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DIPLOMARBEIT

Titel der Diplomarbeit

Invasion Potential of *Vibrio cholerae* O1/O139
in Lake Neusiedler See and Three Alkaline Saline Lakes.

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser:	Rupert Bliem
Matrikel-Nummer:	0348742
Studienrichtung /Studienzweig (lt. Studienblatt):	A 441
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dr. Witte Angela Mag. Dr. Kirschner Alexander
Wien, Dezember 2009	Die Diplomarbeit wurde durchgeführt am Institut für Hygiene und Angewandte Immunologie Abteilung für Wasserhygiene Medizinische Universität Wien.

Ein Bakterium aus der Sicht meines dreijährigen Sohnes.

An erster Stelle möchte ich mich bei meiner Frau und meinem Sohn für die nötige Energie und ihr Verständnis während der Studienphase und der Diplomarbeit bedanken. „Ohne euch wäre ich nie so weit gekommen.“

Ein großer Dank geht an Mag. Dr. Kirschner Alexander für seine unermüdliche Hilfe und Betreuung während der Diplomarbeit und vor allem aber für seine große Unterstützung beim Verfassen dieser Diplomarbeit.

Vielen Dank auch an Frau Prof. Sommer Regina und ihr ganzes Team aus der Abteilung für Wasserhygiene, welches mich in vielen Bereichen mit Rat und Tat unterstützt hat.

Mein besonderer Dank gilt auch den Mitarbeitern der biologischen Station Illmitz, Prof. Herzig Alois, Schalli Rudolf, Haider Richard und Rauchwarter Franz.

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Abbreviations

µg	microgram
µl	microliter
µM	micromolar
ADP	adenosine diphosphate
bp	base pair
cfu	colony forming units
DNA	deoxyribonucleic acid
dNTP	2’3’-dideoxyribonucleoside 5’-triphosphate
e.g.	for example (abbr. from *exempli gratia*)
EDTA	ethylenediamine tetraacetic acid
et al.	and others (abbr. from *et alii*)
etc.	*et cetera*
f	forward
FAM	6- carboxyfluorescein
Fig.	figure
h	hours
kb	kilobase
M	molar
mg	milligram
mM	millimolar
ng	nanogram
nM	nanomolar
nt	nucleotide
Ø	diameter
OD	optical density
PFA	paraformaldehyde
pmol	picomol
r	reverse
rcf	relative centrifugation force
SD	standard deviation
SDS	sodium dodecylsulfate
V	volt
W	watt

1 Abstract

Vibrio cholerae is a facultative anaerobic bacterium, which belongs to the family Vibrionaceae. Toxigenic strains of *Vibrio cholerae* are the etiological agent of cholera, an acute dehydrating, life-threatening diarrhea, that occurs in many countries all over the world. Although its activity in human hosts has received great scientific attention, the behaviour of the microbe in its natural environment has received much less consideration. *V. cholerae* is, besides being a pathogen, a component of the normal aquatic flora in brackish, estuarine and seawater. It occurs in two environmental main life modes, free-floating or particle/zooplankton attached. Of the more than 200 serogroups only two, O1 and O139, are able to cause epidemic cholera, whereas the others are responsible for less severe watery diarrhea, septicemia, otitis, wound and respiratory tract infections. During the period from 2000 to 2005, 13 infections of *V. cholerae* nonO1/nonO139 were documented in Austria. Out of these, five infections, including one case of death by an immunosuppressed fisherman could be directly related to the lake Neusiedler See.

The aim of the study was to estimate the invasion potential of toxigenic *V. cholerae* strains into the lake Neusiedler See and adjacent smaller alkaline saline lakes. Therefore, laboratory batch culture microcosm experiments were performed, to which pandemic *V. cholerae* strains and naturally colonized zooplankton were added. The data showed that, with the exception of *V.cholerae* O1 ElTor, all tested strains were able to proliferate to high numbers. Due to the fact, that the national park Neusiedler See is a protective area for migratory and aquatic birds, and an important recreational area for humans, which may both transport toxigenic strains to this region, a possible invasion of pandemic *V. cholerae* has to be considered.

In the course of this study, the abundance of environmental free-floating and zooplankton attached *V. cholerae* in five selected sampling areas were also investigated from May to September by means of cultivation on selective agar, to look for the presence of culturable *V.cholerae* O1 /O139 strains. In addition to cultivation based quantification, a second method based on fluorescence *in situ* hybridisation (FISH) and following microscopical detection was performed for the zooplankton attached cells. The results showed that the total *V. cholerae* number can vary from less than 1000 to more than 1 x 10^8 cells L^{-1} in dependence on the

respective sampling area, but no *V.cholerae* O1/O139 strains were detected over the whole study period.

However, the fact of global warming and its effects on the regional environment, as well as the presence of possible transport routes (human, birds) claim the need of an enhanced and continued monitoring of *V. cholerae* in the lake Neusiedler See and its adjacent alkaline saline lakes for a reliable risk assessment.

2 Introduction

The gamma-proteobacterium *Vibrio cholerae* belongs to the family of Vibrionaceae, which consists of 6 different genera (Bergey et al., 2001). In general, Vibrionaceae are Gram-negative, facultative anaerobic and contain the enzyme oxidase to use oxygen as an electron acceptor. The genus Vibrio is in general characterized by the presence of circumpolar flagella, by which *Vibrio* can be motile in various fluids. One of the most famous representatives of this genus is *Vibrio cholerae*, a curved rod-shaped bacterium, with a monopolar flagellum. It was first described in 1854 by Filippo Pacini, an Italian anatomist during an Asiatic pandemic cholera which swept through Florence (Pacini, 1854). However, due to the prevailing belief of Italian scientists in the miasma theory of disease, his work was not noted by others. The famous German microbiologist Robert Koch, rediscovered the bacterium in 1883 during a research trip through Egypt. He isolated and described it as the causative agent which leads to the disease cholera (Koch, 1884).

More than 200 different *V. cholerae* somatic antigen (O) serogroups have been defined till now, but only two serogroups, termed as O1 and O139, are able to cause cholera, an acute life-threatening diarrhoeal disease. The disease occurs primarily in many developing countries, particulary in South Asia, Africa and Latin America (Kaper et al., 1995; Faruque et al., 1998). *V. cholerea* O1 is subdivided into two major serotypes, “Ogawa” and “Inaba” and a rarely reported “Hikojima” serotype. These serotypes have been further categorized into two biotypes, the classical and El Tor, based on certain biochemical properties (Takeya, 1974; Guidolin and Manning, 1987; Watnick et al., 1999). The O139 strains were derived from a *V. cholerae* O1 El Tor strain by genetic changes in some serotype-specific genes (Bik et al., 1995; Faruque et al., 2003). Cholera typically spreads in explosive epidemics affecting large numbers of people. Therefore, these epidemic strains can also spread across countries and continents and may lead to cholera pandemics. All other serogroups, which are pooled and termed as nonO1/nonO139, are not associated with epidemics and may be responsible in some cases for less severe watery-diarrhea, septicemia, otitis, wound and respiratory tract infections (Morris et al., 1990; Cheasty et al., 1999).

Vibrio cholerae genome:

The genome of *V. cholerae* consists of two unique and separate circular mega-replicons (Trucksis et al., 1998). The complete genome of the representative *V. cholerae* O1 El Tor biotype strain N16961 contains 4.033,460 bp (Heidelberg et al., 2000), where the larger chromosome I contains a total of 2.961,146 bp and the chromosome II consists of 1.072,314 bp. The larger chromosome I domiciles genes for essential cell functions, commonly designated as house-keeping genes and furthermore all genes which are involved in pathogenesis, including toxins, surface antigens and adhesion factors (Faruque et. al., 2003).The major virulence genes in *V. cholerae* are clustered in several regions of chromosome I. The *ctxAB* genes encoding the A and B subunit of the cholera toxin (CT), an ADP ribosylating toxin, are part of a lysogenic bacteriophage designated CTXΦ (Waldor and Mekalanos, 1996), whereas the genes for the toxin coregulated pilus (TCP) are clustered at the *Vibrio* pathogenicity island (VPI) (Faruque and Mekalanos, 1996).

V. cholerae has to acquire its *ctxAB* genes, which are part of CTXΦ, by bacteriophage adhesion to the TCP. Aside from these two major virulence genes, which are characteristic for all O1/O139, some other virulence genes are existing like *mshA*, which encodes for a mannose sensitive haemagglutinin (MSHA) to adhere via this pilus to plankton or to make biofilms on abiotic surfaces (Chiavelli et al. 2001), or *stn* which encodes a heat stable enterotoxin of TCP negative CT negative nonO1/nonO139 *V. cholerae* (Heidelberg et al., 2000). ToxR, a transmembrane protein, acts as the master regulator of the virulence genes and is itself regulated by environmental signals and its enhancer ToxS (DiRita and Mekalanos, 1991).

Pathogenesis:

Infection due to *V. cholerae* begins with the ingestion of contaminated food or water containing the bacterium. The reported infectious dose in healthy volunteers is approximately 10^8 bacteria (Hornick et al., 1971). A significantly lower dose (10^5) was found, when frozen *V. cholerae* cells were administered during vaccination studies (Sack et al., 2004). After the passage through the acid barrier of the stomach, *V. cholerae* colonizes the small intestine using TCP, and produces CT, leading to an increase of cyclic AMP and thereby to a rapid efflux of chloride ions and water from the host intestinal cells (Kaper et al., 1995). Symptoms of cholera, namely watery

diarrhea and vomiting, generally occur abruptly after an incubation period between 18 hours and 5 days. Severe cholera patients may loose 500-1000 ml h^{-1} and 10% of their body weight, and may die from volume shock (50% case-fatality) without treatment or sufficient rehydration. Deaths from cholera usually occur in the first day (Sack et al., 2004).

Ecology:

Although *Vibrio cholerae* is a human pathogen, these bacteria are part of the normal aquatic flora in estuarine and brackish waters (Colwell and Spira, 1992; Colwell, 2004; Worden et al., 2006). They are able to persist and grow in the water column in the total absence of a human host (Faruque et al., 2002). New studies strongly indicate the ability of *V. cholerae* to live either planktonic (Kirschner et al., 2008), or in association with crustacean zooplankton (Huq et al., 1983; Schauer, 2008). It has also been isolated from prawns, oysters, chironomid egg masses and crabs (Tamplin et al., 1990; Islam et al., 1994; Colwell, 1996; Broza et al., 2005). Chitin, the most abundant natural polymer in the aquatic environment and furthermore a major component of planktonic organisms serves as a source of carbon and nitrogen for the growth of *V. cholerae* (Nalin, 1979), as well as as surface for the formation of surface-attached communities or biofilms (Watnick, 1999).

V. cholerae is able to attach to chitinous surfaces through TCP and MSHA (Chiavelli et al., 2001), which leads to a protection against external environmental conditions like protozoan grazing, low temperature and acidic conditions (Lipp, et al., 2002) and as suggested most recently, competition by the natural bacterial community (Kirschner et al., 2010). Additionally, the acquisition of new genetic material during the growth on chitinous surfaces may be enhanced (Meibom et al., 2005). Moreover, it has been shown in microcosm experiments that zooplankton attachment leads to significant higher growth and survival rates of *Vibrio cholerae* (Huq, et al., 1983).

Ecology of *V. cholerae* in the lake Neusiedler See:

Since several human infections of bathers occurred in the lake Neusiedler See, a survey program was started in the year 2001 to monitor the seasonal development of *Vibrio* cholerae in combination with a variety of environmental variables. During the period from 2001 to 2005, five infections, including one lethal case of septicaemia

were explicitly associated with recreational activities at the lake Neusiedler See (Huhulescu, et al., 2007).

The lake Neusiedler See has a salinity between 1 and 2 ‰ and a pH between 8.5 and 9.1 and therefore offers perfect conditions for the bacterium to grow (Kirschner et al., 2008). Due to the shallow water column, water temperature changes rapidly in response to weather events. The frequent resuspension of the sediment caused by winds and current also results in a high concentration of suspended solids in the water column. The large scale distribution of reed, the high number of crustacean zooplankton and the high variability of abiotic parameters offer *V. cholerae* a lot of highly specific habitats. Laboratory experiments indicated that endemic *V. cholerae* could also grow rapidly in a free-floating state in natural-lake water (Kirschner et al., 2008). It was also pointed out that the temperature and the quality of the dissolved organic carbon (DOC) had a significant influence on growth of *V. cholerae* (Kirschner et al., 2008). It is widely accepted that the colonization of planktonic copepods by the family of Vibrionaceae is a common phenomenon (Colwell et al., 1977; Huq et al., 1983). Interestingly, it has been demonstrated in a recent study that nonO1/nonO139 *Vibrio cholerae* in Neusiedler See prefer, in contrast to their marine counterparts, cladocerans instead of copepods, which was indicated by a two orders of magnitude higher cell concentration, attached to the cladocerans surfaces (Schauer, 2008).

Aims of the study:

The aim of this study was firstly to evaluate the invasion potential of various pandemic cholera pathogenic *V. cholerae* O1 and 0139 strains into the Neusiedler See and selected adjacent shallow saline lakes pools. This was performed by means of microcosm experiments in the laboratory under controlled conditions. *V.cholerae* cells were detected with a culture independent approach, namely by fluorescence *in situ* hybridization (FISH) in combination with epifluorescence microscopy.

Second, environmental *V. cholerae* were cultivated from the water body and zooplankton of the lake Neusiedler See and three selected shallow alkaline lakes to (i) investigate for the first time the abundance of *V. cholerae* in the shallow saline alkaline lakes and to (ii) search for the possible presence of pandemic *V.cholerae* O1 or O139 strains in the whole lake area. All isolates and enrichment cultures were screened by multiplex-PCR for pathogenic and O1/O139 specific genes.

3 Materials and Methods

3.1 Site Description

The lake Neusiedler See (47°42'N, 16°46'E) (**Fig. 1**) is one of a few steppe lakes in Europe and the largest lake in Austria (115 m above the sea level and a surface area of 321 km^2). The lake is characterized by its high alkalinity (pH 8.1-8.9), moderate salinity (1-2 g L^{-1}) and high turbidity (Secchi depth ~20 cm). Its mean depth is approximately 1.1 m and 55% of the total surface area is covered with the reed *Phragmites australis*. In contrast to the lake, the shallow alkaline saline lakes called "Lacken" are scattered close to the south-eastern coast of the lake. This area of the National Park Neusiedler See-Seewinkel (47°45'-49', 16°47'-53') is situated above the largest mineral water deposit in Europe (Krachler et al., 2000). The mineral solutes ascending with the groundwater flux formed these shallow soda pools. They are characterized by a higher alkalinity (pH 8.8-10.5), a broad spectrum of salinity (3.8-7.4 g L^{-1}) and turbidity from 1 to 25 cm. The shallow lakes' depth depends mainly on rainfall and evaporation during the sunny periods and so they may dry out during a hot summer season. Soda lakes are regarded as being among the worlds most productive environments, as the access to dissolved inorganic carbon in form of HCO^-_3 and CO^{2-}_3 for primary producers is unlimited (Duckworth et al., 1996). Aerobic heterotrophic bacteria have been reported to reach extremely high numbers of 10^7 to 10^9 cells ml^{-1} (Kilham, 1981; Grant, 1990, Eiler et al 2003).

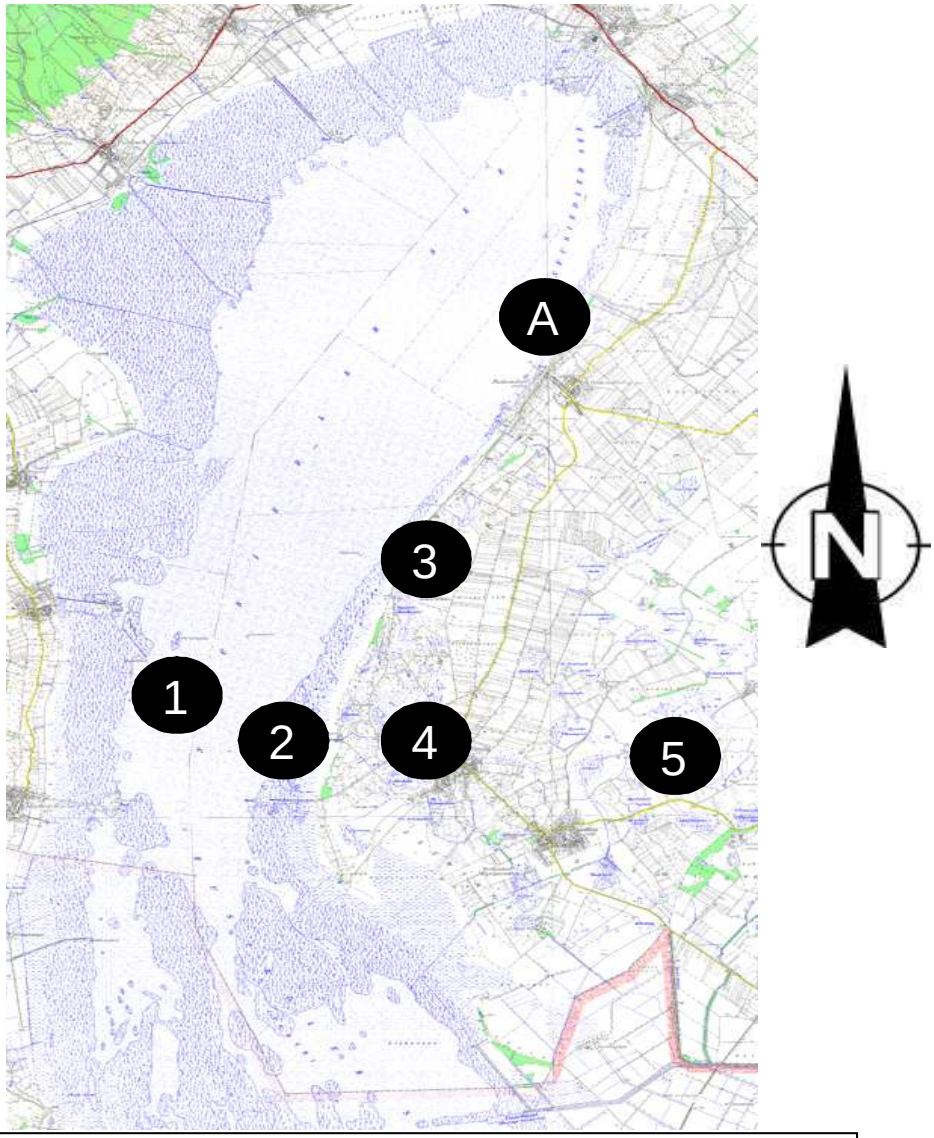


Fig. 1: View of the lake Neusiedler See and the shallow alkaline lakes (Lacken). **1** indicates lake center, **2** reed belt, **3** Oberer Stinker, **4** Zicklacke, **5** Lange Lacke and **A** lake shore at Podersdorf.

