

REVIEW ARTICLE

The Aquatic Flora and Fauna as Reservoirs of *Vibrio cholerae*: A Review

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1. Introduction

Cholera is an endemic disease in Bangladesh and has a regular seasonal pattern of epidemics (1-5). During epidemics, toxigenic *Vibrio cholerae* O1 can be isolated from the local fresh water as well as from patients (6) but disappears from the environment after the epidemic subsides. The reservoirs or sites of survival and multiplication of *V. cholerae* O1 between epidemics are not known. The literature regarding the role of the aquatic environment as a reservoir of *V. cholerae* has recently been reviewed by Drasar and Islam *et al.* (7,8).

One of the novel aspects of the literature is the suggestion that *V. cholerae* O1 can be maintained in an aquatic environment but it is not known in which particular micro- or macro-habitat this occurs. Do the *V. cholerae* survive in the aquatic environment as free floating organisms or do they prefer an epibiotic host as suitable ecological niche?

Hood *et al.* (9) found that the survival of *V. cholerae* is dependent on suspended particulate matter in the water. They observed that the survival time is reduced in filtered and centrifuged sea water, the viable number of cells decreasing in proportion to the filtration and the speed of the centrifugation. Since filtering and centrifugation reduce the particulate load, the viability of the organism may be related to the presence of particulates. They suggested that *V. cholerae* prefer an epibiotic form of habitat. As the organisms can be recovered from filtered estuarine and sea water as well as particulate-free water, they also suggested that *V. cholerae* can survive as a planktonic form for a certain period of life.

Lee *et al.* (10) in a 3-year survey from 1979 to 1981 of the incidence of *V. cholerae* in water, animals and birds in Kent, England, observed that about 6% of all the gulls sampled contained *V. cholerae* non-O1. They also collected the water samples from the same ditches where the birds were caught but could not isolate any *V. cholerae*.

Moreover, *V. cholerae* non-O1 have been isolated from the cavum nasi and pharynx of ducks by Bisgaard *et al.* (11). They could not isolate *V. cholerae* non-O1 from ducks which had never been outside the houses. The prevalence of *V. cholerae* non-O1 in the cavum nasi was very high when ducks were admitted to the open field. However, they could not isolate *V. cholerae* non-O1 from ducks kept in cages but provided with the same drinking water source as in the field. Similar observations were also made by Ogg *et al.* (12). They isolated *V. cholerae* O1 from the cloacal swabs and freshly voided faeces collected from 20 species of aquatic birds in Colorado and Utah during 1986 and 1987. They could not, however, detect the *V. cholerae* O1 from the water samples, collected from the habitat of the birds. These studies might indicate that it is not the water but the plants or animals (e.g. duckweed, algae, crustaceans, etc.) present in the water that had been eaten by the birds, contained *V. cholerae*. So the nature of the conditions in which *V. cholerae* survive during the interepidemic period remains unexplained, but at present plants or animals reservoirs seem quite likely.

2. Aquatic fauna as reservoirs

Aquatic animals and plants may be reservoirs of *V. cholerae* in the environment. We first consider the possibilities of aquatic fauna as possible reservoirs.

Various kinds of aquatic fauna including oysters, zooplankton, crabs, etc. have been considered as potential habitats of *V. cholerae* in the aquatic environment from time to time. Dastidar and Narayanaswami (13) studied the chitinase activity of 7 strains of *V. cholerae* classical biotype, 15 strains of El Tor biotype and 4 NAG (non-agglutinating) vibrios. Detectable amounts of chitinase activity were observed in most of the strains studied. Nalin (14) suggested that *V. cholerae* can survive during the interepidemic period attached to copepods in the Ganges river delta, because of the vibrio's chitinase production and ability to use chitin as a source of nutrients. Kaneko and Colwell (15) examined copepods

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collected from the Chesapeake Bay and isolated *Vibrio parahaemolyticus* from more than 80% of the copepod samples. The number of *V. parahaemolyticus* increased with rising water temperature.

Sochard *et al.* (16) investigated the micro-organisms associated with the surface and gastrointestinal tract of various genera of copepods. They collected the copepods from pelagic, estuarine and freshwater environment. *Vibrio* sp. were isolated from copepods collected in all three types of environment. The highest isolation (94.4%) was made from copepods collected from estuarine environments. Five genera of copepods were collected: *Acartia tonsa*, *Pontellopsis regalis*, *Pleuromamma* sp., *Labidocera aestiva* and *Centropages furcatus*. Among these five genera of copepods, the highest number of *Vibrio* spp. were isolated from *A. tonsa*. This may be due to the feeding habits of *A. tonsa*; among the 5 genera, only *A. tonsa* is truly herbivorous whereas the others are carnivores. It may be that the *Vibrio* sp. were attached to phytoplankton which were eaten by the herbivorous *A. tonsa*, which could explain why this strictly herbivorous copepod was so heavily colonized.

