The survival and stability of water pathogens in artificially contaminated water

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English summary

The aim of this study was to investigate the fate of potential water pathogens in spiked water samples using *Salmonella thyphimurium, S. bongorii, Escerichia coli* (EHEC), *Vibrio cholerae* and *Bacillus cereus*, the latter used as a model organism for *Bacillus anthracis* causing anthrax.

The survival of the different bacteria was studied in filtered and non-filtered raw water and tap water. Plate counting and real-time PCR were used to monitor the survival of the bacteria in water. *Salmonella bongori* remained culturable for more than 3 months in filtered raw water, however, in non-filtered raw water the cells were culturally detected for only 13 days. In drinking water the *S. bongori* and *Salmonella typhimurium* cells remained culturable for 15 and 17 days, respectively. The culturability of *S. typhimurium* was lost within one day in chlorinated drinking water. However, nucleic acid sequence based amplification (NASBA) of mRNA showed that the cells remained viable for 30 days.

This study shows that the bacteria were able to survive and remain culturable in spiked water samples for at least one to two weeks. In chlorinated drinking water NASBA analysis showed that RNA in the cells remained for 30 days, which indicate that the *S. typhimurium* remained viable. However, they were not culturable.

This study may mimic what happens if these bacterial species were used in a deliberate attack on a limited volume of water, supporting the assumption that such bacterial species can pose a threat to human health if used by terrorist to contaminate water.

Sammendrag

Bevisst spredning av sykdomsfremkallende mikroorganismer er en av de mest alvorlige sceneriene våre militære styrker eller sivile kan bli utsatt for. Derfor kan et biologisk angrep eller trusselen om et biologisk angrep på drikkevannssystemet ha stor samfunnsmessig betydning. Stabilitet og overlevelse av det biologiske materialet er avgjørende for et vellykket "bioterrorangrep". Døde bakterieceller som degraderes i et gitt miljø vil ikke kunne påføre sykdom, skade eller død av de eksponerte. Det er kjent at mikroorganismer kan miste sin smitteeffekt i miljøer som ikke er naturlig for organismen.

Formålet med denne studien var å undersøke stabilitet og overlevelse av sykdomsfremkallende bakterier som *Salmonella thyphimurium, S. bongori, Escherichia coli* (EHEC), *Vibrio cholerae* og *Bacillus cereus* etter tilsetting til drikkevann og råvann. *Bacillus cereus* er brukt som en modellorganisme for *Bacillus anthracis* (miltbrann). Det er vist at vannbåren smitte fra bakterier som *E. coli*, *Salmonella* spp. eller parasitter som *Giardia* kan være en trussel mot drikkevannssystemet, men bevisst kontaminering av store reservoar er mindre sannsynlig pga stor fortynningseffekt og behandling av drikkevannet. Kontaminering via forsyningsnettet eller mindre volum som f eks flaskevann er mer sannsynlig.

Formålet med denne studien var å undersøke hvor lenge de ulike bakteriene kunne overleve i drikkevann og råvann. Bakterier ble tilsatt vann og prøver tatt ut regelmessig for å undersøke stabilitet og overlevelse. Analysemetoder benyttet er utplating på vekst agarskåler, mikroskopi og molekylære teknikker.

Contents

Preface

Different pathogenic microorganisms may appear naturally or accidentally in water sources including drinking water (tap water). Some of these agents can also be used for bioterrorism. This report describes the stability and survival of several water pathogens inoculated into water. The potential of artificially contamination of water in bioterrorism is discussed. The report is a part of the ongoing FFI-project “Evaluering av biologisk trussel “P1091.

