Growth of *Vibrio cholerae* O1 Ogawa Etor in freshwater

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Growth of *Vibrio cholerae* O1 Ogawa Etor was studied with a growth assay in which autoclaved and filtered (0.22 µm) freshwater was inoculated at low cell density (5×10^3 cells ml⁻¹) and proliferation was followed with flow cytometry. Against the common view, *V. cholerae* was able to grow extensively in different kinds of freshwater. The bacterium multiplied in river water, lake water and effluent of a wastewater treatment plant up to a cell density of 1.55×10^6 cells ml⁻¹. In these samples, apparent assimilable organic carbon (AOC_{app}) concentrations ranged from 52 up to 800 µg l⁻¹ and the results demonstrate a positive trend between the AOC_{app} concentration and final cell concentration, suggesting that AOC was a key parameter governing growth of *V. cholerae*. No growth was observed in waters (tap and bottled drinking water) containing less than approximately 60 µg AOC_{app} l⁻¹. When pure cultures of *V. cholerae* were grown on identical lake water at different temperatures (20, 25 and 30 °C) the maximum specific growth rates ($\mu_{\max}$) achieved were 0.22 h⁻¹, 0.32 h⁻¹ and 0.45 h⁻¹, respectively. In addition, growth was characterized in lake water samples amended with different concentrations of NaCl. The highest $\mu_{\max}$ of *V. cholerae* was recorded at moderate salinity levels (5 g NaCl l⁻¹, $\mu_{\max}=0.84$ h⁻¹), whereas at 30 g NaCl l⁻¹ ($\mu_{\max}=0.30$ h⁻¹) or 0 g NaCl l⁻¹ ($\mu_{\max}=0.40$ h⁻¹) specific growth rates were significantly reduced. In the water tested here, $\mu_{\max}$ of *V. cholerae* was always around 50 % of that exhibited by a freshwater community of indigenous bacteria enriched from the water sampling site. Direct batch competition experiments between *V. cholerae* and the lake water bacterial community were performed at different temperatures in which *V. cholerae* was enumerated in the total community using fluorescent-surface antibodies. In all cases *V. cholerae* was able to grow and constituted around 10 % of the final total cell concentration of the community. No significant effect of temperature was observed on the outcome of the competition. Mathematical modelling of the competition at the different temperatures based on the calculated $\mu_{\max}$ values confirmed these experimental observations. The results demonstrate that *V. cholerae* is not only able to survive, but also able to grow in freshwater samples. In these experiments the bacterium was able to use a large fraction (12–62 %) of the AOC_{app} available to the bacterial AOC-test community, indicating that *V. cholerae* has the ability to gain access to the substrates present in freshwater even in competition with an autochthonous bacterial lake water consortium.

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INTRODUCTION

Vibrio cholerae is able to adapt to both copiotrophic and oligotrophic environments: on the one hand, *V. cholerae* can promote disease by extensive growth in the human intestine; on the other hand it is also found amongst the

autochthonous microbial flora of natural aquatic systems (West & Lee, 1982). In natural waters the bacterium can be present in both a free-living state (Worden *et al.*, 2006) or attached to copepods, zooplankters and algae (Lipp *et al.*, 2002; Reidl & Klose, 2002).

Previous laboratory studies have emphasized the influence of salinity on growth and, hence, the bacterium was considered to be naturally abundant in waters characterized by moderate or high salinity (Miller *et al.*, 1982; Singleton *et al.*, 1982a, b). Accordingly, *V. cholerae* O1 was

Abbreviations: AOC, assimilable organic carbon; AOC_{app}, apparent assimilable organic carbon; DOC, dissolved organic carbon.

Two supplementary figures are available with the online version of this paper.

frequently detected in estuary and coastal waters, and growth in sterile-filtered natural seawater under defined laboratory conditions has been reported (Binsztein *et al.*, 2004; Louis *et al.*, 2003; Mourino-Perez *et al.*, 2003; Worden *et al.*, 2006). In addition, laboratory studies using artificial seawater demonstrated growth of *V. cholerae* O1 down to a salinity of 5 g l⁻¹ (Singleton *et al.*, 1982a, b). However, *V. cholerae* O1 has been detected frequently in freshwater as well (Yamai *et al.*, 1996; Borroto, 1997), even in the absence of faecal index organisms (Bourke *et al.*, 1986; Jesudason *et al.*, 2000). Although freshwater systems are considered to be an environmental reservoir for *V. cholerae* (Shapiro *et al.*, 1999), there is, as far as we are aware, no information available demonstrating the growth of this bacterium in freshwater.

In addition to salinity, temperature is reported to be a second important parameter controlling growth of *V. cholerae* in estuarine environments. Laboratory experiments showed a positive correlation between temperature and the metabolic activity of *V. cholerae* O1 in defined sea salt solution in a temperature range from 10 to 25 °C (Singleton *et al.*, 1982a). Furthermore, a more frequent detection of *V. cholerae* O1 in estuary waters and a higher morbidity rate among people in Bangladesh was shown to be associated with increasing temperatures (Louis *et al.*, 2003; Rodo *et al.*, 2002).