3.2 Isolation, identification and quantification of presumptive Vibrio cholerae

3.2.1 Isolation and quantification of presumptive *V. cholerae* from water samples

Triplicate samples from 5 sampling points (**Fig. 1**) were collected during a period from April to September 2009. Each water sample was filled into a sterile 500 ml glass-bottle and samples were transported to the laboratory in a cool box within 3 h at *in situ* temperature (±2 ℃). Electric conductivity (EC), p H, Secchi depth, FTX and temperature were measured immediately, whereas the leftover of the environmental and chemophysical parameters were measured in the laboratory, as described in Eiler et al. (2003) and Kirschner et al. (2004). Subsamples (up to 200 ml) were filtered through a 0.22 µm pore size cellulose nitrate filter (Ø 47 mm, Sartorius, Vienna, Austria). The filter was introduced into 3 ml alkaline peptone water (APW, 1% peptone; 1% NaCl; pH 8.8-9) and incubated at 37 ℃ for 12 h. The filters were removed and 1.5 ml of each sample was centrifuged with 10000 rcf. The pellets were resuspended and washed with 1 ml 1 x PBS. After a second centrifugation and washing step, the pellet was resuspended with 0.5 ml 1 x PBS and stored at -20 ℃ for further analysis (Das et al., 2009)

All triplicate samples from lake and reed-belt were filtered through 0.45 µm pore size cellulose nitrate filters. One, ten and hundred ml of each water sample were filtered and the filter-attached bacteria were placed bottom up on Thiosulfate Citrate Bile Sucrose (TCBS, Merck, Darmstadt, Germany) agar plates and incubated for 18 h at 41 ℃. Samples from the shallow alkaline lakes were analyzed direct by plating 100 µl of 10-fold dilutions onto TCBS agar plates. Yellow, flat 1 to 3 mm diameter colonies were picked and streaked onto nutrient agar without NaCl (3% beef, 5% peptone, 15% agar) plates and incubated overnight at 37 ℃. Colonies were counted and chosen for further testing.

3.2.2 Isolation and quantification of presumptive *V. cholerae* from zooplankton

The amount of zooplankton attached *Vibrio cholerae* was determined by plating 100 µl of a 10-fold dilution series of 10 washed (0.85% NaCl) pestled copepods or cladocerans onto TCBS agar plates according to the above explained procedure.

3.2.3 Identification of presumptive *V. cholerae*

Cytochrome-oxidase tests were performed for each positive isolate. One drop of the oxidase reagent (N,N,N`,N`Tetramethyl-1,4phenylendiammoniumchlorid) was brought onto a *Vibrio cholerae* colony, which was streaked onto a cotton swab. In the case that a blue color appeared within 30 seconds, the test was interpreted as positive. All positive isolates were stored in 0.5 ml cryo-medium (CASO medium; glycerine) at -80 ℃ for further analysis. The genes responsible for O-antigen biosynthesis and for the generation of serotype-specific determinants are located in the *wb* region on the *V. cholerae* chromosome. The *wb* genes specific for *V. cholerae* O1 (wbe) and 0139 (wbf), the *ctxA* gene, encoding subunit A of cholera toxin, the *ompW* gene, encoding a outer membrane protein and the *tcpA* gene, encoding a major subunit of TCP were amplified using multiplex-PCR.

All isolates were boiled for 10 min and after centrifugation at 13,000 rcf for 10 min at 4 ℃, the supernatant was used directly as a DNA template for multiplex-PCR (Sarkar et al., 2009). The used primers (Thermo Scientific, Karlsruhe, Germany) are listed in **Table 1** and the multiplex-PCR was performed in a T 3, Biometra Gradient Thermo-cycler (Biometra, Göttingen, Germany). The following reagents were added in a reaction mixture volume of 25 µl:

Each reaction contained of 2.5 µL of a 10x reaction buffer B (Mg^{2+} free, Solis BioDyne, Tartu, Estonia), 2 µL of 25 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 0.5 µL of 10 mM dNTP mix (Promega, Mannheim,Germany), 1.2 µl each of the forward and reverse primer of *ompW* and *tcpA* (10 pmol μL^{-1}), 1 µl each of the O1 *wbe* primer pair (10 pmol μL^{-1}) and O139 *wbf* primer pair (10 pmol μL^{-1}) and 0.9 µL for the *ctxA* primer pair (10 pmol μL^{-1}) 0.4 µL of primer pair (10 pmol μL^{-1}) and 0.9 µL for the *ctxA* primer pair (10 pmol μL^{-1}) 0.4 µL of hot FIREPol DNA polymerase (Solis BioDyne,

Tartu, Estonia). Finally 5 µL of DNA template and Mili-Q water were added to a final volume of 25 µL.

The T3 thermocycler conditions used consisted of initial denaturation at 94 ℃ for 15 min, followed by 30 cycles consisting of 94 ℃ for 1 min, 59 ℃ for 1 min and 72 ℃ for 2 min and a final extension step at 72 ℃ for 1 0 min.

As positive control, a *Vibrio cholerae* O1 strain (ML-1148) designated as Vc49 and a *Vibrio cholerae* O139 strain (MJ-5791) designated as Vc52 were used.

Multiplex-PCR products were separated in a 2% agarose gel elctrophoresis for 70 min with 90 V in 1 x TAE buffer (40 mM Tris, 1 mM EDTA, pH 8), stained in 1 µg ml^{-1} ethidium bromide solution and visualized under UV light (GelDoc 2000 System; Biorad, Hercules, CA, USA).

Table 1: Multiplex-PCR primers used in this study.

Target	Nucleotide sequence	bp	Reference
ompW (F)	CAC CAA GAA GGT GAC TTT ATT GTG	304	Goel et al.,
ompW (R)	GGT TTG TCG AAT TAG CTT CAC C		2006
ctxAB (F)	GCC GGG TTG TGG GAA TGC TCC AAG	536	Goel et al.,
ctxAB (R)	GCC ATA CTA ATT GCG GCA ATC GCA TG		2006
wbe O1 (F)	TCT ATG TGC TGC GAT TGG TG	638	Goel et al.,
wbe O1 (R)	CCC CGA AAA CCT AAT GTG AG		2006
wbf O139 (F)	AGC CTC TTT ATT ACG GGT GG	449	Hoshino et
wbf O139 (R)	GTC AAA CCC GAT CGT AAA GG		al., 1998
tcpA (F)	CGT TGG CGG TCA GTC TTG	805	Goel et al.,
tcpA (R)	CGG GCT TTC TTC TTG TTC G		2006

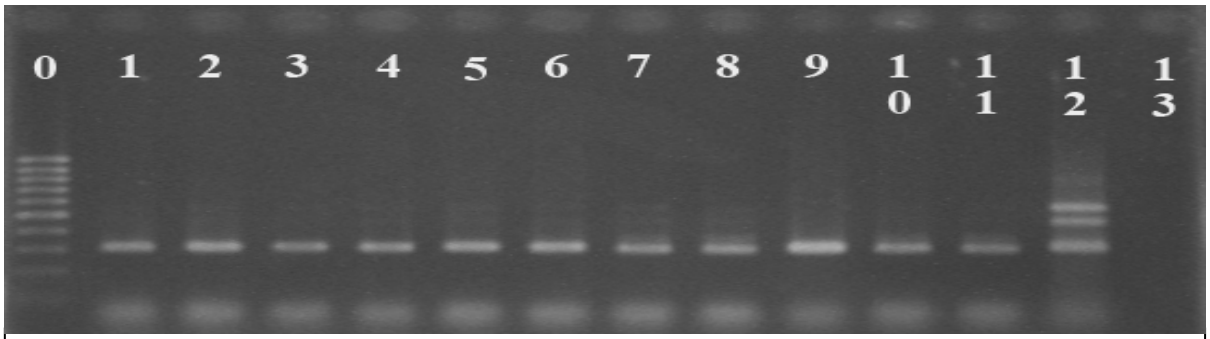


Fig. 2: PCR results: Lane O 100 bp mass ruler low range (Fermentas), lane 1-11 presumptive environmental *V. cholerae*, lane 12 positive control (O139), lane 13 negative control (Milli-Q water)

3.2.4 Zooplankton-associated *V. cholerae* quantification by means of FISH

Due to the high variability of the native bacterial number in the saline shallow lakes, a second counting procedure was applied to even out typical cultivation problems like rarefying or overgrowing bacteria.

Therefore, FISH was done simultaneously to the cultivation procedure. Ten cladocerans or copepods were washed twice with 10 ml 1 x PBS. The collected zooplankton was fixed with 2.5 ml 4% PFA (para-formaldehyde) for 12 -16 h at 4 ℃, following addition of 8.5 ml 0.118 mM tetra-sodium pyrophosphate and gently shaking on ice for 2 h. Zooplankton were then puls-sonicated (Branson Sonifier S-450A) with 15 W for 3 min and filtered on a white polycarbonate membrane filter (Ø 25 mm, pore size 0.2 µm). The filters were dried for 1 h and stored at -20 ℃ in sterile 1.5 ml tubes for FISH.

3.2.5 Fluorescence *in situ* hybridisation

For the hybridization following materials were needed:

- 6-FAM-labeled probe (Thermo Electron, Karlsruhe, Germany), Vchomim1276 (5´-[6FAM]ACTTTGTGAGATTCGCTTCCACCTCG-3´), concentration 50 ng μl^{-1}
- 1 ml hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS, 35% formamide, 450 µl Milli Q)
- 50 ml washing buffer (80 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 5 mM EDTA, 47750 µl sterile Milli Q-water)
- DAPI mix (5.5 parts Citifluor, 1 part Vectashield, 0.5 parts 1 x PBS, DAPI final concentration 1 µg ml^{-1})

A quarter of the filter was cut out with a scalpel and the remaining filter was immediately stored at -20 ℃.

In the absence of light, 270 µl of the hybridization buffer were mixed with 30 µl of the 6-FAM labelled probe in a 0.7 ml tube followed by adding the filter pieces and hybridization was performed at 46 ℃ for 2 h.

The filters were transferred into the pre-warmed washing buffer and incubated for 10 min at 48 °C. The washing buffer with the filters was poured through a suction filter. The filter sections were washed for 1 min in an ascending ethanol order (50-96%) and dried in the dark on a paper. The filter was counterstained with DAPI-mix (5.5 parts Citifluor, 1 part Vectashield, 0.5 parts 1 x PBS, with DAPI at a final concentration of 1 µg ml^{-1}) by placing a drop on the top of a microscope slide, adding the filter and covering the filter with another drop of DAPI-mix. After mounting a cover slip the filter was incubated for 10 min in the dark and finally stored at -20 °C.

3.3 Microcosm experiments

During the summer period 2009 five microcosm experiments were carried out to test the invasion potential of various pandemic *V. cholerae* strains. Each water sample was filled in a sterile 2 L glass-bottle, where pH, EC and temperature were measured immediately. The rest of the chemical parameters were measured in the laboratory. In the lake Neusiedler See, zooplankton was collected with a vertical net haul (mesh size 250 µm) from each sampling point and collected in sterile 250 ml flasks. Due to the shallow depth of the soda pools, zooplankton was collected with a beaker and a zooplankton net (mesh size 250 µm). All samples were transferred to the laboratory in a cool box within 3 h at *in situ* temperature (±2 °C). The water of each sampling point was filtered through a 0.45 µm pore size cellulose nitrate filter (Ø 47 mm, Sartorius, Vienna, Austria) with a vacuum pump (max. 400 mbar) and filled into four 400 ml flasks. Selected pandemic *V. cholerae* strains were grown in 50 ml brain heart infusion broth (BHI, Merck, Darmstadt, Germany) at 37 °C on a shaker to an OD of 0.8 which equals approximately 10^9 cells ml^{-1}. Twenty ml of the liquid enrichment medium was transferred into sterile centrifuge tubes and spun down for 10 min at 5,000 rcf at 25 °C. The supernatant was discarded and the cell pellet was resuspended and washed with 10 ml 1 x PBS. The last step was repeated and after discarding the supernatant the pellet was resuspended in 10 ml 1 x PBS.

From each *V. cholerae* suspension, 4 µl were injected into one 400 ml flask from each sampling point, to a final concentration of approximately 10^3 - 10^4 cells ml^{-1}. For each sampling point, one flask was used as a blank. Each flask was gently shaken to scatter the cells homogeneously. Twenty ml of the samples containing the homogenized inoculum were taken out and fixed with 5 ml 4% PFA for 12- 18 h at 4

℃ before the addition of zooplankton. Fifty cladocerans or a mixture of cladoceran and copepod individuals with a total amount of 50 were inoculated to each flask and incubated at 28 ℃ for 24 h. Twenty ml of each sample were fixed with 5 ml 4% PFA and fixed as described above. The PFA fixed samples were filtered through a white polycarbonate membrane filter (Whatman, Ø 25 mm, pore size 0.2 µm) and further prepared for FISH or stored at -20 ℃. To calculate the amount of zooplankton-associated *V. cholerae* duplicates of 10 cladocerans and 10 copepods of each sampling point were stained with FISH. The zooplankton was washed twice with 10 ml 1 x PBS, followed by fixation with 2.5 ml 4% PFA for 12 – 18 h. Afterwards, 8.5 ml 0.118 mM tetra-sodium pyrophosphate was added and shaken gently for 2 h on ice. The samples were puls-sonicated with 15 W for 3 min and filtered on a white polycarbonate membrane filter (Ø 25 mm, pore size 0.2 µm). For FISH, the filters were dried for 1 h and stored at -20 ℃ in sterile 1.5 ml tubes.

At the end of the experiment the remaining water in the sample flasks were filtered through a 5 µm polycarbonate membrane filter (Ø 47 mm, Nucleopore, Whatman) with a vacuum pump (max. 400 mbar) and the obtained zooplankton was washed twice with 10 ml 1 x PBS, followed by the same procedure as described above.

3.4 Dissolved organic carbon (DOC) analysis

Water (up to 750 ml) of the sampling points was filtered through 0.45 µm precombusted (450 ℃, 4 h) Whatman GF/F filters and 10 ml of each sample was withdrawn, acidified to pH 2 with 50 µl of 6 N HCl and stored in combusted-glass scintillation vials with Teflon-lined caps at -20 °C for subsequent DOC concentration analysis (kindly processed by Ing. Schimpf Eva). The absorbance characteristics of the DOC were measured against those of double distilled water at 250 and 365 nm using a Perkin Elmer Lambda 40 UV/VIS Spectrophotometer (Norwalk, USA) and a 1 cm quartz cuvette. The absorbance ratio at 250 nm to that at 365 nm was calculated to determine possible shifts in the molecular size spectrum of the DOC during the experiments. A higher ratio indicates a higher percentage of low molecular weight (LMW) substances than high molecular weight (HMW) substances (Reitner et al., 1999).

3.5 Microscopy

V. cholerae cell concentrations and total bacterial numbers were counted by using epifluorescence microscopy (NIKON, Eclipse 80i, equipped with a digital camera) at 1250 x magnification (**Fig. 3**). All FISH sample counts were done with a stained quarter of the filters. For total bacterial numbers, 1 ml was fixed with 37% formaldehyde (final conc. 2%), filtered onto 0.2 µm white polycarbonate filter (Cyclopore, Whatman) and stained with Sybr Gold. The filters were put on a microscope slide, overlaid with a drop of paraffin oil and mounted with a cover slip. For the determination of cell numbers and cell morphology 20 microscopic fields were inspected.

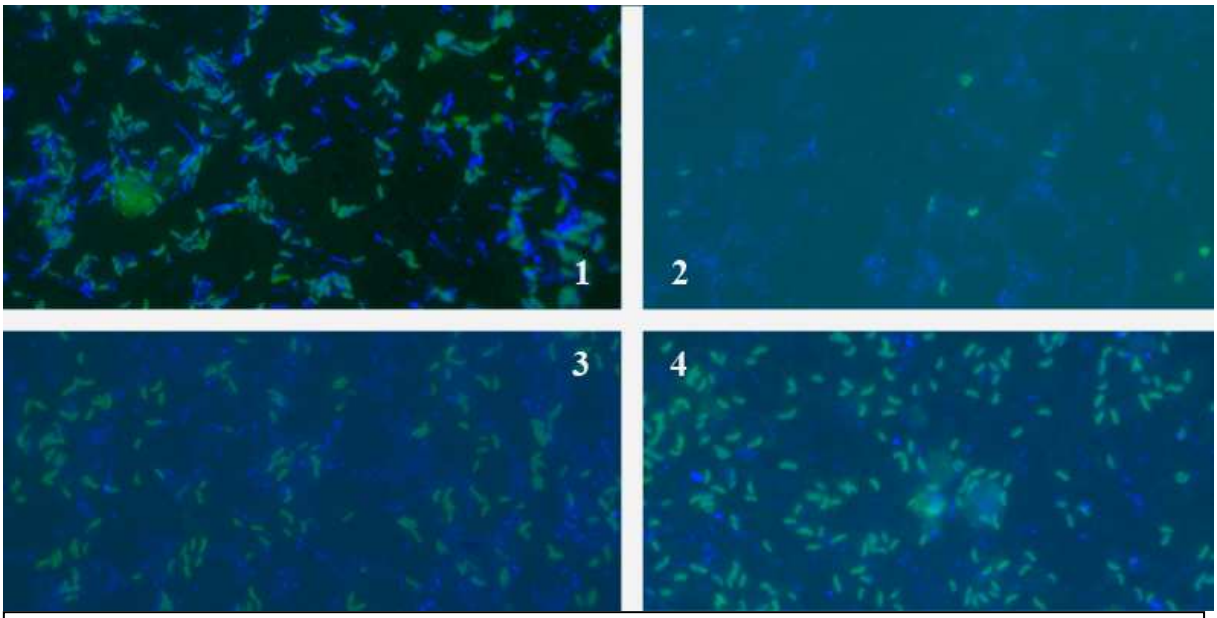


Fig. 3: Epifluorescence micrograph of *Vibrio cholerae* O139 (Vc55) in batch cultures from various sampling sites at the same sampling time; **1** lake, **2** reed, **3** Zicklacke, **4** Lange Lacke.