Twedt *et al.* (17) examined freshly harvested oysters from estuarine waters in Florida, USA, from June 1979 to May 1980. They isolated *V. cholerae* non-O1 from 111 of 790 oysters. *V. cholerae* O1 serotype Inaba was also isolated from seven samples during the months of May to July. All *V. cholerae* O1 isolates were non-toxigenic. *V. cholerae* non-O1 were also isolated from oysters collected from the Chesapeake Bay (18).

Hood *et al.* (19) isolated two strains of *V. cholerae* O1 from oysters, *Crassostrea virginica*, collected during April 1980 in estuarine waters in the Apalachicola Bay, Florida. None of these strains was toxin positive.

Huq *et al.* (20) studied the role of copepods on survival and multiplication of *V. cholerae* O1 in laboratory microcosms. They found that *V. cholerae* survive longer and multiply in the presence of copepods. *V. cholerae* was isolated for 312 h from the copepod samples. Patuxent river water (Maryland) was used in the experiments with the salinity ranging from 0.2%-2.2%. The salinity in this water was above fresh water levels so it was not clear what would have happened if lower salinity water had been used. Moreover, 312 h is not considered enough to cover the interepidemic period.

Bourke *et al.* (21) isolated *V. cholerae* O1 El Tor Inaba from a river in Australia at least once in every year from 1977 to 1983. They investigated the possible reservoirs and natural ecological niches of *V. cholerae* O1. They studied 127 rock oysters (*Crassostrea commercialis*), 15 mud crabs (*Scylla serpata*) and 5 unidentified fish from the mouth of the two rivers which contained *V. cholerae*; they could not isolate any *V. cholerae* from any of the samples. About 120 sea mullet (*Mugil cephalus*) and a similar number of freshwater mullet (*Trachystoma petardi*) were netted and tested. *V. cholerae* O1 was isolated from two sea mullet from two rivers. The mullet is also an herbivore. They also collected a total of 456 batches of plants from rivers and tested them. *V. cholerae* O1 was isolated from four batches of plants.

Amako *et al.* (22) studied the role of chitin in survival of *V. cholerae* O1 in laboratory microcosms. They found that *V. cholerae* could survive more than a week longer in the presence of chitin at low temperature (0°C) than in the absence of chitin. Venkateswaran (23) studied the seasonal variation of *V. cholerae* non-O1 and the role of zooplankton in their distribution for 1 year in Fukuyama Coastal waters of Japan. They observed that zooplankton samples did not harbour more *V. cholerae* non-O1 than did the water column. DePaula *et al.* (24) investigated the ecology of vibrios in Mobile Bay on the US Gulf Coast. They implemented an intensive sampling programme and isolated *V. cholerae* O1 serotype Inaba, biotype El Tor from intestinal contents of fish and four Mobile Bay Oyster samples. All isolates were toxigenic as determined by DNA probe, enzyme-linked immunosorbent assay and tissue culture (Chinese hamster ovary).

The first case in the USA was detected in 1973, an old man living in Port Lavaca, Texas. *V. cholerae* El Tor Inaba was isolated in the stool sample of this patient. This is the first reported case in the USA since 1911 (25). After 5 years in 1978, another case of a 44-year-old man was detected in Louisiana. Then 10 more cases were detected in four additional clusters (26) in the same area surrounding the Gulf of Mexico. It was found that the only meal which was common to all infected persons was crab (26).

An attempt was made to isolate *V. cholerae* O1 from seafood and the environment in the sites from which the crabs came along the entire area between Vermillion Bay and Mud lake. They cultured 110 live crabs from 60 geographic sites, 400 shrimps from 41 sites, 27 oysters from four sites and 165 Moore swabs that had been submerged in canals, lakes, bays, and rivers for 24 or more. They also cultured frozen cleaned crabs, whole boiled crabs leftover after eating and shrimps from the freezers of patients. *V. cholerae* O1 was isolated from one of the three boiled crabs, from a shrimp caught in a canal and from two Moore swabs from two canals.

It has been hypothesized that *V. cholerae* O1 had been able to survive along the Gulf Coast for years in absence of human disease in the area. The strongest evidence is the fact that isolates from Texas and all isolates from Louisiana five years later were of a single phage type unique to the United States. This toxigenic *V. cholerae* O1 strain is designated as the Gulf Coast strain. The Gulf Coast isolates are haemolytic and have vibriophage VcA-3 and a unique cholera toxin gene pattern on Southern-blot analysis (27). These endemic toxigenic *V. cholerae* O1 strains are clearly different from the seventh pandemic El Tor O1 strains isolated worldwide in the past 30 years. Although cholera caused by this strain has usually been associated with seafood consumption, that strain is rarely isolated from seafood or environmental samples.