As well as salinity and temperature, nutrients are considered to control growth of *V. cholerae* in aquatic systems (Colwell, 1996). In natural waters a key parameter governing microbial growth is assimilable organic carbon (AOC) (Van der Kooij, 1992). AOC is the small fraction (0.1–10 %) of the dissolved organic carbon, which is readily available for consumption by micro-organisms, resulting in cell proliferation (Hammes & Egli, 2005). AOC is composed of low molecular mass compounds such as amino acids, sugars or organic acids (Egli, 1995; Münster, 1993). It is constantly produced through photosynthesis, viral lysis of bacterial cells, and biological and chemical hydrolysis of natural organic matter, and it is continuously consumed by the microbial flora. Residual AOC concentrations from 1 µg l⁻¹ up to 2000 µg l⁻¹ can typically be observed in natural waters (LeChevallier *et al.*, 1991). AOC has been directly linked to bacterial regrowth in drinking-water distribution systems (Van der Kooij, 1992). A relationship between the occurrence of coliform bacteria and AOC concentrations has been also shown in a full-scale study of drinking-water systems (LeChevallier *et al.*, 1996). As most aquatic environments are usually oligotrophic and carbon/energy-limited (Morita, 1997), bacteria are continuously competing for AOC constituents. AOC is therefore an important factor in determining the microbial community composition in aquatic ecosystems.

Contaminated drinking water is one of the important transmission routes of the enteric pathogen *V. cholerae* and, therefore, data on the growth characteristics of this bacterium in freshwater are essential. The better the

behaviour of *V. cholerae* in the natural environment is understood, the better hygienic measures can be taken in order to prevent the spread of this disease. For this reason, the growth of *V. cholerae* in freshwater was investigated and the influence of several selected environmental factors (salinity, AOC concentration and temperature) was studied. In addition, the growth of *V. cholerae* on nutrients in freshwater in competition with an autochthonous lake water bacterial community was investigated.

METHODS

Strains. *Vibrio cholerae* O1 (Biovar Eltor, serotype Ogawa, strain Nent 720-95) and *Salmonella typhimurium* ATCC 14028 were used in this study.

Preparation of carbon-free materials. Carbon-free glassware (bottles and vials) was prepared as described by Greenberg *et al.* (1993). In short: all glassware was first washed with common detergent, and thereafter rinsed three times with deionized water. These were then submerged overnight in 0.2 M HCl and subsequently rinsed with deionized water again and finally air-dried. The bottles and vials were heated in a muffle furnace at 550 °C for at least 6 h. Teflon-coated screw caps for the glassware were similarly washed and treated with acid. Caps were thereafter soaked in a 10 % sodium persulphate solution at 60 °C for at least 1 h, rinsed three times with deionized water and finally air-dried.

Pre-cultivation of bacteria and growth in natural water. *V. cholerae* and *S. typhimurium* were cultivated in carbon-limited minimal medium (Ihssen & Egli, 2004) containing 10 mg glucose l⁻¹. Bacterial cells were sampled after 4 days of incubation during late stationary phase and directly inoculated to an initial concentration of 5 × 10³ cells ml⁻¹ into carbon-free glass vials (40 ml) that contained autoclaved and 0.22 µm-filtered freshwater (30 ml) originating from different sources. These vials were incubated at 30 °C and the total cell concentration was determined directly after inoculation (time 0) and at time 96 h when the stationary phase had been reached.

Preparation of natural water. Natural water was sampled with a 500 ml Duran flask (Schott) and autoclaved within 30 min. Subsequently, aliquots of 30 ml were filtered using a 50 ml Luer-Lok Syringe (BD) through a 0.22 µm Millex syringe filter (Millipore) into 40 ml carbon-free glass vials capped with a carbon-free PTFE/silicone septa cap (Supelco). It should be pointed out that autoclaving (20 min at 121 °C) was used as the method for sterilization, since it has been shown that filtration processes allow the passage of a fraction of bacteria present in freshwater (Hahn, 2004). After sterilization, samples were filtered in order to remove crystalline particles formed during autoclaving, which can interfere with the flow cytometric analysis. Different types of freshwater were used: tap water (community of Dübendorf, Switzerland), river water (Glatt River, Dübendorf, Switzerland), lake water (Lake Greifensee, Switzerland) and wastewater treatment plant effluent (WWTP; Glattbrugg, Switzerland). In addition, a dilution series of natural water (0, 10, 25, 50, 75 and 100 % of Lake Greifensee water) was prepared using filtered (0.22 µm Millex filters) mineral water (Evian) as the diluent. These dilutions were subsequently autoclaved and again 0.22 µm-filtered.