3.6 Statistics

Statistical analysis was carried out with PASW statistics 18 for Windows XP. Significance of the difference between sample and blanks was tested with the Wilcoxon-test. Predictive values (p) < 0.05 were considered as statistically significant.

4 Results

4.1 Quantification of environmental V. cholerae from the water samples

During the summer period, the concentrations of environmental *V. cholerae* in the lake center ranged between 6.8 x 10^2 cells L^{-1} in May and a maximum of 2.73 x 10^4 cells L^{-1} in August. Cell numbers in the reed belt varied from 1.2 x 10^3 cells L^{-1} in May to 1.89 x 10^4 cells L^{-1} in July. In the Zicklacke, the *V. cholerae* cell numbers showed their minimum with 1.4 x 10^4 cells L^{-1} during June and their maximum with 3.54 x 10^5 cells L^{-1} in August. The Lange Lacke exhibited minimum *V. cholerae* concentrations of 7.2 x 10^3 cells L^{-1} in June and a maximum concentration of 9.24 x 10^4 cells L^{-1} in August (**Fig. 4**). Surprisingly, in the Oberer Stinker, no planktonic *V.cholerae* cells were found.

Preliminary study of Steinberger (2008) indicate that cfu ml^{-1} corresponds to an equal number of cells L^{-1} in the lake, while in the shallow lakes cfu ml^{-1} may lead to a slight underestimation of cells L^{-1}.

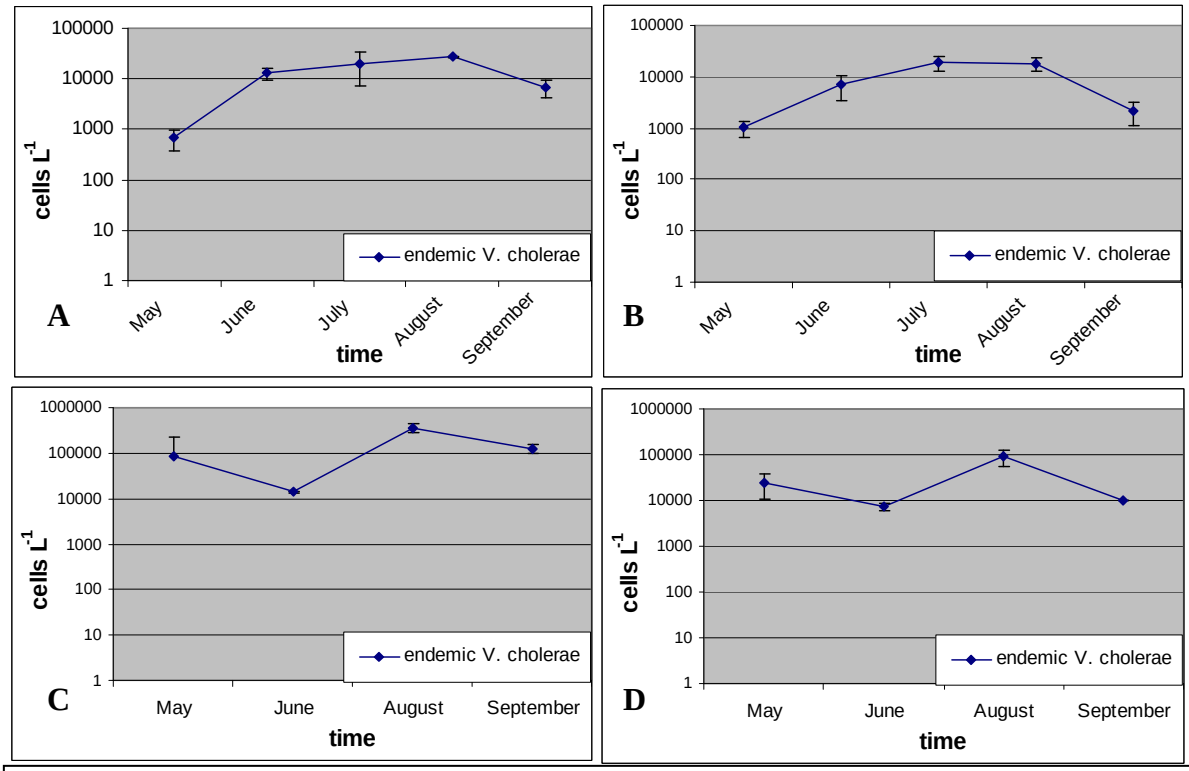


Fig. 4: Cell numbers of environmental *V. cholerae* cells L^{-1} during the sampling period at 4 monitored areas; lake center (**A**), reed belt (**B**), Zicklacke (**C**) and Lange Lacke (**D**). Bars represent standard deviation of triplicate samples.

4.2 Quantification of endemic V. cholerae attached to zooplankton

Due to the ability of *V. cholerae* to adhere to various zooplanktonic organisms, the endemic zooplankton of the 5 sampling areas was counted and named (data kindly provided by Prof. Alois Herzig). The most abundant crustacean zooplankton was divided into two main subclasses. *Daphnia magna*, *Moina brachiata*, *Diaphanosoma mongolianum*, *Bosmina longirostris*, *Ceriodaphnia quadrangular* were pooled in the cladoceran subclass and *Arctodiaptomus ssp*. and *Cylopidae* were pooled in the copepod subclass. The attached *V. cholerae* were visualised by FISH and quantified by microscopy, due to the reason that classical cultivation methods lead to hardly reliable results. Attached *V. cholerae* cells were observed from May to September (**Fig. 5**).

In May, the maximum of copepod-attached *V. cholerae* was 4.52 x 10^5 cells L^{-1} at Oberer Stinker, and the highest number of cladoceran-attached *V. cholerae* was 8.88 x 10^4 cells L^{-1} in Zicklacke. In June, maximum cell numbers of copepod-attached *V. cholerae* reached 3.71 x 10^4 cells L^{-1} in the Oberer Stinker. Highest *V. cholerae* numbers attached to cladocerans were found in the lake center with 1.43 x 10^4 cells L^{-1}. In July, the highest numbers of zooplankton-attached *V.cholerae* were recorded at the Oberer Stinker for both copepods (6.4 x 10^7 cells L^{-1}) and cladocerans (1.95 x 10^7 cells L^{-1}). During August a maximum of 1.47 x 10^5 *V. cholerae* cells L^{-1} was found at Oberer Stinker, whereas the highest number of cladoceran-attached *V. cholerae* was counted at Lange Lacke with 9.28 x 10^4 cells L^{-1}. In September, the maximum cell numbers were found at Zicklacke with 1.43 x 10^5 (copepods) and 4.18 x 10^4 cells L^{-1} (cladocerans).

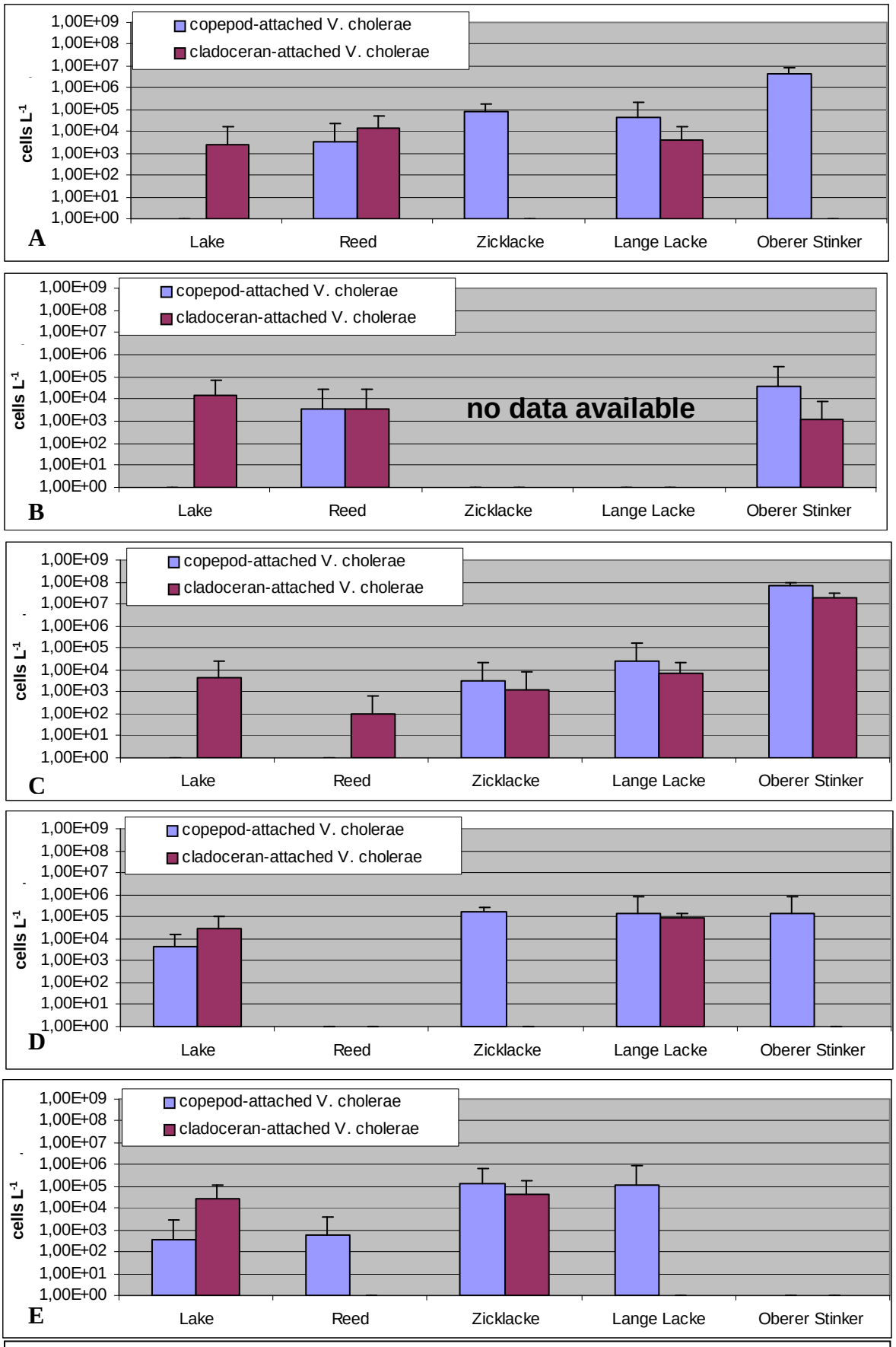


Fig. 5: Environmental *V. cholerae* cells L^{-1} attached to zooplankton monitored over the whole sampling period, May (**A**), June (**B**), July (**C**), August (**D**) and September (**E**). Bars represent standard deviation of triplicate samples.

4.3 Microcosm experiments

All experiments were carried out at 28 °C with various *V. cholerae* strains. Strains which were used during the summer period are listed in **Table 2** below.

Table 2: *Vibrio cholerae* (Vc) strains used for microcosm experiments.

Strain	Source	Notation	Serotype
Vc33	University of Maryland, USA	ATCC 39315	O1 El Tor
Vc34	University of Maryland, USA	O395	O1 Classical, 6th pandemic strain
Vc35	University of Maryland, USA	MO10	O139
Vc55	ICDDR, Bangladesh	AI-1852	O139

4.3.1 Microcosm experiment I (June)

The inoculated cell concentration of Vc33 was on average 1.22 x 10^7 L^{-1}. Growth was observed in all cultures inoculated with Vc33, but in no culture was a significant difference to the control flask observed (**Fig. 6**). In contrast, Vc34 showed in the batch cultures of the reed belt and the Lange Lacke a higher yield than in the respective controls. The inoculum concentration was on average 3.32 x 10^7 cells L^{-1} and the highest net yield (i.e. cell numbers in batch culture minus control) of 2.02 x 10^9 cells L^{-1} was detected in the batch culture from lake (**Fig. 6**). For Vc35, the inoculated number was on average 1.63 x 10^7 cells L^{-1}, and net growth (i.e. cell number increase was significantly higher than in the controls) was observed for the reed belt and the Lange Lacke. The highest net yield was detected with 1.3 x 10^9 cells L^{-1} for reed belt (**Fig. 7**).

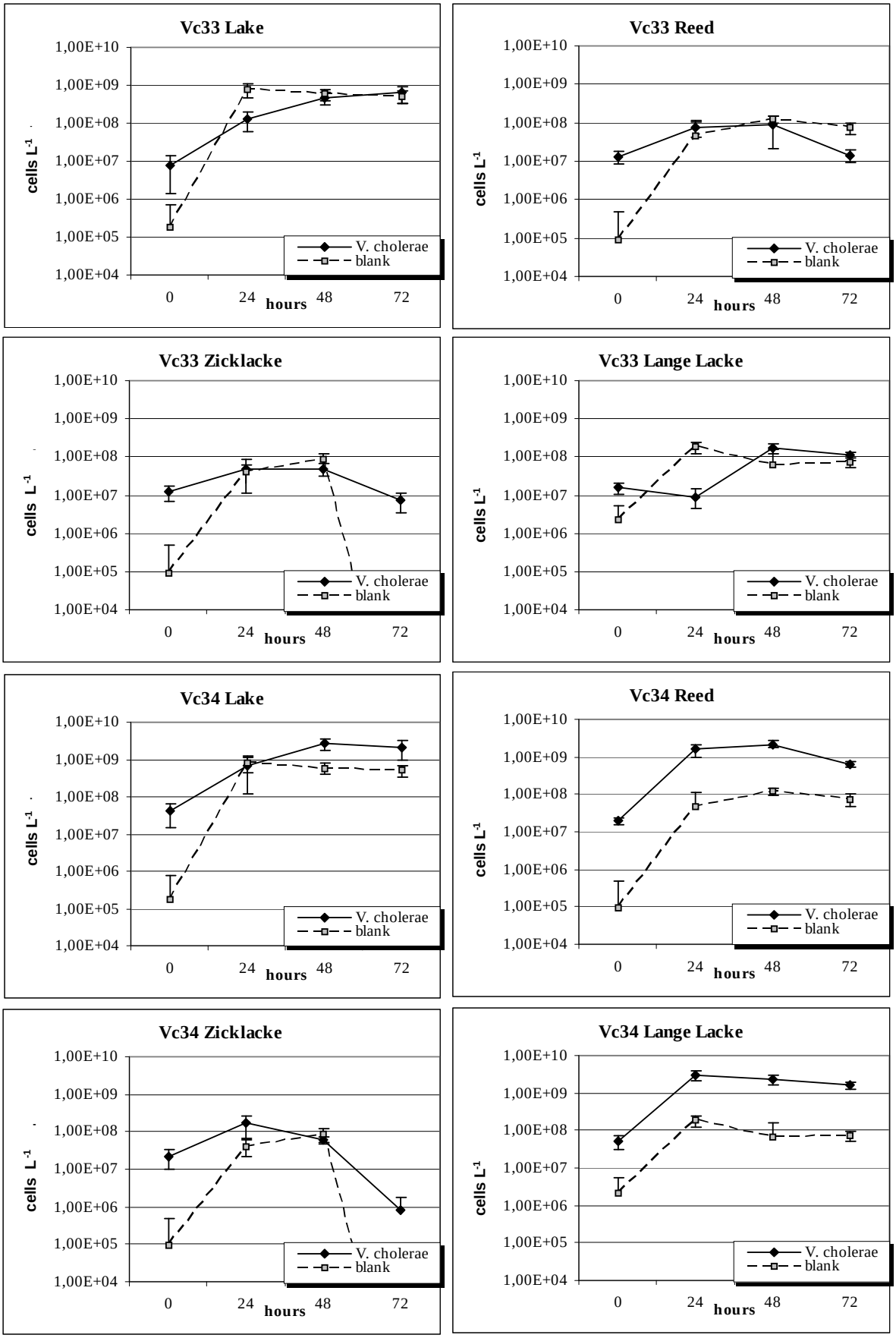


Fig. 6: Cell growth $L^{-1}$of *V. cholerae* Vc33 (upper graphs) and Vc34 (lower graphs) in batch cultures prepared with water from 4 sampling stations during June. Error bars indicate the standard deviation of 20 microscopic fields.

