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Ciliate-*Vibrio cholerae* interactions within a microbial loop: an experimental study

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ABSTRACT: Interactions between *Vibrio cholerae* and 2 Mexican athalassohaline-lake bacteria assemblages were studied in laboratory microcosms with the presence or absence of protists. Pre-cultivated and harvested vibrios were added as the food for protists at a concentration of 10^7 cells ml^{-1}. Total direct bacterial counts, colony forming units on a selective (thiosulphate-citrate-bile salts-sucrose) medium, and *V. cholerae* indirect fluorescent antibody (FA) direct counts were measured. Numbers of *V. cholerae* decreased faster in the microcosms inoculated with protists and, moreover, the proportion of non-culturable vibrio counts increased. Comparison between the ciliate feeding rates upon fluorescently labelled *V. cholerae*, *Salmonella typhi* and *Shigella* sp. showed that the ciliates fed preferably upon vibrios. Calculated grazing rates of *Cyclidium glaucoma* related to non-culturable but FA-positive *V. cholerae* were correlated to observed decrease in *V. cholerae* numbers.

KEY WORDS: Non-culturable *Vibrio cholerae* · *Cyclidium glaucoma* · *Colpoda steinii* · Clearance rate

INTRODUCTION

Vibrio cholerae is a human pathogen belonging to the autochthonous microbial assemblages in estuaries and brackish waters (Colwell et al. 1981), i.e. it is able to survive or even to flourish in a natural environment. Unfortunately, it cannot be easily isolated from water samples, excluding periods of epidemics, because it fails to grow on selective media. The presence of high numbers of viable but non-culturable cells was confirmed using an immunofluorescence technique (Xu et al. 1984, Colwell et al. 1985, Brayton et al. 1987, Huq et al. 1990).

The position of *Vibrio cholerae* within food webs is not yet fully understood. Present knowledge (Brayton et al. 1987, Huq et al. 1990, Tamplin et al. 1990, McKay 1992) has been summarised in Fig. 1. In the presence of other bacteria, faster disappearance of *V. cholerae* was observed (Singleton et al. 1982). The general relationship of *V. cholerae* to phytoplankton was not specified but both colonisation of some phytoplankton species and long-term persistence in the mucilaginous sheath have been observed (Huq et al. 1990, Islam et al. 1990, Tamplin et al. 1990). The relationship of vibrios with planktonic copepods and chitin, which comprises the structure of their carapaces, was described by Kaneko & Colwell (1975). Filter feeding rotifers can also be colonised by vibrios (Muroga & Yasunobu 1987, Huq et al. 1990, Tamplin et al. 1990). However, *V. cholerae* distribution relative to zooplankton has not yet been clearly documented using the bacteria-selective cultivation method in seawater (compare Kaneko & Colwell 1978, Sochard et al. 1979, Huq et al. 1983 vs Venkateswaran et al. 1989).

Vibrio cholerae as a common saline water bacterium could serve as a source of food for planktonic bacterivorous organisms, particularly for ciliates and flagellates (compare Berk et al. 1976 with Porter et al. 1985, Sherr et al. 1986, Beaver & Crisman 1989), and thus enter a 'microbial loop' (Pomeroy 1974, Azam et al. 1983) within the plankton food web. However, many pathogenic bacteria are known as toxic to ciliates, or as not being able to meet all their carbon requirements. Moreover, some bacterial strains are protected against

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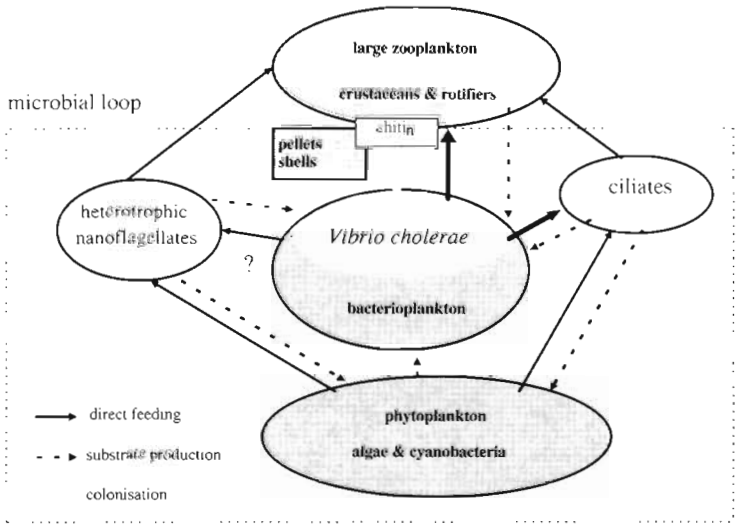

Fig. 1. Position of *Vibrio cholerae* in a microbial loop within the plankton food web

being digested by protozoans by the hydrophobicity of their cell walls; they can pass through the feeding vacuole without losing viability (King et al. 1988, Gurijala & Alexander 1990). In the presence of other suitable bacteria they can be ingested as an alternative prey (Curds & Vandyke 1966, Dive et al. 1974, Mallory et al. 1983, González et al. 1992).