AOC determination. AOC was determined with a batch growth assay as described previously (Hammes *et al.*, 2006; Hammes & Egli, 2005). In short: autoclaved and filtered water samples (30 ml) were inoculated with 10 µl (1 × 10⁴ cells ml⁻¹ final concentration) of a

bacterial AOC-test community. These suspensions were then incubated at 30 °C for 96 h (until stationary phase was reached) and the resulting growth was measured with flow cytometry. The AOC inoculum originated from river water (Chriesbach River, Dübendorf, Switzerland) and was prepared as described by Hammes & Egli (2005). The same bacterial community was used for all AOC determinations throughout the present study. As standard quality control prior to use, the performance of this inoculum was compared to bacterial AOC-test communities used in previous studies in our group (Hammes *et al.*, 2006), using different types of natural surface waters as media. A difference of less than $\pm 10\%$ in the mean AOC values was deemed acceptable for use. AOC ($\mu\text{g l}^{-1}$) is estimated from cell concentrations (cells l^{-1}) using a theoretical conversion factor (Hammes *et al.*, 2006; Van der Kooij, 2002); we will henceforth use the term 'apparent AOC' (AOC_{app}) to express the data obtained with this method (equation 1). All assays were performed in triplicate. The detection limit of the method was 10–20 $\mu\text{g AOC}_{\text{app l}^{-1}}$ and the SEM was $\pm 10\%$.

$$\text{AOC}_{\text{app}} [(\mu\text{g C}) \text{ l}^{-1}] = \frac{[(\text{net grown cells}) \text{ l}^{-1}]}{[1 \times 10^7 \text{ cells } (\mu\text{g C}^{-1})]} \quad (1)$$

Enumeration of total cell concentration. Absolute cell counting was performed with flow cytometry. Ten microlitres of SYBRgreen (Molecular Probes), 100 $\times$ diluted in dimethylsulfoxide (Fluka Chemie AG), was added to 1 ml of a bacterial suspension and incubated in the dark at room temperature for 15 min before analysis. All samples were measured on a PASIII flow cytometer (Partec) equipped with a 25 mW argon laser emitting at a fixed wavelength of 488 nm and volumetric counting hardware. Green fluorescence signals were collected at 520 nm. The detection limit was 1000 cells ml^{-1} with an SEM of $\pm 5\%$.

Specific detection of *V. cholerae* O1 by flow cytometry with antibodies. A new method for specific detection of *V. cholerae* O1 with immunostaining was developed. Samples (1 ml) collected from growth experiments were UVC-irradiated in a collimated beam apparatus (Metanor AG) with a fluence of 96 J m^{-2} to arrest growth. Then the sample was transferred into a flow cytometry vial (Sarstedt) and 10 μl of the *V. cholerae* O1 DFA reagent (a fluorescent surface antibody) was added (New Horizons Diagnostics Corporation). The bacteria/antibody suspension was incubated for 30 min at 35 °C to let the antibody pre-adsorb to the bacterial surface. Subsequently, suspensions were further incubated for 12–17 h at room temperature in the dark to obtain a strong fluorescent signal. Finally, the concentration of immunostained *V. cholerae* O1 was determined by flow cytometry (as described above). To evaluate the staining efficiency, autoclaved and filtered freshwater samples were spiked with different concentrations of *V. cholerae* in the range of 10^3 – 10^5 cells ml^{-1} and the above procedure was applied immediately. Results were compared with cell counts obtained flow cytometrically with SYBRgreen staining. A linear correlation ($R^2=0.99$) was observed over the whole range of cell concentrations tested. As a negative control, a sample (10^6 cells ml^{-1}) of the lake water bacterial community used for competition experiments was treated in exactly the same manner with the fluorescent antibody. No fluorescence was detected and thus it can be concluded that significant cross-reaction of the antibody with the lake water bacterial community did not occur. Note that staining efficiency of all immunostained samples was evaluated with epifluorescence microscopy as well.

Growth of *V. cholerae* at different salt concentrations. For growth kinetic experiments at different salt concentrations, different volumes of an autoclaved 25% NaCl stock solution were added to autoclaved and 0.22 μm -filtered freshwater samples of Lake Greifensee (Switzerland) to achieve the following final concentrations of NaCl: 0, 1, 5, 10, 20 and 30 g l^{-1} . The NaCl-amended water was

subsequently inoculated with stationary-phase *V. cholerae* bacteria at an initial concentration of about 5×10^3 cells ml^{-1} and incubated at 30 °C. Cells of *V. cholerae* for inoculation were pre-grown in autoclaved and 0.22 μm -filtered freshwater of Lake Greifensee (Switzerland) amended with the same NaCl concentration as used for each specific experiment. The growth of the indigenous lake water bacterial community in autoclaved and 0.22 μm -filtered freshwater was recorded by removing 1 ml samples at regular intervals and total cell concentration was measured as described above. All assays were performed in triplicate samples. The specific growth rate (μ) based on cell concentration increase was determined as follows:

$$\mu = [\ln(N_t) - \ln(N_0)] / \Delta t \quad (2)$$

where N_t and N_0 are the cell concentrations measured at two subsequent time points and Δt is the expired time interval between these points. We have measured an increase in cell concentration and not in biomass; thus, growth could also be described using the cellular division rate (k), defined as the number of divisions per unit time. Since k is not often used and values differ from that of μ ($\mu = \ln 2 \times k$), we decided to use the specific growth rate (equation 2) throughout the whole study.

Competition experiments. For these experiments a lake water bacterial community of natural origin and *V. cholerae* O1 grown on autoclaved and 0.22 μm -filtered lake water were used. The lake water bacterial community was sampled from the surface water (4.1 mg l^{-1} DOC; 22 °C, pH 8.3) of the eutrophic Lake Greifensee (Switzerland) and pre-cultivated as follows: 30 ml autoclaved and 0.22 μm -filtered lake water was inoculated with 100 μl untreated lake water from the same original sample. Suspensions were incubated at 20, 25 and 30 °C, respectively, for 1 week and subsequently stored at 4 °C until further use. Culture density of the community was monitored with flow cytometry (see above).