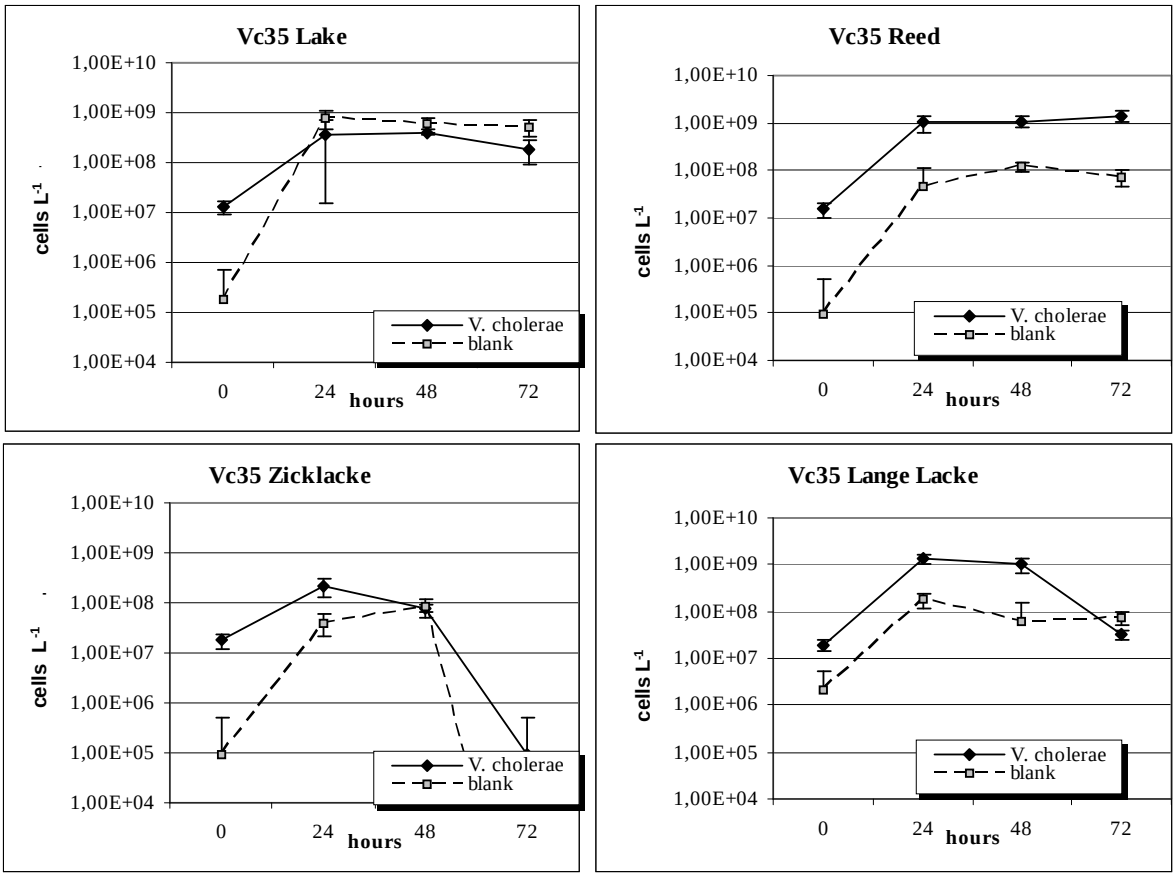


Fig. 7: Cell growth $L^{-1}$of *V. cholerae* Vc35 in batch cultures prepared with water from 4 sampling stations during June. Error bars indicate the standard deviation of 20 microscopic fields.

4.3.2 Microcosm experiment II (July)

The inoculated number of Vc34 cells was on average 4.1 x 10^7 cells L^{-1}, the highest yield was detected with 1.81 x 10^9 cells L^{-1} for Zicklacke with an absolute increase of 1.54 x 10^9 cells L^{-1} (**Fig. 8**). At Lange Lacke and lake center no significant difference between sample and control were observed. The inoculated cell concentration of Vc35 was on average 3.04 x 10^7 cells L^{-1}. A significant net growth was only observed for Zicklacke with an absolute increase of 2.85 x 10^8 cells L^{-1} (**Fig. 8**). On average, strain Vc55 started with a cell concentration of 9.88 x 10^6 cells L^{-1} and showed net growth for all stations except for the lake center (**Fig. 9**). The highest yield was obtained for Lange Lacke with 2.3 x 10^9 cells L^{-1}.

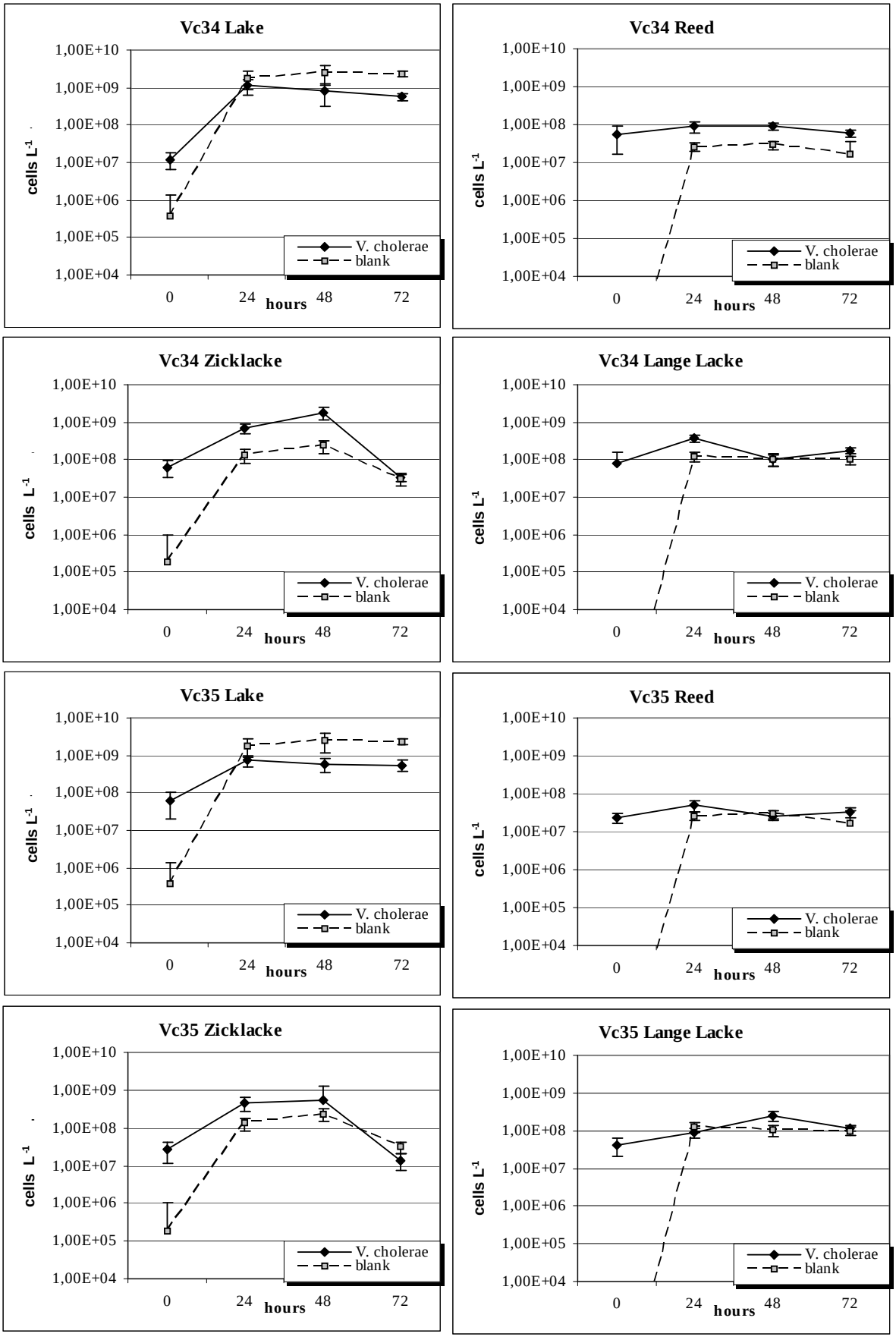


Fig. 8: Cell growth $L^{-1}$of *V. cholerae* Vc34 (upper graphs) and Vc35 (lower graphs) in batch cultures prepared with water from 4 sampling stations during July. Error bars indicate the standard deviation of 20 microscopic fields.

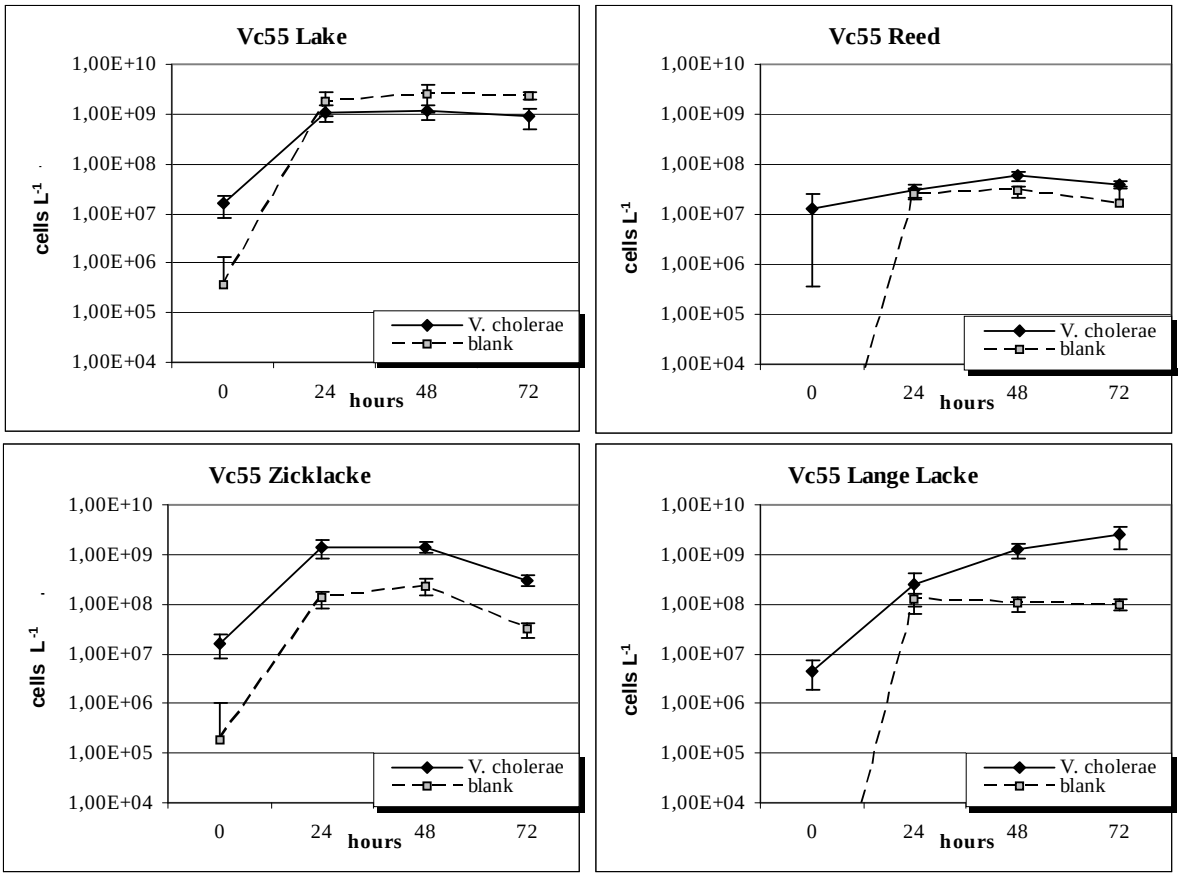


Fig. 9: Cell growth $L^{-1}$of *V. cholerae* Vc55 in batch cultures prepared with water from 4 sampling stations during July. Error bars indicate the standard deviation of 20 microscopic fields.

4.3.3 Microcosm experiment III (August)

The strain Vc34 showed significant net growth for reed, Zicklacke and Lange Lacke From an initial average cell concentration of 3.62 x 10^7 the highest net yield was obtained at Zicklacke with 3.87 x 10^9 (**Fig. 10**). The inoculated Vc35 strain showed net growth for reed and Zicklacke with an absolute increase of 1.5 x 10^9 cells L^{-1} (**Fig. 10**). Strain Vc55 was able to grow in all batch cultures from all four sampling stations. From an initial average inoculum concentration of 2.45 x 10^7 cells L^{-1}, highest net yield was observed at Zicklacke with an absolute increase of 4.75 x 10^9 cells L^{-1} (**Fig. 11**).

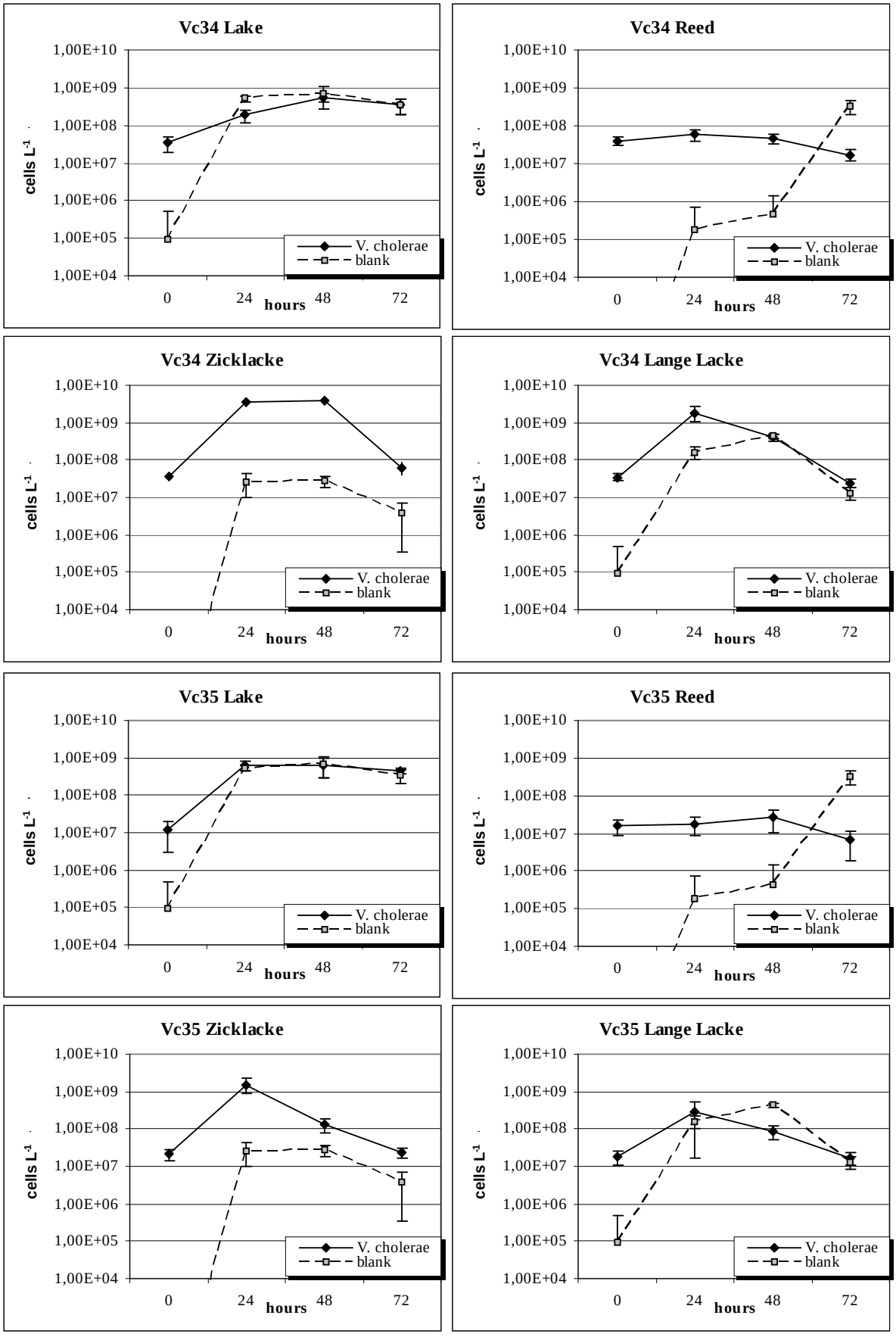


Fig. 10: Cell growth $L^{-1}$of *V. cholerae* Vc34 (upper graphs) and Vc35 (lower graphs) in batch cultures prepared with water from 4 sampling stations during August. Error bars indicate the standard deviation of 20 microscopic fields.

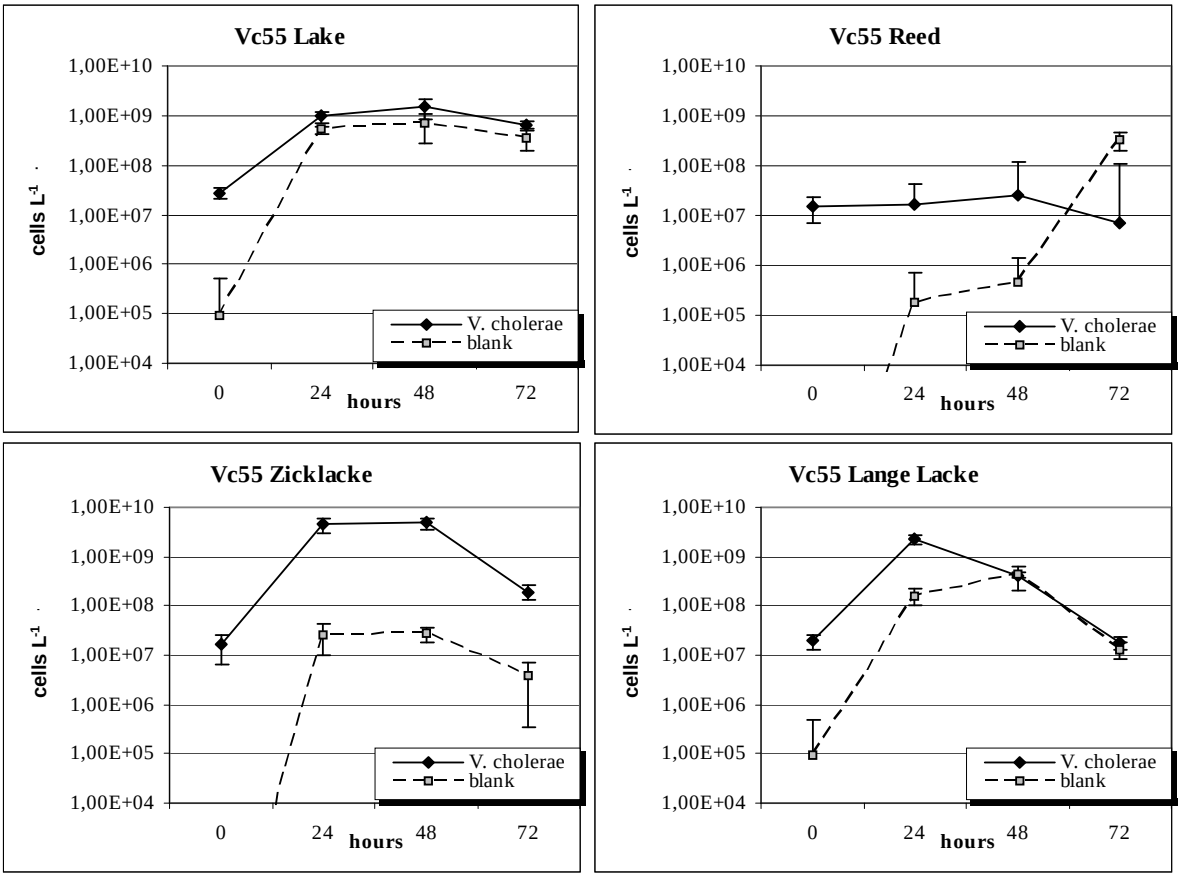


Fig. 11: Cell growth $L^{-1}$of *V. cholerae* Vc55 in batch cultures prepared with water from 4 sampling stations during August. Error bars indicate the standard deviation of 20 microscopic fields.

