar *et al.*, (1973) for *V. cholerae* population.

During unfavourable environmental conditions *V. cholerae* survives for longer period within clusters of biofilm (Alam *et al*., 2006a). The observations of the present study appear in concordance as the individual planktonic cells in the MW microcosm gradually united to form aggregates, reduced in size by becoming coccoid and eventually turned to clusters of biofilms. These clusters of coccoid cells were unequivocally visible within the biofilms, confirming the observations of Watnick *et al*. (2001), and Kierec and Watnick (2003). In our laboratory microcosm experiments, *V. cholerae* cells secreted exopolysaccharides, either as capsules or as extracellular matrices in their immediate environment (Davey and O'Toole, 2000), which is proposed to be a function to protect *V. cholerae* from adverse external conditions (Flemming, 1993).

Environmental signals including temperature, pH, osmolarity, iron availability, oxygen tension have been proposed to influence the initial bacterial attachment during the process of biofilm formation (Fletcher, 1996; Nyvad and Kilian, 1990; O'Toole *et al*., 2000b; O'Toole and Kolter, 1998b; Pratt and Kolter, 1998). In the present study, *V. cholerae* O1 has exhibited different survival capabilities under physicochemical conditions such as temperature, pH, salinity etc. *V. cholerae* O1 was actively growing for extended period of 68 days in a laboratory microcosm maintained at 4°C as opposed to 26 days at 45°C. This observation appears to contradict with the generally observed positive role of higher temperature for higher survival of *V. cholerae*, as shown by many previous studies (Johnston and Brown, 2002; Miller *et al*., 1985). A possible explanation of the extended active growth of *V. cholerae* at lower temperature is that the bacterium has lower biofilm forming capacity at lower temperatue, as the biofilm formation is negatively linked with the culturablity of *V. cholerae* in the environment (Alam *et. al*. 2007). The data obtained in the present study also revealed that *V. cholerae* can survive for longer periods in microcosms having higher pH; 8.0-9.0. This observation appears concordant with the findings of study carried out by Miller *et al*. (1985) suggesting that toxigenic *V. cholerae* O1 could remain culturable for longer periods at a higher pH of 8.0 as compared to a lower pH. The data suggest the supposition as vibrios are halophilic organisms, higher salinity would aid in their higher survivality in the natural

aquatic ecosystem. Interestingly, in the present study *V. cholerae* O1 could remain culturable for longer periods at a salinity of 1% in comparison to higher salinities, i.e., 2% and 4% (Miller *et al*., 1985), a reason why toxigenic *V. cholerae* O1 has been established as an estuarine and brackish water bacterium. The extended culturability of *V. cholerae* at 1% salinity, as observed in this study, can be due to lower biofilm formation. Environmental surveillance studies in the coastal areas of the USA also indicated that *V. cholerae* abundance can be higher at a salinity range of 0.2 to 1.4% (Louis *et al*., 2003), supporting the findings of the present study.

V. cholerae O1 cells in the present study have shown increased amount of biofilm formation on borosilicate glass tube maintained under moderate temperature (30°C), neutral to alkaline pH (7.0-9.0) and moderate salinity (2%). Thus, our data suggest that biofilm formation may predominantly occur both in natural water reservoirs where pH is neutral/alkaline and in human intestine, which is also highly alkaline. *V. cholerae* can significantly enhance biofilm formation in response to bile salts, and the biofilm-bound bacteria are more resistant to toxicity of biles compared to the planktonic (free-living) cells (Hung *et al*., 2006).

Results of the present laboratory-based microcosm study suggest that biofilm formation provides a powerful mechanism for the persistence of vibrios in the natural environment. In this study, increasing concentration of Calcium and Magnesium was found supportive for the formation of biofilm by *V. cholerae* cells, a result suggesting the requirement of these metal ions in biofilm formation. Our results appear to support previous observations in which increasing concentration of Ca^{2+} in growth media was shown to enhance biofilm development by diverse bacteria (Keirec and Watnick, 2003; Kim *et al*., 1999; Konobloch *et al*., 2001; Rose and Turner, 1998; Sheikh *et al*., 2001). As the marine water is rich in Ca^{2+} whereas fresh water is Ca^{2+}-poor, *V. cholerae* biofilms was shown to be of marine origin (Keirec and Watnick, 2003). This may be the basis that the cholera cases of an epidemic may occur initially at the coastal villages befdore cases occur inland (Ramamurthy *et al*., 1993; Siddique *et al*., 1991).