Carbon-free 50 ml glass vials containing 30 ml autoclaved and 0.22 μm -filtered freshwater from Lake Greifensee were inoculated with either *V. cholerae* or the lake water bacterial community at an initial concentration of about 5×10^3 cells ml^{-1} , each. The cells used as an inoculum were pre-cultured on the same lake water at 30 °C and growth was monitored by recording the total cell concentration flow cytometrically at regular intervals. Cells were then sampled in the exponential-growth phase and directly inoculated into competition assays at 5×10^3 cells ml^{-1} of each partner (thus 10^4 cells ml^{-1} initial total cell concentration). Assays were incubated at 30 °C and performed in triplicate. Samples of 2 ml were collected at different time points throughout the competition experiment. Of these samples 1 ml was used for determination of the total cell concentration and 1 ml for immunological determination of the cell concentration of *V. cholerae*.

For competition experiments performed at different temperatures, the above pre-cultivation was performed at the relevant temperatures. The competition assays were started as described above. Samples were incubated at 20, 25 and 30 °C, respectively, for 48 h, at which time the stationary phase had been reached. Subsequently, final concentrations of total cells and of *V. cholerae*, respectively, were determined as described above.

In addition, separate vials, containing the same autoclaved and 0.22 μm -filtered lake water as used for the competition experiments, were inoculated with exponential-phase cells from the pre-cultures of either *V. cholerae* or the lake water bacterial community at an initial concentration of 5×10^3 cells ml^{-1} . The vials were then incubated at 20, 25 and 30 °C, respectively. Samples were taken at different time points from batch cultures and subsequently total cell concentrations were measured flow cytometrically. Specific growth rates (μ) were determined as described above (equation 2).

Relative cell number yield. The relative cell number yield for *V. cholerae*, $Y_{Vd/AOC}$, for growth on identical freshwater AOC is defined in equation 3. The total concentration of cells formed in freshwater samples was determined for *V. cholerae* and the bacterial AOC-test community in separate assays (as described above).

$$Y_{Vd/AOCapp} = \frac{[(\text{cells formed of } V. \text{ cholerae}) \text{ ml}^{-1}]}{[(\text{cells formed of a bacterial AOC-test community}) \text{ ml}^{-1}]} \quad (3)$$

Simulation software. The simulation of growth and parameter estimation was carried out using AQUASIM software (Reichert, 1998). AQUASIM allows estimation of parameters by fitting predicted model data to experimental results. This feature was used to estimate kinetic parameters, which resulted in the best fit of simulations (s_{pred}) and measurements (s_{obs}). The program estimated the best fit by minimizing χ^2 values ($\chi^2 = \sum [(s_{obs} - s_{pred})/\sigma]^2$). For the estimation the standard deviation (σ) was set to 10% (if not determined experimentally).

RESULTS

Growth of *V. cholerae* in different types of freshwater

Stationary-phase cells of *V. cholerae* were inoculated into autoclaved and 0.22 μm -filtered mineral, tap, river, lake, and effluent water from a wastewater treatment plant in order to investigate its growth potential in different types of freshwater. Total growth was measured after 96 h by enumerating total cell concentration with flow cytometry. With the exception of mineral and tap water, *V. cholerae* showed extensive growth in all the freshwater samples tested (reaching final cell concentrations from $2.9 \times 10^5 \text{ ml}^{-1}$ to $1.55 \times 10^6 \text{ ml}^{-1}$). The growth of *S. typhimurium* was also tested in the same waters. In contrast to *V. cholerae*, *S. typhimurium* was unable to grow in any of the natural waters tested.

Effect of salinity on the growth of *V. cholerae*

When *V. cholerae* cells were inoculated into autoclaved and 0.22 μm -filtered lake water amended with different concentrations of NaCl, exponential growth of *V. cholerae* was observed after a short lag phase of about 2–4 h (Fig. 1a). The growth pattern of *V. cholerae* in freshwater followed the typical pattern observed for pure and mixed microbial cultures growing on complex media, consisting of an initial exponential growth phase at a constant μ , followed by a continuously decreasing specific growth rate until stationary phase is reached. A considerable fraction of the cells was formed in the exponential growth phase (16–80%), where cells exhibited a constant μ . In the batches amended with NaCl, *V. cholerae* occasionally showed a prolonged decelerating phase. In Fig. 2, the maximum specific growth rates (μ_{max}) exhibited in the initial growth phase and the final cell concentration formed by *V. cholerae* are visualized as a function of NaCl concentration. The highest μ_{max} was achieved at moderate salinity levels (μ_{max} at 5 g NaCl l^{-1} was as high as that of the lake water bacterial community in

