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DIPLOMARBEIT

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Quantification of *Vibrio cholerae* in Lake Neusiedler See by means of classical and molecular biological methods

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„Die Reize der Wissenschaft, so wie die der Tugend, werden erst dann empfunden, wenn man in beiden schon beträchtliche Fortschritte gemacht hat."

Christian Grave

An erster Stelle möchte ich mich bei meiner Familie bedanken, die mich während meines Studiums stets unterstützt und an mich geglaubt hat. Mein größter Dank gilt hier aber meiner Mutter: „ Danke dafür, dass du immer da warst, deinen Zuspruch und dein Vertrauen."

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TABLE OF CONTENTS

1 ABSTRACT

Vibrio cholerae is a motile, Gram-negative, heterotrophic rod that belongs to the family Vibrionaceae. Besides being an important human pathogen, it is a component of the normal bacterial flora of aquatic environments, including fresh, brackish and sea water. In the environment, at least two modes of life have been discussed, either particle/zooplankton associated or free living (planktonic). Of the 200 currently recognized serogroups of *V. cholerae*, only strains belonging to serogroups O1 and O139 can cause cholerae, a fatal diarrhoeal disease. NonO1/nonO139 *V. cholerae* strains are responsible for less severe watery diarrhoe and infections of blood, wound, ear and the respiratory tract. Between 2000 and 2007, the World Health Organization (WHO) reported of 249 imported O1/O139 *V. cholerae* cases in 14 different European countries. During the period from 2000 to 2005, 13 infections due to environmental nonO1/nonO139 *V. cholerae* strains were documented in Austria. Five infections thereof could be directly related to Lake Neusiedler See, including one case of death.

Vibrio cholerae is usually detected from environmental samples by means of cultivation on selective media. However, under certain circumstances, like nutrient deprivation and low temperature *V. cholerae* changes to the so-called VBNC status (viable but non cultivable) where it is undetectable by cultivation. In this study, environmental *V. cholerae* from the lake Neusiedler See were quantified by a cultivation based and a molecular biological method (fluorescence in situ hybridisation = FISH) at two different sampling sites (lake center and reed belt). *V.cholerae* were detected in three different size fractions (> 12 µm, 3-12 µm and 0.2-3 µm) as well as on zooplankton (copepods *Arctodiaptomus spinosus* and the cladocerans *Diaphanosoma mongolianum)* from February till August, 2008. Therefore a specific FISH protocol was developed for its detection in environmental samples. By relating its abundance to a variety of environmental parameters it was attempted to trace the preferred ecological niche of this human pathogen in the Lake Neusiedler See.

The results indicate that the selected FISH protocol was suitable for detection of the bacteria in laboratory experiments and environmental samples even when the cells were in the VBNC status (February to mid May). However, one major disadvantage of the established FISH protocol was that the presence of high particle concentrations in the water column at periods of high wind speed (mainly in the lake center) limited the accuracy and reliability of the method which further led to a masking of possible relationships of *V. cholerae* concentrations to environmental variables. Both, FISH and cultivation showed cell number peaks in July with 22.000 - 26.000 CFU L^{-1} and 45.000 - 46.000 CFU L^{-1}, and decreasing values on the two

following sampling dates, which could be attributed to a decrease in particles in the water column due to calm weather situations. From May to August, both methods yielded similar results and there was a significant positive correlation between the FISH and cultivation based *V. cholerae* concentrations. Significantly higher *V. cholerae* concentrations were present in the lake center than in the reed belt. In both, the lake center and the reed belt most of the cells could be detected in the size fraction 3-12 µm by cultivation indicating that this particle fraction contains certain environmental features that enhance *V. cholerae* cultivability. In contrast, by means of FISH most *V. cholerae* cells occurred in the size fraction 0.2-3 µm. Because the reliability and recovery of cells by the FISH method proved to be dependent on the particle concentration in the water and the 0.2-3 µm size fraction was the poorest in particles this may be due to a methodical bias. Surprisingly, in contrast to common knowledge, the nonO1/nonO139 *V. cholerae* strains isolated from the lake Neusiedler See showed very weak association with copepods but a strong association with the dominant cladocerans species. This implicates that there is a specific interaction between certain zooplankton species and specific *V. cholerae* strains. Moreover, the large difference in cladoceran associated *V. cholerae* numbers between the FISH method and the cultivation based approach indicates that most of the zooplankton attached cells colonize inner surfaces and are not present on the exoskeleton.

Summing up, the *V. cholerae* strains in Lake Neusiedler See seem to have several life strategies (attachment to cladocerans, association with the 3 – 12 µm particle fraction, planktonic growth) and do not occupy a single specialized ecological niche.

2 INTRODUCTION

The recent issue of the *Bergey`s Manual of Systematic Bacteriology* (Bergey et al. 2001) classifies the family Vibrionaceae into six genera, namely Vibrio, Allomonas, Enhydrobacter, Listonella, Photobacterium and Salinivibrio. The vibrios are a group of Gram-negative, curved or straight motile rods that normally inhabit the aquatic environments. Currently, the genus consists of 51 species. *Vibrio cholerae*, the most prominent one, is classified into more than 200 somatic antigen (O) serogroups. The serogroups O1 and O139 cause endemic and epidemic cholera, while the other *V. cholerae* serogroups, not associated with epidemics or pandemics, are referred to as nonO1/nonO139 *V. cholerae* (Uma et al. 2003), which are responsible for less severe watery diarrhoe and infections of blood, wound, ear and the respiratory tract (Morris et al. 1990; Cheasty et al. 1999).

V. cholerae was first identified with a microscope by Felipe Pacini, an Italian anatomist (Pacini 1854). Thirty years later, Robert Koch isolated the bacterium as the bacillus causing cholera (Koch 1884).

The *V. cholerae* genome is bi-chromosomal. The larger one has a size of 2.4 Mb, while the smaller one is 1.6 Mb. The larger chromosome contains most of the genes involved in cell division, transcription and translation. The major pathogenicity-associated elements like the VPI (= vibrio pathogenicity island) and CTX (= cholera toxin) are located on this chromosome. The smaller chromosome has a lot of hypothetical genes, which are not yet well understood (Uma et al. 2003). The CTX element corresponds to a phage genome (Waldor et al. 1996; Pruzzo et al. 2008) and consists of two regions, RS2 and core, which contains the genes *ctxA* and *ctxB* encoding the cholera toxin, an ADP ribosylating toxin. The VPI element comprises the TCP (= toxin co-regulated pilus) operon, which contains the *tcpA* gene, which constitutes the pilus structure for both the colonization of the host (Uma et al. 2003) and the attachment of the CTX phage at the cell surface. The majority of the nonO1/nonO139 serogroups do not contain CTX and/or TCP, although *ctxAB*- and *tcp*-related genes have been shown to be present in certain strains of nonO1/nonO139 (Sarkar et al. 2002).

Although *V. cholerae* is known to be a human pathogen, the bacteria constitute part of the normal aquatic flora in estuarine and brackish waters (Colwell et al. 1992) and are able to persist in the absence of the human host (Faruque et al. 2002). Studies have revealed the existence of *V. cholerae* as free-living planktonic bacteria (Worden et al. 2006; Kirschner et al. 2008) or in association with phytoplankton (Eppstein 1993; Eiler et al. 2006), crustacean

zooplankton (Huq et al. 1983), molluscs (Islam et al. 1994), and chironomid egg masses (Halpern et al. 2001), which may provide some protection from the harsh environmental conditions of planktonic bacterial cells. Yet, it is not clear whether such association is specific for pathogenic strains or a general phenomenon for all *V. cholerae* (Faruque et al. 2002).

The basis for these recoverings were the studies of Colwell and colleagues (1977). They proved that *V. cholerae* naturally occurred in the aquatic environment and proposed that there is a global association between *V. cholerae* and chitinous plankton because they isolated the bacterium from plankton samples from both Bangladesh and Chesapeake Bay water (1980). Three years later Huq et al. (1983) proposed that once *V. cholerae* attach to zooplankton (i.e. copepods or cladocerans), they are protected from the external environment and begin to proliferate because of the increased surface area and improved conditions of nutrition. That conclusion was confirmed by Colwell (1996), who showed that *V. cholerae* could survive longer in seawater when adhering to external surfaces and the gut of copepods. Chitinaceous organisms, e.g. copepods and cladocerans, are prevalent among zooplankton populations (Lipp et al. 2002), and chitin is one of the most abundant and important sources of nutrients and energy (Gooday 1990; Pruzzo et al. 2008). *V. cholerae* produces an active chitinase and therefore is able to metabolise chitin. Chitinases are enzymes that randomly cleave glycosidic linkages of N-acetylglucosamine (subunit of chitin) to produce soluble oligosacharides (Hood et al. 1977; Pruzzo et al. 2008). Additionally, *V. cholerae* is able to build up large multicellular structures on solid surfaces (living hosts or detrital aggregates (Cottingham et al. 2003)) known as biofilms, which provides increased resistance to various stress factors (e.g. chlorine, antibiotics) (Reidl et al. 2002) and enhances bacterial productivity (Cottingham et al. 2003). The biofilm formation is dependent on the production of an exopolysaccharide (EPS), which is encoded by the *Vibrio* polysaccharide (*vps*) genes. *Vps* expression is modulated through a quorum-sensing signalling cascade (Heithoff et al. 2004).

V. cholerae measures approximately 3 µm long by 1 µm wide when active and less than 1 µm in diameter when in VBNC status (viable but non cultivable) (Cottingham et al. 2003). In response to rapid transition in environmental conditions, including temperature and osmolarity, *V. cholerae* can change into the VBNC state, where it loses its flagellum and changes to a smaller, spherical shape (McDougald et al. 1998; Cottingham et al. 2003). VBNC is a term that describes a condition in which bacteria exhibit detectable metabolic function, but are not cultivable by conventional laboratory culture methods (Colwell et al. 1985; Chaiyanan et al. 2007), in contrast to starved cells which may remain cultivable (Colwell et al. 1985). The VBNC cells have a decreased nucleic acid content and reduced

respiration and metabolic rates. As mentioned before, the cells are reduced in size and become coccoid, which is interpreted as a strategy to minimize requirements for cell maintenance and both, size reduction and coccoid cell formation appear to constitute protection against adverse environmental conditions (McDougald et al. 1998; Cottingham et al. 2003).
Ravel and colleagues (1995) found that temperature change alone was insufficient for resuscitation of VBNC *V. cholerae*. Resuscitation of non-cultivable cells appears to require a significant length of time, independent on the presence of exogenous nutrients (Oliver et al. 1993; Chaiyanan et al. 2007).

According to WHO (World Health Organization), 249 cases of imported O1/O139 *V. cholerae* incidents were documented in Germany, France, the United Kingdom, Ireland, Russia, Spain, Czech Republic, the Netherlands, Sweden, Belgium, Denmark, Norway, Finland, Poland, Italy, Switzerland, Slovenia, Ukraine and Austria between 2000 and 2007. Officially, in Austria two cases were reported. Theses numbers are probably just a smallish fraction of the real amount of *V. cholerae* cases because many incidents were and are not reported by the persons concerned. (WHO, The Weekly Epidemiological Record, 2000-2007).
In Austria, 13 infections due to nonO1/nonO13 *Vibrio cholerae* were documented from 2000 to 2005. Eight of these people did not have a travel history outside of Austria, with symptoms like otitis media, otitis externa and a lethal case of septicaemia of an immunsupressed fisherman. For 5 of these 8 patients a travel history inside of Austria was available and everybody had visited or lived near Lake Neusiedler See (Huhulescu et al. 2007). As a result of the incidences in 2000, the lake has been tested for the presence of *V. cholerae* at regular intervals since 2001.
The lake Neusiedler See offers, with a salinity between 1 and 2 ‰ and a pH between 8.5 and 9.1, perfect conditions for the bacterium to grow (Kirschner et al. 2008). Kirschner and colleagues (2008) proved that nonO1/nonO139 *V. cholerae* are able to grow in sterile lake water at high growth rates depending on temperature and DOC (dissolved organic carbon) quality, indicating that *V. cholerae* may be able to grow planctonically, in addition to zooplankton association. They showed that in laboratory batch culture experiments the growth rate of *V. cholerae* increased with the temperature and the concentration of low molecular weight nonhumic organic carbon exuded by phytoplankton and decreased with the concentration of high molecular weight humic organic carbon produced by the reed *Phragmites australis,* covering more than 50% of the lake Neusiedler See. Furthermore, they could detect the bacterium by means of cultivation in all size fractions (with particle sizes

ranging between 0.2 to > 250 µm (zooplankton)) in the lake water. The results showed that the bacterium was not more frequently isolated from zooplankton than from other size fraction filtrates. But these results were just semi-quantitative and restricted to the warm season because of the cultivation.

These studies built the basis of my diploma thesis and so the aim was the establishment of Fluorescence In Situ Hybridisation (FISH) to detect and quantify nonO1/O139 *V. cholerae* in the lake Neusiedler See at two different sampling sites (lake center and reed belt) and in three different size fractions (> 12 µm, 12-3 µm and 3-0.2 µm) as well as on zooplankton (copepods *Arctodiaptomus spinosus* and the cladocerans *Diaphanosoma mongolianum)* from February to August 2008 by means of FISH. The principle of FISH is that an rRNA-targeted oligonucleotide probe, which is fluorescently labelled, specifically detects cells of its target species in a fixed environmental sample. The probe does not bind to ribosomes of an other species when the correct hybridisation and washing conditions are applied. The cells of the target species can be detected by epifluorescence or confocal laser scanning microscopy (Amann et al. 1995; Daims et al. 2005). Concurrently to FISH, *V. cholerae* was quantified by cultivation on TCBS (Thiosulfate Citrate Bile Sucrose) agar at the same sampling sites and in the same size fractions as for FISH from April to August. A variety of environmental variables were also determined in order to relate changes in *V.cholerae* concentrations to changes in environmental conditions.

3 MATERIALS AND METHODS

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 sea level, surface area 321 km²). It is suited in eastern Austria and shared by Austria as well as Hungary. It has a high alkalinity (pH 8.5-9.1), a moderate salinity (1 - 2 ‰) and a high turbidity (Secchi depth ~ 0.2 m). About 55% of the lake is covered with reeds (*Phragmites australis*).

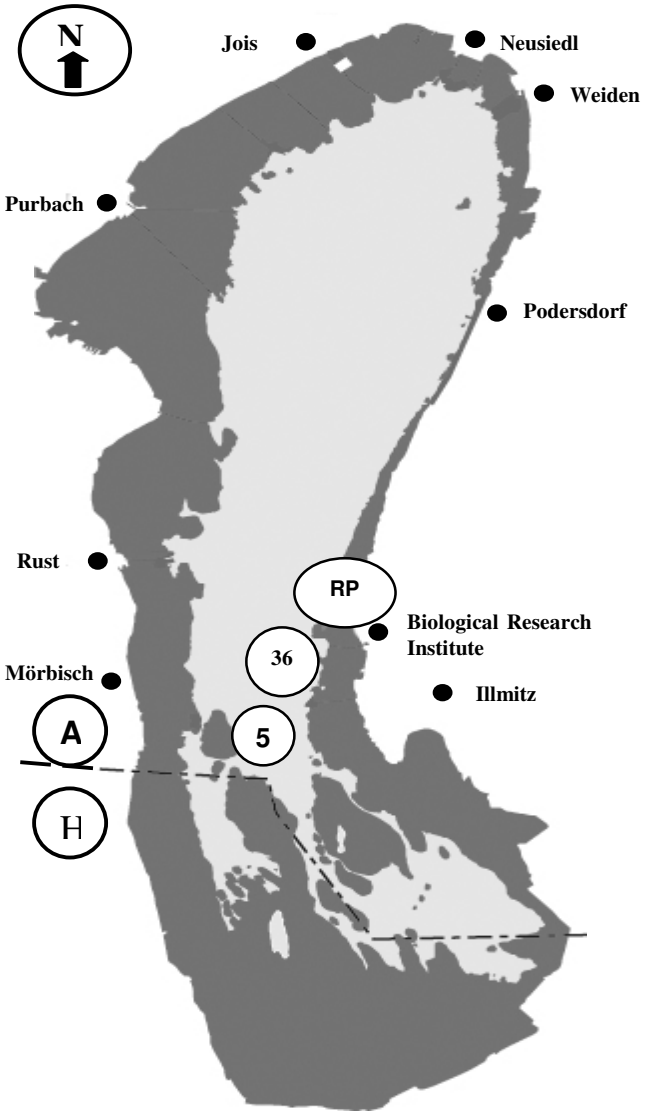

Fig. 1: View of lake Neusiedler See with two routine sampling points (5 and 36) and the extra sampling point (Ruster Poschen [RP]) for the isolation of *V. cholerae*. The dashed line indicates the border between Austria and Hungary. The darkly shaded area indicates the reed belt; the lightly shaded area indicates the open water area

ESTABLISHMENT OF THE FLUORESCENCE IN SITU HYBRIDISATION METHOD (FISH) FOR DETECTION OF *VIBRIO CHOLERAE* IN ENVIRONMENTAL SAMPLES

The *V. cholerae* strain (VC4) for the establishment of the FISH method was isolated from lake Neusiedler See during May 2007 according to Choopun et al. (2002) by Mag. Sonja Schauer (Schauer 2008). The isolates were confirmed by the cytrochrome oxidase test and further identified with API 20E (Biomerieux, Nürtingen, Germany). After biochemical identification the isolates were checked for the presence of ISR (16S - 23S intergenic spacer region) specific for *V. cholerae* via PCR (Chun et al. 1999).