Although a correlation between the seasonal plankton blooms in the Bay of Bengal and occurrence of cholera in Bangladesh has been proposed previously (Colwell, 1996a), the precise mechanism of how plankton blooms contribute to the life cycle of *V. cholerae,*

bacterium that can produce chitinase enzyme, in the aquatic environment is not fully understood. In this regard, the microcosm experiment of the present study has clearly demonstrated that chitin derived from shrimp, which occurs in abundance during seasonal plankton blooms in the estuarine ecosystem of Bangladesh, is a very important component for the survival of toxigenic *V. cholerae* O1 by serving both as food and shelter, thereby, allowing biofilm formation and the cells to remain in an active growth stage for an extended period of time. It has been demonstrated for *V. parahemolyticus* (Kaneko and Colwell, 1973), and *V. cholerae* O1 and non O1 (Colwell *et al*., 1977), pandemic strains of *V. cholerae* adsorb and multiply on chitinous fauna, including crabs, shrimp, and zooplankton (Nalin *et al*., 1979).

Chitinase, an enzyme secreted by *V. cholerae*, is employed by the bacterium to colonize and degrade the insoluble polysaccharide component of chitin comprising the exoskeleton of fauna in the aquatic ecosystem (Gooday *et al*., 1991). Tamplin *et al*., (1990) had reported earlier that the chitinous surface of plankton served to concentrate *V. cholerae* O1, increasing the number of *V. cholerae* in a given unit of water. In the present study, the strong affinity of *V. cholerae* for chitin was demonstrated by *V. cholerae* cells colonizing the shrimp chitin, extensively along the chitin surface, suggesting that chitin plays an important role in the aquatic life cycle of *V. cholerae*.

While chitin serves as an abundant source of carbon and nitrogen for *V. cholerae*, and provides protection from environmental challenges, chitinous zooplankton has been established as an important reservoir for the bacterium (Huq *et al*., 1984). Thus, it is assumed that chitin may enhance the environmental fitness of *Vibrio* spp. by providing attachment substrate, an important pre-requisite for biofilm formation, and allowing *V. cholerae* to protect themselves against killing at low temperature (Amako *et al*., 1987). The data obtained in the present study reveal that *V. cholerae* on shrimp chitin can remain in an active growth phase for up to six months, with strong evidence of utilization of the chitin as the source of nutrient. In contrast, *V. cholerae* O1 was active for only 49 days in the same water but without chitin, suggesting crucial role of chitin in the natural life cycle of the bacterium.

In the previous study, microcosms prepared with freshwater supported the active growth of *V. cholerae* O1 for only 15 days, a result that appeared analogous to what has been reported by this study (Huq *et al*., 1983; Islam *et al*., 1997). Thus, brackish and estuarine water supported the active growth of *V. cholerae* O1 for extended period of time (Alam *et al*., 2006a; 2006b). More to the point, *V. cholerae* O1 can remain active for an extended period of time when they colonize on the surface of shrimp chitin, which occurs in abundance along with other chitinous crustaceans in the estuarine ecosystem of Bangladesh. Thus, the combination of estuarine water and chitinous fauna such as zooplankton, shrimp, and crabs, can explain, in part, the survival strategy of toxigenic *V. cholerae* which can persist as actively growing for extended period of time in the estuarine ecosystem of Bangladesh (Alam *et al*., 2006a; 2006b), as opposed to the fresh water habitats where chitinous fauna are less abundant, but not absent (Huq *et al*., 1983; Huq *et al*., 2005; Islam *et al*., 1997).