1 Introduction

Outbreak of waterborne disease has demonstrated the vulnerability of water supply and the public's health to biological contamination of drinking water. Different pathogenic microorganisms may appear naturally or accidentally in water sources including drinking water (tap water) (Leclerc *et al*. 2002). However, some of these agents can also be used for bioterrorism. Therefore they are listed by The Center for Disease Control and Prevention (CDC), USA as potential bioterror agents (Rotz *et al*. 2002). Examples of such agents are *Bacillus anthracis*, *Escerichia coli* (EHEC), pathogenic *Vibrio*, *Shigella*, *Salmonella* and *Campylobacter* species, viruses such as Norovirus or Hepatitis A and protozoa including *Cryptosporidum* or *Giardia* species (Leclerc *et al.* 2002; Rotz *et al*. 2002; Pappas *et al*. 2006). A biological attack or even a threat of an attack on the drinking water supply system can have great impact on the public health and the economics of a community. Intentional contamination of large reservoirs (drinking water supply) such as lakes or rivers may pose only a limited risk due to factors such as massive dilution, specific inactivation from chlorine or filtration or inactivation by different environmental factors. However, access point in the distribution system or smaller bottles of drinking water could be more vulnerable (Khan *et al*. 2001; Craun *et al*. 2006; Nuzzo, 2006). *Salmonella typhimurium* and *Salmonella typhi* are listed as a potential bacterial species of public concern that can be used for intentional contamination of drinking water and food (Meinhardt, 2005). In 1984 an intentional contamination of a salad bar in a restaurant caused a large community outbreak of salmonellosis in the USA (Török *et al*. 1997). However, the outbreak was not solved until a religious group took the responsibility after one year, and this illustrates the difficulties in distinguishing between natural and deliberate or accidental outbreaks of disease. A large outbreak of salmonellosis on a cruise ship in the Caribbean in 1974 that involved several hundred people was probably caused by the potable water system onboard (Craun, 1977). In 2000, a water borne disease outbreak was caused by accidental contamination of a municipal water supply (Ontario, Canada) with *E. coli* O157:H7, resulting in 2300 symptomatic residents and seven deaths (Meinhardt, 2005). During the period 1988-2002, it was reported 72 water borne outbreak of diseases in Norway. A total of 10.616 people became ill and in all except one outbreak more than 200 people were affected (Nygård *et al*. 2003)

The viable but non-culturable (VBNC) state is a survival strategy adopted by bacteria when they are exposed to hostile environmental conditions, a dormancy state with loss of culturability (Barer *et al*. 1993; Barer and Harwood, 1999). *Vibrio* spp., *S. typhimurium*, *Shigella* spp., *Legionella pneumophilia*, *Francisella tularensis*, *E. coli* and *Campylobacter* spp. may all enter a VBNC form (Xu *et al.* 1982; Oliver, 2005). However, is the VBNC cell still pathogenic and can they be resuscitated and initiate an infection? At least in some bacterial species resuscitation occurs. Oliver and Bockian (1995) showed that *Vibrio vulnificus* VBNC cells are able to infect mice and that culturable *V. vulnificus* cells are recovered after infection. Colwell *et al*. (1996) showed that *Vibrio cholerae* VBNC cells are able to enter a culturable state in the human intestine. In an *E. coli* O157:H7 outbreak in Japan in 1998, it was suspected that cells in the VBNC form caused the infection (Makino *et al.* 2000). In 1999 *Salmonella* Oranineburg in dried squid caused an

outbreak of septicemia and gastroenteritis (Tsuji and Hamada, 1999). Asakura *et al*. (2002) showed that *S*. Oranineburg becomes non-culturable under osmotic stress, but retains their pathogenicity. This makes VBNC cells a potential threat to human health and using only culture methods may fail to detect these cells. The nucleic acid sequence based amplification (NASBA) method is based on RNA amplification and may therefore be a useful tool for detecting live and VBNC cells (Keer and Birch, 2003; Fykse *et al*. 2007).

The outcome of a deliberate attack on the water infrastructure in a society is dependent on the stability and survival of the microorganisms in water as well as the dose needed for an infection. Therefore, the aim of this study was to investigate the fate of potential water pathogens in spiked water samples using two different *Salmonella* species as model organisms. The fate of the *Salmonella* species was compared to *E. coli*, *V. cholerae* and *Bacillus cereus*, the latter as an example of a spore-forming Gram-positive bacterium and as a model organism for the pathogenic *B. anthracis*. Plate counting and real-time PCR were used to monitor the fate of the bacteria in the water samples. Since PCR only detects DNA, NASBA was used to separate dead cells from viable or VBNC cells of *S. typhimurium*.