STARVATION EXPERIMENTS

Primarily, the starvation experiments were carried out to know if starved cells are even able to be detected by means of FISH and secondarily, to determine the limit of detection and quantification. The experiments were performed with the isolated *V. cholerae* strain 4 (VC4) as working culture. The lake Neusiedler See strain collection contains *V. cholerae* isolates since 2006 and is kept at the Clinical Institute of Hygiene and Medical Microbiology at the Medical University of Vienna.

***V. cholerae* strain 4** - For the generation of the working culture, one Eppendorf stock culture tube (VC4) of the lake Neusiedler See strain collection was streaked onto BHI (brain heart infusion) agar (Merck, Darmstadt, Germany) and incubated for 16–18 hrs at 37°C. Three to five single colonies were taken from the plate with a sterile tip, transferred to a 500 ml sterile Erlenmeyer flask containing 50 ml sterile BHI broth and put on a shaker for 5 hrs at 37°C. Afterwards, the liquid enrichment medium was mixed with 10 ml glycerine (plus peptone). The working culture was divided up into sterile Eppendorf tubes (each containing about 1 ml) and stored at –80°C (ISO 10705-1:1995(E)).

Water for the experiments – 600 ml lake Neusiedler See water (NSW) was sterile filtered (filtered through 0.2 µm [GTTP, Millipore, Vienna, Austria] and 0.1µm [Anodisc 47, Whatman, Dassel, Germany]) and filled into a sterile 1 L Schott flask. On the other side, 600 ml artificially generated lake Neusiedler See water (ALW; 823 mg/L $NaHCO_3$, 36.5 mg/L $CaCO_3$, 19,7 mg/L $NaCO_3$, 436 mg/L $NaSO_4^{2-}$, 91 mg/L KCl, 402 mg/L $MgCl_2.6H_2O$ and 406 mg/L $MgSO_4.7H_2O$) was filled in another sterile 1 L Schott flask.

For the starvation experiment, 1 ml of working culture was mixed with 50 ml pre-warmed BHI broth and put on a shaker at 37°C until OD_{600} 0.6 – 0.8. 20 ml of that liquid enrichment

medium was transferred into a sterile centrifugation tube and centrifuged for 10 min at 7.500 x g at 25°C. The supernatant was discarded and the pellet was resuspended in 1 ml sterile filtered Lake Neusiedler See water (SFNSW) and again centrifuged for 10 min at 7.500 x g at 25°C. After the supernatant was discarded, the pellet was resuspended in 10 ml pre-warmed SFNSW and centrifuged for 10 min at 7.500 x g at 25°C. The last resuspension and centrifugation procedure was repeated and after discarding the supernatant again, the pellet was resuspended in 10 ml SFNSW.

6 ml of the prepared cells were added each to both prepared 1 L flasks (final concentration 8 x 10^6 cells ml^{-1}) and gently bubbled with sterile filtered air (0.2 µm PFTE, Sartorius) to avoid anoxic situations. Both 1 L flasks were incubated for 15 days at 10°C and afterwards, they were further incubated for another 83 days at 4°C. At intervals ranging from 1 to 14 days, 1 ml samples were taken and stained with acridine orange to compare the cell number, cell volume and cell form between SFNSW and ALW over these 98 days. Therefore, 1 ml samples were fixed with 37% formaldehyde (final conc. 2%) and stored at 4°C till use.

FISH dilution series - 150 µl of prepared cells were transferred on the one hand, to 60 ml SFNSW and on the other hand, to 60 ml original NSW (final conc. 2 x 10^6 cells ml^{-1}). After that, 40 ml of both SFNSW and original NSW were diluted decimally to a final concentration of 2 cells ml^{-1}. Additionally, another dilution row was generated with SFNSW and original NSW. Therefore, 13.2 ml of the SFNSW and original NSW plus bacteria were mixed with 26.8 ml SFNSW and original NSW (final concentration 6.6 x 10^5 cells ml^{-1}) and diluted decimally to a concentration of 6.6 cells ml^{-1}. As controls SFNSW and original NSW were taken without addition of cells. FISH was performed with every sample for determining the limit of detection and the limit of quantification.

The same dilution procedure was performed after 29 and 98 days of incubation. Therefore, 15 ml of bubbled SFNSW were mixed on the one hand with 45 ml SFNSW and on the other hand with 45 ml original NSW (final conc. 2 x 10^6 cells ml^{-1}). ALW was not considered because SFNSW better reflects the natural conditions of the lake. Again, SFNSW and original NSW served as controls.

FLUORESCENCE IN SITU HYBRIDISATION

Every sample of the dilution row was fixed with 4% para-formaldehyde (final concentration 1%). After incubation for 12–18 hrs at 4°C, the samples were filtered on a white

polycarbonate membrane filter (Whatman, Ø 25mm, pore size 0.2 µm). The filters were put into petri dishes and air dried for about 1 h. Dried filters were either used immediately or transferred into sterile 1.5 ml Eppendorf tubes (the side holding the bacteria cells should face inside) and stored at –20°C till use.

A quarter of the filer was cut out with a scalpel and the remaining filter was further stored at –20°C. For the hybridisation, 1 ml hybridisation buffer was prepared: 0.9 M NaCl, 0.02 M Tris-HCl, 35% formamide, 450 µl sterile MilliQ and 0.01% SDS.

270 µl of this hybridisation solution were mixed with 30 µl 6-FAM (Fluorescein phosphoramidite) labelled probe (Vchomin 1276 (5`-[6-Fam] ACTTTGTGAGATTCGCTCCACCTCG-3`); working concentration 50 ng μl^{-1}) in a 0.7 ml Eppendorf tube and the filter quarters were added (maximal 10 – 15 filter sections per 0.7 Eppendorf tube). Hybridisation was performed for 1–1.5 hrs at 46°C in a hybridization oven by gentle mixing. The filters were transferred to 50 ml pre-warmed washing buffer (0.08 M NaCl, 0.02 M Tris-HCl, 5 mM EDTA, 35% formamide, 47.500 µl sterile MilliQ and 0.01% SDS) and incubated for 30 min at 48°C. Filters plus buffer were poured through a suction strainer; the filters were rinsed with ethanol (80–100%) and air dried on a paper in the dark. Afterwards, the filters were counterstained with DAPI-mix (5.5 parts Citi-fluor, 1 part Vectashield, 0.5 parts 1 x PBS and DAPI at a final concentration of 1 µg ml^{-1}). Therefore, the filter was placed on the top of a drop of DAPI-mix on a microscope slide, covered with another drop of DAPI-mix, mounted with a cover slip and incubated for 5–10 min in the dark. Microscope slides can be stored at –20°C for several months.

METHODS FOR QUANTIFICATION OF *VIBRIO CHOLERAE* IN LAKE NEUSIEDLER SEE

V. cholerae abundance was determined for three size fractions, namely > 12 µm, 3-12 µm and 0.2-3 µm, and on zooplankton by means of FISH and cultivation on TCBS (Thiosulfate Citrate Bile Sucrose) agar (Merck, Darmstadt, Germany). Additionally for cultivation, the total bacterial cell number was determined by filtering the original NSW onto 0.2 µm nitrate cellulose membrane filter (Ø 47 mm, Sartorius, Germany). That was not possible for FISH because it was not feasible to filter a significant amount of original water onto white polycarbonate membrane filter (Ø 25mm, pore size 0.2 µm) due to the high amount of suspended solids.

For zooplankton analysis the copepods *Arctodiaptomus spinosus* and the cladocerans *Diaphanosoma mongolianum* were examined, which are the dominating crustaceans in the lake.

I. Quantification by means of FISH

Water as well as zooplankton samples were taken by members of the Biological Research Insitute Burgenland (Rudolf Schalli and Richard Haider) in duplicates from the lake centre (5) and the reed belt (36) at 11 time points from February till August, 2008. Samples were taken by boat along a longitudinal transect through the lake in sterilized 500 ml glass bottles with a sampling rod from about 30 cm below water surface. Zooplankton samples were collected with vertical net hauls (mesh size: 250 µm). The zooplankton was collected from the net and filled into 250 ml plastic bottles. All samples were transferred to the laboratory in an isolated box in the dark within 4 hours at in situ temperature (± 2°C) (Alam et al. 2005). All samples were processed in duplicates.

On average, 600 ml NSW was filtered through a 12 µm polycarbonate membrane filter (Ø 47 mm, Nuclepore, Whatman) with a vacuum pump (max. 400 mbar) and collected in a sterile feeding bottle. 200 ml of the filtered water was further filtered through a 3 µm polycarbonate membrane filter (Ø 47 mm, Whatman) and collected also in a sterile feeding bottle. The collected water (200 ml) was fixed with 4% para-formaldehyde (final conc. 1%; PFA), incubated for 12–18 hrs at 4°C and filtered on a white polycarbonate membrane filter (Ø 25 mm, pore size 0.2 µm). The filter was air dried for 1 h in a petri dish and immediately used or stored at –20°C in a sterile 1.5 ml Eppendorf tube for FISH (see above).

The 12 µm and 3 µm filters were suspended in 15 ml 1 x PBS and associated bacteria were detached with 3.3 ml 4% PFA and incubated for 6 hrs at 4°C. Afterwards, 11.3 ml of 0.118 mM tetra-sodium pyrophosphate (final conc. 10 mM) was added and incubated for another 6 h while gentle shaking on ice. Samples were then sonicated (20W) for 30 s (Branson Sonifier S-450A). Filters were washed with 10 ml SFNSW twice. In case the NSW was rich in sediments, the sample was centrifuged for 20 min at 100 x g after the washing step and the supernatant was further used. The samples were filtered on a white polycarbonate membrane filter (Ø 25 mm, pore size 0.2 µm), dried for 1 h in a petri dish and immediately used or stored at –20°C in sterile 1.5 ml Eppendorf tubes for FISH (Heidelberg et al. 2002).

Zooplankton samples were rinsed with SFNSW, put into petri dishes, checked under a binocular microscope and sorted out (if possible 10 cladocerans and 10 copepods per

sampling site). Zooplankton was suspended in 10 ml 1 x PBS and 2.5 ml 4% PFA and after 6 hrs incubation at 4°C, a volume of 8.5 ml 0.118 M sodiumtetrapyrophosphate (final conc. 10mM) was added, before gentle shaking on ice for another 6 hrs. After sonification (20W, 30 s), large particles were removed by filtration through a 10 µm gaze net. The samples were further processed in the same way as the 12 µm and 3 µm polycarbonate filters (see above).

II. Quantification by means of cultivation

For the cultivation based quantification of *V. cholerae* in the lake Neusiedler See, duplicate water samples and zooplankton were analysed parallel to FISH samples from April to August (9 time points). TCBS agar was used as cultivation medium and yellow, flat 1 to 3 mm diameter colonies were considered as presumptive *V. cholerae*.

100 ml, 10 ml and 1 ml original NSW was filtered each through a 12 µm nitrate cellulose membrane filter (Ø 47 mm, Whatman) and collected in a sterile feeding bottle. The collected water was divided into 100 ml, 10 ml and 1 ml samples and further filtered through a 3 µm nitrate cellulose membrane filter (Ø 47 mm, Whatman). The filtered water was divided the same way as before and filtered through a 0.2 um nitrate cellulose membrane filter (Ø 47 mm, Sartorius). Similarly, 100ml, 10ml and 1ml original NSW was filtered directly through a 0.2 µm nitrate cellulose membrane filter (Ø 47 mm, Sartorius) for determining the total *V. cholerae* cell number. Each filter was put on a TCBS plate and incubated for 18 hrs at 41°C (Baron et al 2007). TCBS plates with 3 µm and 12 µm filters were opened under the laminar air flow for half an hour for drying before incubation. Zooplankton was washed and sorted out the same way as for FISH. 10 cladocerans/copepods per sampling site were homogenized with 1 ml saline solution (0.85% NaCl) in a sterilized micro-mortar with a glass pestle, and diluted twice 1:5 in SFNSW (final dilution: 1: 25). 500 µl were spread onto TCBS agar plates and incubated for 18 hrs at 41°C.

Presumptive *V. cholerae* colonies were streaked onto nutrient agar plates without NaCl and incubated at least 24 hrs at 37°C. Only colonies which were able to grow on these plates were considered as positive, because *V. cholerae* is able to grow without NaCl, and further streaked onto BHI or again in nutrient agar without NaCl and incubated for 24 hrs at 37°C. The colonies were further tested biochemically (cytochrome oxidase test) and molecular biologically (polymerase chain reaction).

BIOCHEMICAL AND MOLECULAR BIOLOGICAL IDENTIFICATION OF PRESUMPTIVE ENVIRONMENTAL *VIBRIO CHOLERAE*

Cytochrome-oxidase test - Each isolate was firstly tested with cytochrome oxidase test. A presumptive *V. cholerae* colony was streaked onto a filter paper and covered with a drop of the oxidase reagent (N,N,N`,N`Tetramethyl-1,4 phenylenediammoniumcholoride). The test was positive when the colony appeared dark blue. Oxidase positive isolates were further identified with polymerase chain reaction (PCR).

PCR - DNA was extracted from a loopful of pure culture of presumptive *V. cholerae* taken from nutrient agar (without NaCl) or BHI agar plates and stored at –20°C in a sterile microcentrifuge tube (1.5 ml). The culture was resuspended into 300 µl sterile water and diluted 1:1000 in sterile water to a final volume of 1 ml in a microcentrifuge tube with safety lock. The tube was placed in a thermomixer at 99°C for 10 minutes. Afterwards, the microcentrifuge tube was cooled to room temperature and stored at –20°C till use. 2 µl of this suspension served as template DNA during PCR. To each template DNA, a PCR mastermix of 38 µl was added.

The mastermix consisted of following reagents: 4 µl amplification buffer (100 mM TrisHCL, 500 mM KCl, 1% Triton X100, pH 9; Promega, Mannheim, Germany), 0.8 µl dNTPs Mix (200 µM final concentration, Fermentas, Germany), 0.84 µl of forward (VC-F) and 0.79 µl of reverse primer (VCM-R) (1 µM final conc; Thermo Scientific, Germany), 4.8 µl of $MgCl_2$ (3.0 mM final conc.), 0.4 µl of Taq polymerase (5U/µl; working conc. 2U/µl; Promega, Austria) and 26.37 µl sterile MilliQ water. The mastermix solution was mixed with the template DNA and placed in a Primus 25 thermocycler (MWG Biotech, Germany).

The amplification conditions used, were as followed: Lid heater at 104°C, 4 min at 94°C for DNA denaturation, 30 cycles consisting of 1 min at 94°C for melting, 1 min at 57°C for primer annealing, and 1 min at 72°C for DNA extension, final extension 10 min at 72°C. The PCR product was stored at 4°C until gel electrophoresis.

Finally, the products were separated by agarose gel electrophoresis at 90 V for 50 min. The gel strength was 2% and 1 x TAE (0.004 M Tris, 0.002 M EDTA, pH 8) served as running buffer. The gel was stained in 1 x TAE ethidium bromid solution (1 µg/ml) and visualized under UV light (GelDoc 2000 System; Biorad, Hercules, CA) (Kirschner et al. 2008). The primers used for PCR are specific for 16S - 23S intergenic spacer region of *V. cholerae* (Chun et al.1999).

Primer sequence:

Primer	primer sequence (5`-3`)	size
VC-F	TTA AGC STT TTC RCT CAG AAT G	295
VCM-R	AGT CAC TTA ACC ATA CAA CCC G	

V. cholerae O1 and *V. cholerae* nonO1/nonO139 strains were used as positive control and sterile water, *Vibrio alginolyticus,* and *Vibrio vulnificus* was chosen as negative control.

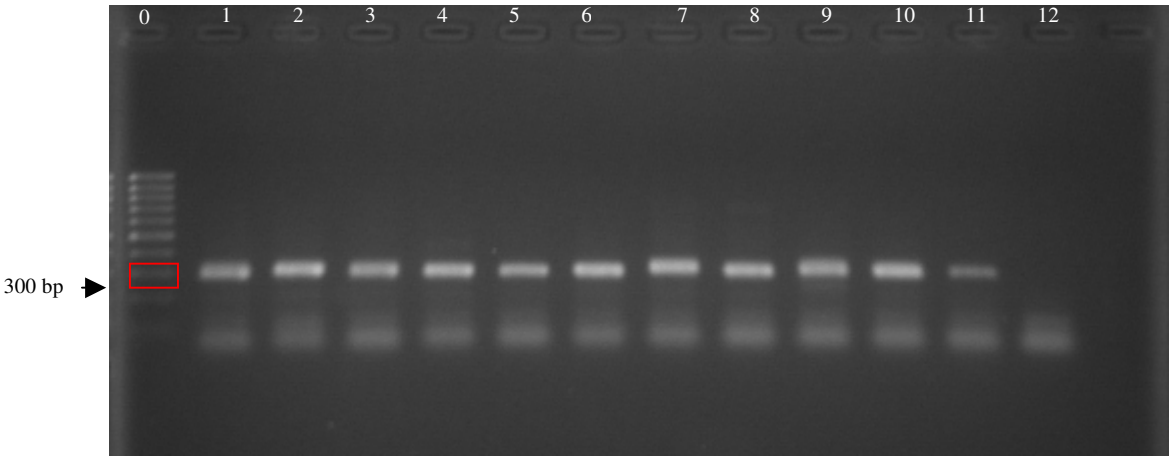

Fig. 2. PCR results. Lane 0: Ladder, lane 1–10: presumptive environmental *V. cholerae*, lane 11-12: positive control (O1 El Tor) and negative control (sterile MilliQ)

MICROSCOPY

For the measurement of cell volume, cell form and cell number of *V. cholerae* in the starvation experiments, 1 ml samples were fixed with 37% formaldehyde (final conc. 2%), stained with acridine orange (1 min) and filtered onto 0.2 µm black polycarbonate filters (Cyclopore, Whatman) (**Fig. 3**). After drying, filters were put on a microscope slide, overlaid with a drop of paraffin oil and mounted with a cover slip. For estimation of total cell number and cell form 20 microscopic fields were counted and for determination of cell length and width 100 bacterial cells were sized. For defining the limit of detection and the limit of quantification of the FISH method to detect *V. cholerae* in the course of the starvation experiments as well as enumeration of free living *V. cholerae* and particle/zooplankton associated *V. cholerae*, FISH was performed according to the protocol (**Fig. 4-5**).