Some of these studies are summarized in Table 1.

3. Aquatic flora as reservoirs

Various aquatic fauna have been investigated but as yet nothing of unequivocal epidemiological importance as a reservoir of pathogenic *V. cholerae* O1 has been reported.

Thus it would be useful to examine the role of aquatic flora as possible reservoirs of *V. cholerae* in the water systems.

Dr. Robert Koch in 1884 (28) suggested that aquatic flora and fauna might be reservoirs of cholera in cholera endemic areas. He examined the maintenance of cholera in the lower part of the Ganges delta by pointing out the geographical location and condition of habitation. He explained:

"The lower part of the delta seems entirely uninhabited. This uninhabited district called Sunderbunds, comprises an area of 7500 English square miles. A luxuriant vegetation and an abundant variety of animal life have developed in this uninhabited district. One can easily imagine what quantities of vegetable and animal matter are exposed to putrefaction in the boggy districts of Sunderbunds and that an opportunity scarcely to be found in any other place in the world is offered here for the development of microorganisms."

Then he postulated:

"Under peculiar circumstances a thoroughly special fauna and flora of microorganisms must develop there to which in all probability the comma bacillus belongs."

To find out the role of vegetative matter as nutrients for the comma bacillus he made following observations:

"I have often formerly made such experiments and it has often happened that a water contains almost no bacteria at all whilst remains of plants especially roots or fruits swimming in it, teemed with bacteria, especially kinds of bacilli and spirilla even in the immediate neighbourhood of these objects, the water was rendered turbid by swarm of bacteria which clearly received their nourishment from the nutritive matter scattered by diffusion at a very small distance."

Thus there was an indication from the time of the discovery of *V. cholerae* that aquatic flora may be possible reservoirs of cholera in endemic areas.

The aquatic flora are divided into two groups, i.e. macrophytes and microphytes.

3.1. Macrophytes as reservoirs. Macrophytes are divided into two groups for convenience of discussion, marine macrophytes and fresh water macrophytes.

3.1.1. Marine macrophytes. Chan and McManus (29) carried out an investigation to estimate the number of bacteria on two marine algae, *Polysiphonia lanosa* and *Ascophyllum nodosum*, and their environmental sea water. They isolated the bacteria and identified them to the genus level from these sources. They observed that the bacterial count associated with *P. lanosa* and *A. nodosum* was 100 to 10,000 times higher than that from the environmental waters. Vibrios were the predominant organism on *A. nodosum*. Nutrition requirement experiments showed that most of the vibrios isolated had absolute requirements for amino acids. It is known that marine algae produce a number of extracellular products which contain peptides, amides and free nitrogen (30,31); thus, this may explain why the amino-acid-requiring bacteria were predominant on algal surfaces.

Laycock (32) investigated the bacterial population

associated with the fronds of the sublittoral brown alga *Laminaria longicruris* (*L. longicruris*) collected at monthly intervals from Shag Bay, Nova Scotia, in Canada. He isolated two major groups of bacteria from the seaweed: vibrios and pseudomonas. He observed a distinct variation in the count of these two groups of bacteria with the time of the year and the region of the frond from which they were isolated. It was observed that a high proportion of vibrios was maintained by *L. longicruris* throughout the winter. The isolated vibrios could hydrolyse laminaran (an excretion product of *Laminaria* spp.) which may be why this group of bacteria was observed on this seaweed.

Shiba and Taga (33) investigated the bacterial flora on various seaweeds and those of the environmental sea water from December 1973 to June 1974. They collected seaweeds and sea water samples from the Nabeta inlet and Otsuchi Bay, Japan. Two green algae, *Monostroma nitidum* (*M. nitidum*) and *Enteromorpha linza* (*E. linza*), one red alga *Porphyra suborbiculata* and a brown alga *Eisenia bicyclis* were collected. The viable counts of bacteria attached to the green algae, *M. nitidum* and *E. linza* ranged from 10^4 to 10^6 /cm² and on the red alga *P. suborbiculata* from 10^3 to 10^4 /cm². The counts in sea water samples were 10^3 /ml. The bacterial counts on the brown alga *E. bicyclis* were generally smaller than on red and green algae. The counts on brown alga fluctuated considerably; the largest number was 2.2×10^4 /cm² in March and the smallest (2.9×10^1 /cm²) in January. This variation in bacterial numbers was not influenced by the variation of the bacterial population in sea water which was relatively constant during the study period, but was considered to be dependent on the physiological conditions of the sea weed. The bacterial concentration was smallest when the largest number of germinating leaves were observed.