2 Materials and methods

2.1 Bacterial strains and culture conditions

The bacterial strains and their sources used in this study are shown in Table 1. The concentration of bacterial cells in these cultures was estimated by direct microscopic counting (Zeiss, Germany) using a counting chamber (Hawksley). Enumeration of colony forming units was performed by plating of serial dilutions of each culture. To determine the number of CFU ml^{-1} of the native micro-flora in raw water R2A agar supplemented with 2.5 g l^{-1} cycloheximide (Sigma, USA) was used. The R2A agar plates were incubated at 20 ºC for 6-9 days. The total CFU count of the raw water was 599 CFU ml^{-1} (February 2006) 713 CFU ml^{-1} (March 2006), 3730 CFU ml^{-1} (April 2006) and 1833 CFU ml^{-1} (May 2006). The native micro-flora contained among others *Clostridium perfringens*, *E. coli*, coliforms bacteria and intestinal *Enterococcus* detected by cultivation on selective agar (results not shown).

Table 1 Bacterial strains and their sources, media and agar used.

Bacterium	Strain	Source	Pre-enrichment, 37 °C, 210 rpm for 20 hrs	Plating agar (survival experiments) 37 °C, for 20 hrs
Salmonella bongori	ATCC 43975	NorAnalyse Lillestrøm, Norway	BPW[a]	BGA[b] (selective) XLD[c] R2A[d]
Salmonella typhimurium	CCUG 18375		BPW	XLD
Bacillus cereus	ATCC 14579		BHI[e]	PEMBA[f] (selective) BHI R2A
Escherichia coli O157:H7	CCUG 44857		LB	LB
Vibrio cholerae	O1 Inaba El Tor CIP 106855	ATCC 393	TSB[g]	TCSB[h] (selective) TSB
Vibrio cholerae	VC503, non-O1	The Norwegian School of Veterinary science, Norway	TSB	TCSB (selective) TSB

[a] BPW: Buffered peptone water (Oxoid, England)
[b] BGA: Brilliant Green agar (Oxoid, England)
[c] XLD: xylose lysine desoxycholate agar (Oxoid, England)
[d] R2A: Merck, Germany
[e] BHI: Brain hearth infusion broth (Fluka, Germany)
[f] PEMBA: *Bacillus cereus* agar (Oxoid, England)
[g] TSB: Tryptose soya broth (Oxoid, England)
[h] TCSB: Thiosulphate citrate bile sucrose agar (Oxoid, England)

2.2 Survival experiments

The water samples used for the survival studies (Table 2) were drinking water from the tap (FFI, Kjeller, N 6650560, E 614280) and raw water from the river Glomma; sample point Hauglifjell (N 6652345, E 617916). The raw water was used in a filtered (0.20 µm) and a non-filtered state. The drinking water samples were supplemented with sodium thiosulfate (0.12 g l^{-1}) to inactivate traces of chlorine if not otherwise stated. The test bacteria were pre-enriched in the media shown in table 1, washed once in water and the water samples (Table 2) were spiked with 10^6-10^8 cells ml^{-1} (the exact number of cells is stated in the legends to the figures) in a total of 50 ml in an Erlenmeyer flask at room temperature (20 ºC). Non-spiked water samples were used as negative controls. Bacteria inoculated in growth medium used for pre-enrichment, buffered peptone water (BPW) or phosphate buffered saline (PBS) was used as positive controls. Samples were plated out in duplicates in several dilutions immediately after inoculation, later every 24 hours and then occasionally until the CFU ml^{-1} was no longer detectable (< 10 CFU ml^{-1}). For each bacterial strain, except *E. coli* and *S. typhimurim*, selective and non-selective media were used for plating (Table 2). The survival of *S. bongori* was studied in drinking water, filtered raw water and non-filtered raw water. The survival of *S. typhimurium* was studied in drinking water containing no chlorine as well as drinking water with traces of chlorine. The survival of *E. coli* and the two *V. cholera* strains, the environmental non-toxigenic strain 503 and the toxigenic strain CIP 106855 was studied in drinking water. The survival of *B. cereus* cells was investigated in drinking water and raw water (Table 2).

2.3 Cell count and viability determination

Total cell counts were determined by direct microscopic counting in a counting chamber. The culturability was determined by plate counting. When the number of CFU ml^{-1} was no longer detectable (< 10 CFU ml^{-1}), the viability of the *S. bongori* cells in drinking water, *B. cereus*, *E. coli* and *V. cholera* cells was examined by direct fluorescence staining using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes) according to the manufacturers recommendations. The stained bacteria were observed in a fluorescence microscope (Axioskop 2 plus, Zeiss) with FITC filter set (488009-0000). The viability of the non-culturable *S. bongori* cells in non-filtered raw water was tested in a recovery experiments in BPW. The bacterial viability of *S. typhimurium* cells in drinking water was analysed using NASBA.