For microscopic examination, an epifluorescence microscope (NIKON, Eclipse 80i, equipped with a digital camera) was used at 1250 x magnification.

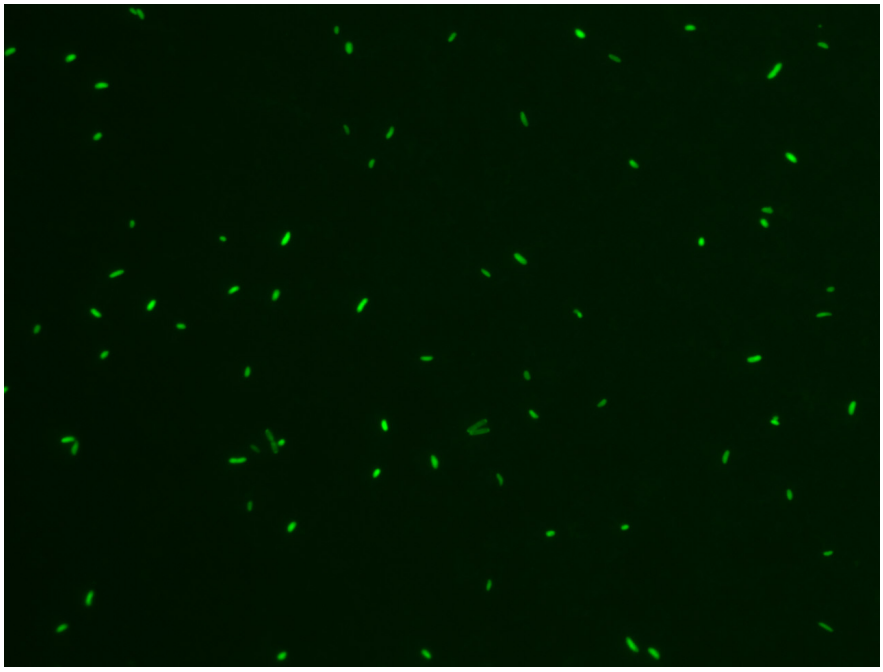

Fig. 3. Epifluorescence micrograph of starved culture *V. cholerae* cells (green) stained with acridine orange

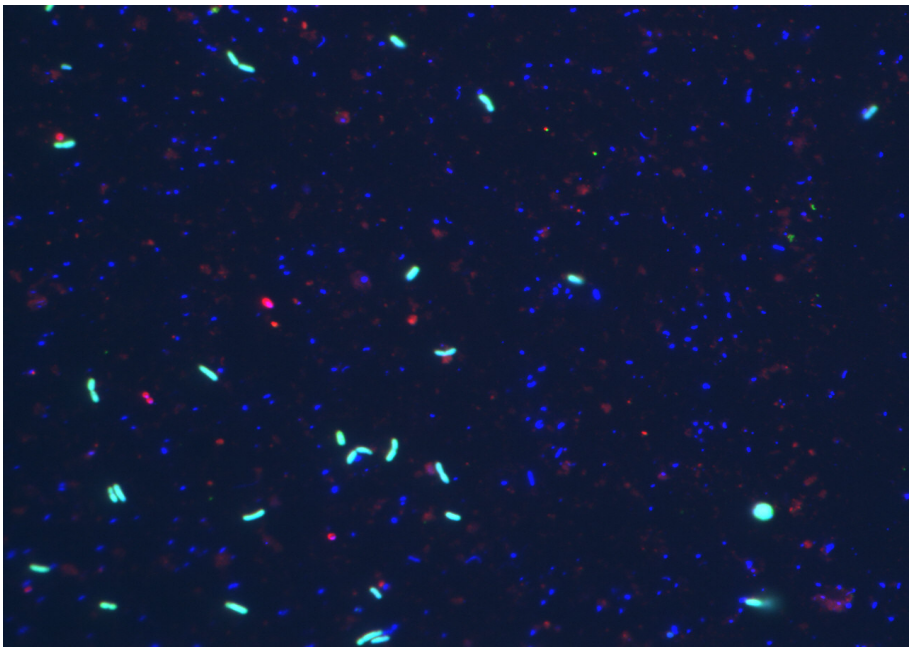

Fig. 4. Epifluorescence micrograph showing VC4 (green), concomitant flora (blue) and autofluorescencing cyanobacteria (red) in a spiked environmental sample

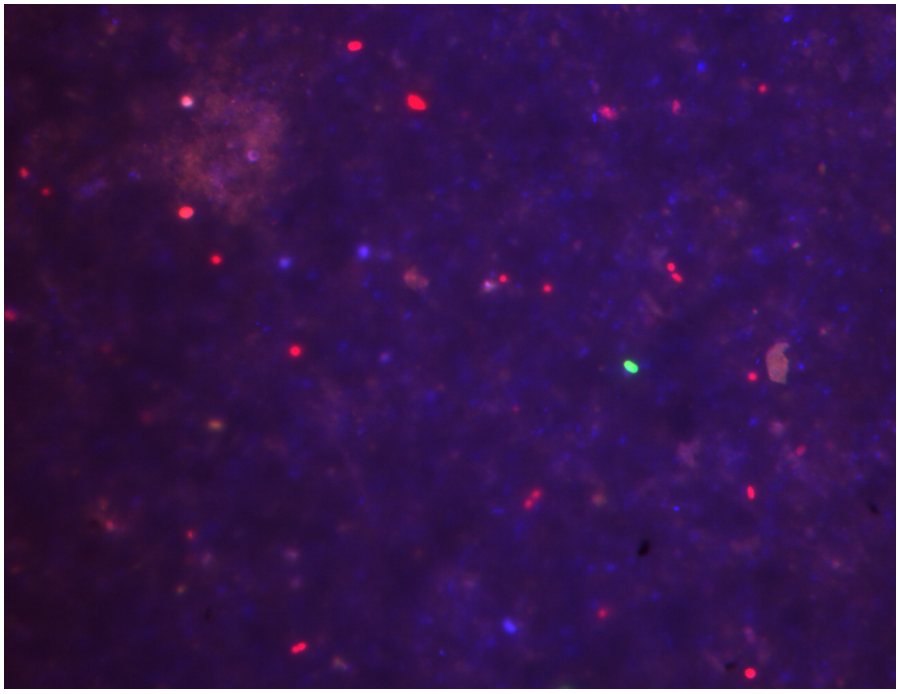

Fig. 5. Epifluorescence micrograph of an environmental sample: *V. cholerae* (green), concomitant flora (blue) and autofluorescencing cyanobacteria (red)

STATISTICS

For the statistic analysis SPSS 14.0 for Windows was used. The Spearman-Rank-Test was applied for examining correlations between *Vibrio cholerae* concentrations and environmental variables. P-values < 0.05 were considered as significant. For the statistics, environmental variables were used as followed: day, temperature (°C), conductivity (µS cm^{-1}), total phosphorus (µg L^{-1}), chlorophyll a (µg L^{-1}), dissolved organic carbon (mg L^{-1}), total organic carbon (mg L^{-1}), Secchi depth (cm) and maximal average wind speed (km h^{-1}). Environmental variables were measured by members of the Biological Research Insitute Burgenland (Prof. Dr. Alois Herzig, Franz Rauchwarter, Peter Gisch) according to routine protocols.

In the statistic tables, the first value indicates the coefficient of correlation (rho), the second value stands for the p-value and the third one for n (amount of sample number). The values in red are correlating with each other either positively or negatively depending on the rho-value.

For multiple-regression analysis, log-transformed values were used. The R^2-value (coefficient of determination) represents the net effect of the independent (influencing) variables on the dependent variable.

4 RESULTS

STARVATION EXPERIMENTS

As can be seen from **Figure 6**, the starting concentration was 3 x 10^6 *V. cholerae* mL^{-1} in ALW and SFNSW and the bacteria doubled their starting concentration within the first two weeks. After that, the concentration varied from 6.5 x 10^6 *V. cholerae* mL^{-1} to 9.7 x 10^6 *V. cholerae* mL^{-1} in ALW as well as SFNSW.

The cell size decreased from 1.0 μm^3 to 0.08 μm^3 per cell in SFNSW and from 0.9 μm^3 to 0.07 μm^3 per cell in ALW. The highest rate of cell size reduction was within the first 20 days (**Fig. 7**), afterwards *V. cholerae* cell size remained more or less constant.

On the first day, the fresh culture cells had rod shaped morphology in SFNSW and ALW. Except for the first enumeration on day 5, when there were 30% cocci and 70% rods in ALW, cocci ranged between 55% and 65% and over 70% after the day 66 (**Fig. 8**). In SFNSW more than 70% cocci and less than 30% rods were observed at day 5 and till the end of the experiment cocci varied from 50% to approximately 70% (**Fig. 9**).

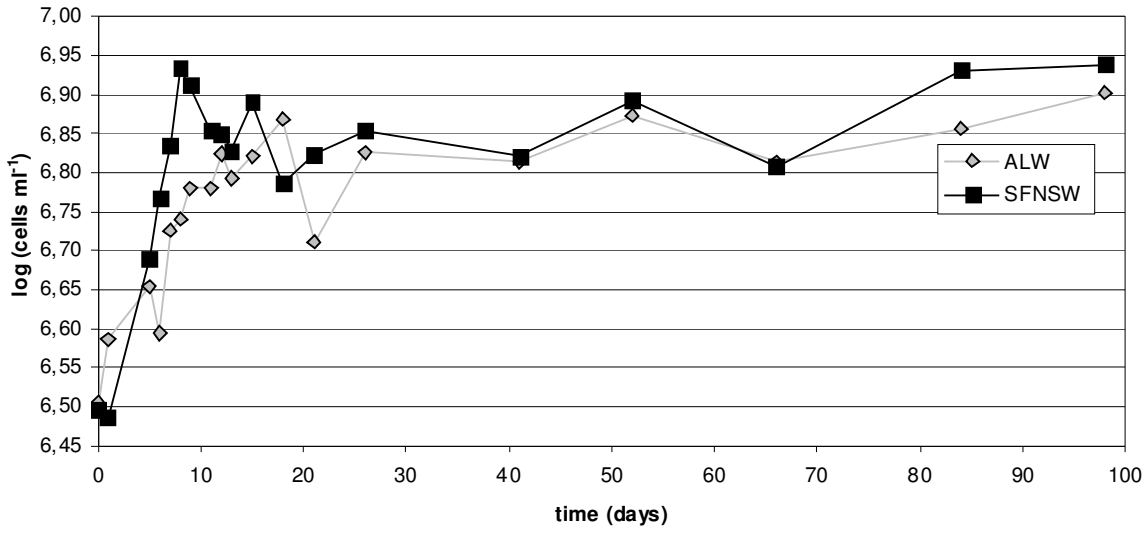

Fig. 6. Cell numbers of *V. cholerae* in ALW and SFNSW starvation experiments

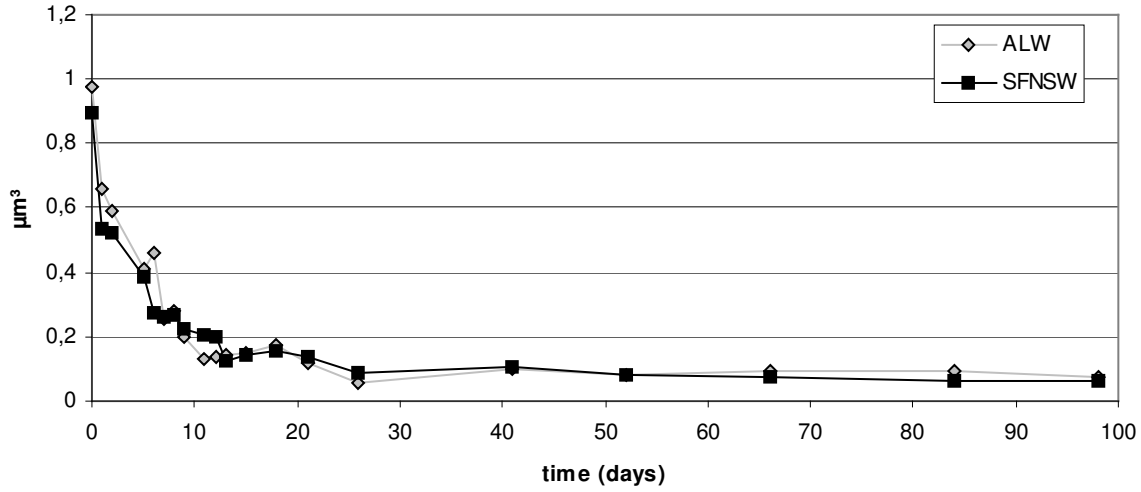

Fig. 7. Median cell volume of *V. cholerae* in ALW and SFNSW

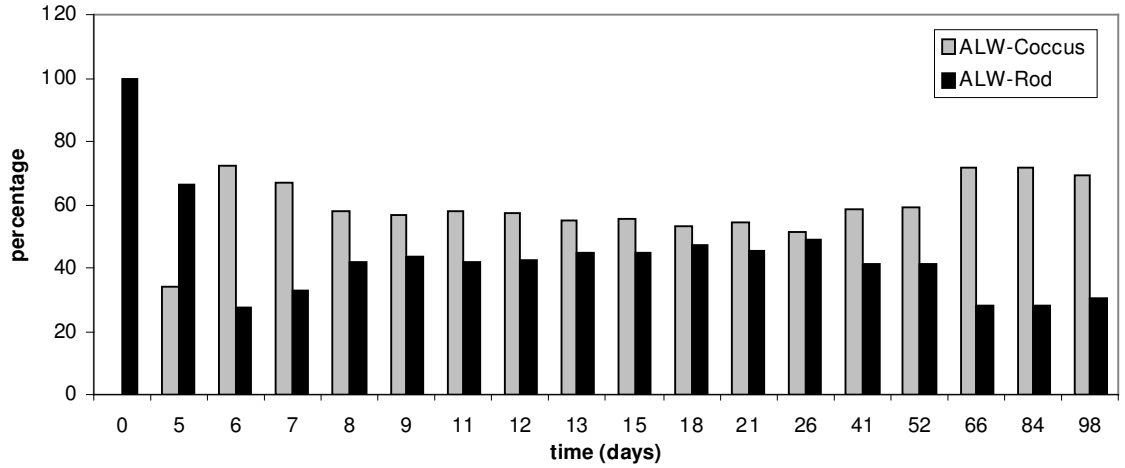

Fig. 8. Percentage of coccus and rod shaped *V. cholerae* in ALW

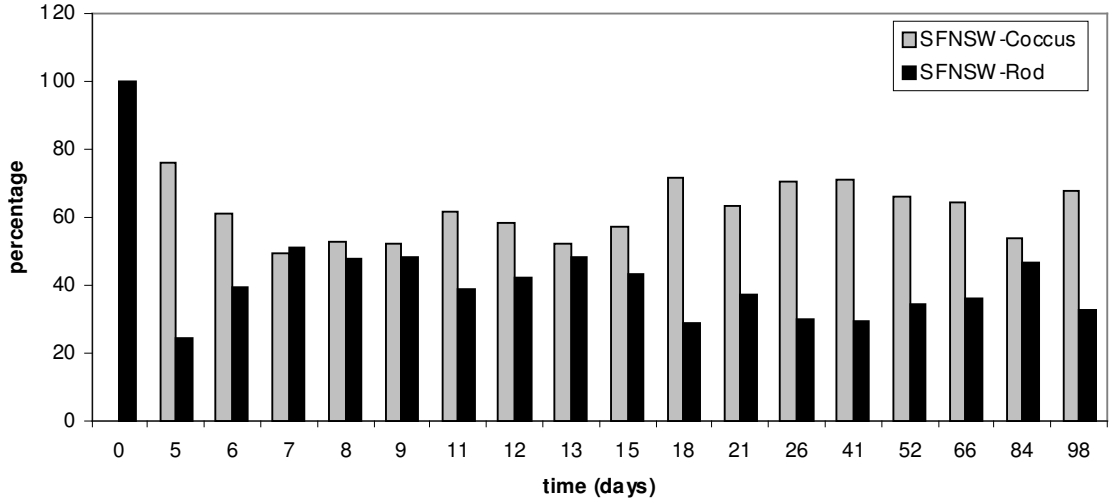

Fig. 9. Percentage of coccus and rod shaped *V. cholerae* in SFNSW

FISH dilution series were performed with samples from the SFNSW starvation experiments three times: at the beginning, after 29 days and after 96 days. Water samples were diluted decimally with original NSW and SFNSW from a theoretical starting concentration of 2×10^6 *V. cholerae* ml^{-1} to two cells ml^{-1} (**Fig. 10–12**) for defining the limit of detection (LOD) and the limit of quantification (LOQ).

As can be seen in **Figure 10-12**, there was always a starting concentration of about $1.5 – 1.7 \times 10^6$ *V. cholerae* ml^{-1} and the limit of quantification was 6.6×10^2 *V. cholerae* ml^{-1} in the first dilution row at the beginning and 6.6×10^1 *V. cholerae* ml^{-1} after 29 and 96 days . The limit of detection was 3.3 *V. cholerae* ml^{-1}.