4.3.4 Microcosm experiment IV (September)

The inoculated number of Vc34 was on average 3.23 x 10^7 cells L^{-1}, a significant net growth was observed only for the two shallow alkaline pools. For Zicklacke, an absolute increase of 2.67 x 10^9 cells L^{-1} was observed (**Fig. 12**). Similarly, significant net growth of strain Vc35 was observed only for Lange Lacke and Zicklacke. The inoculated cell concentration of Vc35 was on average 1.85 x 10^7 cells L^{-1}, where highest net yield was detected for Zicklacke with an increase of 1.42 x 10^9 cells L^{-1} (**Fig. 12**). Strain Vc55 started with an initial cell concentration of 2.42 x 10^7 L^{-1} and was able to show significant net growth at all sampling stations. Again, for Zicklacke, the highest net yield of 3.87 x 10^9 cells L^{-1} was observed (**Fig. 13**).

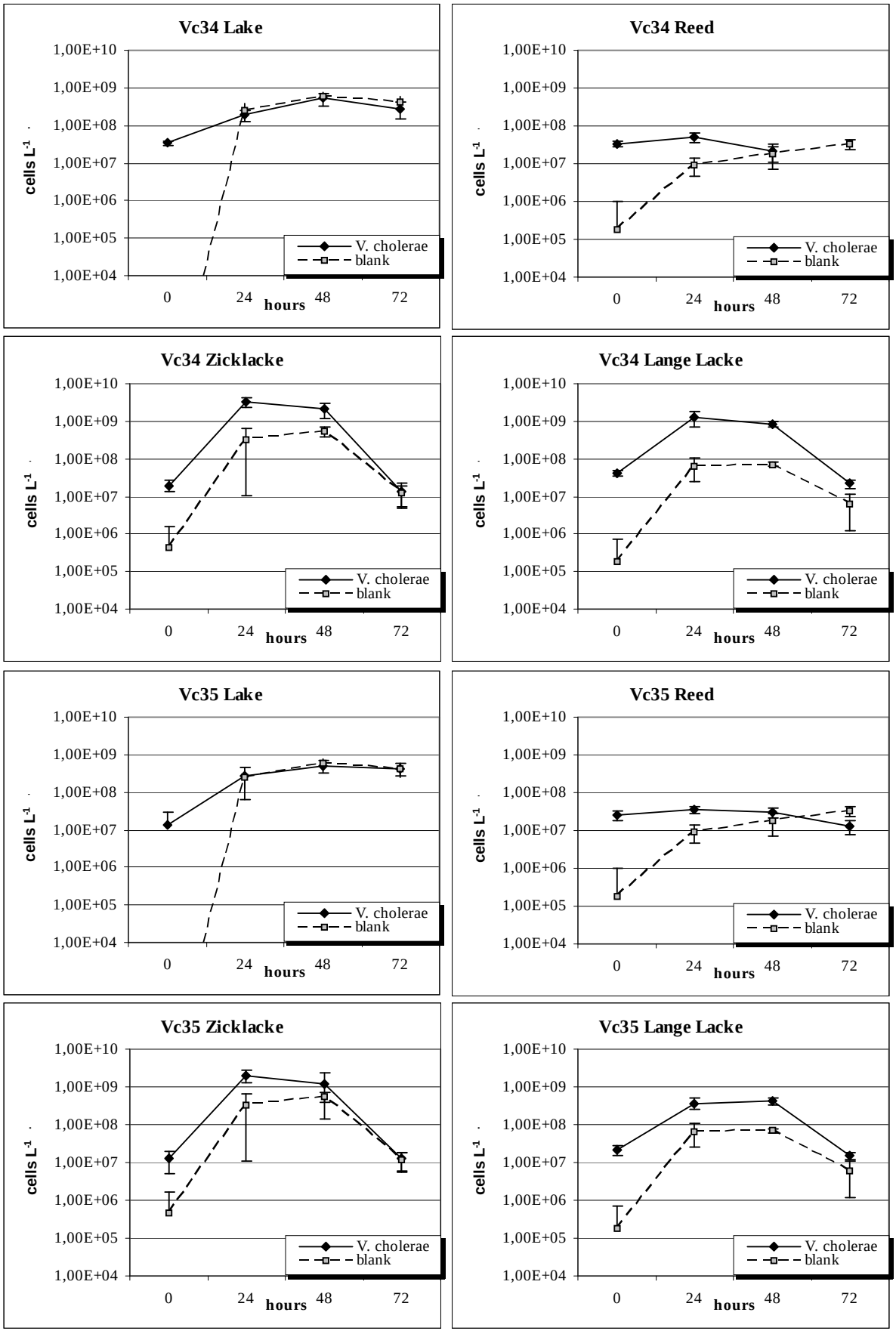


Fig. 12: Cell growth $L^{-1}$of *V. cholerae* Vc34 (upper graphs) and Vc35 (lower graphs) in batch cultures prepared with water from 4 sampling stations during September. Error bars indicating the standard deviation of 20 microscope fields.

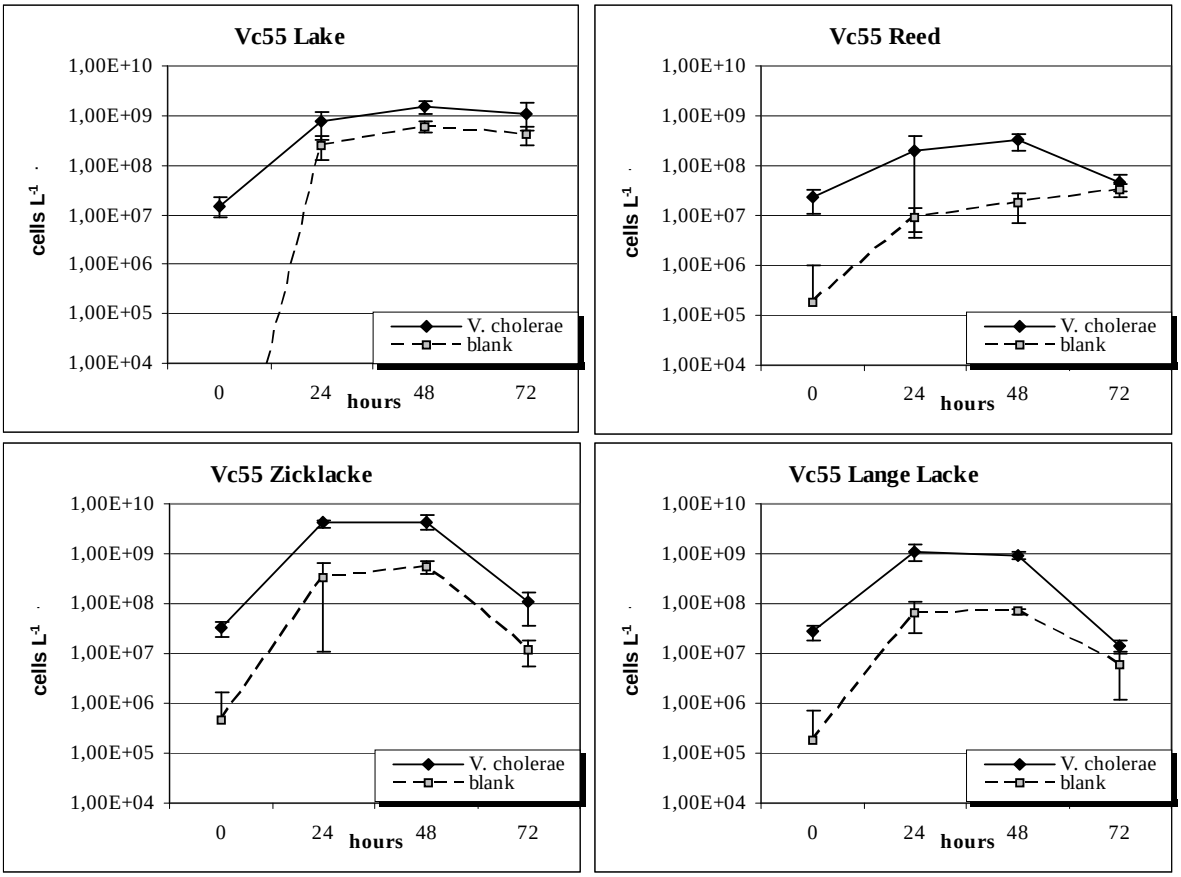


Fig. 13: Cell numbers $L^{-1}$of *V. cholerae* Vc55 in batch cultures prepared with water from 4 sampling stations during September. Error bars indicate the standard deviation of 20 microscopic fields.

4.4 Detection of pathogenic strains

During the sampling period, 181 isolates from free-floating samples and 123 isolates from zooplankton as well as a total of 51 APW enrichment cultures were screened by multiplex-PCR for the presence of genes specific for cholera causing *Vibrio cholerae* O1 or O139 strains. Out of these samples, 164 (free-floating, 91%), 109 (zooplankton, 89%) and 34 (APW enrichment cultures, 67%) were tested *ompW* positive and confirmed the identification as *Vibrio cholerae*. Neither one of the isolated strains, nor of the enrichment cultures were *tcpA*, *ctxAB*, *wbe* and *wbf* positive.

5 Discussion

5.1 Quantification of environmental V. cholerae cells

The crustacean component of the zooplankton community of the lake Neusiedler See and the alkaline saline lake comprises 7 cladoceran and 4 copepod species. The high alkalinity which occurs in the monitored area is tolerated by all of these species.
The highest numbers of both planktonic crustaceans (*A. spinosus* and *D. mongolianum*) varies strongly during summer in the monitored area from 70 crustaceans L^{-1} to maximum value of 1800 crustaceans L^{-1} (A. Herzig, personal communication), with a pure copepod community present in winter.
It is widely accepted, that *V. cholerae* is either a free living bacterium (Worden et al. 2006, Kirschner et al. 2008) or associated with phytoplankton (Epstein 1993), crustacean zooplankton (Huq et al. 1983) in coastal, estuarine and brackish environments (Colwell et al. 1992, Islam et al. 1994). Attachment to surfaces serves as a better protection from bad environmental conditions (Faruque et al. 2002) and furthermore chitinaceous organisms are a nutrient source because *V. cholerae* is able to metabolise chitin by chitinase (Pruzzo et al. 2008).
Beside biotic parameters, a variety of abiotic parameters seem to have important influences on the *V. cholerae* abundance. Culture based detection of *V. cholerae* is usually possible above a temperature of 10 ℃, whil e below this value it turns into a viable but not cultivable state (VBNC), where it can be detected only with molecular or microscopical methods (Huq et al. 1990, Colwell 1996, Chun 1999; Steinberger, 2008). Furthermore, the influence of salinity and pH on the abundance of *V. cholerae* was demonstrated, where estuarine and brackish environments at salinities between 2 and 14‰ are necessary for optimal growth (Patel et al. 1995; Jiang et al. 2001, Louis et al. 2003). The 5 various sampling areas were selected due to their highly different biotic and abiotic constitution which are listed in **Table 3**. Because of the different environmental conditions of the sampling areas investigated in this study they were regarded as 5 distinct ecosystems.

Table 3: pH: pondus Hydrogenii, EC: electric conductivity, Secchi: Secchi depth, FTX: faecal taxation, TSS: total suspended solids, DOC: dissolved organic carbon, POC: particular organic carbon; n.d.: not determined

	Lake	Reed
temperature C°	21.9 (17.5-27.3)	22.2 (17.6-27.2)
pH	8.8 (8.7-8.9)	8.3 (8.2-8.4)
EC (µS)	2220 (2000-2700)	2360 (2100-2900)
Secchi	37.4 (28-61)	64.8 (44-90)
FTX (faecal pellets $100m^{-2}$)	n.d	n.d
TSS (mg/L)	15.2 (5-29.9)	7.8 (4-15)
DOC (mg/L)	20.2 (18.1-24.6)	22.2 (20.3-24)
POC (mg/L)	6.5 (3-10)	2.5 (1-4)
absorbance ratio	10.6 (4.1-14.2)	8.1 (7.7-8.9)
alkalinity (mmol/L)	9.5 (9.3-9.8)	10.6 (10-11.2)
Chlorophyll a (µg/L)	6.2 (2.7-10.6)	7.1 (3.5-10.2)

	Zicklacke	Lange Lacke	Oberer Stinker
temperature C°	25.6 (21.2-28)	24.1 (20.2-27.5)	24 (19.5-27.7)
pH	9.5 (9.1-9.8)	9.3 (9.2-9.5)	9.7 (9.5-9.8)
EC (µS)	4986 (4080-6400)	4192 (3260-6520)	12732 (8150-18310)
Secchi	4.2 (3-5)	4.5 (2-7)	2.4 (1-5)
FTX (faecal pellets $100m^{-2}$))	150 (6-313)	440 (10-1200)	67 (0-210)
TSS (mg/L)	182 (13-550)	445.4 (67-1250)	3066 (800-7500)
DOC (mg/L)	73.4 (51.9-93.8)	46.1 (30.7-78)	100.2 (53.2-148)
POC (mg/L)	315 (180-450)	456 (62-850)	3665 (2130-5200)
absorbance ratio	9.6 (6.4-11.3)	10.4(8.3-12.3)	13.2 (9.9-15.9)
alkalinity (mmol/L)	26.7 (21.2-34)	24.8 (19-35.2)	111.8 (58-137)
Chlorophyll a (µg/L)	88.6 (14.4-249.3)	31.3 (2.5-113.6)	56.3 (4.6-86.8)

5.2 Quantification of free-floating and zooplankton associated *V. cholerae*

In general, little is known about the ecological niches that are occupied by endemic *V. cholerae* strains and by which biotic and abiotic factors a possible switch in its main life mode is triggered. Steinberger (2008) reported that many of these environmental parameters simultaneously interact, their positive or negative influence on the growth of *V. cholerae* is often compensated and it is extremely difficult to unveil statistically proved relationships of *V. cholerae* concentrations with environmental variables. Furthermore, it is well known that *V. cholerae* occurrence is often associated with algae blooms (Eppstein 1993, Eiler et al. 2006) and crustacean zooplankton blooms (Colwell et al. 1977, Sochard et al. 1979, Huq et al. 1983). The following figures show the seasonal changes in the abundance of free-floating *V. cholerae*, zooplankton-attached *V. cholerae* and the crustacean community, which probably serves as an important environmental reservoir for *V. cholerae*.

In the investigated sampling area lake center (**Fig. 14-A1**) the seasonal abundances of the free-floating and zooplankton attached *V. cholerae* are following a similar pattern. Zooplankton attached *V. cholerae* cells are hardly influenced by the absolute abundance of zooplankton (**Fig. 14-A1**), but *V. cholerae* clearly prefers cladocerans (94.7%) over copepods (**Fig. 14-B1**). The highest number of 6.28 x 10^4 *V. cholerae* cells L^{-1} was measured in August (**Fig. 14-A1**). In contrast, sampling area reed belt (**Fig. 14-A2**) is clearly dominated by free-floating *V. cholerae* over the surveillance period. Although the abundance of zooplankton is higher than in lake center during May and June, (**Fig. 14-A1**), total *V. cholerae* cell numbers are nearly the same as in the lake center. During July, zooplankton concentration decreases drastically from 82 (**Fig. 14-A2**) to a total of 7 zooplankton individuals L^{-1}. In parallel, zooplankton-attached cells decreased from 91 *V. cholerae* cells zooplankton^{-1} to 13 *V. cholerae* cells zooplankton^{-1}, while the concentration of free-floating *V. cholerae* was unaffected. The highest number of 1.43 x 10^4 *V. cholerae* cells L^{-1} was measured in June (**Fig. 14-A2**) and in general, 71.1% of all attached *V. cholerae* cells were found on cladocerans. The preference of *V. cholerae* to attach to cladocerans in the Neusiedler See, firstly reported by Schauer (2008), is indicated by the high percentage of cells attached to cladocerans. The calculated absolute cell numbers per individual (2.37 x 10^3 – 3.13 x 10^4) coincide to numbers (3.04 x 10^4 – 7.66 x 10^4) reported by Schauer (2008).

Interestingly, the lower zooplankton abundance in the reed belt comes along with an increase of HMW substances (data not shown). HMW may have an inhibitory effect on the zooplankton and their attached cells, while free-floating *V. cholerae* seem to be unaffected. The reed belt is hardly influenced by wind and currents indicated by a higher Secchi depth. Due to this fact the reed belts' sediment can act as a sink and may serve as a microbial reservoir.