The environment as a reservoir for toxigenic *V. cholerae* in water and plankton has been well documented in earlier studies conducted in the USA (Rivera *et al*., 2003) and in Bangladesh (Alam *et al*., 2006a; 2006b). *V. cholerae* causing Asiatic cholera has been established as the autocthonous flora of the natural estuarine habitat, close to the mangrove forest of the Bay of Bengal (Alam *et al*., 2006a; 2006b). The ability of *V. cholerae* to colonize on, and degrade chitin, as documented in the present study, appears concordant with results reported for vibrios (Colwell, 1970) and many other marine bacteria (Keyhani and Roseman, 1999). Degradation of chitin involves four main steps: (i) sensing chitin; (ii) attachment to chitin; (iii) enzymatic degradation; and (iv) utilization of carbon and nitrogen-containing breakdown products (Keyhani and Roseman, 1999; Somerville *et al*., 1989). Chitin was shown to serve in the formation of surface-attached communities or biofilms by Watnick *et al*., (1999). The present study demonstrated that planktonic *V. cholerae* O1 can effeciently colonize on the surface of the shrimp chitin, gradually increase their number by degrading chitin, and eventually form clusters of biofilms. The chitin particles and residues can be heavily colonized by *V. cholerae* by embedding themselves in a structured matrix. It can be inferred that chitin utilization supports *V. cholerae* to remain actively growing while biofilm formation is a simultaneous process that helps them to remain together in a consortia.

In the present study, DFA detection of *V. cholerae* O1 cells in chitin-bound biofilms has revealed the presence of mostly atypical and coccoid cells, together with a few typical curved rods in biofilm samples. The isolation of culturable *V. cholerae* after prolonged treatment of the chitin chips of microcosm with HCl, indicates that HCl treatment might have killed the planktonic or free swimming cells but not the ones bound within the consortium of a biofilm. If ingested, the biofilm-bound cells in drinking water may overcome the gastric acid barrier of the human stomach and reach the small intestine where conditions are alkaline, an inducible habitat for the growth of the bacterium (Colwell *et al*., 1996a). Since chitin can resist acidic digestion, vibrios can survive gastric transit if adherent to chitin ingested during a crab meal (Nalin *et al*., 1979). Therefore, chitin present in aquatic plankton play a vital role in the disease occurrence by pathogenic *V. cholerae*.

Another observation of the present study is that the nonculturable *V. cholerae* O1 cells in MW microcosms without chitin can be also present in aggregates within biofilms. Interestingly, the present study also demonstrate that after animal challenge experiments, the nonculturable *V. cholerae* cells in biofilms can be converted to actively growing cultivable *V. cholerae* O1, even after having been nonculturable for more than 1 year. The results presented in this study explicitly demonstrate the resuscitation of nonculturable cells, after exposure to MW microcosms for 495 days, into cultivable state, indicating the undoubt occurrence of viable cells within biofilms. It has been proposed that pre-epidemic enrichment of nonculturable *V. cholerae* in the human host provides a method of amplification of epidemic *V. cholerae* immediately before onset of an epidemic (Colwell *et al.*, 1996a; Faruque *et al.*, 2005) and contributes to subsequent transmission via the fecal-oral route of the human-amplified clones. However, this phenomenon does not account for the natural environmental reservoir of *V. cholerae* O1 nor for the centuries long persistent pattern of cholera epidemics historically documented for this disease (Duncan *et al.*, 1994). On the other hand, biofilm-bound nonculturable *V. cholerae* cells that have been shown in this study to occur in the natural estuarine environment can play important role in the seasonal epidemics of cholera (Alam *et al.*, 2006b; Rahman *et al.*, 1996).

The ability of pathogenic *V. cholerae* to cause diseases depends primarily on the expression of two virulence factors: encoding cholera toxin (CT), a potent enterotoxin,

and a pilus colonization factor known as toxin coregulated pilus (TCP). The *ctxA* and *ctxB* genes that encode cholera toxin (CT) subunits A and B, respectively, are constituents of a lysogenic bacteriophage CTXф, whose genome consists of a core region encoding the cholera toxin responsible for the severity of the illness of cholera and other genes that code for virion morphogenesis (Alam *et al*., 2007; Waldor and Mekalanos, 1996). Under appropriate conditions, CTXф is self-transmissible and replicates as a plasmid (Waldor and Mekalanos, 1996). The filamentous CTXф propagates horizontally by infecting susceptible strains of *V. cholerae* and *Vibrio mimicus* (Faruque *et al*., 1999). However, it is not known whether *ctx*$^-$ strains exist in the environment as a separate clonal lineage, originally lacking CTXф, or whether CTXф previously had been excised. Evidence is provided in the present study that while persisting in biofilm the *ctxA, ace, zot* and *rstR2* genes of CTXф can be lost simultaneously from toxigenic *V. cholerae* O1, whereas the *wbe* gene encoding the O1 antigen can be retained, confirmed by M-PCR. As *ctxA* and *rstR2* are located at two opposite termini of the CTXф genome (Waldor and Mekalanos, 1996), it can be assumed that the entire CTXф can be excised from bacterial chromosome while surviving by evolving into variant forms from the original clone of the bacterium. Existence of *ctx* progenitors of pandemic strains in the aquatic environment of Mathbaria has been already documented (Alam *et al*., 2006a), which support this hypothesis. Evidence for the loss of CTXф prophage, resulting in *ctx*-negative *V. cholerae* O1, as observed in this study, indicates that toxigenic strains in the environment can become nontoxigenic during interepidemic periods. Thus, our results have suggested a mechanism whereby *ctx*-negative *V. cholerae* O1 can occur in the environment and serve as progenitor.