2.4 Primers and probes

The specific primers and probes used for real-time PCR and NASBA are listed in Table 3. The *iroB* primers and probe targeting *S. typhimurium* were specifically designed for this study. Selection of the gene was based on Bäumler *et al*. (1997) and the Primer3 program was used for primer and probe design (Rozen *et al.* 2000). The primers and probes were synthesized by Invitrogen and Eurogentec, respectively.

Table 2 Water samples used in survival studies.

Bacterial species added	Raw water filtered	Raw water nonfiltered	Drinking water (thiosulfate[a])	Drinking water chlorine present[b]
	Survival of the bacterial species, demonstrated by CFU ml^{-1} as a function of time (days)			
Salmonella bongori	> 97	13	17	
Salmonella typhimurium			15	< 1
Bacillus cereus		Sporulation within 3	Sporulation within 2	
Escherichia coli O157:H7			30	
Vibrio cholerae O1 Inaba El Tor			7	
Vibrio cholerae non-O1, VC503			7	

[a] 0.12 g/liter thisulfate added to inactivate residual chlorine
[b] Chlorine added to purified drinking water was 10.1 mg/l on March 5, 2007 (http://www.nrvra2.no/analyseresultater_vannkvalitet/cms/5)

2.5 Real-time PCR analysis

Samples for real-time PCR analysis were collected at different time points as indicated (Table 4). The cells were washed with PBS and frozen at -20 °C until use. Amplification of a PCR product was indicated by the Ct-value. The Ct-value represent the cycle at which the fluorescence value crosses a fixed threshold that is 10 times the standard deviation of the baseline intensity. The Ct-value is proportional to the amount of DNA in the sample, which means that an increase in the Ct-value reflects a decrease in the amount of DNA. Melting point analysis and analysis by electrophoresis (BioAnalyser) of the amplified products were performed to identify a specific product. In the real-time PCR analysis of *S. bongori*, *E. coli*, *V. cholerae* and *B. cereus* cells it was investigated if an increase in the Ct-values corresponded to the decrease in number of CFU ml^{-1} in the survival experiments. In the case of *S. typhimurium* the concentration of the amplified product was calculated using the BioAnalyser and plotted as a function of the number of incubation days. The amplification reaction was performed as described (Fykse *et al*. 2008). In all PCR reactions, the Light Cycler® Fast Start DNA Master^{PLUS} SYBR-Green I kit (Roche Applied Science) was used, and the PCR reactions were performed on a Smart Cycler® II (Cepheid, USA). The temperature programs used for each primer set are listed in table 3. Briefly, the reaction mixture contained deoxynucleoside thriphosphates, $MgCl_2$ (5 mM), Taq DNA polymerase, reaction buffer, primers [1 µmol l^{-1} (each)], SYBR Green I and DNA template in a final volume of 20 µl.

Table 3 Primers and probes used for real-time PCR and NASBA.