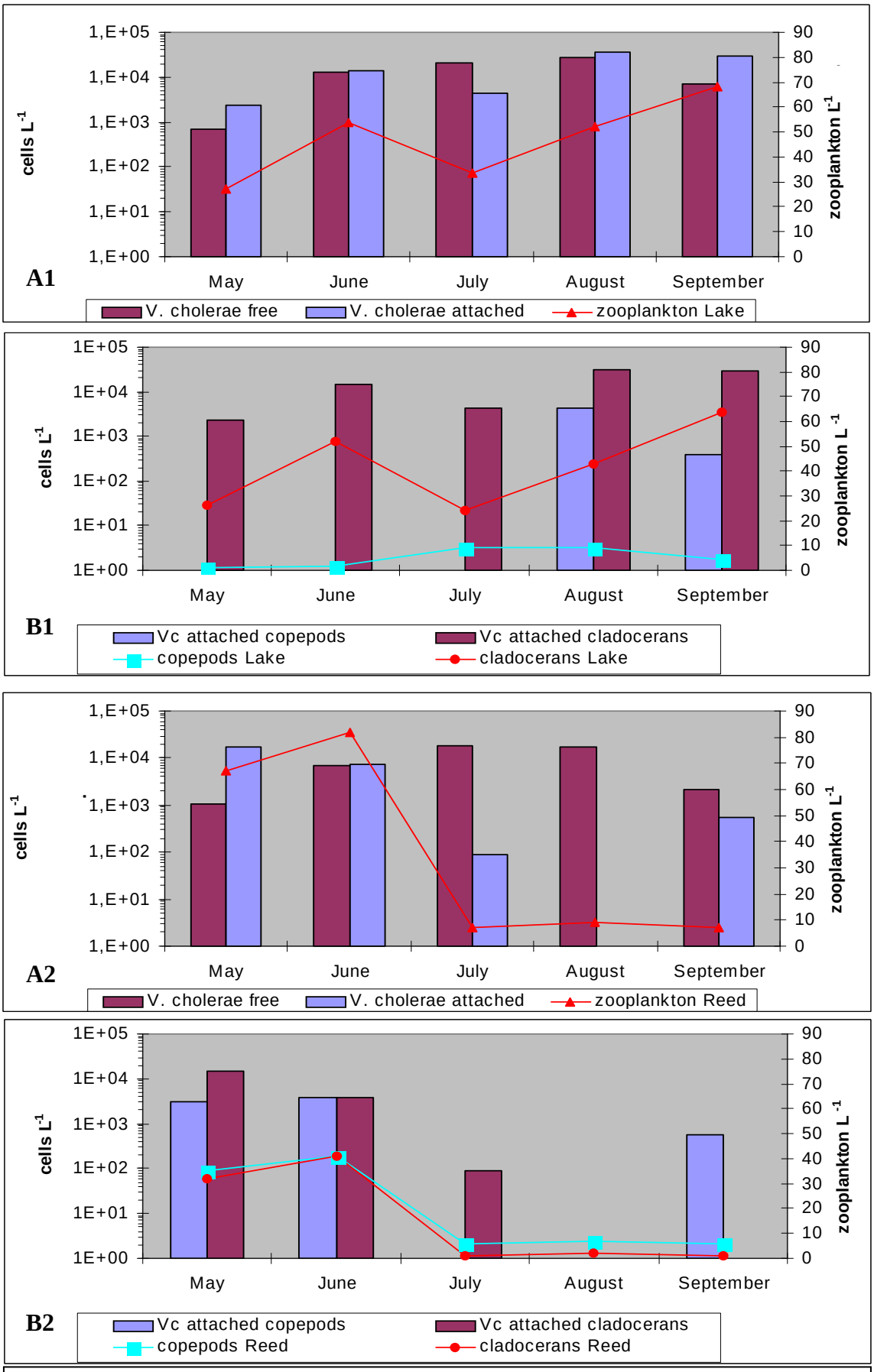


Fig. 14: Free-floating *V. cholerae* cells L^{-1} and zooplankton attached *V. cholerae* cells L^{-1} in comparison with zooplankton abundance L^{-1} (**A**) and *V. cholerae* cells L^{-1} attached to either copepods or cladocerans L^{-1} (**B**) in lake center (**1**) and reed belt (**2**).

In contrast to the well examined lake Neusiedler See, data of the shallow alkaline saline lakes are scarce. In all measured parameters the three examined sampling areas differ clearly. The sampling area Zicklacke is characterized (**Table 3**) by an average salinity of 5.4 (2.7-17.8) g L^{-1} (Eiler et al., 2003). In parallel to the observation that heterotrophic bacterial numbers in the shallow soda pools are much higher than in the lake Neusiedeler See (Eiler et al., 2003), total *V. cholerae* cell numbers in the soda pools were also markedly higher. The maximum *V. cholerae* cell number of 5.14 x 10^5 cells L^{-1} (**Fig. 15A**) was recorded in August, the average cell number was 1.01 x 10^5 cells L^{-1}. In contrast to lake center and reed belt, the proportional distribution of zooplankton-attached *V. cholerae* is completely inverted, indicated by a low percentage (9.8%, **Fig. 15B**) of cladoceran-attached *V. cholerae* cells. The maximum zooplankton number of 676 organisms L^{-1} (**Fig. 15A**) occurred in September, when *V. cholerae* concentrations remained on a high level.

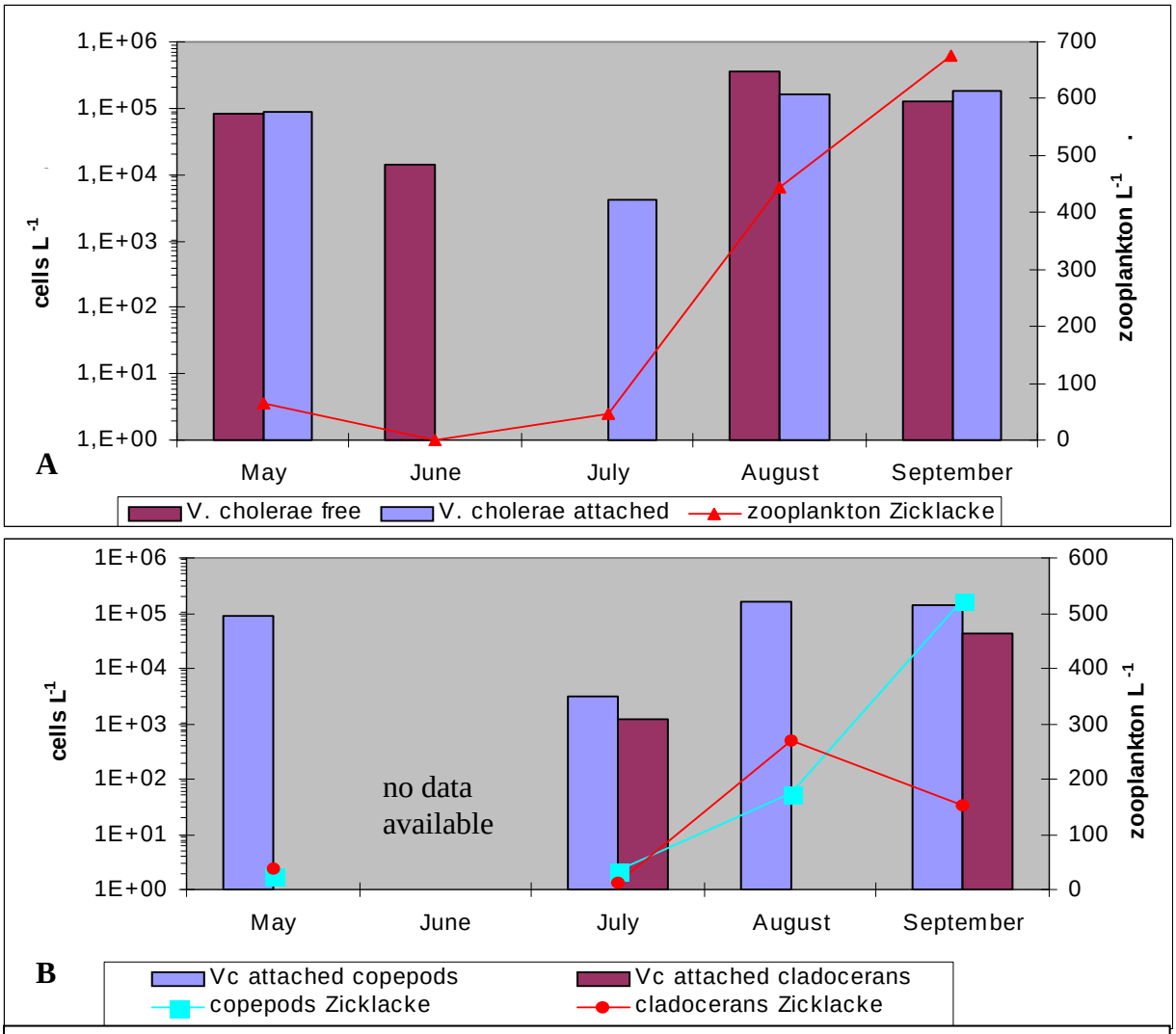
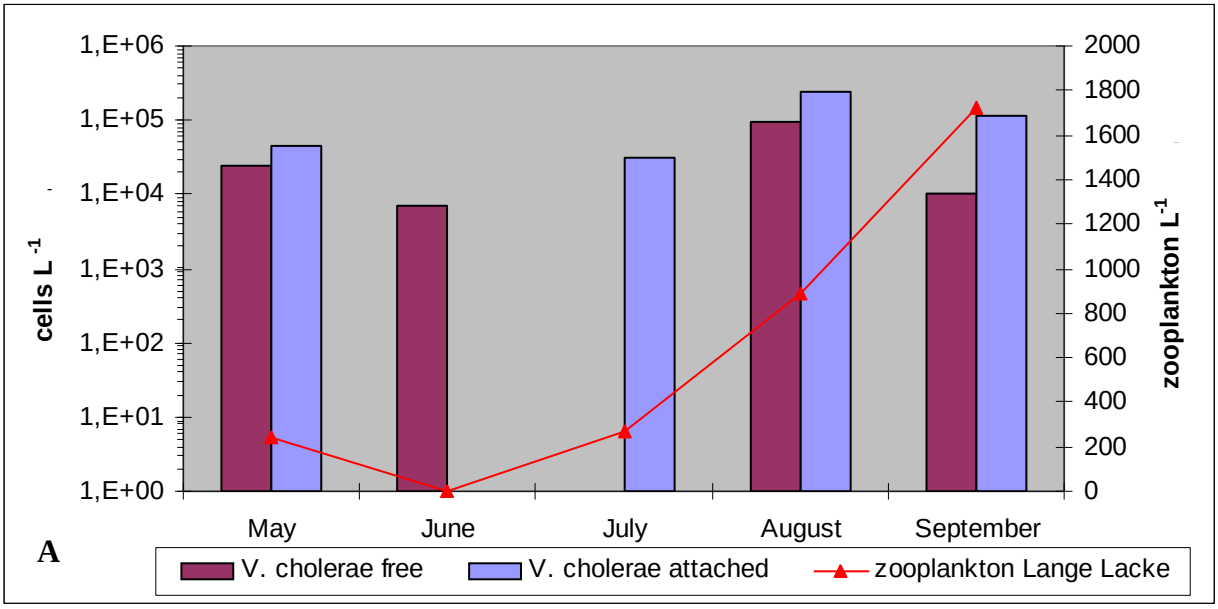


Fig. 15: Free-floating *V. cholerae* cells L^{-1} and zooplankton attached *V. cholerae* cells L^{-1} in comparison with zooplankton abundance L^{-1} (**A**) and *V. cholerae* cells L^{-1} attached to either copepods or cladocerans L^{-1} (**B**) in Zicklacke.

The soda pool Lange Lacke is characterized (**Table 3**) by an average salinity of 3.8 (2.4-8.4) g L^{-1} (Eiler et al., 2003). The highest *V. cholerae* concentrations were measured in August with a total of 3.28 x 10^5 cells L^{-1} (**Fig. 16A**). Over the investigation period an average of 5.59 x 10^4 cells L^{-1} was calculated. Both values are smaller than in Zicklacke which may be explained by the absolute amount and the composition of the DOC. The DOC concentration is much higher in Zicklacke than in Lange Lacke indicating a higher amount of organic substances utilisable for *V. cholerae*. Because the absorbance ratio in Lange Lacke is similar as in Zicklacke this means that more LMW substances in Zicklacke are present than in Lange Lacke.

Figure 16B shows the seasonal development of copepods and cladocerans in comparison to their attached *V. cholerae* cells. The abundance of cladocerans is far smaller than the one of copepods but the number of attached *V. cholerae* is clearly different to this pattern. On average, 127 *V. cholerae* cells are attached to one copepod and 175 *V. cholerae* cells are attached to one cladoceran. Interestingly, in August 182 *V. cholerae* cells are attached to one copepod and 910 *V. cholerae* cells are attached to one cladoceran.

These values clearly indicate *V. choleraes'* preference for cladocerans. This may be one reason for higher concentrations of attached *V. cholerae* cells L^{-1} than free-floating *V. cholerae* cells L^{-1}. If cladoceran blooms are enhanced by various reasons like an increase of water temperature by global warming a concomitant *V. cholerae* bloom can be expected.



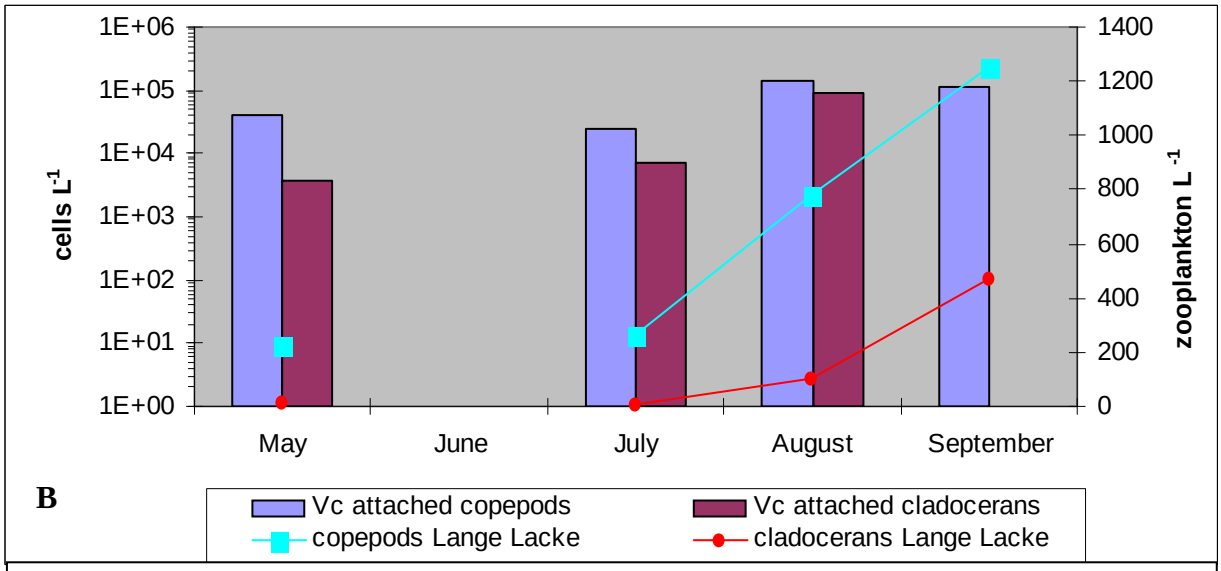


Fig. 16: Free-floating *V. cholerae* cells L^{-1} and zooplankton attached *V. cholerae* cells L^{-1} in comparison to zooplankton abundance L^{-1} (**A**) and *V. cholerae* cells L^{-1} attached to either copepods or cladocerans L^{-1} (**B**) in Lange Lacke.

The shallow alkaline saline lake Oberer Stinker is characterized (**Table 3**) by the highest salinity of 7.4 (4.2-31.3) g L^{-1}, highest alkalinity (average 112 mmol L^{-1}) measured during this survey program and a wind-independent permanent turbidity. As mentioned in the results section, no planktonic *V. cholerae* cells could be isolated from the water body of the Oberer Stinker (**Fig. 17A**). The high alkalinity may either inhibit the cultivation of *V. cholerae* or no *V. cholerae* cells are present in a free-floating modus vivendi in this shallow alkaline lake. Considering this observation, a new method for detection and identification of *V. cholerae* is necessary to overcome a possible misinterpretation. Nevertheless, zooplankton abundance climbed upwards constantly during the surveillance period, whereby only 5.2% (**Fig. 17B**) of the total

zooplankton number consisted of cladocerans. Though, the average number of cladoceran attached *V. cholerae* cells L^{-1} amounted to 22.1%. Despite no free-floating *V. cholerae* cells were detected, the sampling area Oberer Stinker harboured the highest concentrations of zooplankton and zooplankton attached *V. cholerae* cells of all investigated waters. The maximum cell concentration was measured at July with 8.35 x 10^7 *V. cholerae* cells L^{-1} and an average of 8.82 x 10^6 cells L^{-1} was calculated. These outstanding values may be based on a special composition of various parameters. An average DOC (100 mg L^{-1}) and an absorbance ratio of 13.2 clearly indicate that this shallow alkaline saline lake is a major reservoir for an active and abundant bacterial community (Kirschner et al. 2002, Eiler et al. 2003).