The aim of this study was to identify ciliates as a possible means of decrease of *Vibrio cholerae* in the natural environment, using laboratory microcosms with saline-lake waters. Disappearance of *V. cholerae* was compared with ciliate grazing rates, measured using fluorescently labelled *V. cholerae*.

MATERIALS AND METHODS

Microcosm design. Experimental microcosms were handled in 2 parallel batches (aerated and mixed using an electromagnetic stirrer ~200 rpm), with addition of the *Vibrio cholerae* cells as a major organic carbon source. Acid-washed and autoclaved Erlenmeyer flasks of 1.5 l were filled to 0.5 l, using 0.8 µm pore sized membrane-filtered water. Waters from the Mexican (State of Puebla) maar crater athalassohaline lakes (of freshwater origin) Atexcac (Atx) and Alchichica (Alch) were used (salinity of 6 and 8 g l^{-1} and pH of 8.5 and 8.8, respectively; for more details see Vilaclara et al. 1993). The microcosms were kept in the dark at a temperature of 27 ± 1°C (Atx) or 22 ± 1°C (Alch) that was in concordance with the respective lake *in situ* temperatures during the summer. For more details see Table 1.

Bacterial cultures. Stock cultures of *Vibrio cholerae* O1 El Tor Ogawa (C-12-CDC, V-12-CDC, INDRE: Mexican patient isolate) were used. To provide a reproducible quality of bacterial cells, mostly 2-step preincubation was used as follows (Table 1): 10 ml of 1% peptone solution in 0.2 µm membrane-filtered lake water was inoculated with the stock culture (kept on nutritive agar) and pre-incubated at 37°C. Bacterial culture was added quantitatively into 100 ml of the same broth and incubated at 37°C. Finally, the *V. cholerae* cells produced were harvested by centrifugation (15 000 × *g*, 10 min), resuspended in filtered lake water, and proportionally added to the 2 parallel microcosms.

Table 1. List of the experiments, showing cultures, their pre-incubation, and inoculation

Lakewater	Microcosm	Pre-incubation of *V. cholerae*		Inoculation (Day)	Protists
		First step	Second step		
Atexcac	Atx 1	6 h	15 h	0	*Monas* sp.
	Atx 2	6 h	15 h	0	Mixed assemblage
	Atx 3	6 h	15 h	0	Absent
		6 h	15 h	7	*Cyclidium glaucoma*
	Atx 4	6 h	15 h	0	*Colpoda steinii*
		6 h	15 h	7	*Cyclidium glaucoma*
	Atx 5	6 h	15 h	0	Absent
	Atx 6	6 h	15 h	0	*Cyclidium glaucoma*
Alchichica	Alch 1	6 h	15 h	0	Absent
	Alch 2	6 h	15 h	0	*Cyclidium glaucoma*
	Alch 3	6 h	15 h	0	Absent
	Alch 4	6 h	15 h	0	*Cyclidium glaucoma*
	Alch 5	15 h	No	−3 (before)	Absent
	Alch 6	15 h	No	−3 (before)	*Cyclidium glaucoma*
	Alch 7	6 h	No	0	Absent
	Alch 8	6 h	No	0	*Cyclidium glaucoma*

Mixed bacterial assemblages originated both from membrane-filtered water used for the experiments and from protist cultures used for inoculation. Thus the bacteria coming from filtered (all 0.8 µm membrane except for 1.2 µm in Atx 1, 2) protist cultures were inoculated into the ciliate-free parallels.

Protist cultures. Protists were incubated for 48 h, inoculating 40 ml of a wheat-grain medium (1 grain per 10 ml) with 1 ml of the stock culture. A 20 ml volume of protist culture was added along with the addition of harvested *Vibrio cholerae* cells. The following protist cultures were used: (1) a mixed assemblage from Lake Atexcac dominated by the flagellate *Monas* sp. and ciliates *Oxytricha* sp. and *Cyclidium* sp.; (2) *Colpoda steinii* Maupas, 1883, isolated from a reed-bed pilot waste water treatment plant (State of San Luis Potosí, México); (3) *Cyclidium glaucoma* O. F. Müller, 1773, isolated from Lake Chapultepec (México D.F., México).

Culturable bacterial counts. For enumeration of the colony forming units (CFU) of *Vibrio cholerae*, TCBS (thiosulphate-citrate-bile salts-sucrose) medium was used. Prior to processing, the samples were diluted with lake water (sterilised by passage through 0.22 µm membrane filter; Millipore, USA). To obtain optimum CFU on a dish (20 to 200), at least 4 dilutions within 3 orders of magnitude were used. The method of Venkateswaran et al. (1989) was adopted: the samples were filtered onto sterile membrane filters (0.45 µm pore size; Sartorius, Germany, or Millipore) using a Millipore filtration apparatus (diameter 47 mm). The filters were put on a tryptone broth-soaked pad, incubated for 6 h at 37°C, and finally put on TCBS agar (Difco, USA) plates and incubated at 37°C for 18 h before counting.

Direct counts of bacteria. Total numbers of bacteria were determined by direct microscopic counting (DC), using the DAPI method of Porter & Feig (1980). An epifluorescence microscope (Carl Zeiss, Germany) was also used for all following methods.