Bacteria	Primer/ probe	Sequence 5' – 3'	Target gene size (bp)	Temperature program	Reference
S. bongori PCR	*inv*A F	GTGAAATTATCGCCACGTTCGGGCAA	*inv*A 260 bp	95 ºC for 5 s 55 ºC for 5 s 72 ºC for 20 s 35 cycles[i]	Rahn *et.al*. (1992)
	*inv*A R	TCATCGCACCGTCAAAGGAACC			
S. typhimurium PCR and NASBA	Ps90-1 *iroB*	GTTTGTCGGTCCACCACTGTA	*iroB* 153 bp	95 ºC for 5 s 58 ºC for 10 s 72 ºC for 20 se 45 cycles	This study
	Ps92-2 *iroB*	aattctaatacgactcactataggg[j] AGAAGGCGCTTCCGAATCGAAACCA			
	MBs[k] *20iroB*	ccgatc[l]CAAGCGTTTCGTGTTAATGGgatcgg		65 ºC for 4 min 41 ºC for 90 min (NASBA)	
B. cereus PCR	Bc*groEL*-1	CAATTAATCGCTGAAGCAATGGA	*groEL* (*B. cereus group*) 140 bp	95 ºC for 5 s 58 ºC for 5 s 72 ºC for 15 s 35 cycles	This study
	Bc*groEL*-2	AGAAGGGTAATCATGTAAGGAGATGCATA			
E. coli O157:H7 non-toxic PCR	ECO157*per*-f	TCTGCGCTGCTATAGGATTAGC	*per* 225 bp	95 ºC for 5 se 58 ºC for 5 s 72 ºC for 15 s 35 cycles	O´Hanlon *et.al*. (2004)
	ECO157*per*-r	CTTGTTTCGATGAGTTTATCTGCA			
V. cholera PCR	Vc*groEL*-f	GGTTATCGCTGCGGTAGAAG	*groEL* (*V. cholerae*) 116 bp	95 ºC for 2 s 58 ºC for 5 sec. 72 ºC for 20 sec. 40 cycles	Fykse *et.al*. (2007)
	Vc*groEL*-r	ATGATGTTGCCCACGCTAGA			

[i] The optimal number of cycles were run for each assay

[j] T7 RNA polymerase promotor sequence used in NASBA. The reverse primer in PCR is performing as the forward primer in NASBA and vice versa.

[k] MB molecular beacon. 5' end of the molecular beacon was labeled with 6-carboxyfluorescein and 3' end with dabsyl. MB is used for NASBA.

[l] Stem sequence.

2.6 NASBA analysis

Real-time NASBA analysis was performed on *S. typhimurium* cells. The samples for NASBA analysis were prepared by adding one part of the culture to two parts of RNAprotect (Qiagen). The mixture was mixed and incubated at 20 ºC for 5 min, followed by centrifugation at 8700 rpm for 10 min. The pellets were frozen and stored at -80 °C until used for RNA isolation. RNA was isolated using the NucliSens® Isolation Reagents Kit (BioMérieux Ltd., Boxtel, The Netherlands) following the NucliSens Basic Kit User Manual, Version 1.4. The RNA was eluted in 70 µl elution buffer. The NASBA amplification was performed using the NucliSens Basic Kit Amplification Reagents (BioMérieux Ltd., Boxtel, The Netherlands) according to NucliSens Basic Kit User Manual, Version 1.4 and Fykse *et.al*. (2007). Primers and molecular beacon probe were targeted to the *iroB* gene (this study). Real-time measurements were made with a Smart Cycler® II thermocycler as described in Fykse *et al.* (2007). The concentration of the amplified product was calculated using the BioAnalyser and plotted as a function of the number of incubation days.

To determine if NASBA could be used to detect viable *S. typhimurium* cells analysis of viable versus non-viable *S. typhimurium* cells were investigated. Samples of *S. typhimurium* cells were subjected to heating at 98 °C for 10 min and further incubated for 12 hours. The viability was tested by plating on XLD-agar. Nucleic acid isolated was subjected to real-time PCR and NASBA analysis.

3 Results

3.1 Survival of *S. bongori* cells

Drinking water from the tap, filtered and non-filtered raw water samples were spiked with *S. bongori* cells and its survival was analysed by plating regularly on a selective and non-selective agar. *S. bongori* was used as a non-pathogenic model organism for *S. typmimurium* in the initial experiments. In filtered raw water (0.2 µm) (not containing the native micro-flora) the CFU counts of the *S. bongori* cells decreased slowly the first 17 days (10-fold), followed by approximately a 10-fold increase (Figure 1), which was probably due to nutrient in the water and the dead bacterial cells. Sampling was continued for 97 days and at that point the number of cells was 6 x10^4 CFU ml^{-1}. For comparison, the initial concentration was 2 x10^5 CFU ml^{-1}. Selected samples were analysed by real-time PCR (Table 4). Surprisingly, melting point analysis indicated that no specific PCR product was amplified after nine days. However, after 17 (a minimum in CFU ml^{-1} was detected) and 37days specific PCR products were amplified, which is consistent with the increasing number of culturable cells. In general, in all samples the PCR products were amplified at a late stage with a high Ct-value (Table 4), indicating that the number of cells were close to the detection limit of the PCR analysis. This might explain the failed PCR analysis after nine days. In non-filtered raw water containing the native micro-flora, the number of CFU ml^{-1} decreased gradually and was at a minimum after nine days and within 13 days the cells were no

longer culturable. Real-time PCR analysis showed that no specific product was amplified after nine days and recovery experiments in BPW failed, indicating that the cells were not viable at the time.