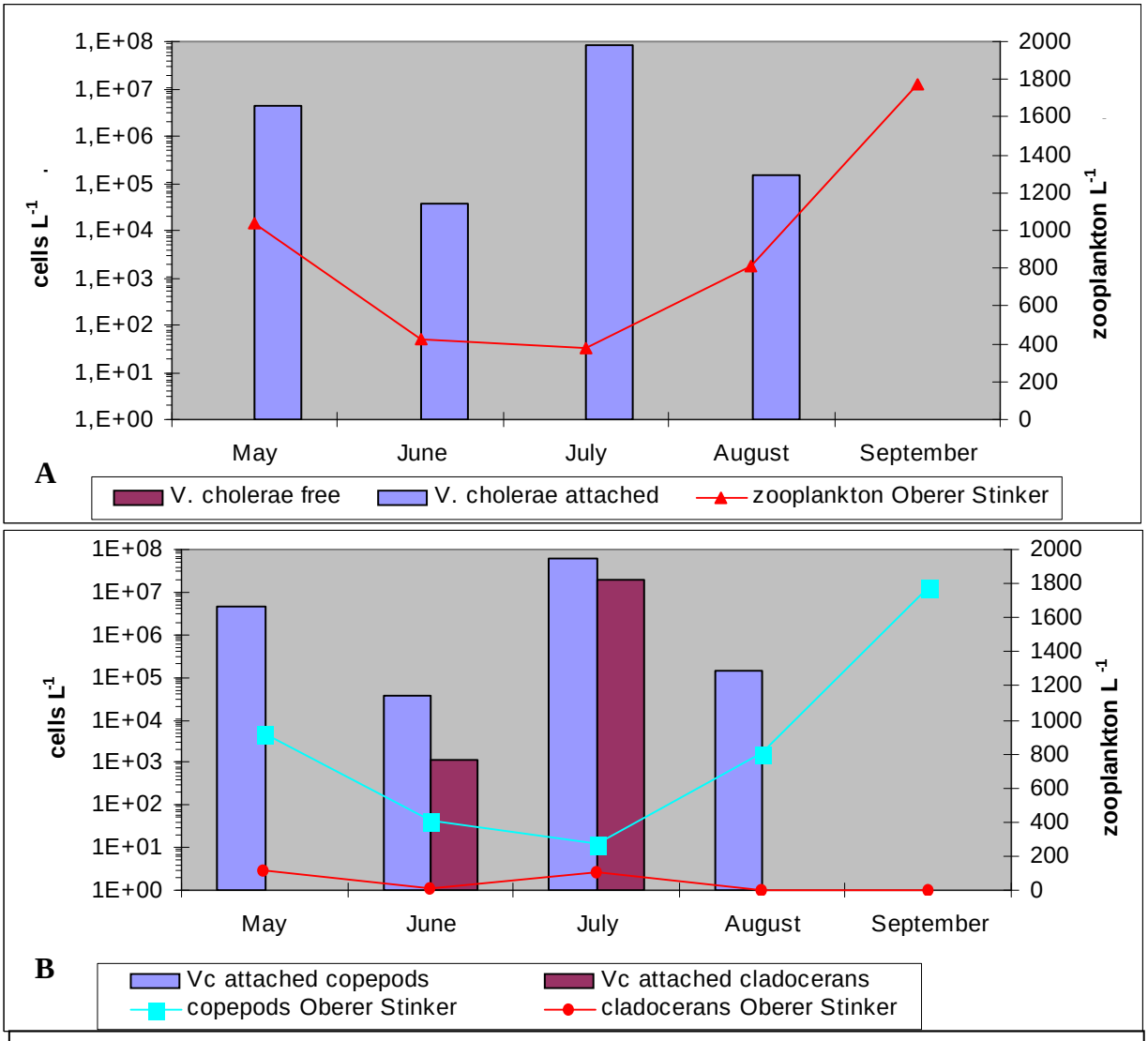


Fig. 17: Free-floating *V. cholerae* cells L^{-1} and zooplankton attached *V. cholerae* cells L^{-1} in comparison to zooplankton abundance L^{-1} (**A**) and *V. cholerae* cells L^{-1} attached to either copepods or cladocerans L^{-1} (**B**) in Oberer Stinker.

5.3 Microcosm experiments

From May to September, five microcosm experiments were performed to evaluate the invasion potential of selected pandemic cholera-pathogenic strains into the lake Neusiedler See and two representative shallow alkaline lakes. The lakes were chosen according to represent a broad spectrum of ecological parameters **(Table 4).** All selected areas are more or less used by migratory birds as breeding grounds and feeding habitats.

Table 4: pH: pondus Hydrogenii, EC: electric conductivity, Secchi: Secchi depth, amount of inoculated cladocerans and copepods in one sample flask, DOC: dissolved organic carbon.

	lake	reed	Zicklacke	Lange Lacke
temperature C°	20,3 (15,5-24,8)	21,3 (17,5-25,4)	25,9 (23,5-28,8)	23,2 (20,3-26,8)
pH	8,8 (8,7-8;9)	8,3 (8,2-8,4)	9,3 (9,1-9,5)	9,3 (9,2-9,5)
EC (µS)	2100	2160 (2000-2300)	3250 1290-5500)	2848 (1020-5300)
Secchi (cm)	25 (12-35)	70,4 (48-98)	3,8 (2-5)	2,7 (1-5)
Copepodes /flask	0	36 (0-50)	31 (0-50)	45 (40-50)
Cladocerans /flask	50	14 (0-50)	19 (0-50)	5 (0-10)
DOC (mg/L)	17,6 (15,8-18,9)	20 (16,2-21,9)	71,9 (44,2-95,8)	44,4 (24,2-62,9)
humic ratio	12,6 (10,5-13,5)	18 (8-33)	12,6 (8,3-15,6)	15,4 (10,9-22)
alkalinity (mmol/L)	9,5 (9,2-9,6)	10,5 (9,4-11,1)	25,7 (15,2-34)	23,4 (15,6-28,8)

Earlier investigations lead to the suggestion that birds may transport incorporated *Vibrio cholerae* cells in their gut to new unseated areas (Ogg et al., 1989). It is suggested, that these strains may be able to invade and occupy an ecological niche, displace an endemic strain or interchange some genetic material by horizontal gene transfer to a nonO1/non139 strain, which probably leads to a pathogenic highly locally adapted *V. cholerae* strain. Another scenario may be that nonO1/nonO139 *V. cholerae* may undergo antigenic changes and convert to an O1 serogroup while in the intestines of a bird (Ogg et al., 1989; Blokesch et al., 2007). Thus, given the likelihood of more recombination events leading to new toxigenic strains, it becomes increasingly clear that there is a need to understand the occurrence of and monitor for *V. cholerae* nonO1 in the environment (Lipp et al., 2002).

5.3.1 Microcosm experiments with *V. cholerae* O1 El Tor (Vc33)

Vibrio cholerae O1 El Tor is the causative agent of the seventh pandemic which is the most extensive of the pandemics in geographic spread and duration. The pandemic started 1961 on the island of Sulawesi and spread to the entire Southeast Asien archipelago, by 1970 the pandemic has invaded the Soviet Union, Irak, Iran and nearby republics. In the 1990s, the pandemic reached South America in form of an explosive epidemic which began in Peru and was spread nearly over the whole continent in a short time. The seventh pandemic is still ongoing and it continues to cause seasonal outbreaks in many developing countries (Faruque et al., 1998), especially in Africa (WHO, 1996 – 2008). Other studies indicate, that rugose colony variants of *Vibrio cholerae* O1 El Tor produce an exopolysaccharide, that confers chlorine resistance and biofilm-forming capacity (Yildiz et al., 1999). Due to these facts *V. cholerae* O1 El Tor, designated as Vc33, was selected for microcosm experiments. On the basis of the results from the experiments performed in June (**Fig. 18**), which indicated no significant difference between maximum cell numbers in the blank and the Vc33 inoculated flasks for all four sampling sites the decision was made to exclude this strain from further experiments.

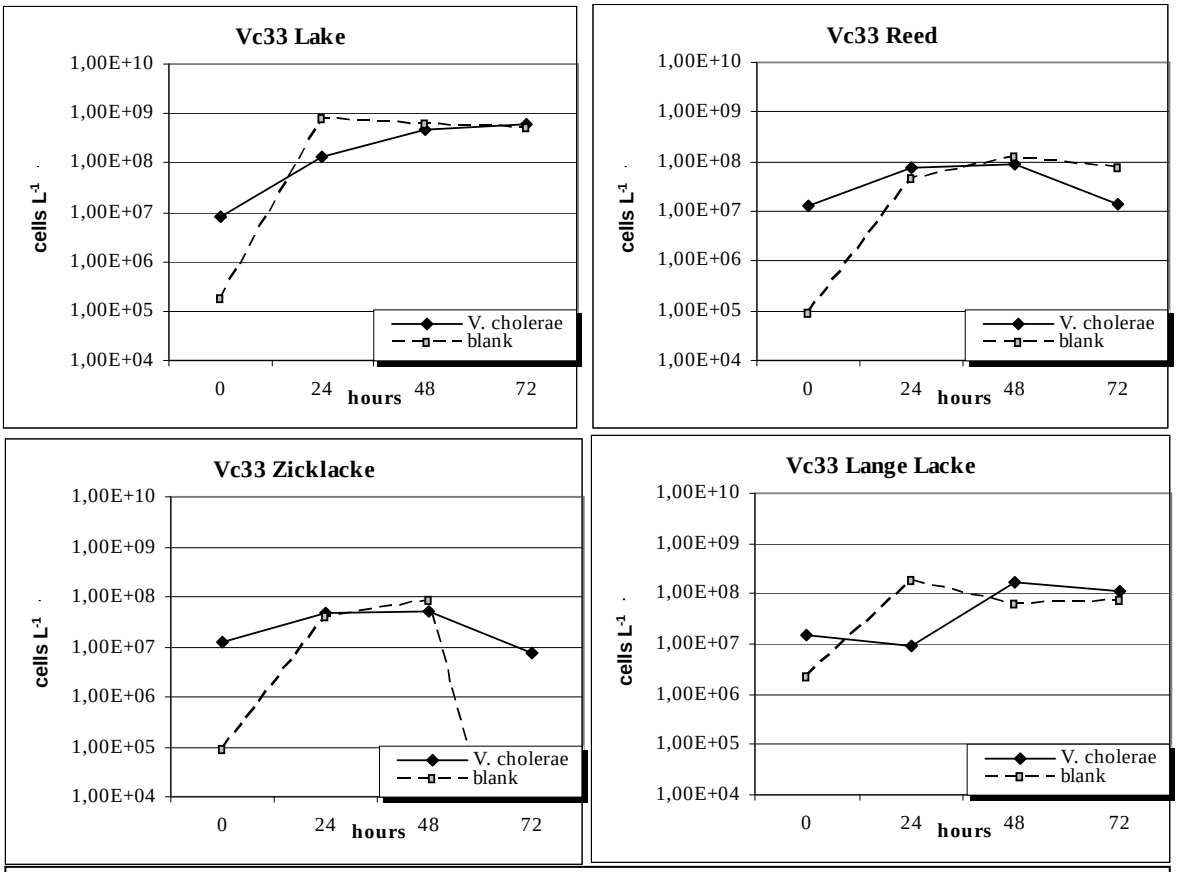


Fig. 18: Grow patterns of Vc33 in microcosms with water taken from lake center, reed belt, Zicklacke and Lange Lacke during a 72h microcosm experiment.

5.3.2 Microcosms experiment with *V. cholerae* O1 classical (Vc34)

The sixth and presumably the fifth pandemic were caused by *V. cholerae* O1 classical biotype, which was spread in the near and middle East, the Balkan and in large scale in the south south-east Asian subcontinent during 1900 up to 1923. After that time *V. cholerae* classical was more and more replaced by a new emerging and more severe biotype called El Tor (Faruque et al., 1998). Pooling data from all experiments, Vc34 (**Fig. 19**) showed in all batch cultures except for lake center ($p > 0.1$, $n = 4$) significant net growth (i.e. growth in the batch culture was significantly higher than in the respective control). Starting from an average initial concentration of 3.83×10^7 cells L^{-1}, net yields of 1.71×10^9 cells L^{-1} (Zicklacke; $p < 0.01$, $n = 4$), 4.33×10^8 cells L^{-1} (reed belt; $p < 0.01$, $n = 4$) and 1.41×10^9 cells L^{-1} (Lange Lacke; $p < 0.01$, $n = 4$) were observed.

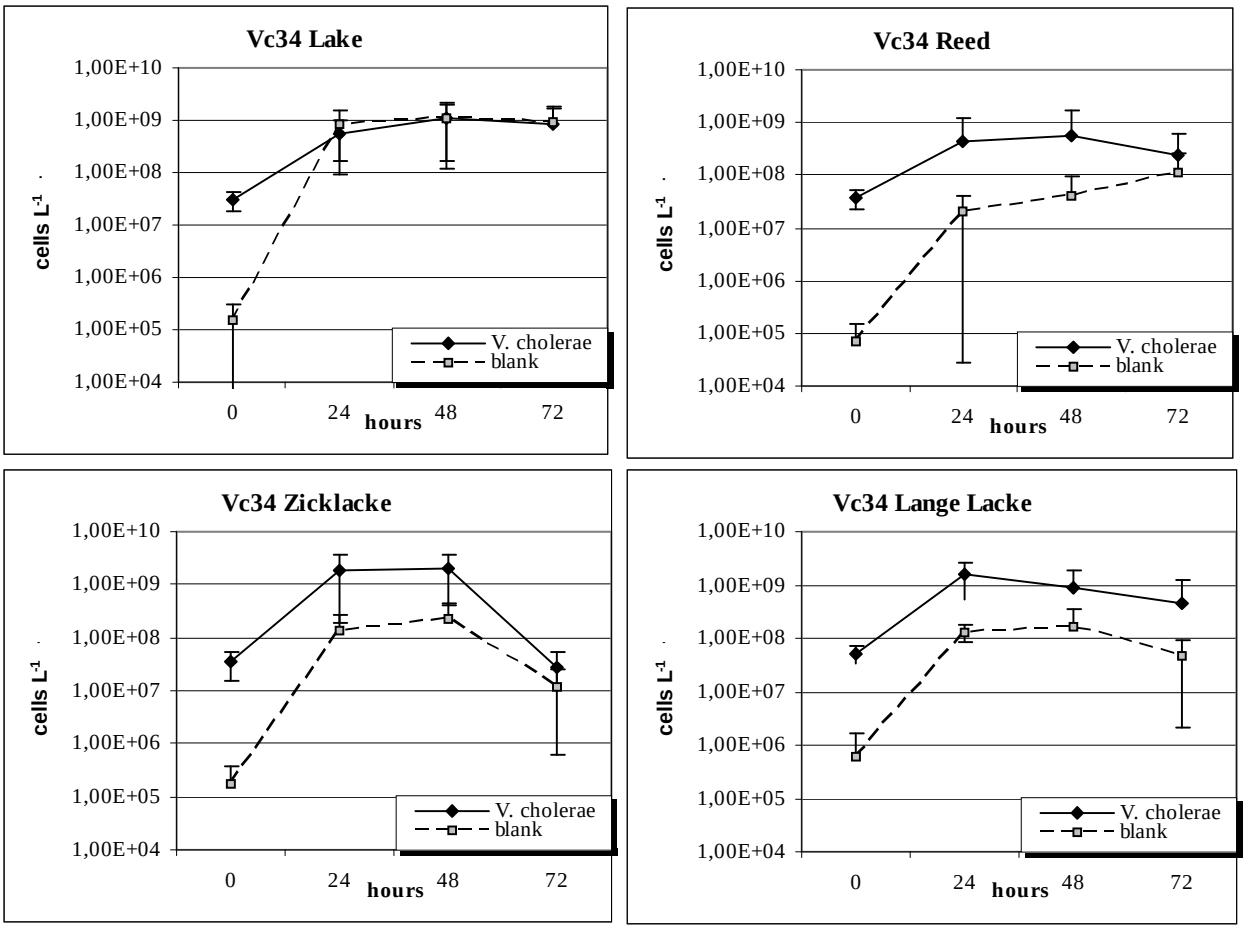


Fig. 19: Averaged (June, July, August, September) Vc34 pattern of growth in lake center, reed belt, Zicklacke and Lange Lacke during a 72h microcosm experiment. Error bars represent standard deviations of the 4 independent replicate experiments performed between June and September).

5.3.3 Microcosm experiments with *V. cholerae* O139 (Vc35)

Prior to 1992, the origin of all cholera epidemics had been strains of *V. cholerae* O1. However, during that year cholera outbreaks were associated with a new toxigenic serogroup, *V. cholerae* O139, in India and for a short time this group replaced *V. cholerae* O1 El Tor. Various researchers have now demonstrated that O139 strains associated with epidemics arose via a homologous recombination event. Ribotyping of both toxigenic and nontoxigenic strains of *V. cholerae* O139 revealed that isolates might have arisen from at least two different progenitors, including nonO1 serogroups (Lipp et al., 2002).

Based on this knowledge, Vc35 was selected for microcosm experiments and (**Fig. 20**) showed for all sampling areas except lake center ($p > 0.1$, $n = 4$) significant net growth. Starting from an average inoculation concentration of 2.24×10^7 cells $L^{-1,}$ net

yields of 8.09 x 10^8 cells L^{-1} (Zicklacke; $p < 0.01$, n =4), 2.36 x 10^8 cells L^{-1} (reed belt; $p < 0.01$, n =4) and 3.26 x 10^8 cells L^{-1} (Lange Lacke; $p < 0.01$, n =4) was calculated.