Fig. 1. Selected growth curves of *V. cholerae* cultivated in autoclaved and 0.22 μm -filtered freshwater. (a) Growth of *V. cholerae* in freshwater amended with different concentrations of NaCl (g l^{-1}): 0 ($\square$); 5 (Δ); 30 ($\bullet$) g NaCl l^{-1} . For comparison, growth for the lake water bacterial community at 0 g NaCl l^{-1} is shown ($\times$). (b) Growth of *V. cholerae* in lake water at different temperatures ($^{\circ}\text{C}$): 20 ($\bullet$), 25 (Δ), 30 ($\blacksquare$). For comparison, the growth curve for the lake water bacterial community at 30 $^{\circ}\text{C}$ is shown as well ($\times$). The error bars indicate the standard deviation for triplicate samples. For the whole set of data see the supplementary material available with the online version of this paper.

freshwater), whereas at higher or lower salt concentrations, μ_{max} was significantly reduced (Fig. 2). Furthermore, the maximum cell concentrations formed by *V. cholerae* followed a similar pattern as a function of the salt concentration as μ_{max} , with the highest cell concentration reached at moderate salinity (Fig. 2).

Relationship between AOC_{app} and growth of *V. cholerae*

Growth can be affected by both the quality and quantity of nutrients. To assess this we compared *V. cholerae* growth in a dilution series of lake water (AOC quantity only varied) and in water from different sources (where both AOC quantity and quality vary). To assess the effect of AOC quantity, water from Lake Greifensee was diluted with a water sample that did not support growth of *V. cholerae* (Evian, 52 μg AOC_{app} l^{-1}). This resulted in a dilution series that ranged from approx. 52 to 300 μg AOC_{app} l^{-1} . A linear correlation ($r^2=0.98$) between AOC_{app} concentration originating from Lake Greifensee and final cell concentration of *V. cholerae* was observed (Fig. 3a).

In a subsequent experiment, water from different sources was sampled, the AOC_{app} concentration was determined

Fig. 2. Growth of *V. cholerae* in autoclaved and 0.22 µm-filtered river water amended with different concentrations of NaCl (g l⁻¹). Maximum specific growth rates (h⁻¹) of *V. cholerae* (●) and the maximum cell concentration of *V. cholerae* (△) reached as a function of the NaCl concentration are shown. For comparison $\mu_{\max}$ (■) and the final cell concentration (□) for the lake water bacterial community at 0 g NaCl l⁻¹ in the same water are shown as well. The maximum specific growth rate was determined from the linear part of the exponential growth curves shown in Fig. 1 (and the supplementary data).

and the growth of *V. cholerae* was examined (Fig. 3b). Here also, a positive trend between AOC_{app} concentration and the total growth of *V. cholerae* was observed, although the correlation was much lower ($r^2=0.36$). Nevertheless, with increasing AOC_{app} concentrations, higher final cell concentrations of *V. cholerae* were formed. The cell number yields of *V. cholerae* in relation to that of the bacterial AOC-test community (equation 2) were rather variable, ranging from $Y_{Vc/AOC_{app}}$ 0.12 to 0.62 with a mean of 0.28. Waters containing very low amounts of AOC_{app} (tap water with 44 µg l⁻¹, and bottled drinking water with 52 µg l⁻¹) did not support growth of *V. cholerae*.

Competition of *V. cholerae* with a lake water bacterial community

The ability of *V. cholerae* to compete in batch culture with a lake water bacterial community was tested in autoclaved and filtered lake water at 30 °C (Fig. 4). The results show that *V. cholerae* was able to use a part of the available substrates for growth and could even grow when competing with the indigenous lake water bacterial community (starting from a similar initial concentration of 4.13×10^3 *V. cholerae* cells ml⁻¹ and 2.84×10^3 cells ml⁻¹ of a lake water bacterial community, respectively). A simultaneous exponential increase in the concentration of cells was observed for both *V. cholerae* and total bacterial cells. In these experiments, a lag phase was successfully avoided by pre-culturing the two inocula in the same freshwater sample as used also in the competition experiment and by inoculating cells from the exponential-growth phase. The mean final cell concentration of *V. cholerae* after 22 h (stationary phase) was $9.73 \times 10^4 \pm 1.31 \times 10^4$ cells ml⁻¹,

Fig. 3. Correlation between AOC_{app} concentration and the final cell concentration of *V. cholerae* reached in the stationary phase of batch cultures. The final cell yield of *V. cholerae* is dependent on both the AOC concentration and the composition or quality of the AOC present in a water body. (a) Correlation between growth of *V. cholerae* (●) and different concentrations of AOC_{app} of the same quality. Autoclaved, sterile-filtered lake water was used. The concentration of AOC_{app} resulting from the diluent (bottled mineral water) was subtracted in the individual dilutions, indicated on the x-axis. It is therefore not considered in the linear regression analysis ($r^2=0.98$). (b) Correlation between AOC_{app} concentration and growth of *V. cholerae* on autoclaved sterile-filtered freshwater from different sources (i.e. with AOC of different quality). The different water samples [tap water Eawag, Dübendorf (■), bottled drinking water (□), river water (●), Lake Greifensee water (◇) and effluent of a sewage treatment plant (▲)] were collected on different days. Error bars indicate the standard deviation for triplicate samples. AOC_{app} concentration was determined according to Hammes & Egli (2005) with a standard bacterial AOC-test community as described in Methods.

which represents 9.7 % of the total bacterial concentration ($1 \times 10^6 \pm 3.50 \times 10^4$ cells ml⁻¹).