The number of cells inoculated into drinking water were increased about 10 fold compared to raw water to improve the PCR detection. The CFU counts of *S. bongori* in drinking water remained relatively stable during the first nine and then rapidly decreased, and within 17 days the CFU ml^{-1} was no longer detectable (Figure 1). The two-colored fluorescent staining Live/Dead kit indicated that the cells were not viable since most of the cells appeared as red under the fluorescence microscope. However, the results were not conclusive due to low cell concentration. Therefore, the cell concentrations were further increased 10 fold in the fate studies of *E. coli* and *V. cholerae*. Drinking water samples selected from the entire period were analysed using real-time PCR, including samples having no detectable CFU ml^{-1}. The analyses showed that the Ct-values increased according to the decrease in CFU ml^{-1}, indicating that the concentration of DNA was decreasing (Table 4). After 17 days the PCR product was only weakly detected at a late stage, indicating low concentration of DNA. Melting point analyses indicated that no specific PCR product was amplified after 23 and 35 days; only primer-dimers appeared supporting the results that no CFU ml^{-1} was detectable after 17 days. In general, the real-time PCR analysis showed that the DNA concentration in the samples varied according to the number of CFU ml^{-1} detected, which indicated that DNA was gradually degraded in samples containing non-cultuarble cells.

The present results show that *S. bongori* cells can survive in sterile filtered raw water for more than 97 days, indicating that the water contained enough nutrients. However, in raw water containing the native micro-flora and in drinking water the survival was approximately seven fold shorter. In table two a summary of the survival studies of all bacteria is shown. PCR analysis, the Live/Dead staining and failed resuscitation experiment in BPW indicated that the non-culturable cells were dead.

3.2 Survival of *S. typhimurium* cells

The survival of the pathogenic *S. typhimurium* was studied in drinking water from the tap (Table 2). As an additional parameter the survival was studied in samples containing traces of chlorine (not treated with thiosulphate). The culturability of *S. typhimurium* in drinking water containing traces of chlorine decreased rapidly, and was lost within one day (Figure 2A). In contrast, the culturability of the cells in drinking water without chlorine fell gradually and within 15 days the CFU ml^{-1} was not detectable. In both cases the number of cells ml^{-1} was reduced 10 fold (10^7 to 10^6) immediately, and was further kept constant throughout the experiment (47 days). However, the amplified DNA decreased gradually in both cases (Figure 2B). The viability of *S. typhimurium* cells in drinking water was investigated using NASBA analysis. Several targets, *invA* (this study), *dnaK* (D'Souza and Jaykus, 2003) and *iroB* (this study) was tested since the correlation between RNA present in the cells and viability is not clear, due to variable persistence of RNA in dead cells, and each target needs to be tested (Keer and Birch, 2003, Fykse *et al*. 2007). NASBA amplification targeting the *invA* and *iroB* genes failed to detect heat-inactivated non-culturable cells (98 °C for 10 min), indicating that NASBA detects viable *S.typhimurium*

cells and not dead cells. The mRNA level of the *S. typhimurium* cells in drinking water was not detectable by NASBA after 20 days in the absence of chlorine (Figure 2C). However, in the presence of traces of chlorine the mRNA could be detected for as long as 30 days. These results showed that the culturability of the *S. typhimurium* cells is highly sensitive to traces of chlorine, but using the NASBA method, as an indicator for cell viability, the cells were still detectable after 30 days of incubation.