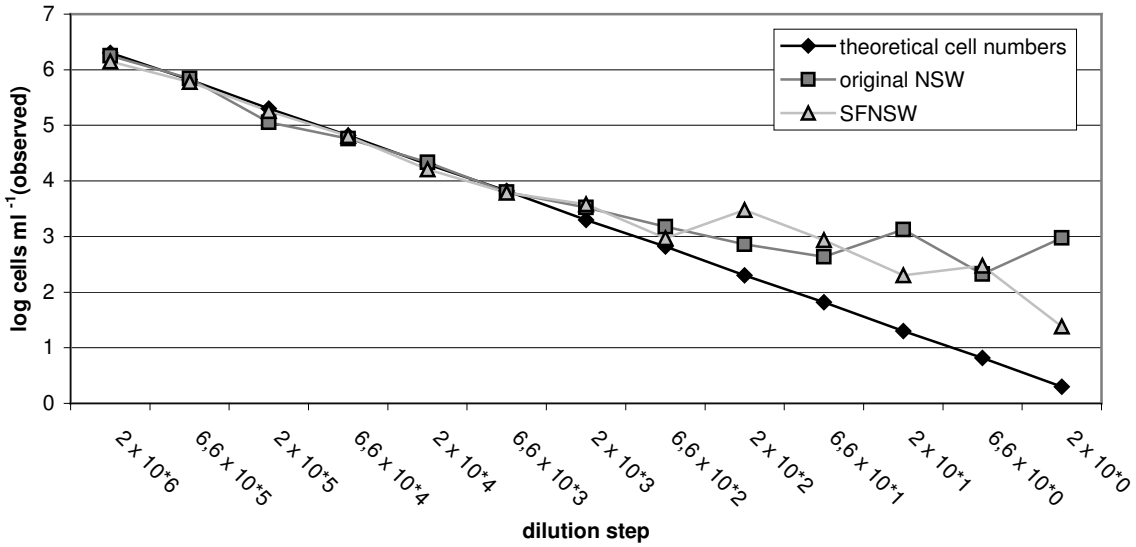

Fig. 10. Cell numbers of *V. cholerae* (taken from SFNSW starvation culture) in SFNSW and original NSW over different dilution steps at the beginning of the experiment

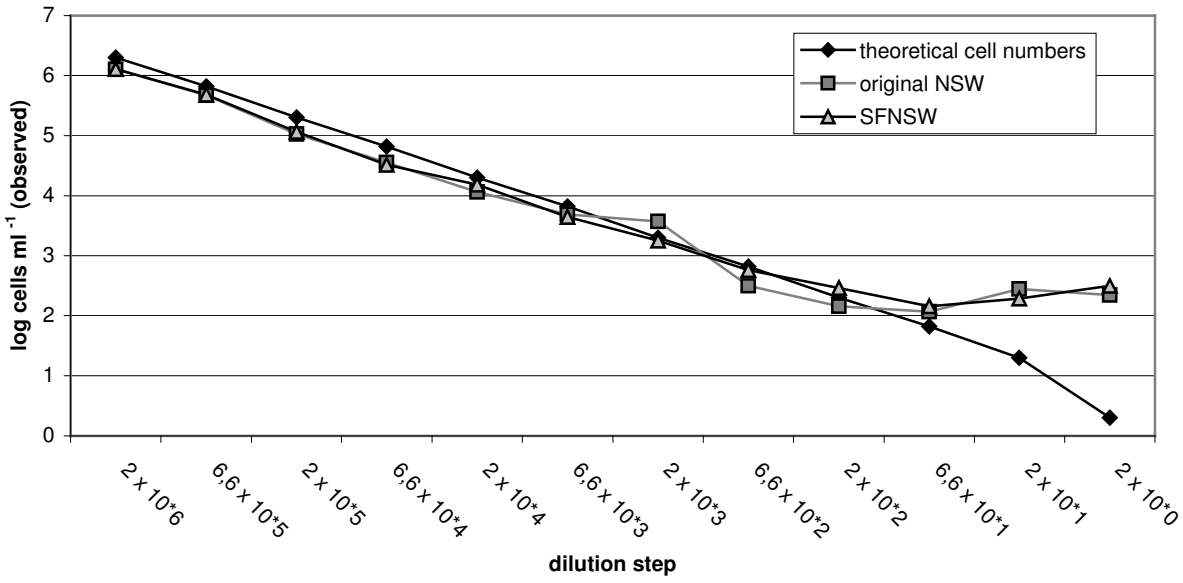

Fig. 11. Cell number of *V. cholerae* in SFNSW and original NSW over different dilution steps after 29 days

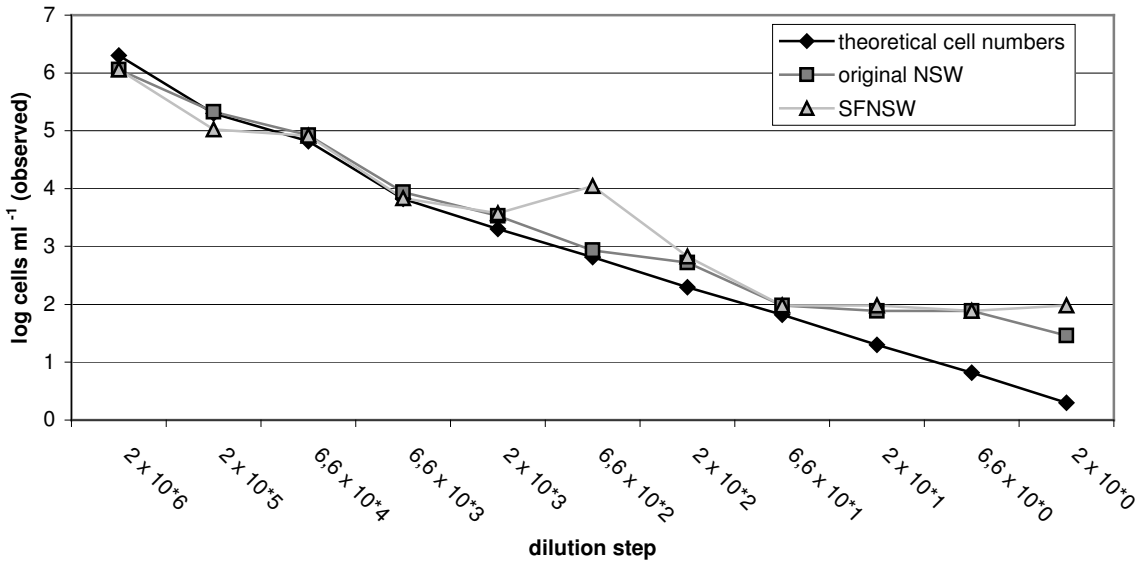

Fig. 12. Cell number of *V. cholerae* in SFNSW and original NSW over different dilution steps after 96 days

QUANTIFICATION OF V. CHOLERAE IN LAKE NEUSIEDLER SEE

I. Quantification by means of FISH

As can be seen in **Figure 13-14**, *V. cholerae* concentrations in the reed belt ranged between 10.000 cells L^{-1} in February to the highest number of 22.500 cells L^{-1} in the middle of July. In the lake center *V. cholerae* numbers varied from 5.000 cells L^{-1} at the beginning of April to the highest concentrations of 26.000 cells L^{-1} in the middle of July. After that peak, the bacteria decreased to about 15.000 cells L^{-1} both in the lake center and the reed belt. The LOD ranged between 230-1200 cells L^{-1} (depending on the filtration volume of 100-510 ml).

In the lake center, on average about 50% of *V. cholerae* were found in the 0.2-3 µm fraction, 40% in the 3-12 µm fraction and only 10% in the size fraction > 12 µm. In the reed belt, about 45% of *V. cholerae* appeared in the size fraction 0.2-3 µm and 3-12 µm and again only 10% in the size fraction > 12 µm. In the lake center, *V. cholerae* were not associated and in the reed belt hardly any *V. cholerae* (0.2 %) were associated with copepods. In contrast, about 3% of total *V. cholerae* were associated with cladocerans in the reed belt and 1% in the lake center (**Fig. 15**).

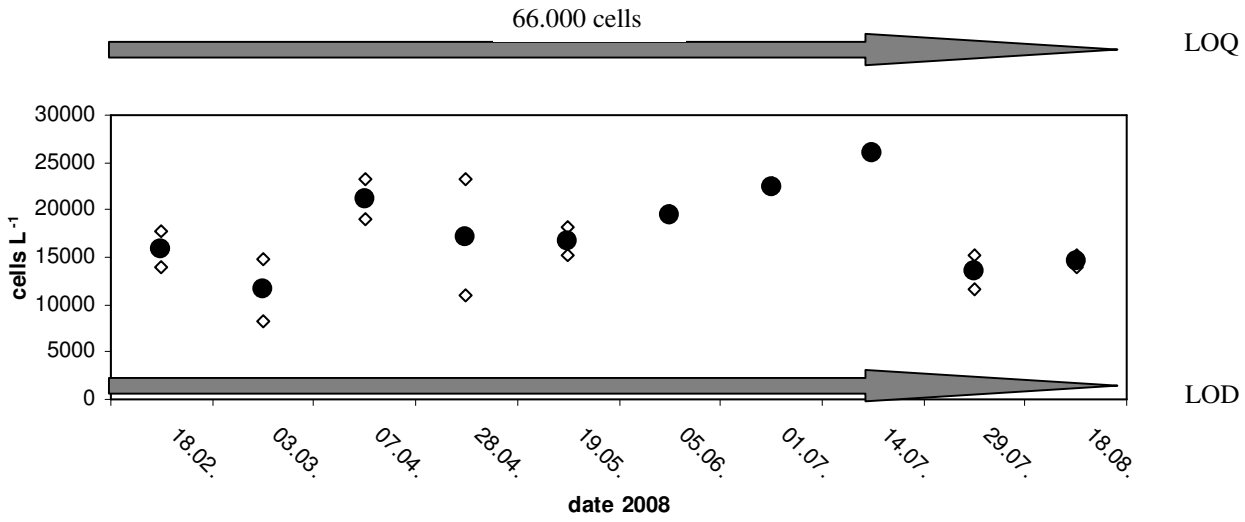

Fig. 13. *V. cholerae* L^{-1} in the lake center from February to August. Black dots show the average of duplicate independent measurements (white diamonds). LOQ: limit of quantification; LOD: limit of detection

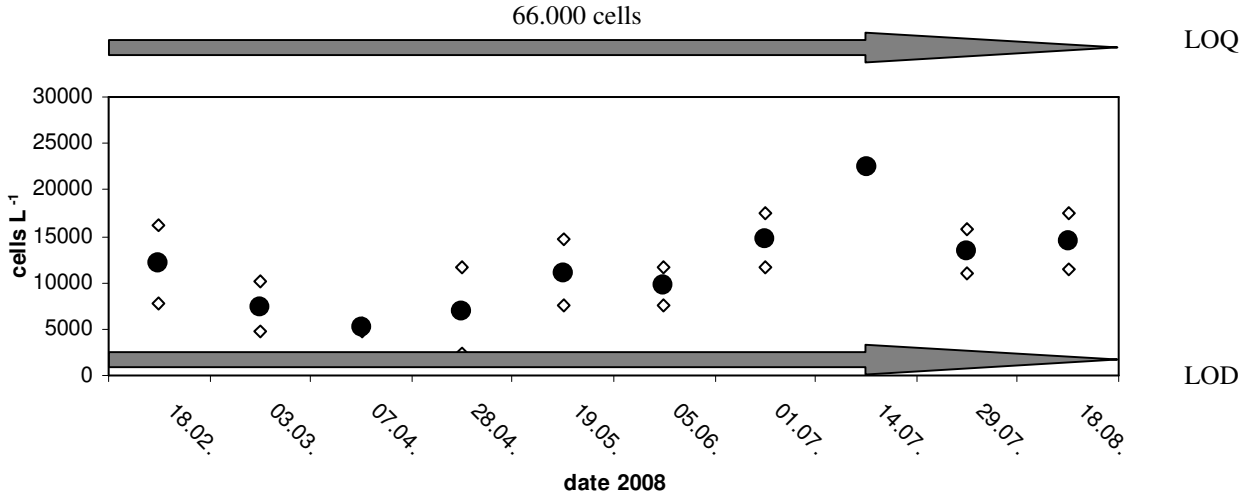

Fig. 14. *V. cholerae* L^{-1} in the reed belt from February to August. Black dots show the average of duplicate independent measurements (white diamonds). LOQ: limit of quantification; LOD: limit of detection

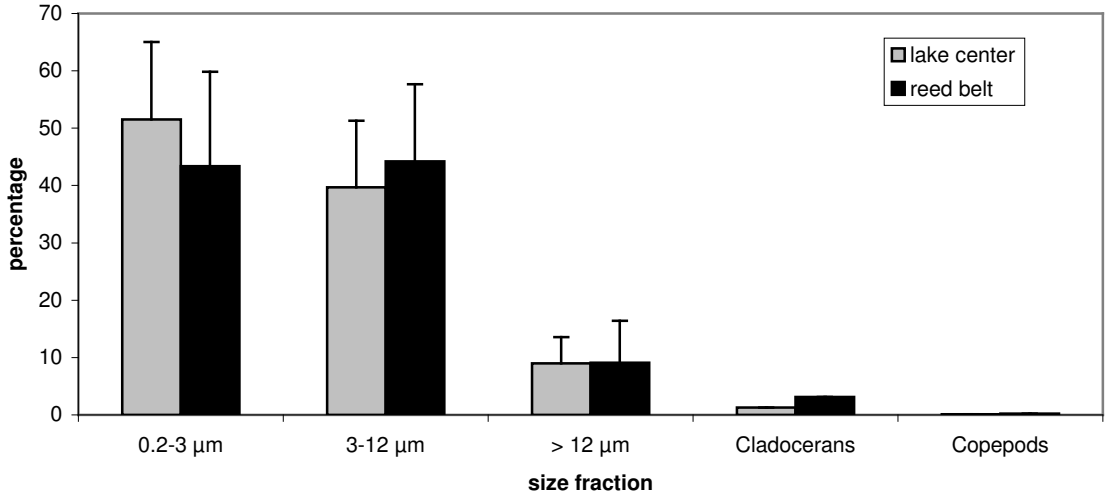

Fig. 15. Average percentage of *V. cholerae* cells in the three different size fractions and associated to zooplankton in the lake center and the reed belt. Data from all sampling events were pooled (n = 11). Bars represent one standard deviation.

II. Quantification by means of cultivation

In the lake center, about 40% of suspicious *V. cholerae* appeared in the size fraction 3-12 µm and 15-20% in the size fraction 0.2-3 µm and > 12 µm. In the reed belt about 25% of suspicious *V. cholerae* were found in the size fraction 3-12 µm and 10-15% in the size fraction 0.2-3 µm and > 12µm. Interestingly, over 53% of the suspicious reed belt *V. cholerae* could be attributed to cladoceran samples but no bacteria to copepods samples. In the lake center about 30% could be ascribed to cladocerans and only 1.5% to copepods (**Fig. 16**). In **Table 1** and **Table 2** the total numbers of suspicious *V. cholerae* colonies on TCBS, and the percentage of presumptive *V. cholerae*, which were able to grow on nutrient agar plates without NaCl and which were oxidase positive are listed. At both stations (lake center and reed belt), a lower percentage of presumptive *V. cholerae* was identified as *V. cholerae* by PCR for the first sampling points.

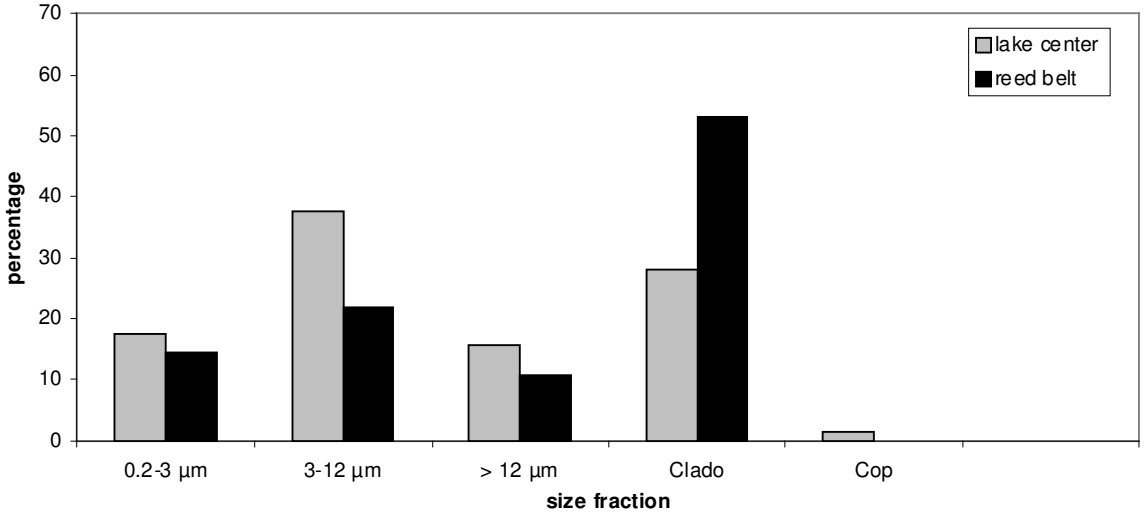

Fig. 16. Percentage of *V. cholerae* within three different size fractions and zooplankton associated *V. cholerae* in lake center and reed belt. Values of all sampling dates were pooled for each size fraction and zooplankton, and the percentage was calculated from the total sum. By this procedure no standard deviations can be calculated.