To test whether or not it is possible to predict the outcome of the competition with a simple growth model, the individual maximum specific growth rates of *V. cholerae* and of the lake water bacterial community were determined in a separate experiment during growth in sterilized, identical freshwater samples and were found to be 0.34 h⁻¹ and 0.50 h⁻¹ for *V. cholerae* and the lake water bacterial community, respectively. It was possible to simulate the population dynamics by applying the simplest growth model ($\mu=\mu_{\max}$) using the previously determined $\mu_{\max}$ of the pure cultures over the whole batch experiment until all the available nutrients were used up (Fig. 4). This is a strong hint that this is a case of 'pure and simple' competition for nutrients and that no other interaction was taking place between *V. cholerae* and the lake water bacterial community.

Fig. 4. Growth of *V. cholerae* in autoclaved and 0.22 µm-filtered lake water in competition with a lake water bacterial community at 30 °C in batch culture. Experimental data of *V. cholerae* (●), total counts (▲) and mathematical simulations (dashed lines) using $\mu_{\max Vc}=0.34 \text{ h}^{-1}$ and $\mu_{\max LWBC}=0.50 \text{ h}^{-1}$. Error bars indicate the standard deviations for triplicate samples.

Effect of temperature on growth of *V. cholerae* and competition with the lake water bacterial community

To investigate the effect of temperature on growth and competition, similar experiments were performed at 20, 25 and 30 °C. First, batches of autoclaved and 0.22 µm-filtered lake water were inoculated separately with either a lake water bacterial community or *V. cholerae*, respectively, and $\mu_{\max}$ was determined. Selected corresponding growth curves are presented in Fig. 1(b) and the respective values for $\mu_{\max}$ are listed in Table 1. In all these experiments an extended phase of exponential growth at $\mu_{\max}$ was observed for both *V. cholerae* and the lake water bacterial community, with only 50 % of the total cells formed in the second decelerating phase of growth.

The fact that the ratio of the maximum specific growth rates of *V. cholerae* and the lake water bacterial community (LWBC), i.e. $\mu_{\max Vc}/\mu_{\max LWBC}$, was virtually the same at all temperatures suggests little advantage or disadvantage of *V. cholerae* to compete for nutrients with the lake water bacterial community within the temperature range tested.

In addition, competition experiments were performed in the same sterile freshwater samples at 20 °C, 25 °C and 30 °C. As before, equal cell concentrations were inoculated and the final concentrations of *V. cholerae* and total bacterial cells were enumerated in the stationary phase after 48 h when all substrates had been consumed (Table 1). In all experiments *V. cholerae* proliferated from initially $5 \times 10^3 \text{ cells ml}^{-1}$ during competition with the lake water bacterial community to $1.3 \times 10^5 \text{ cells ml}^{-1}$ (20 °C), $1.76 \times 10^5 \text{ cells ml}^{-1}$ (25 °C) and $1.97 \times 10^5 \text{ cells ml}^{-1}$, respectively. The final fraction of the bacteria was around 10 % of the total bacterial cells at all temperatures tested. All this suggests that within the temperature range tested, the fitness of *V. cholerae* was not affected compared to that of the lake water bacterial community. In Table 1, the results of the simulation using the same simple growth model used in Fig. 4 by applying the previously determined $\mu_{\max}$ of the pure cultures are also listed. They are in good agreement with the experimentally determined values.

DISCUSSION

Although determination of bacterial growth rates in nature – particularly in the marine environment – is an intensively investigated and much discussed research area (Ducklow & Carlson, 1992; Hagström *et al.*, 1979, 1984; Moriarty, 1986; Robarts & Zohary, 1993; Simon *et al.*, 2002), studies concerning the kinetics of growth of waterborne bacterial

Table 1. Competition in batch culture of *V. cholerae* (Vc) and a lake water bacterial community (LWBC) for nutrients present in lake water at different temperatures

Autoclaved and 0.22 µm-filtered water from Lake Greifensee was inoculated with similar concentrations of *V. cholerae* and a lake water bacterial community ($5 \times 10^3 \text{ cells ml}^{-1}$, each). The concentrations of total cells and of *V. cholerae*, respectively, were enumerated 48 h after inoculation when stationary phase had been reached. The predicted fraction of *V. cholerae* at the end of growth is based on a simulation with previously determined $\mu_{\max Vc}$ and $\mu_{\max LWBC}$ on the same water. Maximum specific growth rates were determined in separate assays and corresponding growth curves for *V. cholerae* are shown in Fig. 1(b) (and in the supplementary figures available with the online version of this paper).