It was also observed that the counts of heterotrophic bacteria were different in different algal species. The bacteria belonging to the vibrios and *Flavobacterium-Cytophaga* group were present on the green algae collected at both stations but were not dominant in environmental sea water. It was suggested that a beneficial relation existed between the green algae and their epiphytic bacteria.

Islam *et al.* (34) studied the survival and attachment of toxigenic *V. cholerae* O1 to four marine algae, e.g. *Ulva lactuca*, *Enteromorpha intestinalis*, *Ceramium rubrum* and *Polysiphonia lanosa*, in artificial aquatic ecosystems. Of all the plants tested, *V. cholerae* O1 survived longest in association with *U. lactuca*.

These studies are summarized in Table II.

3.1.2. Fresh water macrophytes. Plant surfaces can influence the distribution of bacteria in several ways, e.g. topography of the leaf surfaces, excretion of metabolites which can act as nutrients for bacteria and by the presence of stimulatory or inhibitory substances (35-38).

More bacteria in mature leaves may be due to multiplication of bacteria or due to attachment of bacteria over time from the water column. So the growth rates of epiphytic bacteria were studied on a common duckweed, *Lemna minor* (36). Detachment experiments were carried

out with 20 *Lemna* plants. They observed that in one hour the bacterial population increased in size by approximately 4%. It was evident that these bacteria can multiply on *Lemna* plant surfaces.

Islam et al. (39) carried out a survival study of toxigenic *V. cholerae* O1 with *Lemna minor* in artificial aquatic eco-systems. Survival of both environmental and clinical strains of *V. cholerae* O1 was assessed by viable bacterial counts on thiosulfate citrate bile salt sucrose agar. It was observed that both strains survived longer on *L. minor* than in water on which *L. minor* was floating or in control water. *V. cholerae* secretes an enzyme, mucinase, (40) which may play a role in the environment by degrading naturally present mucin and mucin-like substances present in the plant cells and thus further help to form an association with the plants. It was suggested that plants may serve as an effective environmental reservoir for *V. cholerae* either through a non-specific association or by interaction with *V. cholerae* in a commensal relationship (41).

supports the idea that carbon fixed by the macrophyte is transferred to heterotrophic epiphytic bacteria.

The search for vibrios in association with fresh water macrophytes started in the late 1970s when Islam and Aziz (43) studied fresh water macrophytes in cholera endemic areas in Bangladesh. They first reported the association of vibrios with some fresh water macrophytes from Bangladesh (43).

As part of a study to investigate the association of vibrios with hydrophytic plants (44), plants and water samples were collected at 15 day intervals from various locations in Dhaka, Bangladesh from July 1977 to December 1977. Eleven different kinds of water plants were collected throughout the study period. The association of vibrios with four macrophytes was observed. The macrophytes were *Eichhornia crassipes* (water hyacinth), *Monochoria hastata*, *Marsilea quadrifolia* and *Ludwigia repens*. Vibrios were isolated from the roots of these plants.

Spira et al. (45) studied the association of *V. cholerae* El Tor with water hyacinth in endemic areas in Bangladesh as well as in laboratory microcosms. Water and intact water hyacinth plants floating within the boundaries of the same sampling point were collected. In one third of the samples, *V. cholerae* biotype El Tor were present both in plants and water; the rest of the sample showed 52% isolation from plants alone and 16% from water alone. The difference in percentage of isolation from plants alone and water alone was statistically significant. They also observed a significant difference in the number of *V. cholerae* per gram of roots as compared with stems and leaves of water hyacinths at all times, which was simply due to a greater exposed surface area to water as compared to the rest of the plants. This study concluded that virulent *V. cholerae* biotype El Tor can associate with water hyacinth. Vibrios present in water contaminated by stool from cholera victims concentrated on the surface of these plants and increased the length of time the vibrios remained viable in the aquatic environment.

Islam et al. (46) carried out a preliminary survey of isolation of vibrios from water plants, water and soil sediments in three ponds of Dhaka city in Bangladesh. The survey was carried out for one year from September 1980 to August 1981. *Eichhornia crassipes* (water hyacinth), *Telanthera philoxeroides* and *Panicum* sp. were collected every month. Among the three plants, vibrios were found only in the roots of the water hyacinth.

Islam et al. (47) carried out a study to examine the association of *V. cholerae* non-O1 in various components of pond ecosystems in Bangladesh. They collected water, plants, phytoplankton and sediment samples from five ponds in and around Dhaka city, Bangladesh every 15

Table I. Isolation of vibrios from aquatic fauna.