Fluorescent antibody indirect counts of *Vibrio cholerae*. The indirect fluorescent antibody (FA) staining method of Xu et al. (1984) revised by Brayton & Colwell (1987) was modified as follows: the sample was filtered onto a membrane filter (black 0.45 µm pore size; Millipore) and the cells were fixed to the membrane surface by incubation at 55°C for 15 min in a moist chamber. The membrane (surface down) was placed on a 25 µl drop of antiserum on a glass slide (Difco, Bacto-*Vibrio cholerae*-Antiserum Poly and Bacto-FA-Rhodamine Counter Stain; final dilutions of 1:5 and 1:20, respectively). The membrane was then incubated at 35°C for 1 h in the moist chamber, and finally washed for 10 min on a tap-water-soaked filtration paper. Following the same procedure, dried membrane was stained with Bacto-FA-Rabbit-Globulin-Antiglobulin (Goat) in a dilution of 1:160 (Difco) and washed. The dry membrane was mounted in Bacto-FA-Mounting Fluid, pH 9 (Difco) and FA-positive bacteria were counted.

Cross reaction with the following bacterial strains verified selectivity of the method in the negative results obtained for Non-O1 (non-agglutinated) *Vibrio cholerae* NAG, IMBT03, *Vibrio alginolyticus*, *Escherichia coli* (isolates of IMTA Jiutepec), and *Enterobacter aerogenes* 4MBT2EO1.

Ciliate counts. The number of ciliates was determined by direct counts using plankton chambers and a Wild (Switzerland) inverted microscope. Samples were preserved by adding formalin (stabilised by methanol) up to 5% of the final concentration (Bloem et al. 1986).

Ciliate suspension feeding rate. For the measurement of ciliate suspension feeding rates, the protocol of the fluorescently labelled bacteria (FLB) technique of Sherr et al. (1987) was used. The stock cultures of *Vibrio cholerae* (C-12-CDC), *Salmonella typhi*, and *Shigella* sp. (both isolates of ENEP Iztacala) were preincubated as described above for *V. cholerae*.

Ciliate samples taken from microcosms were put into Erlenmeyer flasks and left for 10 min without disturbance. Then the homogenised FLB made from the 3 strains of bacteria were added to respective flasks to obtain roughly the same concentration constituting 5 to 15% of the direct bacterial numbers. Subsamples were taken after 5, 10 and 15 min from each flask and fixed with formalin (Bloem et al. 1986). Ciliates concentrated onto the membrane filter (black 0.8 µm pore-sized nitrocellulose membrane; Sartorius) were visualised via DAPI staining (Porter & Feig 1980) and per ciliate FLB uptake was evaluated in >50 individuals. Ciliate cell-specific volume clearance rate was derived based on uptake of FLB, the concentration of FLB in the mixture, and the incubation time (Sherr et al. 1987). Linearity of ingestion rate was checked comparing different times of incubation.

A comparison between uptakes upon FLB prepared from *Vibrio cholerae*, *Salmonella typhi* and *Shigella* sp. was used as an estimation of ciliate selectivity. A potential maximum grazing rate was derived from the maximum measured FLB uptake and from DC of bacteria. The first day values of FLB uptake and geometrical averages of bacterial and protozoan numbers were used for the integration of the assemblage grazing between 2 sampling points. Grazing rate upon *V. cholerae* was estimated in relation to FA and CFU numbers.

Growth/decline rate of microorganisms. The equation for calculating the specific growth rate based on bacteria or ciliate numbers was used (Legner 1980)

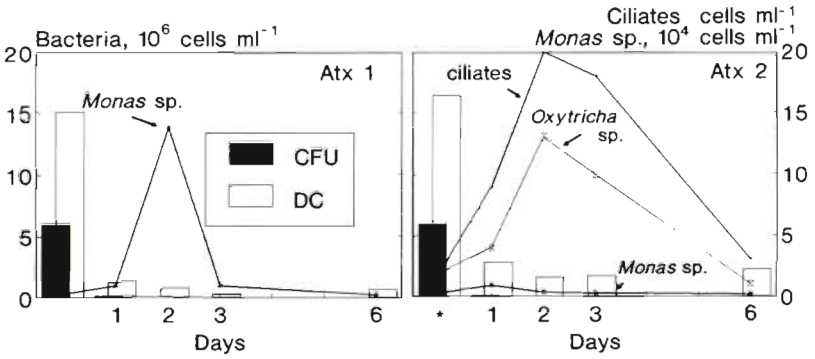

Fig. 2. Development of bacteria and protists in microcosms with Lake Atexcac water, Atx 1 and 2. Total bacteria numbers as DC (direct counts); culturable *Vibrio cholerae* numbers as CFU (colony forming units); numbers of a flagellate, *Monas* sp., a ciliate, *Oxytricha* sp., and total numbers of ciliates

assuming an exponential growth according to the equation: $\mu = (\ln N_t - \ln N_0)/t$ where μ = intrinsic rate of increase (d^{-1}); N_0 and N_t = cell abundances at the beginning and end (cells ml^{-1}); t = time (d).