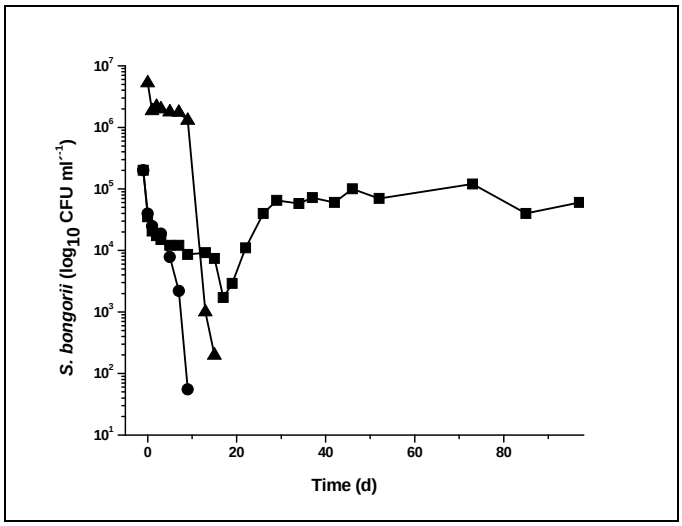

Figure 1 *Changes in the S. bongori culturability (CFU ml^{-1}) in -■- filtered raw water; -●- non-filtered raw water; and -▲-drinking water at 20 ºC as a function of time. The initial concentration of S. bongori cells spiked into drinking water was $5x10^6$ CFU ml^{-1} ($1.5x10^7$ cells ml^{-1}). In filtered and non-filtered raw water the initial concentration was $2\ x10^5$ CFU ml^{-1} ($1.0x10^6$ cells ml^{-1}). BPW was used for positive control.*

Table 4 Real-time PCR[a] of S. bongori cells in drinking water and raw water.

Drinking water		Raw water		
Incubation time, d	Ct-value	Incubation time (days)	Filtered	Non-filtered
			Ct-value	Ct-value
0	20.96	0	28.9	30.8
7	22.58	3	33.1	32.8
11	24.38	9	32.8 [b]	32.6
13	28.68	17	32.9	32.7[c]
15	28.08	37	31.2	
17	33.52[d]			
23	34.18[e]			
35	32.18[f]			
Negative PCR control (water)	0.00		34.5	
Positive PCR control (*S. bongorii*)	24.74		28.9	

[a] Melting point analysis and analysis by electrophoresis (BioAnalyser) of the amplified products were performed to identify a specific product.
[b] No specific PCR product was amplified.
[c]No specific PCR product was amplified.
[d] A small amount of PCR product amplified.
[e] No specific product amplified, only primer-dimers.
[f] No specific product amplified, only primer-dimers.