	20 °C	25 °C	30 °C
Bacterial concentration (total cells ml ⁻¹)	$1.74 \pm 0.22 \times 10^6$	$1.72 \pm 0.20 \times 10^6$	$1.89 \pm 0.13 \times 10^6$
<i>V. cholerae</i> (cells ml ⁻¹)	$1.30 \pm 0.13 \times 10^5$	$1.76 \pm 0.30 \times 10^5$	$1.97 \pm 0.10 \times 10^5$
<i>V. cholerae</i> (% of total cell concn)	7.5 ± 0.6	10.2 ± 1.3	10.4 ± 1.0
Predicted fraction of <i>V. cholerae</i> (% of total cell concn)	7.0	7.1	7.3
$\mu_{\max Vc} \text{ (h}^{-1}\text{)}$	0.218 ± 0.008	0.321 ± 0.004	0.449 ± 0.001
$\mu_{\max LWBC} \text{ (h}^{-1}\text{)}$	0.418 ± 0.018	0.641 ± 0.006	0.884 ± 0.009
Ratio $\mu_{\max Vc}/\mu_{\max LWBC}$	0.52	0.50	0.47

pathogens and their competition with indigenous microbial flora for natural organic carbon are rare. This is mainly due to the lack of methods for easily monitoring the growth of microbial cells of a mixed community (most of them are 'unculturable') at the low concentrations of substrates and biomass typical for environmental conditions. Here we have used flow cytometry in combination with fluorescent nucleic acid staining of cells to monitor the growth of mixed microbial communities. For the specific detection of *V. cholerae* we have developed a flow cytometric method based on cell-surface fluorescent antibodies to enumerate this pathogen in the presence of indigenous freshwater bacterial flora. This combination of techniques allowed us to study the kinetics of growth of the enteric pathogen and its competition for nutrients with a lake water bacterial community in a laboratory system.

Our studies demonstrate for the first time that *V. cholerae* is not only able to survive (Nogueira *et al.*, 2002), but even able to grow in freshwater. In general, *V. cholerae* grew in autoclaved and 0.22 µm-filtered freshwater in batch culture, exhibiting first a short lag, then exponential growth with a constant $\mu_{\max}$ over an extended period of time, which was followed by a decelerating phase of growth and a stationary phase (as commonly seen with, for example, pure cultures of *Escherichia coli* growing on complex medium at much higher substrate and cell concentrations). The specific growth rates achieved by *V. cholerae* during proliferation in sterilized freshwater were roughly 50 % of those observed under optimum salt concentrations (identical freshwater amended with 5 g NaCl l⁻¹). Under such optimum conditions, *V. cholerae* reached a surprisingly high $\mu_{\max}$, which was similar to that exhibited by the lake water bacterial community on the same freshwater ($\mu_{\max}=0.84 \text{ h}^{-1}$).

Earlier reports, based on the distribution of *V. cholerae* in estuarine water, had already indicated the preference of this pathogen for moderate salinity with respect to optimum conditions for growth (Huq *et al.*, 2005; Louis *et al.*, 2003; Miller *et al.*, 1982). Also, microcosm studies, using artificial seawater and tryptone as a carbon source, had previously shown that *V. cholerae* exhibits highest specific growth rates at moderate salinity (Singleton *et al.*, 1982a). However, as far as we are aware, only two studies have been published where growth rates of *V. cholerae* O1 and of natural bacterial seawater flora were investigated and compared in sterile-filtered natural seawater. Reported specific growth rates (determined by microscopic cell counting) ranged from 0.013 to 0.596 h⁻¹ for *V. cholerae* and from 0.004 to 0.404 h⁻¹ for seawater microbial flora, respectively, at temperatures between 15 and 28 °C (Mourino-Perez *et al.*, 2003; Worden *et al.*, 2006). Whereas the specific growth rates of *V. cholerae* compare well with our freshwater results, the rates achieved by the lake water bacterial community were considerably higher in our experiments.

It should also be pointed out that the freshwater samples used were first autoclaved [to kill/inactivate autochthonous

microbes able to pass 0.22 µm membrane filters (Hahn, 2004)] and then 0.22 µm-filtered in order to remove crystalline particles interfering with the flow cytometric analysis. Although several chemical parameters did indicate little change before (pH=8.09±0.04; DOC=100 %) and after the treatment (pH=8.02±0.41; DOC=104.7±18.3 %) the concentration of AOC_{app} increased consistently and was on average 141.5±18.3 % after autoclaving. This can be explained by cell breakage and hydrolysis of organic matter during the treatment. Hence, this treatment certainly increased the concentration of AOC_{app} and probably also affected the composition of the organic matter in the different freshwaters.

Several different approaches for the determination of AOC exist (Greenberg *et al.*, 1993; Hammes & Egli, 2005; Lechevallier *et al.*, 1993), and thus care should be taken with comparison of absolute values from different studies. We therefore used the term 'apparent AOC' for results obtained with the method developed by Hammes & Egli (2005). In general, the absolute values obtained with this method are about two to five times higher than those reported with the conventional method (Greenberg *et al.*, 1993). Interestingly, whereas *V. cholerae* was able to grow at AOC_{app} concentrations as low as 100 µg l⁻¹, *S. typhimurium* did not show detectable growth in any of the freshwaters tested (AOC_{app} concentrations up to 800 µg l⁻¹; Fig. 3b). This confirms recent reports where growth of *V. cholerae* was observed at low environmental carbon concentrations (Mourino-Perez *et al.*, 2003; Worden *et al.*, 2006), whereas *Salmonella* sp. was reported to grow only under highly eutrophic conditions as found, for example, in greywater (Ottoson & Stenstrom, 2003) or in compost (Sidhu *et al.*, 2001). Hence, *S. typhimurium* can be considered a strict copiotrophic enterobacterium unable to grow under environmental oligotrophic conditions (Winfield & Groisman, 2003). This is obviously in contrast to the environmental survival and growth ability of *V. cholerae*.