Further genetic screening of the present study also shows that in addition to the loss of *ctx* prophage, *V. cholerae* O1 mutant strains recovered from thick clusters of biofilms can be also devoid of *tcpA, acf* and *toxT* of pathogenic island of VPI and two of four *mshA* gene-cluster. TcpA and AcfB are considered responsible for colonization of epithelium cells of the intestine that is prerequisite for causing infection in human. ToxT is a transcriptional regulator of the ToxR regulon that can activate *ctx* and *tcp* promoters independently once expression has occurred (DiRita *et al.*, 1991). MSHA are thought to be responsible for formation of three dimentional structures of biofilms on abiotic surfaces that represents a survival strategy utilized by many microbes (O'Toole and

Kolter, 1998a). The obtained mutant strains lacking *mshA* genes, from the animal challenge experiment of the present study, are unable to produce biofilm in the air-water interface and produce less amount of biofilm in the borosilicate tube in comparison to parent strain. MSHA has been extensively investigated as a potential colonization factor that appears to play no role in virulence (Attridge *et al.*, 1996; Tackett *et al.*, 1998; Tamplin *et al.*, 1990) but important for biofilm formation on borosilicate glass tube (Watnick *et al.* 1999). Analysis of the genomic finger printing patterns of parent and mutant strains indicates that the mutant strains are not identical to the parent strain whereas both mutant strains are identical. Therefore, it is evident that genetic rearrangement may occurr in *V. cholerae* cells while surviving within EPS-bound biofilms in the estuarine environment.

Results of this study, obtained from the field-based investigations in estuarine ecosystem of Mathbaria, Bangladesh have shown that only a negligible proportion of bacteria could be enumerated by culture method. Several studies also revealed that bacterial count, employing conventional culture methods, yields only a small portion of the total number of bacteria in environmental samples (Hopton *et al*., 1972; Wright, 1978; Buck, 1979; Fry and Zia, 1982). In this study, micrographs of acridine orange-stained preparation showed that a majority of the bacterial cells occurred within thick clusters of biofilms in the water samples collected from coastal environment. The general observation is that in low nutrient habitats a large number of bacteria can be enumerated by direct microscopic methods but a corresponding low bacterial number will be measured by culture methods (Butkevich, 1938; Jannasch and Jone, 1959), indicating a large component of the microbial population is dormant or requires highly specialized media for successful isolation (Roszak and Colwell, 1987). So, biofilm formation may be a survival mechanism of natural bacteria including pathogens such as *V. cholerae* in the estuarine environment.

In Bangladesh, cholera occurs seasonally, with two distinct seasonal peaks (epidemics), one before and the other after the annual monsoon (Glass *et al*., 1982). Actively growing *V. cholerae* may occur predominantly during seasonal epidemics and as aggregates of structured biofilms during interepidemic periods when they are found mostly as nonculturable cells (Alam *et al*., 2006a; 2006b). When biofilm-associated bacteria detach from a biofilm matrix, they can become culturable (Watnick and Kolter, 2000).