Table 1. CFU concentrations of suspicious *V. cholerae* L^{-1} and percentage of presumptive *V. cholerae* in the lake center: % NaCl negative of total suspicious *V. cholerae* colonies; % oxidase positive of NaCl negative colonies; % PCR positive of NaCl negative, Oxidase positive colonies, i.e. colonies identified as *V. cholerae*

Date 2008	CFU/L (suspicious)	% NaCl – (presumptive)	% Oxidase + (presumptive)	% PCR +
21. 04.	60	25	100	0
28. 04.	80	75	100	25
05. 05.	50	90	100	56
19. 05.	360	100	100	90
05. 06.	14900	60	100	100
01. 07.	45700	100	100	100
14. 07.	13700	75	100	100
29. 07.	1250	100	100	100
18. 08.	2050	100	100	100

Table 2. CFU concentrations of suspicious *V. cholerae* L^{-1} and percentage of presumptive *V. cholerae* in the reed belt: % NaCl negative of total suspicious *V.* cholerae colonies; % oxidase positive of NaCl negative colonies; % PCR positive of NaCl negative, Oxidase positive colonies, i.e. colonies identified as *V. cholerae*

Date 2008	CFU/L (suspicious)	% NaCl – (presumptive)	% Oxidase + (presumptive)	% PCR +
21. 04.	50	90	100	36
28. 04.	40	25	100	100
05. 05.	100	70	100	86
19. 05.	7730	70	100	100
05. 06.	17400	80	100	100
01. 07.	46100	100	100	100
14. 07.	8900	75	100	100
29. 07.	1850	75	100	100
18. 08.	1400	100	100	100

Results from lake centre cultivation samples are shown in **Figure 17-21:**

Total *V. cholerae* CFU concentrations did not correspond with the sum of the three different size fractions at the beginning of July (**Fig. 17**). As can be seen in **Figure 18 - 20**, *V. cholerae* could be detected by means of cultivation not earlier than at the beginning of June. The highest *V. cholerae* CFU concentrations were found in the size fraction 3-12 µm during the whole sampling period and with a peak value of 6.500 CFU L^{-1} in the middle of July. The other two size fractions showed their peak values at this time as well with 3.500 CFU L^{-1}. The size fraction 3-12 µm and > 12 µm could not be evaluated at two sampling time points (**Fig. 19 and 20**). Zooplankton associated *V. cholerae* could be detected from the beginning of July. Cladocerans associated *V. cholerae* showed a peak value of about 10.000 CFU L^{-1} in the middle of July. Copepod associated bacteria were found at only low concentrations (< 420 CFU L^{-1}) (**Fig. 21**)

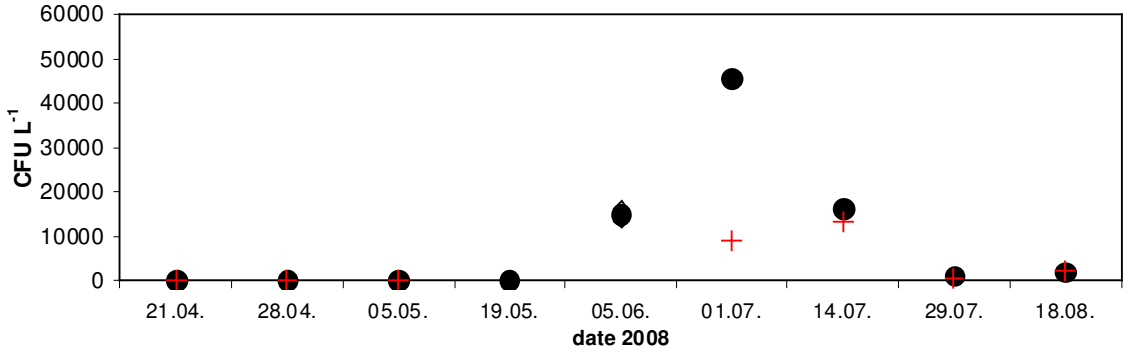

Fig. 17. Total *V. cholerae* CFU concentrations from April to August. Black dots show the average of duplicate independent measurements (white diamonds). The red cross shows the sum of the three different size fractions. Sampling time points with no red cross indicate that the size fraction 3-12 µm and > 12 µm could not be evaluated.

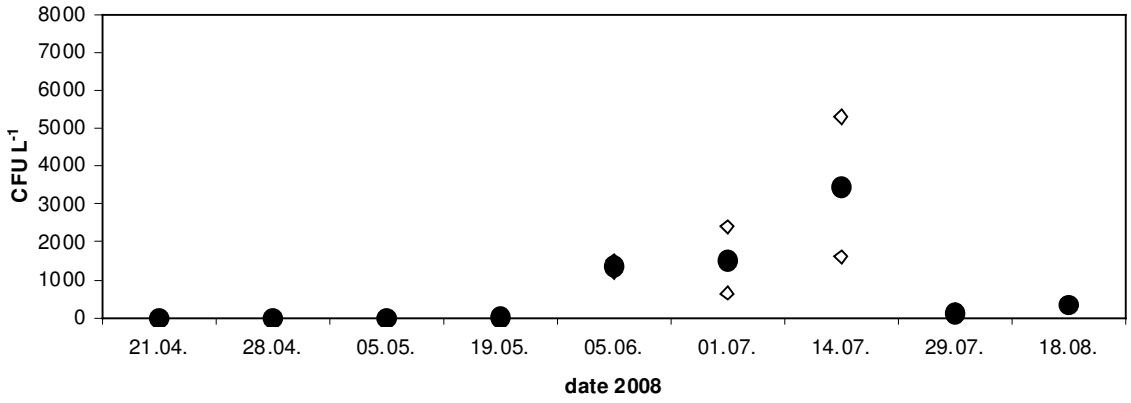

Fig. 18. *V. cholerae* CFU concentrations in size fraction 0.2-3 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

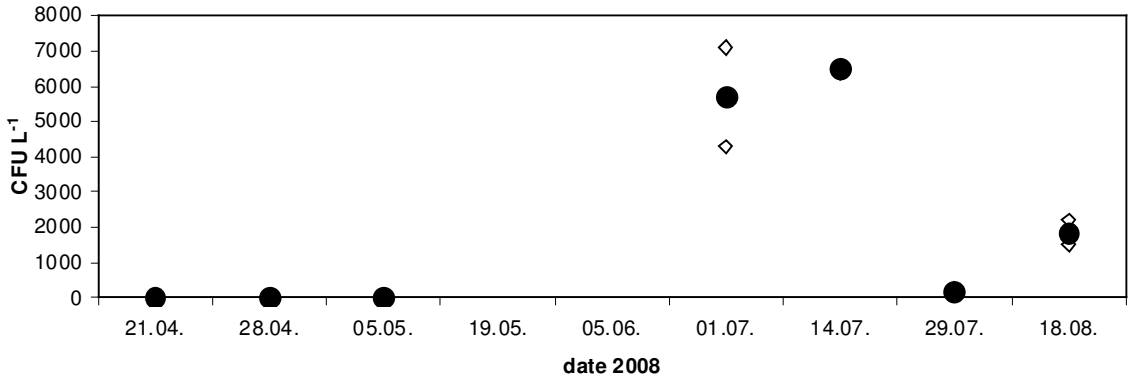

Fig. 19. *V. cholerae* CFU concentrations in size fraction 3-12 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

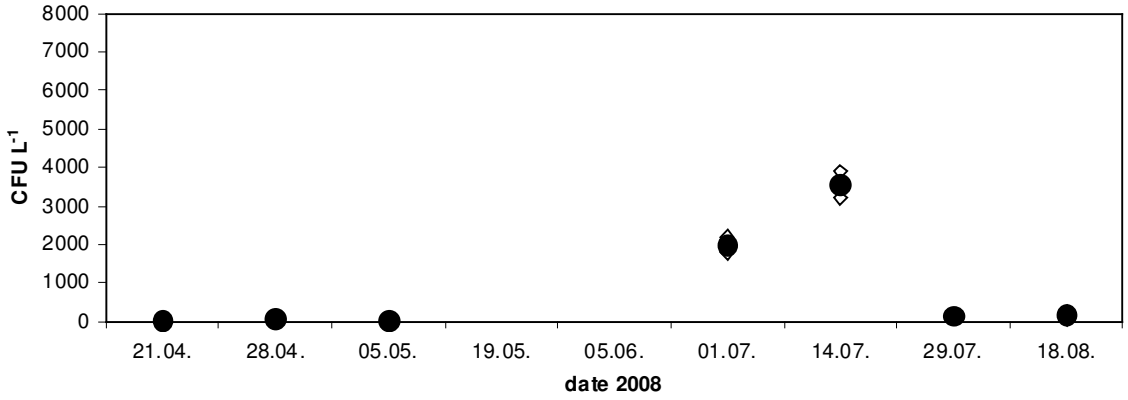

Fig. 20. *V. cholerae* CFU concentrations in size fraction > 12 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

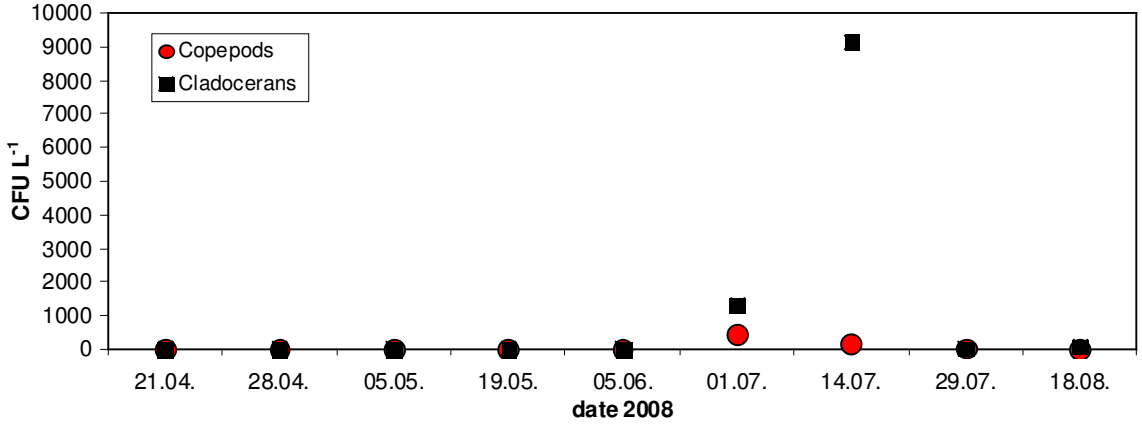

Fig. 21. Zooplankton associated *V. cholerae* CFU concentrations. CFU L^{-1} was calculated by multiplication of zooplankton L^{-1} with CFU zooplankton^{-1}.

Results from reed belt cultivation samples are shown in **Figure 22-26**:

Principally, the results of the reed belt showed similar trends as for the lake center. The total CFU concentrations did not correspond with the sum of the three different size fractions at the beginning of July (**Fig. 22**). A reliable detection of *V. cholerae* by means of cultivation started at the beginning of June. The highest CFU concentrations was always observed in the size fraction 3-12 µm during the whole investigation period, with a peak of 6.600 CFU L^{-1} in the middle of July. The other two size fractions also peaked at this time with concentrations of 3.800 CFU L^{-1} (> 12 µm) and 4200 CFU L^{-1} (0.2-3 µm) (**Fig. 23-25**). The size fraction 3-12 µm and > 12 µm could not be evaluated at two sampling time points (**Fig. 24 and 25**). Only cladoceran associated *V. cholerae* could be found in the reed belt, while no copepod

associated *V. cholerae* were observed. They showed a peak value of 33.000 CFU L^{-1} in the middle of July (**Fig. 26**).

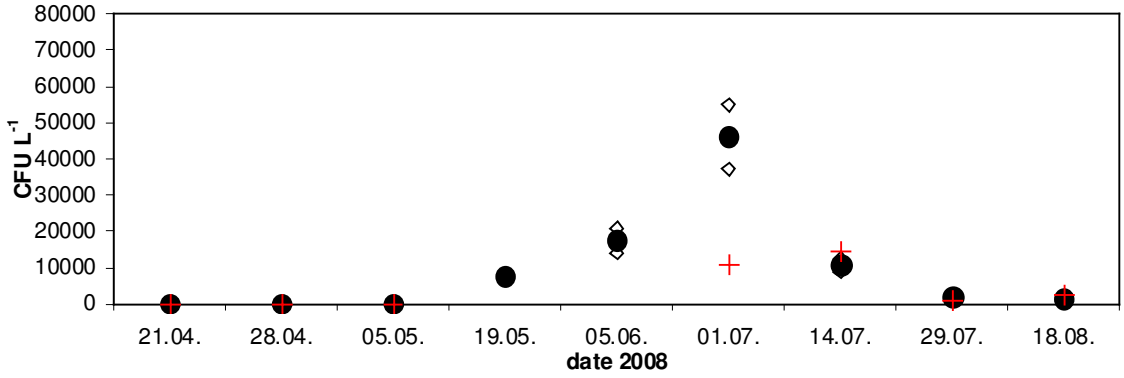

Fig. 22. Total *V. cholerae* CFU concentrations from April to August. Black dots show the average of duplicate independent measurements (white diamonds). The red cross shows the sum of the three different size fractions. Sampling time points with no red cross indicate that the size fraction 3-12 µm and > 12 µm could not be evaluated.

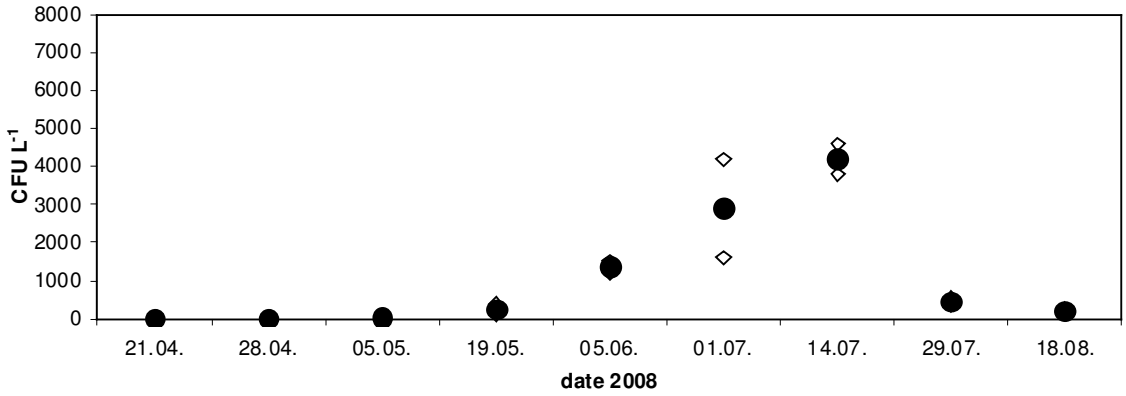

Fig. 23. *V. cholerae* CFU concentrations in size fraction 0.2-3 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

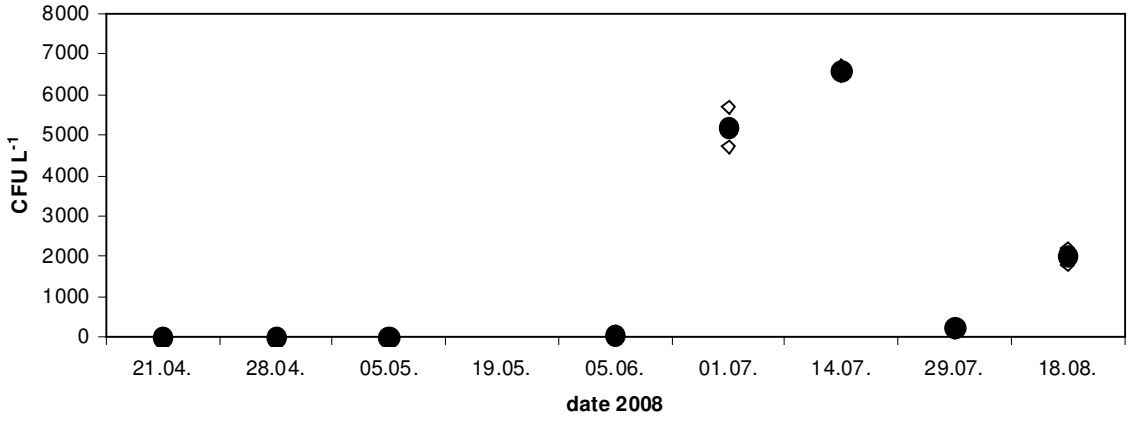

Fig. 24. *V. cholerae* CFU concentrations in size fraction 3-12 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

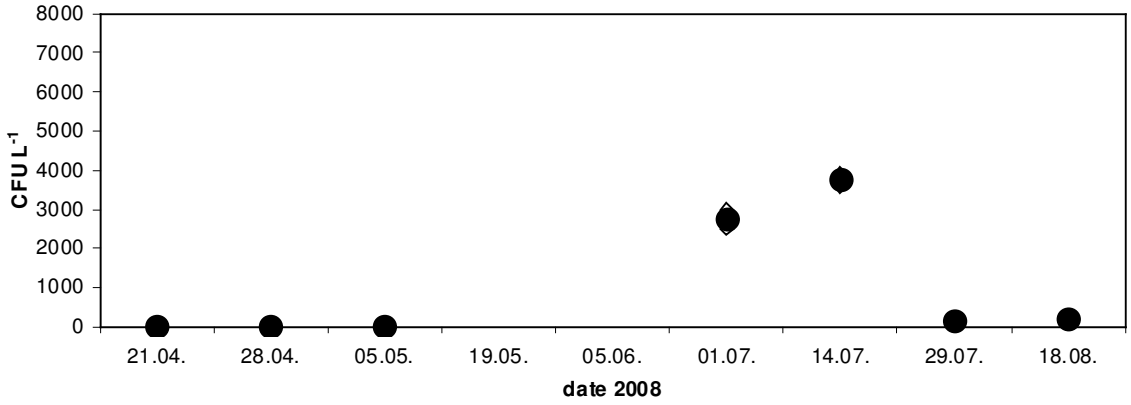

Fig. 25. *V cholerae* CFU concentrations in size fraction > 12 µm from April to August. Black dots show the average of duplicate independent measurements (white diamonds).