Source	Fauna	Vibrios	Date	Country
Kaneko and Colwell (1973)	Copepods	<i>V. parahaemolyticus</i>	1970-1971	Chesapeake Bay, USA
Kaper et al. (1979)	Oysters	<i>V. cholerae</i> non-O1	1976-1978	Chesapeake Bay, USA
Sochard et al. (1979)	Copepods	<i>Vibrio</i> sp.	ND*	Gulf of Mexico
Blake et al. (1980)	Crabs, shrimps	<i>Vibrio Cholerae</i> O1	1978	Area between Vermilion Bay and Mud Lake, USA
Twedt et al. (1981)	Oysters	<i>V. cholerae</i> O1 <i>V. cholerae</i> non-O1	1979-1980	Florida, USA
Hood et al. (1981)	Oysters	<i>V. cholerae</i> O1	1980	Apalachicola Bay, Florida, USA
Bourke et al. (1986)	Sea mullet	<i>V. cholerae</i> O1	1977-1983	River in Queensland Australia
Venkateswaran et al. (1989)	Zooplanktons	<i>V. cholerae</i> non-O1	1988	Fukuyama coastal water, Japan
De Paola et al. (1992)	Oysters	<i>V. cholerae</i> O1	1991	Mobile Bay on the Gulf Coast

*ND = No data

Baker and Farr (42) investigated the amount of dissolved organic carbon (DOC) produced by *L. minor* and how much is utilized by epiphytic bacteria. They measured the DOC produced by *L. minor* in the presence and absence of epiphytic bacteria. DOC was measured by an automated UV photolysis method. They found that approximately 2% of the carbon fixed by *L. minor* is secreted as DOC. The production of DOC by axenic cultures of *L. minor* was greater than that of *L. minor* colonized by bacterial epiphytes. This study strongly suggested the role of epiphytes as a carbon "sink" and

days, between May 1988 and April 1989. Among the plants, *Eichhornia crassipes*, *Nymphoides* sp. and *Telanthera philoxeroides* were collected. Isolation of *V. cholerae* non-O1 was 28% and 47% from plants and phytoplankton respectively. This study demonstrated that *V. cholerae* non-O1 prefer phytoplankton as a better habitat than higher plants in the aquatic environment.

These studies are summarized in Table III.

Table II. Association between marine macrophytes and bacteria

Source	Plants	Bacteria	Date	Country
Chan and McManus (1969)	<i>Polysiphonia lanosa</i> , <i>Ascophyllum nodosum</i>	<i>Vibrio</i> , <i>Flavobacterium</i> , <i>Pseudomonas</i> , <i>Achromobacter</i> , <i>Escherichia</i> , <i>Sarcina</i> , <i>Staphylococcus</i>	1964	Canada
Laycock (1974)	<i>Laminaria longicuris</i>	<i>Vibrio</i> and <i>Pseudomonas</i>	1972-73	Canada
Shiba and Taga (1980)	<i>Enteromorpha linza</i> <i>Porphyra suborbiculata</i> , <i>Monostroma bicyclis</i>	<i>Vibrio</i> , <i>Flavobacterium</i> and <i>Cytophaga</i>	1973-74	Japan
Islam <i>et al.</i> (1988)	<i>Ulva lactuca</i> , <i>Enteromorpha intestinalis</i> , <i>Ceramium rubrum</i> , <i>Polysiphonia lanosa</i>	<i>Vibrio cholerae</i> O1	1986	UK

Table III. Association between freshwater macrophytes and vibrios.

Source	Macrophytes	Vibrios	Date	Country
Islam and Aziz (1978)	Water hyacinth (<i>Eichhornia crassipes</i>), <i>Monochoria hastata</i> , <i>Marsilea quadrifolia</i> , <i>Ludwigia repens</i>	<i>V. cholerae</i> non-O1	1977	Bangladesh
Spira <i>et al.</i> (1981)	Water hyacinth (<i>Eichhornia crassipes</i>)	<i>V. cholerae</i> El Tor	1976-77	Bangladesh
Islam <i>et al.</i> (1983)	Water hyacinth (<i>Eichhornia crassipes</i>), <i>Telanthera philoxeroides</i> , <i>Panicum</i> sp.	<i>V. cholerae</i> non-O1	1980-81	Bangladesh
Islam <i>et al.</i> (1990)	Duckweed (<i>Lemna minor</i>)	<i>V. cholerae</i> O1	1986	UK

3.2. Microphytes as reservoirs. As with macrophytes, microphytes are again divided into two groups for convenience of discussion: marine microphytes and fresh water microphytes.

3.2.1. Marine microphytes. Most bacteria in sea water are attached to marine plankton and small particles in sea water.

Simidu *et al.* (48) investigated the generic composition of bacterial flora of marine phyto and zooplankton. They collected the plankton samples from the Nishiura Bay, on

the Pacific Coast of the Bozo Peninsula 200 km SE of Tokyo. They also enumerated and identified the bacterial flora from the sea water. The viable bacterial number varied from 1.2×10^5 to 1.1×10^7 /ml of plankton sample, whereas the total bacterial number in sea water varied from 7×10^1 - 1.2×10^2 /ml. The main constituents of the bacterial flora from the plankton samples were the *Vibrio-Aeromonas* group with *Pseudomonas* being the next most important constituent. This study revealed that the ecology of marine *Vibrio-Aeromonas* is closely associated with marine plankton.