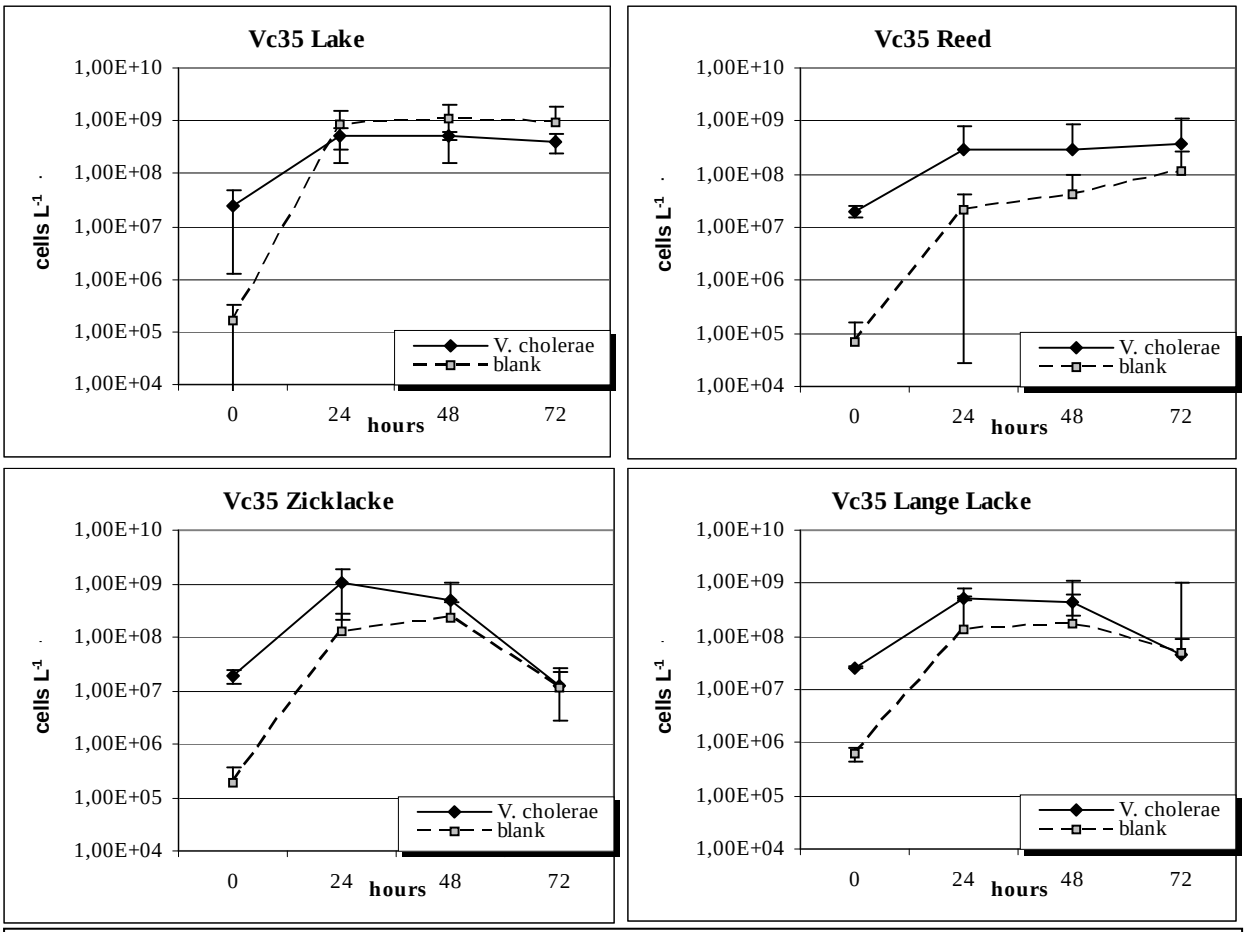


Fig. 20: Averaged (June, July, August, September) Vc35 pattern of growth in lake center, reed belt, Zicklacke and Lange Lacke during a 72h microcosm experiment. Error bars represent standard deviations of the 4 independent replicate experiments performed between July and September).

5.3.4 Microcosm experiments with *V. cholerae* O139 rugose variant (Vc55)

The ability of *V. cholerae* to switch reversibly from a smooth to a wrinkled, corrugated, colonial phenotype termed as rugose, was reported in the early 1920s (Baltenau, 1926). The rugose variant spontaneously appeared in old alkaline peptone water (APW) cultures and was resistant to adverse environmental conditions (White, 1938). Recently, it was observed that rugose variants of *V. cholerae* strains are more resistant to harsh conditions (e.g. exposure to chlorine and UV light) and to serum/ complement-mediated killing than are the strains' smooth variants (Rice et al., 1993; Morris et al., 1996).

Due to these facts Vc55 was chosen for further experiments. Vc55 (**Fig. 21**) showed for all sampling areas except lake center ($p > 0.1$, $n = 3$) significant net growth. Starting from an average inoculation number of 2.03×10^7 cells L^{-1}, net yields of 3.27×10^9 cells L^{-1} (Zicklacke; $p < 0.01$, $n = 3$), 7.8×10^7 cells L^{-1} (reed belt; $p < 0.01$, $n = 3$) and 9.82×10^8 cells L^{-1} (Lange Lacke; $p < 0.01$, $n = 3$) were increased.

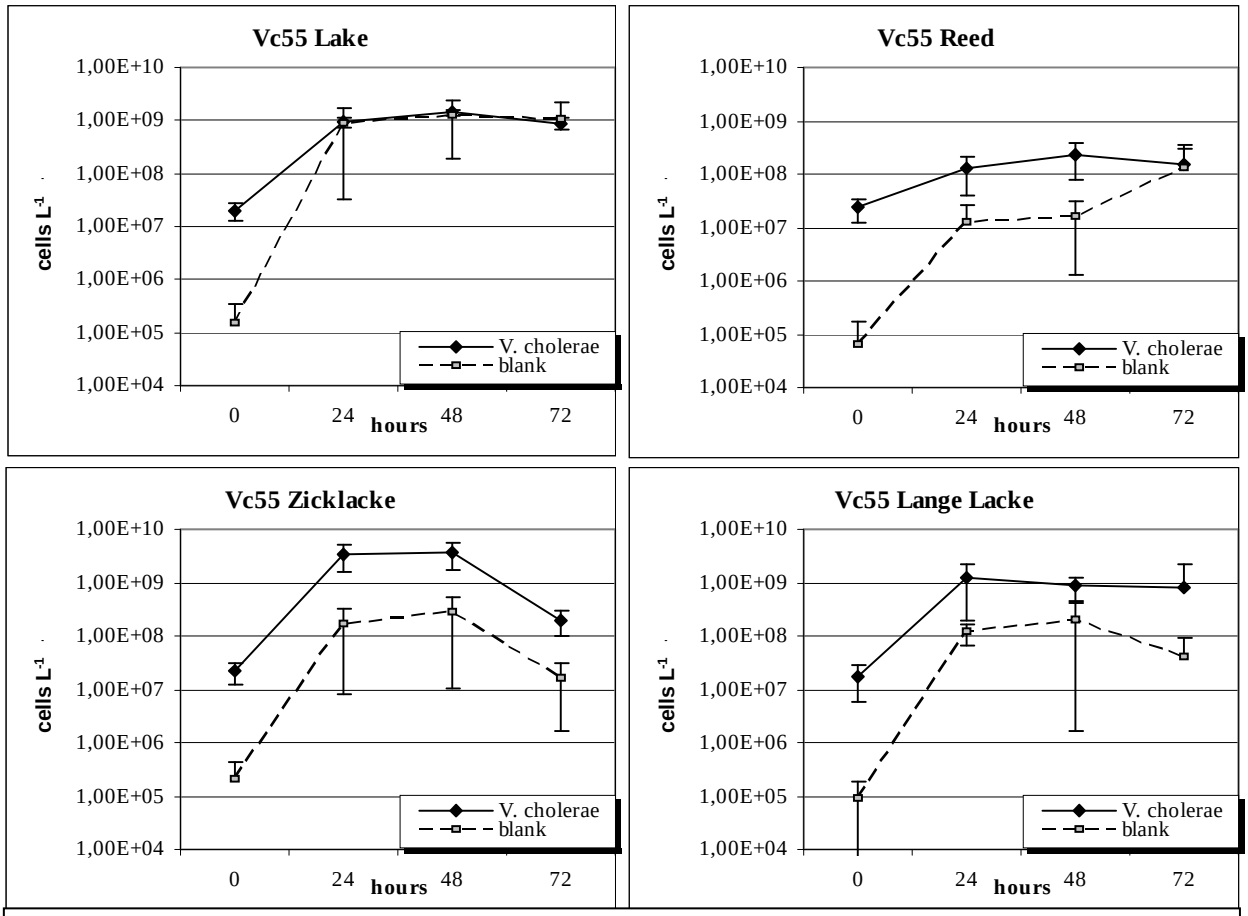


Fig. 21: Averaged (July, August, September) Vc55 pattern of growth in lake center, reed belt, Zicklacke and Lange Lacke during a 72h microcosm experiment. Error bars represent standard deviations of the 3 independent replicate experiments performed between July and September).

5.4 Microcosms review

With the exception of *V.cholerae* O1 ElTor all tested *V.cholerae* O1/ O139 strains showed the ability to grow in lake water from the Neusiedler See and the two tested adjacent shallow alkaline lakes. For the Neusiedler See, only water from the reed belt and not from the lake center did support growth of toxigenic *V.cholerae*. Each of the inoculated strains grew best in water from the Zicklacke. *V. cholerae* O1 classical showed in contrast to the other strains a slight preference of the reed belt and Lange Lacke. In comparison to the other sampling sites, the Zicklacke is characterized by a higher level of electric conductivity, dissolved organic matter, and a higher level of low molecular weight substances supporting growth of *V. cholerae* (Kirschner et al 2008).

In the experiments, a natural population of competitive bacteria was present and zooplankton was added at concentrations as they are found under natural conditions. Thus the experimental design simulates a realistic scenario under which the invasion of epidemic *V.cholerae* strains could happen.

However, there was also an imminent problem in the whole setup of experiments. The idea was to subtract the amount of environmental *V. cholerae* in the blank flask from the amount of the sample flask inoculated with the epidemic strains. It turned out, that in some cases the concentration of *V. cholerae* cells in the blank flask was higher than or at the same level as in the inoculated flask. This fact may lead to various interpretable results because a differentiation between endemic and inoculated strains in the batch cultures was not possible. First, the endemic strains may have inhibited or overgrown the inoculated strain completely and the observed cell number increase was due to the endemic strains alone. Second, both of them coexist and the observed cell number increase in the batch cultures is due to growth of both strains. Third, the inoculated strain may outcompete the endemic strain, leading to enhanced growth of the pathogenic strain and therefore the observed cell number increase in the batch cultures is due to growth of the inoculated pathogenic *V. cholerae* strain alone.

Thus, for a conclusive proof of the results from this study a method for differentiating endemic from inoculated strains has to be applied. This can be done by tagging the inoculated strains via GFP (green fluorescent protein) or a special genetic marker a priori or by applying a differential labelling technique for the endemic and the inoculated strains a posteriori.

6 Conclusions

The present study monitored for the first time the *V. cholerae* concentrations - beside the lake center and the reed belt of the lake Neusiedler See - in three selected adjacent soda pools. By this, a series of snapshots was taken, which enables a direct comparison of the selected sample areas at the same time and the same meteorological conditions. During the surveillance period it could be shown, that the *V. cholerae* concentrations are markedly (one – two orders of magnitude higher in all soda pools compared to the lake. The soda pool Oberer Stinker showed the highest concentration of zooplankton-attached *V. cholerae* cells at all, but in contrast no free-floating cells were detectable. Unfortunately, FISH based microscopic detection of *V.cholerae* could not be performed due to the high amount of disturbing background particles. In general, all the investigated waters are rather turbid, and a high wind speed leads to an increase of resuspended particles. Therefore also the sampling area reed belt may cause troubles during a windy day and the need for improved sensitive, reliable and low time consuming quantitative detection methods are obvious. The use of molecular biological methods, especially quantitative real-time PCR seems to be a suitable tool to overcome the mentioned problems.

In the whole examined area no cholera-causing *V. cholerae* O1/O139 strains could be isolated. It is well known that culturability of O1/O139 strains is extremely low, thus the need for a fast but stable method to screen large amounts of samples is needed for a reliable monitoring of the lake for the presence of epidemic *V.cholerae*.

The batch culture microcosm experiments gave a strong indication that a variety of pandemic *V. cholerae* strains are able to grow to high cell concentrations under conditions similar to what is met in the natural environment. In combination with the findings that *V. cholerae* attach to zooplankton at high concentrations, a potential infection risk cannot be ruled out in the future. However it has to be re-emphasized, that up to now no V. cholerae O1/O139 strain has been detected in the Neusiedler See and the adjacent soda pools.

7 Zusammenfassung:

Vibrio cholerae ist ein bewegliches, kommaförmiges, Gram-negatives, monopolar begeißeltes Stäbchenbakterium, welches zur Familie der Vibrionaceae gezählt wird.
V. cholerae ist der Erreger der Cholera, einer verheerenden Durchfallserkrankung mit einer einhergehenden Letalität von bis zu 10%. Das Bakterium wurde im Jahr 1854 durch Filippo Paccini erstmals entdeckt und in weiterer Folge 1883 durch Robert Koch isoliert und beschrieben. Neben seiner bedeutenden Rolle als pathogener Mikroorganismus, ist *V. cholerae* auch ein natürlicher Bestandteil der aquatischen Bakterienflora von Süß-, Brack- und Salzgewässern. *V. cholerae* weist im Allgemeinen eine partikel- bzw. planktonassoziierte oder eine frei lebende (planktische) aquatische Lebensweise auf. Derzeit sind von *V. cholerae* mehr als 200 verschiedene Serogruppen bekannt, wobei lediglich zwei (O1 und O139) schwere Verlaufsformen der Cholera epidemischen Ausmaßes auslösen können. NonO1/nonO139 *V. cholerae* sind für weniger schwere Durchfallserkrankungen, Blut-, Wund-, Ohr- und Atemwegsinfektionen verantwortlich. In einem Beobachtungszeitraum von 2001 bis 2005 traten 13 *V. cholerae* Infektionen in Österreich auf. Fünf dieser Infektionen, welche auch einen Todesfall eines immunsupprimierten Fischers inkludiert, konnten mit einer Freizeitaktivität am oder im Neusiedler See in Verbindung gebracht werden. Alle diese Fälle wurden aufgrund der Krankheitsgeschichte auf die im Neusiedler See vorkommenden NonO1/nonO139 Stämme zurückgeführt.
V. cholerae wird üblicherweise direkt auf einem Selektivmedium kultiviert, gefolgt von einer Identifizierung mit biochemischen, immunologischen und oder molekularbiologischen Methoden. Eine Quantifizierung kann zum einen durch Auszählung der *V. cholerae* positiven Kolonien erfolgen oder aber zum anderen mittels Epifluoreszenzmikroskopie, wobei unter Verwendung von spezifischen, farbstoffgebundenen Sonden *V. cholerae* Zellen ein fluoreszierendes Signal wiedergeben (fluoreszierende *in situ* Hybridisierung, FISH).
In der Diplomarbeit wurden oben genannte Methoden angewandt um eine Aussage über freilebende und Zooplankton assozierte *V. cholerae* Zellkonzentrationen an fünf ökologisch höchst unterschiedlichen Standorten zu ermitteln. *V. cholerae* positive Isolate wurden unter Verwendung einer multiplex-PCR auf das Vorhandensein von pandemiestammspezifischen und virulenzassoziierten Gensegmenten überprüft. Es

konnte bei keinem der Isolate und Anreicherungskulturen ein *V. cholerae* O1/O139 Stamm nachgewiesen werden.

Neben der Quantifizierung der in den Gewässern vorkommenden *V. cholerae* Stämmen wurde auch das Invasionspotential von ausgewählten pandemischen Stämmen mittels Mikrokosmosversuchen unter kontrollierten Bedingungen im Labor untersucht. Die erhaltenen Ergebnisse lassen darauf schließen, dass einigen pandemischen Stämmen bei einer ausreichenden Menge an Inokulum eine Invasion gelingen könnte. Die erhaltenen Ergebnisse sind wiederum kritisch zu betrachten, da zwischen den bereits im Probewasser befindlichen und den inokulierten Stämmen während des Wachstumsversuchs keine Unterscheidung getroffen werden konnte. Die daraus resultierenden Ergebnisse beruhen auf einem Vergleich zum jeweiligen Blindwert und können in Abhängigkeit der darin befindlichen bakteriellen Gesamtheit zu einer groben Fehleinschätzung des jeweilig inokulierten pandemischen Stammes führen.

Daraus ergibt sich generell die Notwendigkeit einer neuen zeiteffizienteren und genaueren Methode mit höherer Durchsatzleistung um mehrere Proben in kürzeren Zeitabständen analysieren zu können. Die sich ständig weiter entwickelnden molekularbiologischen Methoden, wie etwa quantitative real-time PCR, bieten hier großes analytisches Potential, welches im Zuge weiterer Untersuchungen ermittelt werden soll.

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curriculum vitae

Bliem Rupert

Address: Gestettengasse 18-22/11/6
A-1030 Vienna
Austria

Place and date of birth: Salzburg, Austria 05/07/1978
Citizenship: Austria

Education:

12/2008 – 12/2009	diploma thesis: Invasion Potential of Vibrio cholerae O1/O139 in Lake Neusiedler See and three Alkaline Saline Lakes.
2004 – 2008	diploma study in biology/genetics-microbiology, University of Vienna, Austria
2000 – 2004	high school, Mil. BRG Wr. Neustadt, Austria final examamination: 23.02.2000
1997 - 2000	seargent, 2nd engineer coy, Salzburg
1996 – 1997	basic military service, 2nd engineer coy, Salzburg
1993 – 1996	trained retail salesman final examination: 15.06.1996
1995 – 1996	commercial school, Salzburg, Austria
1991 – 1995	secondary modern school, Viehhausen, Austria
1988 – 1991	primary school, Großgmain, Austria

Experience:

08/2008	Complete an internship at Armanent and Defence Technology Agency, Biological Detection
11-12/2009	Complete an internship at Armanent and Defence Technology Agency, Biological Detection

Languages:

German	mother tongue
English	fluent
French	basic level

Personal skills and competences:

Microbiological methods:	PCR, Fluorescence in situ hybridization, Puls-field gel electrophoresis, DGGE, micro arrays
Computer knowledge:	MS Office, Bionumerics, Visual Basics
other skills:	mil. chief blaster lifeguard driving license A,B,C