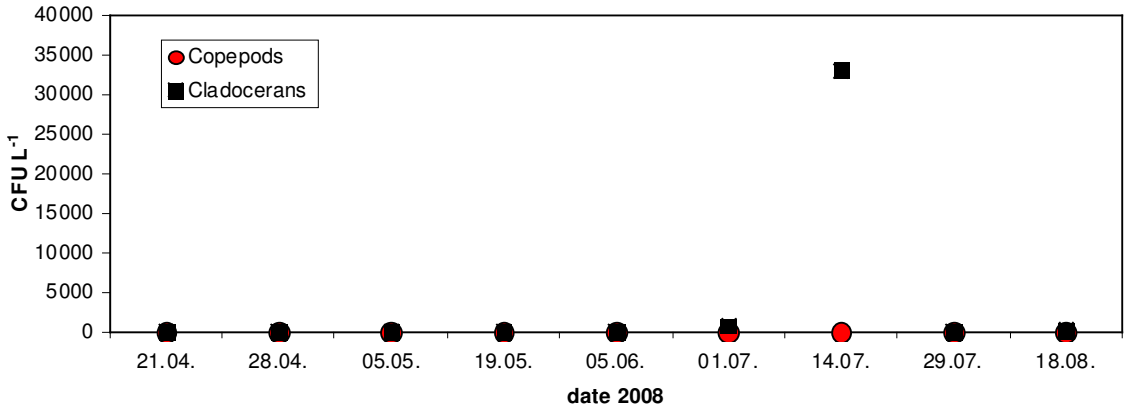

Fig. 26. Zooplankton associated *V. cholerae* CFU concentrations. CFU L^{-1} was calculated by multiplication of zooplankton L^{-1} with CFU zooplankton^{-1}.

STATISTIC RESULTS

I. Spearman-Rank-Test

For statistic analysis, a list of environmental parameters was integrated as described in Materials and Methods. On the one hand, the whole results from February till August and on the other hand, the results from May till August were statistically evaluated, because during this period the first positive results with cultivation were attained.

When all data from lake centre and reed belt were pooled (**Table 3),** $FISH_{tot}$ positively correlated with the FISH size fractions 3-12 µm and 0.2-3 µm and every cultivation size fraction as well as with FISH and cultivation cladocerans and $cultivation_{tot}$. No correlation was observed to any environmental parameter except for a positive correlation with the date.. Cultivation based total *V. cholerae* concentrations positively correlated with the date, temperature and negatively with maximum wind speed. When data for the period from May to August were observed, (**Table 4),** $FISH_{tot}$ and cultivation based total *V. cholerae* concentrations showed no correlation to any environmental parameter.

Table 5 summarizes the correlations for the lake center of the whole investigation period. $FISH_{tot}$ also showed no correlation with any environmental parameter neither with cultivation based *V. cholerae* concentrations. $Cultivation_{tot}$ was positively correlated with date and temperature. For the period from May to August (**Table 6**) $FISH_{tot}$ was positively correlated only with cultivation based total *V. cholerae* concentrations.

Table 7 shows the results of the lake center of the whole investigation period**,** $FISH_{tot}$ was positively correlated with cultivation based total *V. cholerae* concentrations and with date, chlorophyll a, dissolved organic carbon (DOC) and total organic carbon (TOC). $Cultivation_{tot}$ was positively correlated with date, chlorophyll a, DOC and TOC as well as with temperature. For the period from May to August (**Table 8**), $FISH_{tot}$ positively correlated with date and DOC. $Cultivation_{tot}$ showed no correlation with any environmental parameter.

Table 3. Spearman Rank correlation analysis of pooled data from the lake center and the reed belt from February till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12 µm	-0,498	-0,372	0,035	-0,355	0,005	0,030	-0,259	-0,395	-0,132	0,307	0,201	-0,313
	0,018	0,088	0,876	0,105	0,982	0,899	0,299	0,105	0,557	0,164	0,369	0,156
	22	22	22	22	20	20	18	18	22	22	22	22
FISH 3-12 µm	0,253	0,354	0,174	0,156	0,076	0,333	0,133	0,117	-0,074	-0,197	0,729	0,535
	0,256	0,106	0,439	0,487	0,751	0,151	0,598	0,643	0,745	0,380	0,000	0,010
	22	22	22	22	20	20	18	18	22	22	22	22
FISH 0.2-3 µm	0,468	0,269	0,538	0,079	0,186	0,196	-0,272	-0,353	-0,278	-0,021	0,881	0,288
	0,028	0,226	0,010	0,728	0,432	0,407	0,275	0,151	0,210	0,927	0,000	0,194
	22	22	22	22	20	20	18	18	22	22	22	22
FISH cladocerans	0,300	0,198	0,072	0,297	0,169	0,092	0,225	0,060	-0,144	0,300	0,497	0,403
	0,175	0,377	0,750	0,179	0,477	0,699	0,369	0,812	0,523	0,175	0,019	0,063
	22	22	22	22	20	20	18	18	22	22	22	22
FISH copepods	0,000	-0,025	0,090	-0,100	-0,029	-0,260	-0,068	-0,375	-0,062	0,400	0,050	-0,013
	1,000	0,912	0,689	0,657	0,904	0,268	0,788	0,125	0,783	0,065	0,826	0,956
	22	22	22	22	20	20	18	18	22	22	22	22
FISH tot.	0,409	0,304	0,419	0,031	0,207	0,403	-0,186	-0,159	-0,245	-0,038	1,000	0,422
	0,047	0,149	0,042	0,884	0,356	0,070	0,432	0,502	0,250	0,862	.	0,040
	24	24	24	24	22	21	20	20	24	24	24	24
Cult. >12µm	0,844	0,855	0,156	0,639	-0,027	0,074	0,256	0,138	0,166	-0,586	0,518	0,905
	0,000	0,000	0,511	0,002	0,914	0,778	0,339	0,612	0,485	0,007	0,019	0,000
	20	20	20	20	18	17	16	16	20	20	20	20
Cult. 3-12 µm	0,810	0,770	0,155	0,644	-0,126	0,068	0,392	0,098	0,241	-0,438	0,643	0,888
	0,000	0,000	0,502	0,002	0,607	0,789	0,120	0,710	0,294	0,047	0,002	0,000
	21	21	21	21	19	18	17	17	21	21	21	21
Cult. 0.2-3 µm	0,809	0,855	-0,020	0,732	-0,086	0,032	0,484	0,210	0,194	-0,392	0,546	0,937
	0,000	0,000	0,928	0,000	0,703	0,889	0,030	0,373	0,365	0,058	0,006	0,000
	24	24	24	24	22	21	20	20	24	24	24	24
Cult. Sum	0,817	0,895	-0,054	0,710	-0,052	0,064	0,432	0,295	0,230	-0,561	0,387	0,993
	0,000	0,000	0,803	0,000	0,819	0,784	0,057	0,207	0,280	0,004	0,062	0,000
	24	24	24	24	22	21	20	20	24	24	24	24
Cult. cladocerans	0,695	0,507	0,284	0,384	-0,178	0,086	0,253	-0,012	0,159	-0,254	0,566	0,683
	0,000	0,012	0,179	0,064	0,427	0,711	0,282	0,960	0,457	0,231	0,004	0,000
	24	24	24	24	22	21	20	20	24	24	24	24
Cult. copepods	0,376	0,386	0,509	-0,007	0,014	0,103	-0,149	-0,481	-0,008	-0,121	0,376	0,410
	0,070	0,063	0,011	0,975	0,950	0,655	0,531	0,032	0,972	0,572	0,070	0,046
	24	24	24	24	22	21	20	20	24	24	24	24
Cult. tot.	0,828	0,880	-0,045	0,707	-0,033	0,090	0,434	0,298	0,219	-0,524	0,422	1,000
	0,000	0,000	0,835	0,000	0,885	0,698	0,056	0,202	0,304	0,009	0,040	.
	24	24	24	24	22	21	20	20	24	24	24	24

Table 4. Spearman Rank correlation analysis of pooled data from the lake center and the reed belt from May till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12µm	-0,103	0,273	0,039	0,061	-0,097	-0,159	0,097	-0,447	-0,113	0,273	0,381	0,262
	0,726	0,344	0,895	0,837	0,752	0,605	0,777	0,168	0,700	0,345	0,179	0,365
	14	14	14	14	13	13	11	11	14	14	14	14
FISH 3-12 µm	0,283	0,580	0,078	0,229	0,033	0,377	0,389	0,156	-0,120	0,166	0,628	0,950
	0,327	0,030	0,790	0,430	0,914	0,204	0,237	0,648	0,682	0,570	0,016	0,000
	14	14	14	14	13	13	11	11	14	14	14	14
FISH 0.2-3 µm	0,505	0,049	0,439	-0,054	0,028	-0,061	-0,115	-0,395	-0,166	0,174	0,917	0,213
	0,065	0,868	0,116	0,855	0,928	0,843	0,737	0,230	0,570	0,551	0,000	0,465
	14	14	14	14	13	13	11	11	14	14	14	14
FISH cladocerans	0,204	-0,004	0,071	0,209	0,271	0,183	0,202	0,054	-0,292	0,611	0,604	0,408
	0,485	0,990	0,810	0,473	0,371	0,549	0,551	0,875	0,311	0,020	0,022	0,148
	14	14	14	14	13	13	11	11	14	14	14	14
FISH copepods	0,139	0,034	0,391	-0,174	0,309	0,000	-0,300	-0,400	-0,310	0,416	0,448	0,241
	0,636	0,907	0,167	0,553	0,305	1,000	0,370	0,223	0,281	0,139	0,108	0,407
	14	14	14	14	13	13	11	11	14	14	14	14
FISH tot.	0,422	0,194	0,418	-0,039	0,069	0,132	0,036	-0,273	-0,282	0,235	1,000	0,526
	0,133	0,507	0,137	0,895	0,823	0,667	0,915	0,417	0,329	0,418	.	0,053
	14	14	14	14	13	13	11	11	14	14	14	14
Cult. >12µm	0,186	0,348	0,016	0,401	0,243	0,310	0,432	0,198	-0,226	0,310	0,920	0,848
	0,607	0,325	0,965	0,250	0,529	0,417	0,333	0,670	0,531	0,384	0,000	0,002
	10	10	10	10	9	9	7	7	10	10	10	10
Cult. 3-12 µm	0,401	0,159	0,220	0,152	0,213	0,261	0,287	0,036	-0,223	0,244	0,970	0,806
	0,222	0,640	0,515	0,655	0,555	0,466	0,490	0,933	0,509	0,469	0,000	0,003
	11	11	11	11	10	10	8	8	11	11	11	11
Cult. 0.2-3 µm	0,319	0,538	-0,070	0,424	0,027	0,258	0,491	0,164	-0,081	0,248	0,681	0,930
	0,266	0,047	0,811	0,131	0,929	0,394	0,125	0,631	0,782	0,392	0,007	0,000
	14	14	14	14	13	13	11	11	14	14	14	14
Cult. Sum	0,186	0,556	-0,070	0,339	-0,066	0,291	0,473	0,218	-0,011	0,044	0,410	0,965
	0,524	0,039	0,811	0,235	0,831	0,334	0,142	0,519	0,970	0,880	0,146	0,000
	14	14	14	14	13	13	11	11	14	14	14	14
Cult. cladocerans	0,568	0,120	0,332	0,047	-0,084	0,254	0,213	-0,020	-0,016	0,151	0,708	0,636
	0,034	0,684	0,246	0,872	0,785	0,402	0,529	0,954	0,956	0,605	0,005	0,014
	14	14	14	14	13	13	11	11	14	14	14	14
Cult. copepods	0,234	0,260	0,616	-0,447	0,100	0,130	-0,393	-0,665	-0,150	0,111	0,426	0,339
	0,420	0,369	0,019	0,109	0,744	0,672	0,232	0,026	0,609	0,706	0,129	0,235
	14	14	14	14	13	13	11	11	14	14	14	14
Cult. tot	0,239	0,481	-0,048	0,335	-0,016	0,363	0,500	0,236	-0,051	0,151	0,526	1,000
	0,410	0,081	0,872	0,242	0,957	0,223	0,117	0,484	0,864	0,607	0,053	.
	14	14	14	14	13	13	11	11	14	14	14	14

Table 5. Spearman Rank correlation analysis of pooled data from the lake center from February till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12µm	-0,606	-0,396	-0,549	-0,376	-0,114	0,137	-0,104	-0,267	0,009	0,314	0,392	-0,391
	0,048	0,228	0,080	0,254	0,739	0,689	0,776	0,455	0,979	0,346	0,233	0,235
	11	11	11	11	11	11	10	10	11	11	11	11
FISH 3-12 µm	0,129	0,207	0,153	-0,019	-0,327	0,147	0,298	-0,159	0,254	-0,175	0,820	0,363
	0,705	0,541	0,654	0,956	0,326	0,665	0,404	0,661	0,451	0,607	0,002	0,273
	11	11	11	11	11	11	10	10	11	11	11	11
FISH 0.2-3 µm	0,370	0,306	0,445	0,333	0,056	0,056	0,241	-0,049	-0,079	0,204	0,648	0,346
	0,262	0,361	0,170	0,316	0,871	0,871	0,503	0,893	0,818	0,548	0,031	0,298
	11	11	11	11	11	11	10	10	11	11	11	11
FISH cladocerans	0,300	0,200	0,442	0,304	0,000	-0,100	0,175	-0,290	0,000	0,300	0,500	0,404
	0,370	0,555	0,173	0,363	1,000	0,770	0,629	0,416	1,000	0,370	0,117	0,218
	11	11	11	11	11	11	10	10	11	11	11	11
FISH copepods	0,300	0,200	0,442	0,304	0,000	-0,100	0,175	-0,290	0,000	0,300	0,500	0,404
	0,370	0,555	0,173	0,363	1,000	0,770	0,629	0,416	1,000	0,370	0,117	0,218
	11	11	11	11	11	11	10	10	11	11	11	11
FISH tot.	0,067	0,139	0,283	-0,148	0,418	0,491	0,244	0,549	-0,455	0,200	1,000	0,431
	0,855	0,701	0,428	0,684	0,229	0,150	0,497	0,100	0,187	0,680	.	0,162
	12	12	12	12	12	11	11	11	12	12	12	12
Cult. >12µm	0,853	0,865	0,836	0,729	-0,362	-0,119	0,590	-0,186	0,437	-0,595	0,448	0,912
	0,002	0,001	0,003	0,017	0,304	0,761	0,095	0,631	0,207	0,070	0,194	0,000
	10	10	10	10	10	9	9	9	10	10	10	10
Cult. 3-12 µm	0,778	0,792	0,848	0,818	-0,437	-0,201	0,614	-0,495	0,589	-0,410	0,485	0,902
	0,008	0,006	0,002	0,004	0,207	0,604	0,079	0,175	0,073	0,240	0,156	0,000
	10	10	10	10	10	9	9	9	10	10	10	10
Cult. 0.2-3 µm	0,825	0,832	0,507	0,841	-0,403	-0,305	0,655	-0,397	0,564	-0,425	0,485	0,919
	0,001	0,001	0,092	0,001	0,194	0,362	0,029	0,227	0,056	0,168	0,110	0,000
	12	12	12	12	12	11	11	11	12	12	12	12
Cult. Sum	0,861	0,883	0,520	0,743	-0,260	-0,183	0,547	-0,126	0,432	-0,548	0,431	1,000
	0,000	0,000	0,083	0,006	0,415	0,589	0,082	0,713	0,161	0,065	0,162	.
	12	12	12	12	12	11	11	11	12	12	12	12
Cult. cladocerans	0,774	0,620	0,802	0,570	-0,416	-0,190	0,444	-0,486	0,580	-0,337	0,370	0,762
	0,003	0,032	0,002	0,053	0,179	0,577	0,171	0,130	0,048	0,284	0,236	0,004
	12	12	12	12	12	11	11	11	12	12	12	12
Cult. copepods	0,560	0,615	0,580	0,422	-0,211	0,104	0,462	-0,451	0,442	-0,174	0,413	0,682
	0,058	0,033	0,048	0,172	0,510	0,761	0,153	0,164	0,150	0,588	0,182	0,015
	12	12	12	12	12	11	11	11	12	12	12	12
Cult. tot.	0,861	0,883	0,520	0,743	-0,260	-0,183	0,547	-0,126	0,432	-0,548	0,431	1,000
	0,000	0,000	0,083	0,006	0,415	0,589	0,082	0,713	0,161	0,065	0,162	.
	12	12	12	12	12	11	11	11	12	12	12	12