Morphological changes of bacteria, directly or indirectly related to seasonal changes were documented by microscopic observation in the present study. *V. cholerae* O1 cells that can appear as free-swimming and elongated rods to shorter rods during the spring and autumn transform into short rods and coccoid cells and rarely as elongated rods during the winter months (December - January) when bacteria in the aquatic community appear as clusters of cells within the biofilms. Under reduced nutrient conditions, many Gram negative bacteria in the natural aquatic environment may undergo physiological and morphological changes as part of their survival strategy (Tamplin *et al*., 1990). The small ovoid forms of bacteria, normally observed in natural ecosystems by direct microscopy, represent exogenously dormant forms that develop in response to unfavorable chemical or physical conditions in the environment (Stevenson, 1978). Some previous studies also hypothesized that *V. cholerae* O1, a curved rod-shaped bacterium, transforms into resting spore-like coccoid (nonculturable) cells when conditions are not conducive for growth (Colwell *et al*., 1994b; Huq *et al*., 1990; Oliver, 2005; Roszak and Colwell, 1987; Xu *et al*., 1984). Bacterial cellular aggregation and biofilm formation have been proposed as a developmental process, sharing features of the resting spore formation of gram-positive bacteria (Dunny and Leonard, 1997; Watnick and Kolter, 2000), the fruiting body of *Myxococcus xanthus* (Plamann *et al*., 1995; Shimkets, 1999; Wall and Kaiser, 1999; Watnick and Kolter, 2000) and stalked cell formation by *Caulobacter crescentus* (Fukuda *et al*., 1977; Hecht and Newton, 1995; Losick and Youngman, 1984; Quon *et al*., 1996; Watnick and Kolter, 2000; Wu and Newton, 1997). The present study has unveiled that the biofilm-bound bacterial cells outnumber the free-swimming and actively dividing cells in the water samples collected throughout the year and *V. cholerae* O1 cells remain primarily in coccoid morphology within the dense clusters of biofilms during winter and monsoon (inter-epidemic periods).

Plankton and particulate matters in the aquatic environment can provide bacteria with a number of advantages, including food availability, adaptation to environmental nutrient gradients, tolerance to stress and protection from predators (Pruzzo *et al*., 2008). Results of the present study showing the majority of *V. cholerae* attach to solid surfaces within structured biofilms and planktons in the aquatic ecosystem of Banglades corroborate with similar observations in other countries (Costerton *et al*., 1995; Davey and O'Toole, 2000). According to Davey and O'Toole (2000) bacterial cells are not, in general free-

swimming but rather biofilm-associated bacteria attached to suspended particles of organic and inorganic material in the aquatic environment. Vibrios have been shown to adsorb onto and multiply on chitinous fauna, namely crabs, shrimp, and zooplankton (Colwell *et al*., 1977; Kaneko and Colwell, 1973; Keyhani and Roseman, 1999; Nalin *et al*., 1979). Plankton have been documented to play an important role in the seasonal cycle of *V. cholerae* by serving as a reservoir (Brayton and Colwell, 1987; Huq *et al*., 1990) and enhancing disease transmission (Colwell *et al*., 1996a; Huq *et al*., 2005). It may be cloncluded that biofilms attached to both planktons and particles can serve as an important reservoir for *V. cholerae* in the aquatic environment.

In conclusion, the overall data presented in this study may suggest biofilm as an important component in the natural aquatic life cycle of *V. cholerae.* Biofilms formed *in situ* in response to physicochemical, and related abiotic factors of aquatic environment thus serve as an important reservoir where the bacterium can take refuge to, and persist as dormant cell until the favorable season returns for them to be actively growing again to increase the probability of infecting human and spreading cholera epidemics.

5. REFERENCES

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APPENDIX-I

APPARATUS

Autoclave
Bag sealer
Centrifuge, Eppendorff
Deionizer, E-Pure, Branstead
Distilled water plant, Autosil
Disposable micropipette tips
Disposable syringe
Electronic balance
Eppendorff tube
Incubator
Gel chamber and power supply, Life Technologies
Glass wares, Pyrex brand, USA
Inverted Microscope
Fluorescence Microscope, Axioskop 40, Carl Zeiss, Germany

Digital Camera, AxioCam MRc, Carl Zeiss, Germany
Laminar airflow
Magnetic stirrer
Micropipette, Eppendorff
Microoven, Sharp
Petridishes, Sterilin
pH meter
Refrigerated superspeed-centrifuge, Model RC-5B, Sorval
Spectrophotometer
Sterilizer
Slide, Gold seal
Sheff Mapper, Bio Rad
UV- transilluminator, Bio Rad
Vortex, Fisher
Water bath

APPENDIX-II

Microbiological media:

Media used were prepared by standard methods using appropriate compositions. Components used were high grade and were produced either by Sigma or Difco, USA. All media were sterilized by autoclaving for 20 minutes. The composition used for different media have been shown below:

1. Lnria-Bertani (LB) Broth

Ingredients	Amount
Bacto-tryptone	10.0 g
Bacto-yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1.0 L

pH adjusted to 7.4

2. Taurocholate Tellurite Gelatin Agar (TTGA)

Ingredients	Amount
Tryptone	10.0 g
Sodium-uiurocholate	5.0 g
NaCl	10.0 g
Gelatin	30.0 g
Agar	16.0 g
Distilled water	1.0 L

pH adjusted to 8.5-9.0

3. Gelatin Agar

Ingredients	Amount (g/I)
Tryptone	10.0 g
Trypticase (BBL)	10.0 g
Gelatin	30.0 g
Agar	16.0 g
Distilled water	1.0 L

pH was adjusted to 7.4

4. T1N1 broth

Ingredients	Amount
Tryptone	10.0 g
NaCl	10.0 g
Distilled water	1.0 L

pH was adjusted to 7.5

5. Thiosulphate Citrate Bile Sucrose (TCBS) agar

Ingredients	Amount
Yeast extract	5.0 g
Peptone	10.0 g

Sodium thiosulphate	10.0 g
Sodium citrate	10.0 g
Bile	8.0 g
Sucrose	20.0 g
NaCI	10.0 g
Ferric citrate	1.0 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	14,0 g
Distilled water	1 L

pH was adjusted to 8.6

APPENDIX-III

LABORATORY REAGENTS

Reagents which were used in carrying out different methods together with their sources are mentioned below.

Normal saline

Ingredients	Amount
NaCl (sigma)	8.5 g
Distilled water	1.0 L

pH was adjusted to 7.8

Phosphate Buffer Saline (PBS)

Ingredients	Amount
NaCl (sigma)	8.5 g g
KH_2PO_4	0.156 g
Na_2HPO_4	1.28 g
Distilled water	1.0 L

pH was adjusted to 7.4

Polymerase chain reaction (PCR) reagents

(Life technologies Tech-Linesm, Gibco BRL, USA)

10X PCR buffer (200mM Tris-HCl, pH 8.4, 500 mM KC1)
50 Mm MgCl2
lOmMdNTPmix
Taq DNA polymerase
Control primer mix
Sterile water
Siliconeoil
Molecular weight marker

Reaction mixture for simplex PCR of VPI-1, CTXΦ, RS1Φ gene clusters

Reagent	**Amount** (µl)
10X PCR Buffer	2
MgCl2 (50Mm)	1
dNTPs (2.5Mm)	2
Primer F (10 pm/ml)	1
Primer R (10 pm/ml)	1
Taq polymerase	0.2
Template DNA	2
PCR water	10.8
Total	**20**

OmpW PCR

Reagent	Amount (µl)
10X PCR Buffer	2.5
MgCl2 (50Mm)	0.75
dNTPs (2.5Mm)	2.5
Primer 1 (OmpW) (10 pm/ml)	2.5
Primer 2 (OmpW) (10 pm/ml)	2.5
Taq polymerase	0.3
Template DNA	3
PCR water	10.75
Total	**25**

Multiplex PCR for *rfb*O1, *ctxA* and *rstR*2

Reagent	Amount (µl)
10X PCR Buffer	2.5
$MgCl_2$ (50Mm)	1.0
dNTPs (2.5Mm)	0.8
Primer F (*rfb* O1) (10 pm/ml)	1.5
Primer R (*rfb* O1) (10 pm/ml)	1.5
Primer F (*ctxA*) (10 pm/ml)	0.75
Primer R (*ctxA*) (10 pm/ml)	0.75
Primer F (*rstR2*) (10 pm/ml)	1.0
Primer R (*rstR*) (10 pm/ml)	1.0
Taq Polymerase	0.2
Template DNA	5.0
PCR water	9.08
Total	**25**

Gel loading buffer

Ingredients	Amount (g/l)
Sucrose	6.7
Bromophenol blue	0.04

Stored at 4^0 C

Ethidium bromide solution

1.0 g of ethidium bromide was dissolved in distilled water to a final volume of 100 ml. The container was wrapped in aluminium foil and stored at 4^0C.

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