RESULTS AND DISCUSSION

Selective methods for *Vibrio cholerae* quantification

Following the original Venkateswaran et al. (1989) method, pre-incubation of the filters on a tryptone broth-soaked pad was done for 6 h at 37°C. Comparing the results with those obtained using direct TCBS incubation, our results were within the range of possible error of the method (±10%). Hence, a pre-incubation was used routinely only for the first sample taken after inoculation with *Vibrio cholerae* and in samples taken after 5 d. Even the pre-incubation was not sufficient to obtain *V. cholerae* CFU numbers equal to the direct microscopical numbers in the samples taken during the first 2 or 3 h after inoculation of the experiments (cf. Singleton et al. 1982, Xu et al. 1984). At the end of the experiments, the number of bacteria culturable on TCBS but sucrose-negative (small colonies non-sufficiently changing the agar pH) increased by an order of magnitude. Although such colonies do not seem to be *V. cholerae*, several of those were identified as *V. cholerae* using biochemical tests (test kit API 20).

In concordance with published results, the FA method did not show reproducible results in the samples taken immediately after addition of *Vibrio cholerae* into the lake water (Xu et al. 1984). Thus, the first day *V. cholerae* growth rate was calculated based on DC of vibrios added. Other problems appeared when phosphate buffered saline was used for sample dilution and washing the membranes. Precipitation, disturbing bacterial fixation on membranes, appeared and particles showing typical phosphate fluorescence were observed. Thus, the samples had to be diluted with membrane-filtered lake water and washed using filtered tap water.

Persistence of *Vibrio cholerae* within lake assemblages

In the microcosm Atx 1 inoculated with 1.2 µm pore-sized membrane-filtered lake water, flagellates *Monas* sp. developed up to a concentration of 10^5 cells ml^{-1} (Fig. 2). CFU decreased rapidly; however, FLB ingestion by flagellates was not observed within the first 15 min, apparently due to low concentration of the tracer (experiment designed for the ciliates). Flocculation of the bacterial culture occurred, as is typical for bacteria under predation pressure of protists (Güde 1979, 1982, Macek 1989, Macek et al. 1993, Jürgens & Güde 1994). After 6 d of cultivation, a higher concentration of *Vibrio cholerae* (10^1 bacteria ml^{-1}) was observed compared to the ciliate inoculated microcosm Atx 2. Persistence of *V. cholerae* inside the bacterial flocs on one hand and competition with other bacteria on the other seemed to be 2 mechanisms controlling

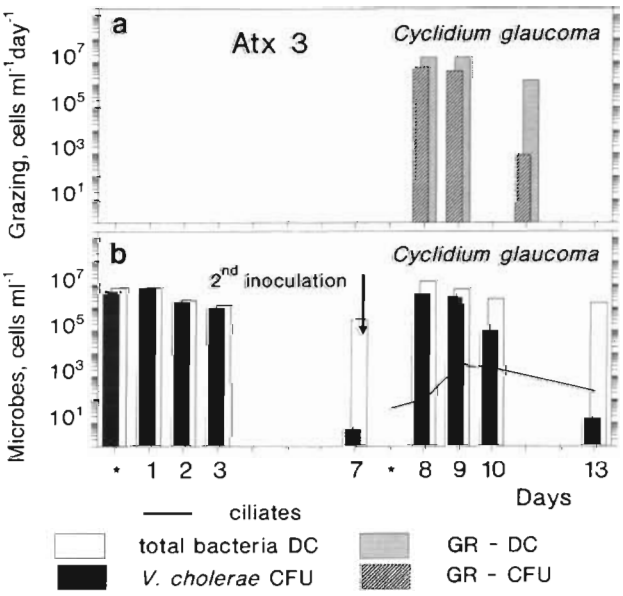

Fig. 3. Development of bacteria and a ciliate, *Cyclidium glaucoma*, in Atx 3. (a) Daily integrated grazing of protists derived from total numbers of bacteria (GR-DC) and from numbers of culturable *Vibrio cholerae* (GR-CFU). (b) Total bacterial numbers as DC, culturable *V. cholerae* numbers as CFU, and numbers of ciliates

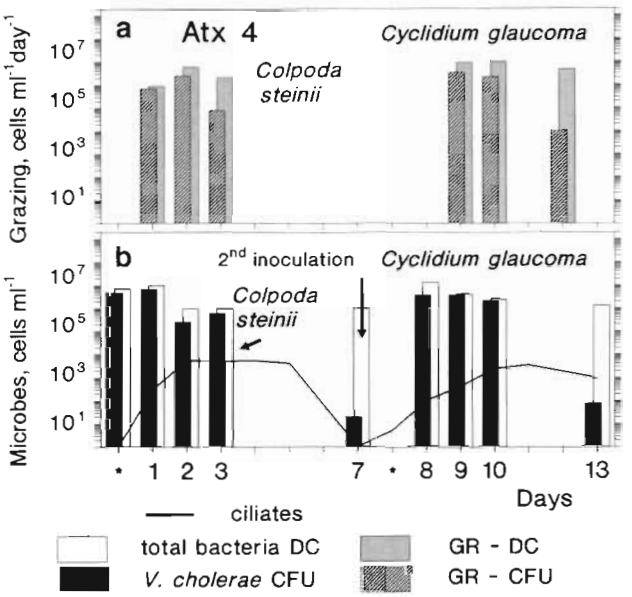

Fig. 4. Development of bacteria and ciliates *Colpoda steinii* and *Cyclidium glaucoma* in Atx 4. (a, b) As in Fig. 3

V. cholerae persistence (Güde 1979, 1982, Mallory et al. 1983, Macek 1989, Macek et al. 1993).

In Atx 2, a mixed-protist assemblage from Lake Atexcac was numerically dominated by a flagellate, *Monas* sp., and by a ciliate, *Oxytricha* sp. Among the ciliates of this assemblage, only *Cyclidium* sp. was recognised as bacterivorous. Thus, direct protist feeding on bacteria was at a low level.