Table 6. Spearman Rank correlation analysis of pooled data from the lake center from May till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12µm	0,126	0,739	-0,117	0,633	-0,234	0,018	0,812	-0,551	0,252	0,090	0,775	0,703
	0,788	0,058	0,803	0,127	0,613	0,969	0,050	0,257	0,585	0,848	0,041	0,078
	7	7	7	7	7	7	6	6	7	7	7	7
FISH 3-12 µm	0,414	0,613	0,448	0,434	-0,342	0,090	0,543	-0,486	0,414	0,018	0,847	0,955
	0,355	0,144	0,314	0,330	0,452	0,848	0,266	0,329	0,355	0,969	0,016	0,001
	7	7	7	7	7	7	6	6	7	7	7	7
FISH 0.2-3 µm	0,148	-0,037	0,360	0,272	0,074	0,000	0,029	-0,232	-0,148	0,519	0,815	0,296
	0,751	0,937	0,427	0,555	0,875	1,000	0,957	0,658	0,751	0,233	0,025	0,518
	7	7	7	7	7	7	6	6	7	7	7	7
FISH cladocerans	0,204	0,000	0,441	0,214	0,204	0,000	-0,131	-0,393	-0,204	0,612	0,612	0,408
	0,661	1,000	0,322	0,645	0,661	1,000	0,805	0,441	0,661	0,144	0,144	0,363
	7	7	7	7	7	7	6	6	7	7	7	7
FISH copepods	0,204	0,000	0,441	0,214	0,204	0,000	-0,131	-0,393	-0,204	0,612	0,612	0,408
	0,661	1,000	0,322	0,645	0,661	1,000	0,805	0,441	0,661	0,144	0,144	0,363
	7	7	7	7	7	7	6	6	7	7	7	7
FISH tot.	-0,371	-0,143	0,339	-0,093	0,600	0,657	0,116	0,406	-0,600	0,771	1,000	0,750
	0,468	0,787	0,510	0,862	0,208	0,156	0,827	0,425	0,208	0,072	.	0,052
	7	7	7	7	7	7	6	6	7	7	7	7
Cult. >12µm	0,200	0,300	0,866	0,447	-0,100	0,100	0,200	-0,400	0,000	0,300	1,000	0,900
	0,747	0,624	0,058	0,450	0,873	0,873	0,800	0,600	1,000	0,624	.	0,037
	5	5	5	5	5	5	4	4	5	5	5	5
Cult. 3-12 µm	0,200	0,300	0,866	0,447	-0,100	0,100	0,200	-0,400	0,000	0,300	1,000	0,900
	0,747	0,624	0,058	0,450	0,873	0,873	0,800	0,600	1,000	0,624	.	0,037
	5	5	5	5	5	5	4	4	5	5	5	5
Cult. 0.2-3 µm	0,464	0,500	0,501	0,524	-0,321	-0,036	0,486	-0,600	0,321	0,107	0,893	0,929
	0,294	0,253	0,252	0,227	0,482	0,939	0,329	0,208	0,482	0,819	0,007	0,003
	7	7	7	7	7	7	6	6	7	7	7	7
Cult. Sum	0,393	0,500	0,501	0,374	-0,321	0,107	0,486	-0,429	0,357	-0,036	0,750	1,000
	0,383	0,253	0,252	0,408	0,482	0,819	0,329	0,397	0,432	0,939	0,052	.
	7	7	7	7	7	7	6	6	7	7	7	7
Cult. cladocerans	0,667	0,259	0,881	0,155	-0,296	-0,037	0,152	-0,577	0,408	0,074	0,593	0,741
	0,102	0,574	0,009	0,739	0,518	0,937	0,774	0,231	0,364	0,875	0,161	0,057
	7	7	7	7	7	7	6	6	7	7	7	7
Cult. copepods	0,335	0,473	0,575	-0,031	-0,059	0,335	0,213	-0,455	0,335	0,217	0,571	0,709
	0,463	0,284	0,177	0,947	0,900	0,463	0,686	0,364	0,463	0,641	0,180	0,074
	7	7	7	7	7	7	6	6	7	7	7	7
Cult. tot.	0,393	0,500	0,501	0,374	-0,321	0,107	0,486	-0,429	0,357	-0,036	0,750	1,000
	0,383	0,253	0,252	0,408	0,482	0,819	0,329	0,397	0,432	0,939	0,052	.
	7	7	7	7	7	7	6	6	7	7	7	7

Table 7. Spearman Rank correlation analysis of pooled data from the reed belt from February till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12µm	-0,452	-0,342	-0,224	-0,244	-0,084	-0,385	-0,216	-0,323	0,227	0,320	-0,493	-0,369
	0,163	0,303	0,507	0,470	0,831	0,306	0,608	0,435	0,503	0,338	0,123	0,265
	11	11	11	11	9	9	8	8	11	11	11	11
FISH 3-12 µm	0,384	0,526	-0,289	0,659	0,326	0,678	0,898	0,898	0,028	-0,229	0,732	0,730
	0,243	0,096	0,388	0,027	0,391	0,045	0,002	0,002	0,936	0,499	0,010	0,011
	11	11	11	11	9	9	8	8	11	11	11	11
FISH 0.2-3 µm	0,722	0,472	0,252	0,562	0,328	0,621	0,548	0,496	-0,261	-0,222	0,848	0,486
	0,012	0,143	0,455	0,072	0,389	0,074	0,160	0,212	0,439	0,513	0,001	0,130
	11	11	11	11	9	9	8	8	11	11	11	11
FISH cladocerans	0,300	0,200	-0,064	0,453	0,411	0,411	0,577	0,412	-0,401	0,300	0,500	0,404
	0,370	0,555	0,852	0,162	0,272	0,272	0,134	0,310	0,222	0,370	0,117	0,218
	11	11	11	11	9	9	8	8	11	11	11	11
FISH copepods	-0,300	-0,300	-0,064	-0,503	-0,274	-0,548	-0,577	-0,577	-0,050	0,500	-0,400	-0,404
	0,370	0,370	0,852	0,114	0,476	0,127	0,134	0,134	0,884	0,117	0,223	0,218
	11	11	11	11	9	9	8	8	11	11	11	11
FISH tot.	0,699	0,566	0,083	0,662	0,200	0,661	0,867	0,850	-0,060	-0,294	1,000	0,648
	0,011	0,055	0,798	0,019	0,580	0,038	0,002	0,004	0,854	0,354	.	0,023
	12	12	12	12	10	10	9	9	12	12	12	12
Cult. >12µm	0,841	0,853	-0,088	0,864	0,342	0,561	0,630	0,593	-0,092	-0,583	0,644	0,925
	0,002	0,002	0,809	0,001	0,408	0,148	0,129	0,161	0,800	0,077	0,044	0,000
	10	10	10	10	8	8	7	7	10	10	10	10
Cult. 3-12 µm	0,838	0,758	0,041	0,804	0,347	0,749	0,873	0,846	-0,114	-0,461	0,872	0,875
	0,001	0,007	0,906	0,003	0,360	0,020	0,005	0,008	0,738	0,154	0,000	0,000
	11	11	11	11	9	9	8	8	11	11	11	11
Cult. 0.2-3 µm	0,812	0,885	-0,229	0,884	0,513	0,757	0,914	0,896	-0,214	-0,384	0,732	0,957
	0,001	0,000	0,474	0,000	0,130	0,011	0,001	0,001	0,504	0,217	0,007	0,000
	12	12	12	12	10	10	9	9	12	12	12	12
Cult. Sum	0,796	0,901	-0,320	0,833	0,436	0,669	0,814	0,848	-0,176	-0,535	0,606	0,993
	0,002	0,000	0,311	0,001	0,208	0,034	0,008	0,004	0,583	0,073	0,037	0,000
	12	12	12	12	10	10	9	9	12	12	12	12
Cult. cladocerans	0,615	0,422	0,224	0,495	0,082	0,619	0,730	0,707	-0,267	-0,165	0,753	0,619
	0,033	0,172	0,485	0,102	0,822	0,056	0,025	0,033	0,402	0,608	0,005	0,032
	12	12	12	12	10	10	9	9	12	12	12	12
Cult. copepods	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.
	12	12	12	12	10	10	9	9	12	12	12	12
Cult. tot.	0,810	0,887	-0,320	0,833	0,460	0,693	0,848	0,865	-0,219	-0,486	0,648	1,000
	0,001	0,000	0,311	0,001	0,181	0,026	0,004	0,003	0,495	0,109	0,023	.
	12	12	12	12	10	10	9	9	12	12	12	12

Table 8. Spearman Rank correlation analysis of pooled data from the reed belt from May till August 2008. The first line indicates the coefficient of correlation (rho), the second line stands for the p-value and the third one for n. Temp.: temperature, P_{tot}: total phosphorus, Chla: cholorophyll a, DOC: dissolved organic carbon, TOC: total organic carbon, Secchi: Secchi depth, Cult. tot.: total cultivation and FISH tot.: total FISH.

Size fraction/Zooplankton	date	temp.	pH	conductivity	Ptot	Chla	DOC	TOC	Secchi	wind speed	FISHtot	Cult. tot
FISH >12µm	-0,396	-0,054	-0,202	0,055	0,174	-0,116	-0,205	-0,462	-0,162	0,468	-0,324	-0,162
	0,379	0,908	0,664	0,907	0,742	0,827	0,741	0,434	0,728	0,289	0,478	0,728
	7	7	7	7	6	6	5	5	7	7	7	7
FISH 3-12 µm	0,164	0,546	-0,204	0,528	0,493	0,986	0,975	0,975	-0,491	0,346	0,491	0,982
	0,726	0,205	0,661	0,223	0,321	0,000	0,005	0,005	0,263	0,448	0,263	0,000
	7	7	7	7	6	6	5	5	7	7	7	7
FISH 0.2-3 µm	0,800	0,145	0,340	0,417	0,118	0,324	0,738	0,580	-0,109	0,000	0,927	0,164
	0,031	0,756	0,455	0,352	0,824	0,531	0,155	0,306	0,816	1,000	0,003	0,726
	7	7	7	7	6	6	5	5	7	7	7	7
FISH cladocerans	0,204	0,000	0,000	0,520	0,393	0,393	0,707	0,354	-0,612	0,612	0,612	0,408
	0,661	1,000	1,000	0,232	0,441	0,441	0,182	0,559	0,144	0,144	0,144	0,363
	7	7	7	7	6	6	5	5	7	7	7	7
FISH copepods	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.
	7	7	7	7	6	6	5	5	7	7	7	7
FISH tot.	0,786	0,214	0,401	0,418	0,086	0,486	0,900	0,800	-0,214	0,000	1,000	0,429
	0,036	0,645	0,373	0,350	0,872	0,329	0,037	0,104	0,645	1,000	.	0,337
	7	7	7	7	6	6	5	5	7	7	7	7
Cult. >12µm	0,200	0,300	0,000	0,600	0,800	0,800	1,000	0,500	-0,700	0,300	0,900	0,800
	0,747	0,624	1,000	0,285	0,200	0,200	.	0,667	0,188	0,624	0,037	0,104
	5	5	5	5	4	4	3	3	5	5	5	5
Cult. 3-12 µm	0,543	0,086	0,131	0,319	0,500	0,800	1,000	0,800	-0,486	0,200	0,943	0,714
	0,266	0,872	0,805	0,538	0,391	0,104	.	0,200	0,329	0,704	0,005	0,111
	6	6	6	6	5	5	4	4	6	6	6	6
Cult. 0.2-3 µm	0,250	0,607	-0,134	0,636	0,543	0,943	1,000	0,900	-0,429	0,321	0,536	0,929
	0,589	0,148	0,775	0,124	0,266	0,005	.	0,037	0,337	0,482	0,215	0,003
	7	7	7	7	6	6	5	5	7	7	7	7
Cult. Sum	0,000	0,536	-0,267	0,400	0,371	0,943	0,700	0,900	-0,393	0,214	0,250	0,964
	1,000	0,215	0,562	0,374	0,468	0,005	0,188	0,037	0,383	0,645	0,589	0,000
	7	7	7	7	6	6	5	5	7	7	7	7
Cult. cladocerans	0,473	0,039	0,369	0,241	-0,030	0,638	0,894	0,783	-0,473	0,236	0,867	0,571
	0,284	0,933	0,416	0,603	0,954	0,173	0,041	0,118	0,284	0,610	0,012	0,180
	7	7	7	7	6	6	5	5	7	7	7	7
Cult. copepods	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.
	7	7	7	7	6	6	5	5	7	7	7	7
Cult. tot.	0,071	0,464	-0,267	0,400	0,429	1,000	0,900	1,000	-0,536	0,357	0,429	1,000
	0,879	0,294	0,562	0,374	0,397	.	0,037	.	0,215	0,432	0,337	.
	7	7	7	7	6	6	5	5	7	7	7	7

II. Multiple Regression

As regression analysis showed, 80% of the variations in total cultivation based *V.cholerae* concentrations could be explained by temperature (positive correlation) alone for both the reed belt an the lake centre (**Table 9** and **10**). The addition of other variables into a multiple regression model did not significantly increase the coefficient of determination. For the total FISH numbers, no suitable explanation model for the lake centre was found. For the reed belt, 84% of the variation in *V. cholerae* concentrations could be explained by DOC (positive correlation) and wind speed (negative correlation) (**Table 11**).

Table 9. Regression analysis of the $cult_{tot}$ results with the temperature in the lake center. R (coefficient of regression), R^2 (coefficient of determination), temp.: temperature and cult. tot.: total cultivation

depending variable: cult. $_{tot}$	R	R^2	Corrected R^2	level of significance
influencing variable: temp.	0.904	0.817	0.799	0.000

Table 10. Regression analysis of the $cult_{tot}$ results with the temperature in the reed belt. R (coefficient of regression), R^2 (coefficient of determination), temp.: temperature and cult. tot.: total cultivation

depending variable: cult. tot	R	R^2	Corrected R^2	level of significance
influencing variable: temp.	0.905	0.819	0.801	0.000

Table 11. Regression analysis of the $FISH_{tot}$ results with the wind speed and the DOC in the reed belt. R (coefficient of regression), R^2 (coefficient of determination), FISH tot.: total FISH and DOC: dissolved organic carbon

depending variable: FISHtot	R	R^2	Corrected R^2	level of significance
influencing variable: wind speed, DOC	0.939	0.882	0.835	0.000

5 DISCUSSION

1. STARVATION

It is well known that under stress conditions (e. g. low temperature and nutrient deprivation (McDougald et al. 1998)) in the natural habitat *V. cholerae* are converted to a dormant life form, the so-called viable but non-cultivable (VBNC) form because these dormant cells are not cultivable by standard culture techniques (Colwell 1985; Chaiyanan et al. 2007). In this state cells are metabolically active but incapable of undergoing the sustained cellular division to form a colony (Wai et al. 1999). Additionally, they are reduced in size and become coccoid (Colwell 1985; Chaiyanan et al. 2007).

The results of the starvation experiments demonstrated that within the first two weeks *V. cholerae* doubled their concentration from 3×10^6 cells mL^{-1} to 6.5×10^6 cells mL^{-1} and then hardly increased their cell number any further in ALW and SFNSW. In the studies of Novitsky et al. (1977) starved cells increased their cell number eight times compared to the initial cell number during the first week. They used a psychrobhilic marine Vibrio and incubated the bacteria in a phosphate-buffered salt mixture at 5°C.

Besides, there was a strong tendency that the bacteria in ALW more quickly become cocci than the bacteria in SFNSW. After 98°C days of starvation, just 70% of *V. cholerae* became coccoid in ALW and SFNSW. In contrast to that, Chaiyanan et al. (2007) postulated that after incubation for 60 days all *V. cholerae* cells became coccoid. They starved one O1 and one O139 *V. cholerae* strain in an artificial sea water medium at 4°C.

Additionally, the cells lost over 90% of their original volume (from 1 μm^3 to 0.08 μm^3) after 30 days and then did not lose volume any further. Baker et al (1983) observed as well that after 30 days the cells lost over 90% of their original volume. There, they exposed one O1 and one nonO1 *V. cholerae* to nutrient-free artificial seawater and filtered natural seawater.

2. FISH METHOD - LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection (LOD is the lowest number of cells in a sample that can be detected but not quantitated as an exact value) was 3.3 *V. cholerae* ml^{-1} and the limit of quantification (LOQ is the lowest number of bacteria in defined sample volume that can be quantitatively determined with suitable precision and accuracy) was 6.6×10^1 *V. cholerae* ml^{-1} in a volume of 36 ml. The LOQ was determined at three different time points (at the beginning, at the middle and at the end) of the starvation experiment and determined by FISH with a specific probe. Besides that, the cell numbers could be determined quite precisely by FISH in all three

time point samples (unstarved vs starved) compared to the theoretical cell number, beginning with 2 x 10^6 *V. cholerae* ml^{-1} to two cells ml^{-1}. This indicated that FISH with a specific probe is suitable to detect starved/VBNC cells and is therefore an appropriate method for quantification of *V. cholerae* in the environment independent of the season.

3. QUANTIFICATION OF *VIBRIO CHOLERAE* IN THE LAKE NEUSIEDLER SEE BY MEANS OF FISH AND CULTIVATION

Studies have proved that *V. cholerae* is a free-living bacterium (Worden et al. 2006, Kirschner et al. 2008) or associated with phytoplankton (Epstein 1993; Eiler et al. 2006), 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 conditions in the environment (Faruque et al. 2002) and additionally, chitinaceous organisms are a nutrient source because *V. cholerae* is able to metabolise chitin (Gooday 1990; Pruzzo et al. 2008). Besides, *V. cholerae* is able to produce a continuous biofilm on the colonized surface (Wai et al. 1999).

Little is known which ecological niche is really occupied by nonO1/nonO139 *V. cholerae* and which is it main life mode (planktonic, particle or zooplankton associated). There is hardly any data in which size fraction of the water the bacterium could be found or if it is associated with the surface of zooplankton or living in its gut. Besides, up to now, no quantitative data of *V.cholerae* in the lake Neusiedler See have been available, a lake where ear, blood and wound infections caused by this human pathogen have been reported in recent years.

Because of the different environmental conditions (average wind speed, Secchi depth, pH, [total phosphorus], [chlorophyll a], [dissolved organic carbon] and [total organic carbon]) in the lake center and the reed belt of the lake Neusiedler See, the two sampling points investigated in this study were regarded separately, as two distinct ecosystems.