Algal extracellular products are the sources of microbial nutrients. These compounds are released as products of cell metabolism, a process sometimes termed as "excretion". These excretion products are the source of extracellular organic materials under natural conditions on which a bacterial population can grow without any additional carbon source.

Martin and Bianchi (49) studied bacterial population dynamics in continuous cultures of marine planktonic algae, maintained by continuous nutrient salt enrichment of natural oligotrophic sea water, in a large volume tank exposed to external climatic conditions. They followed the development of the population during two experimental phytoplankton blooms, in spring and autumn, 1977. They observed a difference in generic identities of bacterial strains at different times of the study period. During spring experiments which started on the first week of April, the percentage of *Pseudomonas* was 54% and *Vibrio* sp. was about 15%. At the end of the first week of May, the *Pseudomonas* percentage had decreased to 22% and the vibrios increased to 74%.

During the autumn experiment which started on the third week of October, *Vibrio*, *Pseudomonas*, *Spirillum* and *Flavobacterium* were 8%, 28%, 5% and 1% respectively. On the first week of November, the percentages of *Pseudomonas*, *Spirillum* and *Flavobacterium* increased whereas vibrios decreased to 1%. After one week, i.e. the second week of November, the *Vibrio* percentage started increasing gradually, whereas all other bacteria showed a gradual decrease. On the fourth week of November, the percentage of vibrio became the highest (32%) of all the bacteria isolated.

In both spring and autumn experiments, the phytoplankton blooms were of *Skeletonema costatum* and *Chaetoceros* sp. So there were complex competitive population changes between different bacterial flora in a natural body of water, and *Vibrio* proportions varied from being at a low level to becoming the dominant genus present.

Hellebust (50) estimated the fraction of photo-assimilated carbon excreted during log phase growth by various classes of marine microphytes. He also investigated the effects of light intensity on excretion of extracellular products by cultures of marine algae. Nine species of algae, i.e. *Cricosphaera carterae*, *Exuviella* sp., *Chaetoceros simplex*, *Skeletonema costatum*, *Thalassiosira fluviatilis*, *Phaeodactylum tricorutum*, *Chlorella* sp., *Pyramimonas* sp. and *Tetraselmis* sp. were included. All the algae were in log-phase growth during the experiment. The amount of carbon excreted by the marine species ranged from 1.5% to 24% of that assimilated through photosynthesis. They also observed that the percentage of excretion varied considerably from species to species. The composition of excretion products also varied from species to species. This study demonstrated that most of the excretion products were of low molecular weight, such as amino acids and sugar alcohols.

Table IV. Association between microphytes and bacteria.

Source	Microphytes	Vibrios	Date	Country
Simidu <i>et al.</i> (1971)	Phytoplankton	<i>Vibrio</i> sp. <i>Aeromonas</i> sp. <i>Pseudomonas</i> sp.	1970	Japan
Martin and Bianchi (1980)	<i>Skeletonema costatum</i> and <i>Chaetoceros</i> sp.	<i>Vibrio</i> sp. <i>Pseudomonas</i> sp. <i>Spirillum</i> sp. <i>Flavobacterium</i> sp.	1977	France
Islam <i>et al.</i> (1989)	<i>Rhizoclonium fontanum</i>	<i>V. cholerae</i> O1	1986	UK
Islam <i>et al.</i> (1990)	<i>Anabaena variabilis</i>	<i>V. cholerae</i> O1	1986	UK
Tamplin <i>et al.</i> (1990)	Phytoplankton	<i>V. cholerae</i> O1	1987	Bangladesh
Islam <i>et al.</i> (1992)	Phytoplankton	<i>V. cholerae</i> non-O1	1988-89	Bangladesh
Islam <i>et al.</i> (1994)	Blue-green alga, <i>Anabaena</i> sp.	<i>V. cholerae</i> O1	1988-89	Bangladesh

3.2.2. Freshwater microphytes. The freshwater microphytes will be discussed under two headings, the green algae (*Chlorophyceae*) and the blue green algae (*Cyanophyceae*).

3.2.2.1. Green algae. Studies showed that bacteria can utilize algal extracellular products as nutrients (30,31,51,52) and can maintain a commensal relationship with algae. These studies also showed that the relationship between bacteria and algae is not a matter of chance. These studies indicate that *V. cholerae* may coexist with some kinds of algae in the environment and may use the extracellular materials secreted by those algae as nutrients.