The present results suggest a 'threshold' AOC_{app} concentration for growth of *V. cholerae* in the range of 50–100 µg l⁻¹. Interesting in this respect is the report of LeChevallier *et al.* (1996) who observed a significant negative correlation between the occurrence of coliforms in drinking-water systems and AOC concentrations lower than 100 µg l⁻¹ (AOC concentration determined according to LeChevallier *et al.*, 1993). Similarly, it was found that *Aeromonas hydrophila* only grew in tap water when starch was added at concentrations higher than 100 µg C l⁻¹ (Van der Kooij *et al.*, 1980). Both authors explained the phenomenon by the existence of a substrate threshold concentration for growth in about the same range as observed for *V. cholerae* growing on natural AOC.

For a particular water source the observed cell increase at the expense of AOC for *V. cholerae* was always less than for the bacterial AOC-test community. This was the case for all waters tested, although the relative numerical cell yields of *V. cholerae* varied strongly between different water samples

(0.12–0.62). There is certainly not a single reason for this variation. A first explanation is the fact that the spectrum of carbonaceous substrates utilizable for *V. cholerae* is narrower in comparison to that of the bacterial AOC-test community. Therefore, the numerical cell yield must be expected to be strongly dependent on quality of the AOC, i.e. the spectrum of compounds it consists of. A second explanation for varying numerical cell yields of *V. cholerae* could be that the average cell size of *V. cholerae* is changing under different conditions and is different from that of the community used for AOC assessment. Therefore, in spite of a positive correlation between AOC_{app} concentration in different types of freshwater and numerical growth of *V. cholerae* (Fig. 3b), it will be very difficult to predict exactly the growth potential of *V. cholerae* in a water sample from the concentration of AOC_{app} only. From an ecological perspective, it would be interesting to investigate the relationship between algal growth, AOC_{app} concentration and growth of *V. cholerae*. It is known that algae release considerable amounts of organic carbon compounds during photosynthesis (Münster, 1993) and that the occurrence of *V. cholerae* during algal blooms is elevated in estuary water systems (Epstein, 1993).

In addition to the concentration of utilizable carbon/energy sources, temperature is a further key parameter that governs growth of heterotrophic microbes in aquatic systems (Moriarty & Bell, 1993). Consequently, it has been proposed that an increase in temperature by a few degrees as a result of global warming might lead to a selective advantage for pathogens (see, e.g. Colwell, 1996). It was argued that a shift of the average environmental temperature towards the range optimal for growth for pathogens, which is typically around 37 °C, would enable pathogenic bacteria to compete better for nutrients with the low temperature-adapted indigenous heterotrophic microbial flora. Supporting this hypothesis is that temperature is frequently reported to influence growth and occurrence of *V. cholerae* O1 in natural waters with low salinity (Huq *et al.*, 2005; Louis *et al.*, 2003). Also, the faecal indicator bacterium *E. coli* was reported to grow outside its host in tropical waters, but not at higher latitudes in the northern hemisphere, where the average annual water temperature is lower (Winfield & Groisman, 2003). Our results clearly demonstrate a positive correlation between increasing temperature and the specific growth rate achieved by *V. cholerae* in freshwater within the range of 20–30 °C (Table 1). However, it appears from our data that in freshwater the ability of *V. cholerae* to compete with the lake water bacterial community for nutrients was not significantly affected. This result correlates with the observation of Louis *et al.* (2003), who found, in contrast to an estuarine environment, no year-to-year variability in the occurrence of *V. cholerae* O1 in a freshwater system. Certainly, it is difficult to extrapolate from our batch microcosm studies (where elevated temperatures did not favour *V. cholerae* when competing with the lake water bacterial community for substrates in freshwater), to the much more complex environmental

situation where many other biotic and abiotic factors influence competitive microbial interactions. For example, temperature might influence competition in an indirect way by promoting algal growth and excretion of utilizable metabolites, grazing or viral lysis of pathogens and heterotrophic competitors. Competition experiments in the laboratory in AOC-limited continuous culture at different temperatures could provide more insight into the dynamics controlling growth and competition of *V. cholerae* with natural bacterial flora. In such a system competition could be followed over more extended periods of time and nutrient fluxes could be manipulated, simulating the varying nutrient supply in nature (Münster, 1993). The results presented here clearly demonstrate that growth of *V. cholerae* under oligotrophic conditions is not restricted to estuary or coastal waters but that this pathogen is able to grow in freshwater. Thus, as suggested earlier (Shapiro *et al.*, 1999), freshwater aquifers – particularly when mesotrophic – might provide an environmental reservoir for *V. cholerae*, resulting in sporadic epidemic outbreaks originating from these waters. Our results also support the recent observation (Worden *et al.*, 2006) that being attached to particles or higher organisms is not compulsory for the survival of this pathogen but that it is able to grow in a free-living state. The techniques described here open a way to well-controlled investigations for enhancing our understanding of the behaviour of *V. cholerae* in the freshwater environment and to improve risk assessment for the disease of cholera.

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