In microcosms Atx 3 and 4 (Figs. 3 & 4), *Vibrio cholerae* persisted for 3 d and CFU decreased only by 1 order of magnitude. In Atx 4 with the ciliates *Colpoda steinii*, numbers of culturable *V. cholerae* decreased by nearly 2 orders of magnitude within 2 d (Fig. 4). Then, however, *C. steinii* encysted quantitatively, which is typical for this species (Proper & Garver 1966). We continued the experiment with these microcosms, adding new *V. cholerae* cells and inoculating *Cyclidium glaucoma*. Similar *C. glaucoma* development was observed, but the growth of *C. steinii* was not induced (cf. Figs. 3 & 4). Results were confirmed in microcosms Atx 5 and 6 (Fig. 5). On the other hand, in ciliate-inoculated Atx 6, *V. cholerae* CFU decreased only by half an order of magnitude within the first 3 d.

The same experiments were repeated with filtered water from the saline Lake Alchichica and the results were confirmed applying the FA method (Figs. 6 & 7). Although decrease in CFU typically appeared in both microcosms after 2 d of incubation, FA were of the same order of magnitude during the first 3 d (double the decrease in the ciliate-inoculated microcosms). By the sixth day, numbers of *Vibrio cholerae* CFU were under a detectable limit.

If pre-incubated, fast-growing *Vibrio cholerae* were used, total bacterial numbers in the ciliate-free microcosm rose until the third day (Alch 5 and 7; Fig. 7). Such DC increases were not observed in the ciliate microcosm and both FA-positive vibrios and other bacteria were suppressed to a greater extent (Alch 6 and 8; Fig 7).

Ciliate feeding upon *Vibrio cholerae*

Colpoda steinii, a ciliate typically found in soil and polluted water (Proper & Garver 1966), as well as the opportunistic ciliate *Cyclidium glaucoma*, frequently seen in pelagic environments (Sanders 1988, Šimek et al. 1994), ingested heat-killed stained *Vibrio cholerae* (FLB). Their suspension-feeding activity depended on both bacterial numbers and on bacteria assemblage composition.

Selectivity of *Colpoda steinii* suspension feeding (Table 2) was tested on a substrate-rich medium. The average clearance of 43 nl $cell^{-1}$ h^{-1} measured for *Vibrio cholerae* FLB (length 1.15 ± 0.36 µm, equivalent

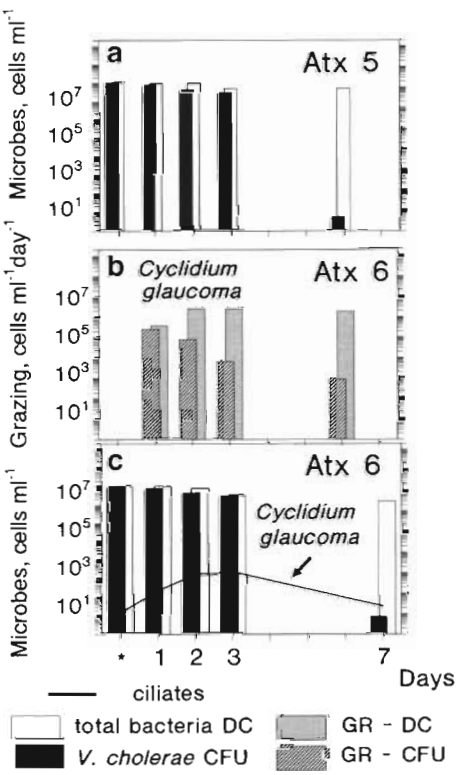

Fig. 5. Development of bacteria and ciliates in Atx 5 and 6. (a, c) Total bacteria numbers as DC; culturable *Vibrio cholerae* numbers as CFU; and numbers of ciliates *Cyclidium glaucoma* in parallel microcosms Atx 5 and 6. (b) Daily integrated grazing of protists in Atx 6, derived from total number of bacteria (GR-DC) and from numbers of culturable *V. cholerae* (GR-CFU)