Islam *et al.* (53) in a laboratory-based study observed that toxigenic *V. cholerae* O1 gain survival advantages in association with a filamentous green alga, *Rhizoclonium*

fontanum in an artificial aquatic environment. *V. cholerae* O1 survived longer with *R. fontanum* ($P < 0.05$) than in water on which *R. fontanum* was floating or in control water (without *R. fontanum*). Though the difference in length of survival of *V. cholerae* O1 between *R. fontanum* and in water on which *R. fontanum* was floating was statistically significant, *R. fontanum* was not considered as a reservoir. Only those flora should be considered as reservoirs with which *V. cholerae* O1 will be able to survive at least nine months to overcome the interepidemic period.

3.2.2.2. Blue green algae. The association between blue-green algae and bacterium during a bloom is well known (54-57) but the functional aspects of the algal-bacterial relationship in the natural communities are not properly defined.

V. cholerae have been detected using the indirect fluorescent antibody technique from both clinical and environmental samples (58-60). Sack and Barua (1964) detected *V. cholerae* O1 from the rice water stools of cholera patients using this technique in Calcutta, India early 1960s. Recently Colwell *et al.* (62,63) also used the same technique to detect *V. cholerae* O1 from laboratory microcosms and environmental water samples.

Brayton *et al.* (64) carried out a field study in a rural cholera endemic area in Bangladesh. They detected and enumerated the *V. cholerae* O1 from the water samples collected from village tubewells, ponds and the adjacent river using fluorescent antibody (FA) direct viable counts and most probable number indices. All water samples yielded higher counts of viable *V. cholerae* O1 by fluorescent-antibody direct viable count than by the most probable number indices. The significant difference in results between the culture and FA procedures provides additional evidence for the existence of viable but nonculturable forms of *V. cholerae* O1 in the natural environment in an area in which cholera is endemic. It is evident from these results that the FA procedure is a more sensitive detection method than standard culture procedures because it allows the enumeration of both culturable and non-culturable cells. Other studies also demonstrated the viable but non-culturable state of *V. cholerae* (65-67).

Huq *et al.* (68) detected *V. cholerae* O1 from 876 plankton samples collected from ponds and rivers between February 1987 and January 1990 in Matlab, Bangladesh by using both the conventional cultural method (CM) as well as fluorescent antibody (FA) techniques. *V. cholerae* O1 was detected in 563 samples (64.27%) by the FA method and in 3 samples (0.34%) by the CM. From the data, it is evident that the FA technique is more sensitive than CM for the detection of *V. cholerae* O1 from the environmental samples.

Tamplin *et al.* (69) carried out both laboratory and field based studies to investigate the attachment of *V. cholerae* O1 to zooplankton and phytoplankton using the fluorescent antibody technique with a monoclonal antibody specific for the A antigen of O1 lipopolysaccharide of *V. cholerae* O1. They collected both zooplankton and phytoplankton from a river and two ponds in Matlab, Bangladesh in April, 1987. Among the zooplankton, 5 species of copepods, 5 species of cladocerans and one species of rotifers were present. Among phytoplankton, 2 species of green-algae (*Volvox* sp. and *Pediastrum simplex*) and two species of blue-green algae (*Spirulina* sp. and an unicellular Cyanobacteria) were studied. None of the zooplankton showed any binding of *V. cholerae* O1 with whole specimens, whereas 3 of 4 phytoplanktons showed binding with *V. cholerae* O1 with whole specimens. *V. cholerae* O1 strains attached preferentially to zooplankton molts (exuviae) rather than to whole specimens. This study indicated that phytoplankton are better habitats than zooplankton for *V. cholerae* O1 because the bacterium have a constant supply of nutrients from the living phytoplankton, a situation not possible from dead zooplankton. The authors did not observe the attachment of *V. cholerae* O1 to any natural zooplankton or phytoplankton (uninoculated) specimens. Islam *et al.* (70) in a similar study also found that *V. cholerae* attached to a blue-green alga, *Anabaena* sp. but not with other algae present in the same sample collected from the natural waters of Bangladesh. The results from these studies may indicate that *V. cholerae* may have some specificity for attachment. The study by Islam *et al.* (70) will be discussed later in details.

Islam *et al.* (71) studied the persistence of *V. cholerae* O1 in the mucilaginous sheath of a blue-green alga, *Anabaena variabilis* in the artificial aquatic environment. They observed that *V. cholerae* O1 can survive in culturable form in association with *A. variabilis* up to 120 h but survived in water on which the alga was floating and in control water without alga more than 144 h. Examination of the alga by phase contrast microscope after 10 days of the survival study showed that *V. cholerae* O1 entered the mucilaginous sheath of *A. variabilis*. *V. cholerae* inside the mucilaginous sheath of *A. variabilis* became nonculturable but found dividing by binary fission and clustering around the heterocysts which are the known sites for atmospheric nitrogen fixation. *V. cholerae* O1 was detected inside the mucilaginous sheath for up to 15 months. This time is considered enough for *V. cholerae* O1 to overcome the interepidemic period. This study suggested that *A. variabilis* may act as a reservoir of *V. cholerae* O1 in the aquatic environment. It was also observed that *V. cholerae* O1 do not lose toxin properties during survival in association with algae (72-74).