Because many of these environmental parameters simultaneaously interact, their positive or negative influence on the growth of *Vibrio cholerae* is often compensated and it is extremely difficult to unveil statistically proved interrelationships of *V.cholerae* concentrations with environmental variables. For example, the wind at the lake Neusiedler See is stronger during winter than during summer. The reed belt is more protected from wind than the lake center. This implicates that it is less turbid (larger Secchi depth), there is more light irradiation and therefore more algae growth that stimulates the *V. cholerae* growth. It is well known that *V. cholerae* occurrence is often associated with algae blooms (Eppstein 1993; Eiler et al. 2006)

but there are no such algae blooms in the lake Neusiedler See because of its turbidity there is less algae growth. On the other hand, the reed belt has much higher concentrations of humic high molecular weight DOC which has shown to be inhibitory for *V.cholerae* growth (Kirschner et al 2008). During summer temperature increases which should have an enhancing effect on *V. cholerae* growth and more obviously – cultivablity. But on the other side, during warm wind-calm summer days, reduced wind speed leeds to a reduction of particles in the water (increased Secchi depth due to lower turbidity) which implicates that less *V. cholerae* cells are present in water, because they are to a large extent associated to particles settling down to the lake bottom.

By means of FISH, *V. cholerae* concentrations in the reed belt ranged between 5.000 cells L^{-1} to 22.500 cells L^{-1} and in the lake center *V. cholerae* numbers varied from 15.000 cells L^{-1} to 25.000 cells L^{-1}. Depending on the filtration volume of 100-510 ml, the LOD ranged between 230-1200 cells L^{-1}. These results show that there are significantly more *V. cholerae* in the lake center than in the reed belt, which may be due to the fact that (i) lower particle concentrations are observed in the reed belt and (ii) higher humic substance concentrations inhibit *V.cholerae* growth. Compared to the concentrations of the lake Neusiedler See, Heidelberg et al. (2002) counted 200.000 to 6.000 000 *V. cholerae* L^{-1} in the Choptank River in Chesapeake Bay from April to December by means of fluorescent oligonucleotide direct-counting. Eiler et al. (2006) quantified 1.000 to almost 10.000 *V. cholerae* L^{-1} in Baltic and Skagerrak Seas, even in the cold and low salinity samples from Bothian Bay, in more than 50% of the samples. They performed their studies during the summer in 2003 and in 2004 by means of quantitative competitive PCR with denaturant gradient gel electrophoresis.

In the lake Neusiedler See, *V. cholerae* could be detected during every sample time point from February to August and the peak value was reached in the middle of July. That indicated that the detection of *V. cholerae* by means of FISH is even possible during the cold season when the cells are probably in the VBNC state. One disadvantage of the applied FISH protocol was that its performance was dependent on the particle concentration in the water column which negatively influenced the microscopic evaluation by blocking the filter. Therefore, it is not surprising that most *V.cholerae* cells occurred in the size fraction 0.2-3 µm, which is the poorest in particles.

In contrast, the detection of *V. cholerae* by means of cultivation on TCBS agar was not possible till the middle of May depending on the temperature. Reliable results were not achieved till the beginning of June. In both, the lake center and the reed belt the total cell number ranged between no cells and 50.000 cells L^{-1}. The peak value was reached at the

beginning of July. Interestingly, the total cell number did not correspond with the sum of the size fractions, which had the peak value with 20.000 cells L^{-1} in the middle of July. The difference in the cell number could be explained by results of additionally performed size fraction experiments (Sonja Schauer, personal communication; data not shown). In these experiments it could be shown, that *V. cholerae* cells resuspended in 0.2 or 2 µm filtered NSW could be cultivated much less effectively than cells resuspended in 5 µm filtered NSW. This indicated that particles within a special size range (2 – 5 µm) may be essential for the cultivation of *V. cholerae* from Lake Neusiedler See. This observation is corroborated by the fact that most of the *V. cholerae* cells were found in the size fraction 3-12 µm in the lake center and the reed belt.

By means of FISH and cultivation, hardly any bacteria were associated with copepods (in case of FISH with cladocerans as well) in contrast to the results of Huq et al. (1983). They firstly observed that O1 and non-O1 *V. cholerae* attach to the surface of copepods. It seems that the association with copepods is more a capacity of O1 than of O139 *V. cholerae* because in laboratory experiments O1 strains consistently achieved higher abundances than O139 strains, when colonizing copepods (Rawlings et al. 2007). By means of cultivation more than half of the detected bacteria were associated with cladocerans in the reed belt and about 30% in the lake center. This may be an evidence that *V. cholerae* is not mainly associated with the surface of the zooplankton but are living in the gut of cladocerans for a better survival. That *V. cholerae* can adhere to the gut of zooplankton was observed by Colwell (1996) as well but in that report the association was with copepods and not cladocerans.

The poor results of zooplankton associated bacteria by FISH may be attributed to the used protocol (sonication; see materials and methods) for the detachment of *V. cholerae* from zooplankton, besides that, maybe most of the bacteria live within the guts of the zooplankton.

The peak value of zooplankton-associated bacteria was reached in the middle of July as well. It could be observed that after this strong increasing of *V. cholerae* in the size fractions by means of FISH and cultivation and of the zooplankton associated bacteria by means of cultivation there was a heavy decreasing of the cell number (50-60%) at the end of July and the middle of August. Zooplankton associated bacteria could not be detected at all. The most reliable explanation for this observation may be the weather conditions, which prevailed on the sampling days and the respective day before. On both days in the middle of July the wind speed reached high values and therefore the lake water had a high turbidity (low Secchi depth) which implicates the presence of many particles and particle associated *V. cholerae*. On the next two sampling days the wind speed was more than 70% lower. Thus the particle

concentrations were significantly lower leading to markedly decreased *V.cholerae* concentrations.

4. STATISTIC ANALYSIS

As mentioned above, the environmental conditions and parameters in the lake center and the reed belt strongly differ from each other and therefore a correlation analysis including both sampling points is not applicable. The only reliable statement that can be made is that the total *V.cholerae* numbers determined via FISH positively correlated with the total CFU concentrations but there was no correlation to any environmental parameter.

According to the correlation and multiple-regression results, 80% of the variation in *Vibrio cholerae* CFU in the lake center and the reed belt from February till August could be explained by temperature. It was observed that the cultivation based *V. cholerae* concentrations strongly positively correlated with temperature which means that the higher the temperature, the more *V. cholerae* are cultivable. In contrast, the variation of the FISH based *V.cholerae* concentrations in the reed belt could be best explained by the DOC and the wind speed together to an extent of 84%. There was a positive correlation with the DOC and negative correlation with the wind speed. This indicates that the more DOC is available and the less wind is present the more *V. cholerae* are detected by FISH. Principally, this observation is in contradiction to the observations stated above, namely that a strong wind resuspends more particles in the water column to which *V. cholerae* cells are attached. The most probable explanation is a methodical one because the more turbid the water, the more difficult is the evaluation of the samples by FISH because filters blocked with particles are difficult to evaluate under microscope.

For the lake centre, the *V. cholerae* concentrations determined via FISH did not correlate significantly with any environmental parameter. The explanation is that depending on the weather conditions (strong wind and hence high turbidity), certain size fractions were not or hardly evaluable and as a result the total cell number was most probably underestimated. Thus statistical evaluations may be biased by this methodical shortcoming and a significantly correlation was not possible (**Fig. 27**).

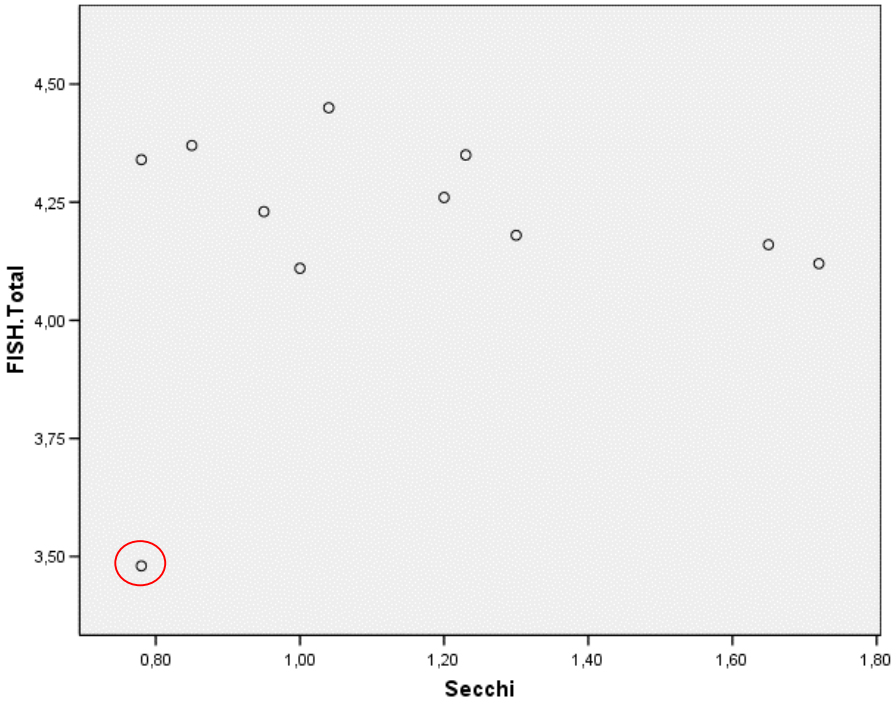

Fig. 27. Negative correlation of FISHtot. with the Secchi depth in the lake center. Red circle indicates an underestimated value due to methodical problems (see text).

6 CONCLUSION AND SUMMARY

Previous studies have proved that *V. cholerae* is able to grow planktonically (Worden et al. 2006; Kirschner et al 2008) or attached to zooplankton (Huq et al. 1983). At harsh environmental conditions (e.g. low temperature) the bacterium can change to the viable but non cultivable status (McDougald et al. 1998; Cottingham et al. 2003) where it can only be detected by molecular biological methods.

In the present study I could show that the FISH method with a specific probe is suitable for detection of environmental *V. cholerae* during every season of the year and that it is not limited to periods of high temperature like the cultivation method. From May to August, both methods yielded similar results and there was a significant positive correlation between the FISH and cultivation based *V. cholerae* concentrations. However, one major disadvantage of the established FISH protocol was that the presence of high particle concentrations in the water column at periods of high wind speed (mainly in the lake center) limited the accuracy and reliability of the method which further led to a masking of possible relationships of *V.cholerae* concentrations to environmental variables.

Nevertheless, the results showed that significantly higher *V. cholerae* concentrations are present in the lake center than in the reed belt. In both, the lake center and the reed belt most of the cells could be detected in the size fraction 3-12 µm by cultivation indicating that this particle fraction contains certain environmental features that enhance *V. cholerae* cultivability. In contrast, by means of FISH most *V. cholerae* cells occurred in the size fraction 0.2-3 µm. Because the reliability and recovery of cells by the FISH method proved to be dependent on the particle concentration in the water and the 0.2-3 µm size fraction was the poorest in particles this may be due to a methodical bias.

Surprisingly, in contrast to common knowledge, the nonO1/nonO139 *V. cholerae* strains isolated from the lake Neusiedler See showed very weak association with copepods but a strong association with the dominant cladocerans species. This implicates that there is a specific interaction between certain zooplankton species and specific *V. cholerae* strains. Moreover, the large difference in cladoceran associated *V. cholerae* numbers between the FISH method and the cultivation based approach indicates that most of the zooplankton attached cells colonize inner surfaces and are not present on the exoskeleton.

Summing up, the *V. cholerae* strains in Lake Neusiedler See seem to have several life strategies (attachment to cladocerans, association with the 3–12 µm particle fraction, planktonic growth) and do not occupy a single specialized ecological niche.

7 ZUSAMMENFASSUNG

Vibrio cholerae ist ein bewegliches, Gram-negatives, heterotrophes Stäbchen, das zur Familie der Vibrionaceae gehört. Neben seiner Rolle als wichtiger pathogener Mikroorganismus, ist es auch Bestandteil der normalen Bakterienflora der aquatischen Umwelt, welche Süß-, Brack- und Salzwasser umschließt. Zusätzlich weist es eine duale Lebensform auf, welche entweder Partikel/Zooplankton assoziiert oder frei lebend (planktonisch) sein kann. Von den 200 momentan bekannten *V. cholerae* Serogruppen können nur Stämme, die zu den Serogruppen O1 und O139 gehören, Cholera, eine schwere Durchfallerkrankung, auslösen. NonO1/nonO139 *V. cholerae* sind für weniger schwere Durchfallerkrankungen und Blut-, Wund-, Ohr- und Atemwegsinfektionen verantwortlich. Zwischen 2000 und 2007 hat die Weltgesundheitsorganisation über 249 importierten O1/O139 *V. cholerae* Fällen, in 14 unterschiedlichen europäischen Ländern, berichtet. Im Zeitraum von 2000 bis 2005 traten 14 Infektionen, die auf nonO1/nonO139 *V. Cholerae* zurückzuführen sind, in Österreich auf. Fünf dieser Infektionen, welche auch einen Todesfall inkludieren, konnten direkt mit dem Neusiedler See in Verbindung gebracht werden.

V. cholerae wird üblicherweise in der Umwelt mittels Kultivierung auf Selektivmedium detektiert. Es ist allgemein bekannt, dass *V. cholerae* unter gewissen Bedingungen, wie Nährstoffmangel und niedriger Temperatur, in den sogenannten VBNC Status (viable but non culturable) wechseln kann, innerhalb dessen es nicht mehr mittels Kultivierungsmethoden detektierbar ist.

In dieser Diplomarbeit wurde *V. cholerae* im Neusiedler See mittels kultivierungsbasierten und molekular biologischen Methoden (Fluoreszenz In Situ Hybridisierung = FISH) an zwei Probenpunkten (Seemitte und Schilfgürtel) quantifiziert. *V. cholerae* wurde in drei unterschiedlichen Größenfraktionen (> 12 µm, 3-12 µm und 0,2-3 µm) sowie auf Zooplankton (Copepoda *Arctodiaptomus spinosus* und Cladocera *Diaphanosoma mongolianum*) von Februar bis August 2008 detektiert. Dafür wurde ein spezifisches FISH Protokoll für dessen Detektion in Umweltproben entwickelt. Mittels Verknüpfung seiner Konzentration mit einer Vielzahl von Umweltparametern, wurde versucht die bevorzugte Nische von diesem Pathogenen im Neusiedler See aufzufinden.

Die Ergebnisse zeigen, dass das ausgewählte FISH Protokoll zur Detektion von Bakterien im Labor und im Freiland geeignet ist, sogar wenn sich die Bakterien im VBNC Status befinden (Februar bis Mitte Mai). Ein großer Nachteil der etablierten FISH Methode war, dass die Gegenwart von hohen Partikelkonzentrationen in der Wassersäule zu Zeiten hoher Windgeschwindigkeit (hauptsächlich in der Seemitte), die Genauigkeit und die

Zuverlässigkeit der Methode limitierte, was weiter dazu führte, dass die mögliche Beziehung zwischen *V. cholerae* Konzentrationen und Umweltvariablen maskiert wurde. Beide, FISH und Kultivierung wiesen die höchsten Zellzahlen im Juli, mit 22.000 – 26.000 CFU L^{-1} und 45.000 - 46.000 CFU L^{-1}, und abnehmenden Werten an den zwei folgenden Probenpunkten auf, was auf einen Abfall von Partikeln in der Wassersäule, aufgrund milder Wetterbedingungen, zurückzuführen ist. Von Mai bis August wurde mit beiden Methoden ähnliche Resultate erreicht und es gab eine signifikant positive Korrelation zwischen FISH und kultivierungsbasierten *V. cholerae* Konzentrationen. Es traten signifikant höhere *V. cholerae* Konzentrationen in der Seemitte als im Schilfgürtel auf. In beiden, der Seemitte und dem Schilfgürtel, wurden mittels Kultivierung die meisten Bakterien in der Größenfraktion 3-12 µm detektiert, was darauf hinweist, dass diese Partikelfraktion bestimmte Umweltbedingungen aufweist, welche die Kultivierung von *V. cholerae* verstärkt. Im Gegensatz dazu, traten mittels FISH die meisten Bakterien in der Größenfraktion 0,2-3 µm auf. Da es sich herausgestellt hat, dass die Zuverlässigkeit und Ausbeute an Zellen mittels FISH von der Partikelkonzentration im Wasser abhängt und die Fraktion 0,2-3 µm die partikelärmste Fraktion war, könnte dieses Ergebnis möglicherweise auf eine methodische Verzerrung zurückzuführen sein. Im Gegensatz zum allgemeinen Wissensstand, zeigten die nonO1/nonO139 isolierten *V. cholerae* Stämme aus dem Neusiedler See eine sehr schwache Assoziation mit Copepoden, aber eine sehr starke Verknüpfung mit der dominierenden Cladoceren Art. Das impliziert, dass es eine spezifische Interaktion zwischen bestimmten Zooplankton Arten und *V. cholerae* Arten geben muss. Außerdem zeigt der große Unterschied an Cladoceren assoziierten *V. cholerae* Konzentrationen zwischen der FISH und kultivierungsbasierten Methode, dass die meisten Zooplankton angehefteten Bakterien die inneren Oberflächen kolonisieren und nicht auf dem Exoskelett vorkommen.

Zusammenfassend ist zu sagen, dass *V. cholerae* Stämme im Neusiedler See einige Überlebensstrategien (Anheftung an Cladoceren, Assoziation mit der 3-12 µm Partikelfraktion, planktonisches Wachstum) zu haben scheinen und anscheinend nicht nur eine, spezialisierte ökologische Nische besetzten.

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