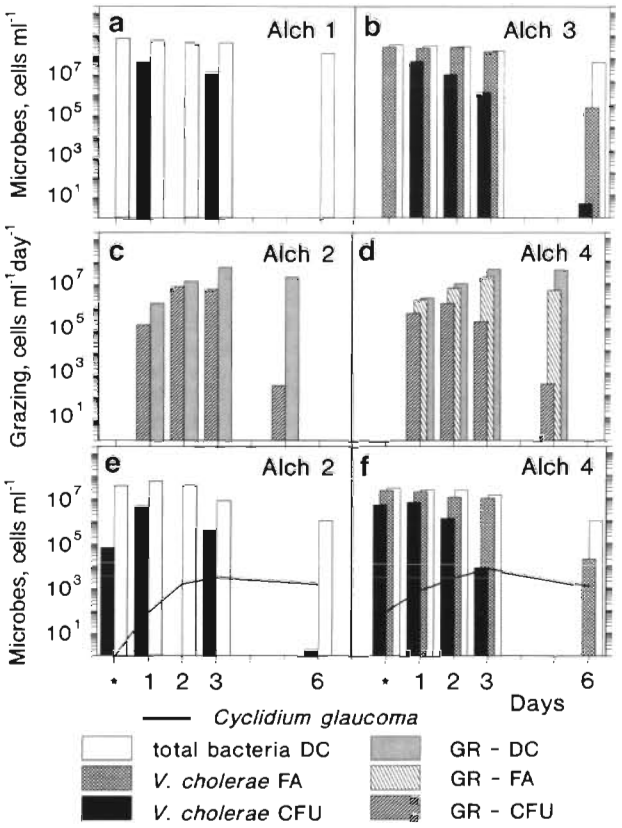

Fig. 6. Development of bacteria and ciliates in microcosms with Lake Alchichica water, Alch 1 to 4. (a, b, e, f) Development of total bacteria numbers as DC; *Vibrio cholerae* numbers as immunofluorescence positive (FA) and culturable cells as CFU; and ciliates *Cyclidium glaucoma* in parallel microcosms. (c, d) Daily integrated grazing of protists, derived from total number of bacteria (GR-DC), immunofluorescence positive *V. cholerae* (GR-FA), and culturable *V. cholerae* (GR-CFU)

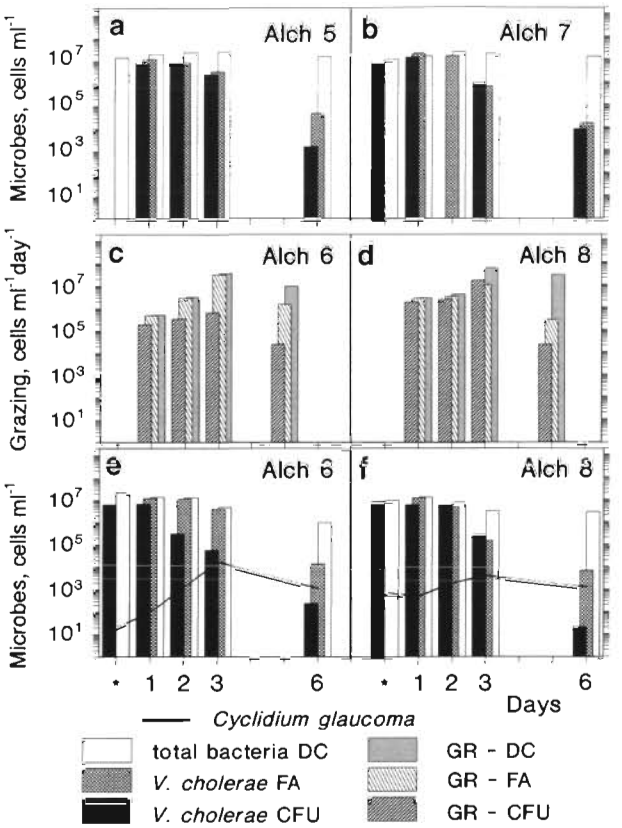

Fig. 7. Development of bacteria and ciliates in Alch 5 to 8. (a to f) As in Fig. 6

spherical diameter, ESD, 0.59 ± 0.08 µm) was similar to that found for a slightly larger *Salmonella typhi* (length 2.26 ± 0.52 µm, ESD 1.00 ± 0.16 µm). The larger cells of *Shigella* sp. (width 1.2 µm, length 2.73 ± 1.25 µm, ESD 1.40 ± 0.26 µm) were ingested at a lower rate. It has been shown that protists prefer prey within a certain size range (Chrzanowski & Šimek 1990, Šimek et al. 1994). In fact, the ciliates had stopped ingestion of *Shigella* sp. FLB after 5 min of incubation (maximum 4, on average 1.50 ± 1.40 ingested FLB ciliate^{-1}).

In microcosm Atx 4, with low bacterial concentrations, *Colpoda steinii* was not able to persist in an active form throughout the 1 wk experiment (cf. Berk et al. 1976), although the first days' clearance on *Vibrio cholerae* and *Salmonella typhi* were on average 40 and 50 nl cell^{-1} h^{-1}, respectively (differences were not significant). The clearance measured on the third day

Table 2. Growth and clearance rates of *Colpoda steinii* and *Cyclidium glaucoma* upon different fluorescently labelled bacteria (FLB)

Ciliate	Microcosm	μ_{cil} (d^{-1})	Clearance (nl cell^{-1} h^{-1}) upon FLB		
			Vibrio cholerae	*Salmonella typhi*	*Shigella* sp.
Colpoda steinii	Atx 4	3.4	43 ± 24	69 ± 26	1.5 ± 1.4
Cyclidium glaucoma	Atx 3	3.5	130 ± 39	30 ± 22	0.9 ± 0.8
	Atx 4	0.3	63 ± 19	32 ± 10	0.9 ± 0.7
	Atx 6	2.5	48 ± 33	–	–
	Atx 6 active[a]	–	100 ± 31	–	–

[a]Large and active ciliate subpopulation

decreased to only 20 nl cell^{-1} h^{-1}, which was insufficient to keep them growing. Maximum (first day) grazing of *C. steinii* upon *V. cholerae* (derived from CFU) reached 430 bacteria ciliate^{-1} h^{-1} (Table 3).