The production of mucinase by vibrios (40) may be a process by which plant and planktonic mucin is degraded in nature. This may be one of the factors which helps to allow the association of *V. cholerae* O1 with mucilaginous blue-green alga, *A. variabilis*.

One important functional aspect of *A. variabilis* and *V. cholerae* O1 is the exchange of carbon dioxide and oxygen. In the association of *A. variabilis* and *V.*

cholerae O1, the algal host provides oxygen during photosynthesis that can be utilized in aerobic respiration of *V. cholerae* O1, which produce carbon dioxide, which may be available for algal photosynthesis. Thus, *A. variabilis* and *V. cholerae* O1 may maintain a symbiotic relationship (71,75,76).

Paerl and Gallucci (77) observed under the microscope that motile bacteria can easily discriminate heterocysts from vegetative cells. Bacteria interacting with *A. oscillarioides* filaments were observed to "bump" onto both vegetative and heterocyst cells but they did not adhere to either. When bacteria encountered heterocysts, flagellar rotation was often increased for 5 to 10 minutes. Then the bacteria attached to the heterocyst-vegetative cell junction. After this attachment to the heterocyst region, flagellar motion stopped. Once attachment took place both hosts and epiphytes started growing. It was observed that bacteria rarely penetrated cyanobacterial cell walls.

Chemotaxis played a key role in the establishment and maintenance of cyanobacterial and bacterial association (77,78). Products of N₂ fixation, which were excreted at heterocyst-vegetative cell junctions are responsible for attracting diverse naturally occurring heterotrophic bacteria. Amino acids are probably chemotactic agents since they are excreted by N₂-fixing *Anabaena*.

These studies demonstrated that heterotrophic bacterial and filamentous blue green algal association may involve two processes. Firstly, the bacteria attach to the filament and then they utilize the algal metabolites as nutrients. Second, the attachment is initiated by chemotaxis and shows site and species specificity.

On the basis of these findings, field studies were carried out by Islam *et al.* (70) to detect *V. cholerae* O1 in blue-green alga from the aquatic environment in Bangladesh. They collected phytoplankton samples every 15 days between May 1988 and April 1989 from a pond in Dhaka city which is used for bathing, washing, swimming and occasionally drinking purposes. The phytoplanktons were mainly *Anabaena* sp., *Euglena* sp. and *Phaeus* sp. *V. cholerae* O1 was detected by immunofluorescence in the mucilaginous sheath of *Anabaena* sp. in 16 of 24 plankton samples. *V. cholerae* O1 could be detected only in association with *Anabaena* sp. and not with other algae collected from the pond, e.g. *Euglena* sp. and *Phaeus* sp.

This study, therefore, clearly demonstrated an association between *V. cholerae* O1 and a blue-green alga, *Anabaena* sp. in the natural aquatic environment in Bangladesh. These findings suggest that *V. cholerae* O1 may have a preference for association with blue-green algae particularly *Anabaena* sp. or related species which have mucilaginous sheaths around them. Therefore, it is evident from these studies that blue-green algae may act as a reservoir of *V. cholerae* O1 in the aquatic environment in Bangladesh Table IV.

4. Conclusion

It now appears that blue green algae may act as a reservoir of cholera. The conventional view that *V. cholerae* O1 is found in the environment only in association with human infections, and surviving for only a short period in the environment is again challenged.

These new observations may provide answers to the longstanding questions about the interepidemic survival of *V. cholerae* and may help to explain how the endemicity and seasonality of cholera in endemic areas are maintained. It has been observed that the peak incidence of cholera in endemic areas of Bangladesh occurs together with the bloom of blue-green algae in their natural aquatic environment (71,79-81). Seasonal occurrence of *V. cholerae* O1 and non-O1 have also been demonstrated by others (47,82,83). However, the implication of these observations for the control of cholera in humans remains to be elucidated.

It now seems that the elimination of cholera would have little impact on the survival of the species *V. cholerae* O1, since it may be primarily an environmental organism.

Though interest has been focused on strains of *V. cholerae* causing disease, most strains of *V. cholerae* must be predominantly environmental organisms. Only in the environment will strains, belonging to the various genotypes including representatives of all known serotypes and biotypes, have an opportunity to interact. Furthermore, the range of biological interactions, the seasonal changes in nutrient availability and physicochemical conditions can generate selective pressures favouring the emergence of novel types. It may be that the development of starved forms is also part of this process.

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