Feeding rates of *Cyclidium glaucoma*, tested in Atx 3 and 4, were apparently higher on *Vibrio cholerae* FLB (clearance up to 190 nl cell^{-1} h^{-1}) than on the 2 other FLB (Tables 2 & 3). The data indicate ciliate feeding selectivity (cf. Fenchel 1986, Sanders 1988). Also this ciliate was able to ingest the large *Shigella* sp. cells (Table 2) but only at a rate of 1 bacterium ciliate^{-1} during a 5 min incubation. Moreover, the number of FLB inside the ciliate did not change during the 15 min incubation, being 0.8 ± 0.6. Grazing rates on *V. cholerae* reached 500 cells cell^{-1} h^{-1}, comparable to data published for *Uronema nigricans* grown on *Vibrio* spp. by Berk et al. (1976), although they used higher bacterial concentrations. In other experiments, maximum grazing rates (Table 3) reached over 2000 bacteria cell^{-1} h^{-1}, showing *C. glaucoma* as a more efficient feeder under these bacterial concentrations (see Fenchel 1986).

The ability to ingest vibrios was never constant during culture development. During the first 2 d of the experiments, *Cyclidium glaucoma* did not ingest FLB at a constant rate during the first 15 min of incubation. The same phenomenon was observed also for uptake rates on *Shigella* sp. Moreover, in the microcosms Atx 6 or Alch 2 and 4 (Table 3), the ciliates did not ingest cells of typical *Vibrio cholerae* shape until the third day, when the maximum clearance rate of 130 to 160 nl cell^{-1} h^{-1} was measured. The maximum clearance coincided with a decrease of *V. cholerae* concentration measured as CFU, although FA was still high. This maximum could have been caused both by the ingestion of vibrios as an alternative prey along with other bacteria (Curds & Vandyke 1966, Mallory et al. 1983, Macek et al. 1993), and by changes of *V. cholerae* cell properties. Starving non-culturable *V. cholerae* should have a different cell composition, e.g. lower phospholipid content (Hood et al. 1986) responsible for the cell hydrophobicity, resulting in a ciliate discrimination among particles sieved (Fenchel 1986, Sanders 1988, Gurijala & Alexander 1990). Indigestibility seems to be the principal property of pathogenic bacteria (King et al. 1988, Gurijala & Alexander 1990).

At the end of all experiments, the ciliates were starving. According to different cell volume and different feeding activity it was possible to divide the population of *Cyclidium glaucoma* into 2 groups, active feeders and passive ones (Table 2). Such a splitting of the population could reflect different feeding patterns — additional grazing on the surfaces was hypothesised for larger, more active individuals, although they also feed on suspensions (Legner 1980, Macek et al. 1993).

The range of calculated *Cyclidium glaucoma* grazing rates (Table 3) was comparable with other data

Table 3. Cell-specific ciliate feeding rates upon *Vibrio cholerae* FLB

Microcosm	Day	μ (d^{-1})	Clearance (nl cell^{-1} h^{-1})	Grazing rate (cells cell^{-1} h^{-1})		
				CFU	FA	DC
Colpoda steinii						
Atx 4	1	3.4	65 ± 23	430	–	640
	2	3.5	19 ± 9	4.8	–	18
Cyclidium glaucoma						
Atx 3	9	3.5	130 ± 39	260	–	500
Atx 4	10	0.3	63 ± 19	13	–	150
Atx 6	1	2.5	48 ± 33	190	–	410
	2	1.8	30 ± 12	0.4	–	190
	3	0.2	47 ± 23	0.1	–	140
	6	−0.5	39 ± 32	0.0	–	60
Alch 2	1	2.8	29 ± 18	150	–	1700
	3	0.6	130 ± 41	58	–	1000
	6	−0.2	83 ± 18	0.0	–	84
Alch 4	1	2.9	20 ± 14	100	460	570
	2	1.3	18 ± 12	23	200	400
	3	1.0	160 ± 40	1.3	1600	2100
Alch 6	1	–	28 ± 18	180	330	380
	2	2.3	42 ± 30	13	480	520
	3	2.5	62 ± 33	3.6	240	280
Alch 8	1	−0.5	18 ± 19	110	220	230
	2	1.3	190 ± 82	1000	880	1400
	3	0.6	170 ± 71	49	26	550

obtained with this ciliate (Fenchel 1986, Sanders 1988) or with feeding of *Uronema* upon *Vibrio* sp. (Berk et al. 1976). Maximum cell-specific ciliate grazing rates of *V. cholerae* (derived from CFU) were frequently found only on the first day while potential maximum grazing (DC-based) did not reach high values. According to the FA data, maximum grazing rates were on the third day along with maximum rates based on DC.

Daily grazing of *Cyclidium glaucoma* was much higher than the daily decrease in DC except for e.g. Atx 6 with a very low concentration of bacteria other than vibrios. Population grazing reached a maximum on the second or third day after inoculation of *Vibrio cholerae* (Figs. 3 to 6). The maximum *V. cholerae* FLB uptake was found when a decrease of *V. cholerae* numbers was apparent (both CFU and FA). It is in concordance with previous findings that ciliates were not able to survive when feeding exclusively upon some growing human pathogens (Curds & Vandyke 1966, Dive et al. 1974, Mallory et al. 1983, Gurijala & Alexander 1990).

General discussion

The calculated maximum CFU growth/decline rates of *Vibrio cholerae* were approximately the same both in protist-free and protist-inoculated microcosms (even 5 d^{-1}). However, comparing the experimental microcosms, the shift of an accelerated induction of a viable but non-culturable state of *V. cholerae* in the protist-containing ones was confirmed. The maximum decrease of vibrios was related to the rapid development of the ciliates but, in protist-free microcosms, the maximum shifted and appeared to be connected with growth of other bacteria in the microcosm (measured as DC).

Changes of *Vibrio cholerae* to a non-culturable form should be induced by starvation (Brayton et al. 1987, McKay 1992). Moreover, due to high experimental temperature, short persistence of *V. cholerae* even in protist-free microcosms was not surprising (Singleton et al. 1982, Xu et al. 1984, Guthrie & Cofie 1991), however, disappearance was very quick compared with enterobacteria (Mallory et al. 1983, González et al. 1992). The longest persistence of *V. cholerae* as CFU (Atx 5 and 6) was observed, in accordance with the literature (Singleton et al. 1982), along with a low concentration of other bacteria.

The maximum ciliate grazing rates were found when the majority of vibrios lost TCBS culturability. Grazing rates calculated from numbers of culturable vibrios (CFU) were roughly equal to a decrease in CFU (Figs. 6 & 7). Since a decrease of CFU was not related directly to a decrease of FA, grazing rates calculated from FA were higher by an order of magnitude. The difference between the *Vibrio cholerae* grazing (calculated from FA) and the apparent decrease of *V. cholerae* (production of non-culturable cells) could be explained as follows: along with direct predation of the ciliates, regeneration of the substrates by protist digestion stimulates the growth of competitive bacteria (Mallory et al. 1983, Brayton et al. 1987, Sambanis et al. 1987, Sanders 1988, Macek et al. 1993) and/or enhances their metabolism (Levrat et al. 1992), suppressing *V. cholerae*. Also chemical cues produced by protists may affect culturability, although this effect was not proved with *Vibrio* sp. and filtrate of *Uronema* feeding upon it (cf. Berk et al. 1976, Levrat et al. 1992).

On the other hand, overestimation of *Vibrio cholerae* ingestion using the FLB method could result due to a qualitative difference between FLB and living *V. cholerae*. It has been shown that living and moving bacteria are preferred over heat-killed FLB by flagellates (González et al. 1993). However, preparation of FLB, when vibrios were heat-denatured and washed several times, could produce such changes of their antigen properties (Singleton et al. 1982, Xu et al. 1984) that FLB might be preferred to living, toxin-producing *V. cholerae* of the same size.

Ciliates were not able to decrease the *Vibrio cholerae* concentration below a threshold concentration (mostly 10^4 bacteria ml^{-1}). The formation of chains composed of several (5 to 8) cells of *V. cholerae* and the formation of bacterial flocs was observed. This formation of non-ingestible agglomerates seemed to be connected with the bacterial defence against protistan attacks (Güde 1979, Jürgens & Güde 1994) that could support a persistence of different bacteria in the same floc matrix (Güde 1982). In accordance with the theory, a longer persistence of vibrios in microcosms inoculated by protists was seemingly promoted by the occurrence of starved-bacteria flocs with a high content of dead organic matter. In substrate-rich microcosms (Alch 7 and 8) inoculated by a young, fast-growing *V. cholerae* culture containing non-exhausted substrates, vibrios were eliminated even inside the flocs. This was probably due to stronger competition between *V. cholerae* and fast-growing bacteria (Güde 1979, 1982, Macek 1989, Macek et al. 1993).

In summary, the following conclusions were drawn from this study:

(1) Maximum feeding rates of ciliates *Colpoda steinii* and *Cyclidium glaucoma* upon *Vibrio cholerae* cells were 600 and 2000 bacteria $cell^{-1}$ h^{-1}, respectively.

(2) Accelerated decline in culturable *Vibrio cholerae* counts was observed in ciliate-containing microcosms earlier than in ciliate-free ones. A decrease in CFU was not directly related to a decrease in antiserum-positive vibrios (FA) that reached its maximum along with a maximum decrease in total bacteria concentration.

(3) Ciliates were not able to directly reduce *Vibrio cholerae* numbers during the first days following their inoculation (majority of TCBS-culturable cells). The ciliate grazing rates calculated from FA-positive but non-culturable *V. cholerae* were related to a calculated *V. cholerae* population decrease.

(4) We hypothesised that *Vibrio cholerae* was changing into a non-culturable form being affected by the ciliate predation.

(5) The results confirm the hypothesis that *Vibrio cholerae* may enter a microbial loop within the plankton food web by being ingested by phagotrophic protists.

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