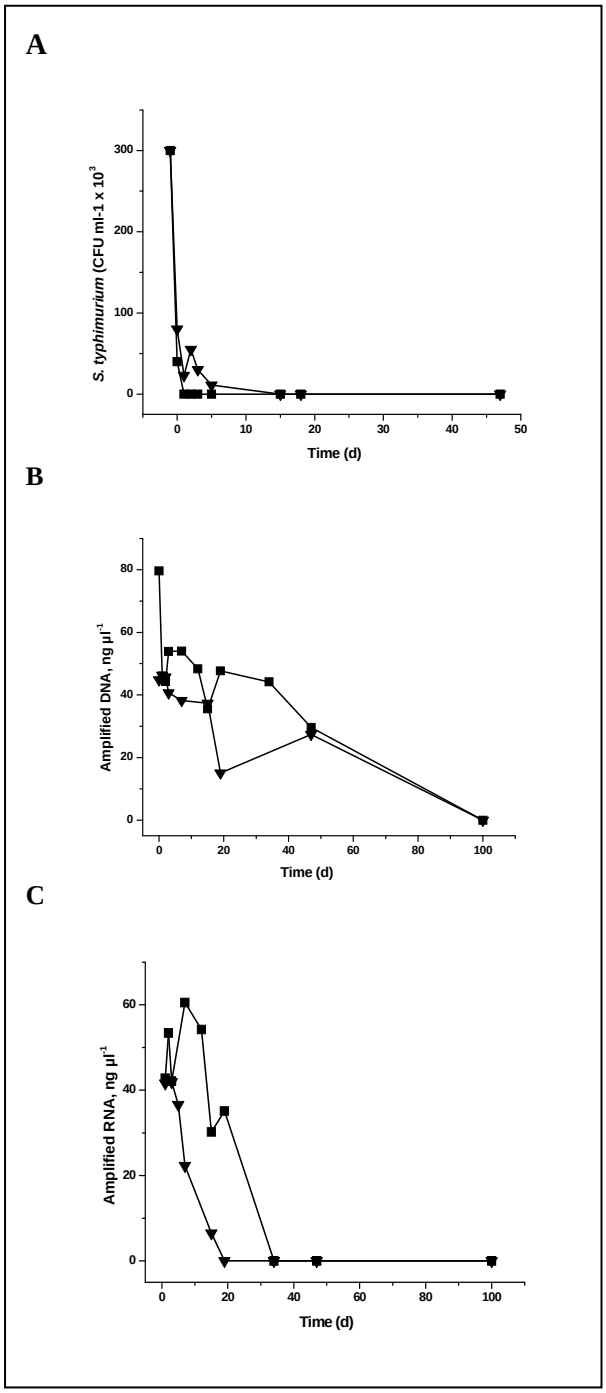

Figure 2 *Fate studies of S. typhimurium cells in drinking water at 20 ºC as a function of time. The initial concentration of S. typhimurium cells was $3x10^5$ CFU ml^{-1} ($1x10^8$ cells ml^{-1}). PBS was used for positive control. -▼-drinking water added thiosulphate to remove traces of chlorine; -■-drinking water with traces of chlorine. A) Decline of S. typhimurium culturability (CFU ml^{-1}). B) Changes in DNA concentration measured by real-time PCR.. C) Changes in mRNA concentration measured by real-time NASBA.*

3.3 Survival of *E. coli* and *V. cholerae* cells

The fate of other Gram-negative bacteria was compared to the fate of the *Salmonella* species (see table 2 for a summary of the results). *E. coli* was one of the bacteria chosen since there are legal restrictions that prohibit *E. coli* in drinking water. *E. coli* cells were inoculated into drinking water, and the number of CFU ml^{-1} decreased gradually and was no longer detectable within 30 days. The total cell count (cells ml^{-1}) did not decrease during the experiments (Figure 3). The viability of the non-culturable cells was verified using the Live/Dead kit (green fluorescence), indicating that the cells may have entered the VBNC form. Real-time PCR analysis also showed a constant level of DNA during the test period supporting the observation that the non-culturable *E. coli* cells remained intact throughout the experiment (results not shown).

V. cholerae, a bacterial species found in fresh, brackish and sea water, was chosen as the second agent for analysis. Two different *V. choleare* strains were tested in drinking water; a toxigenic clinical strain (CIP 106855) and an environmental strain (VC503).The number of CFU ml^{-1} for both *V. cholerae* strains decreased rapidly and were not detectable within seven days. The number of cells ml^{-1} was constant throughout the experiments (Figure 4), and the Live/Dead staining showed that most cells in both microcosms were still alive after 55 days (green fluorescence), indicating that the cells may have entered the VBNC form. Real-time PCR analysis showed a constant level of DNA during the test period supporting that the *V. cholera* cells remained intact throughout the experiment (results not shown).

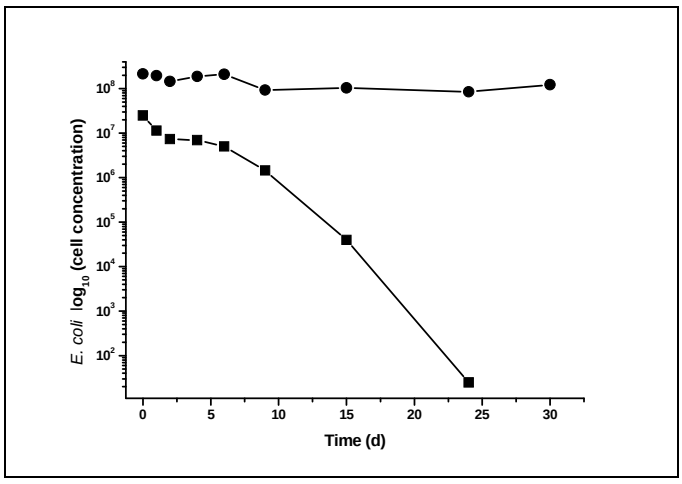

Figure 3 Changes in the number of E. coli cells in drinking water at 20 ºC as a function of time, -■- CFU ml^{-1}; -●- cells ml^{-1}. The initial concentration of E. coli was $2x10^7$ CFU ml^{-1} ($2x10^8$ cells ml^{-1}). LB medium was used for positive control.

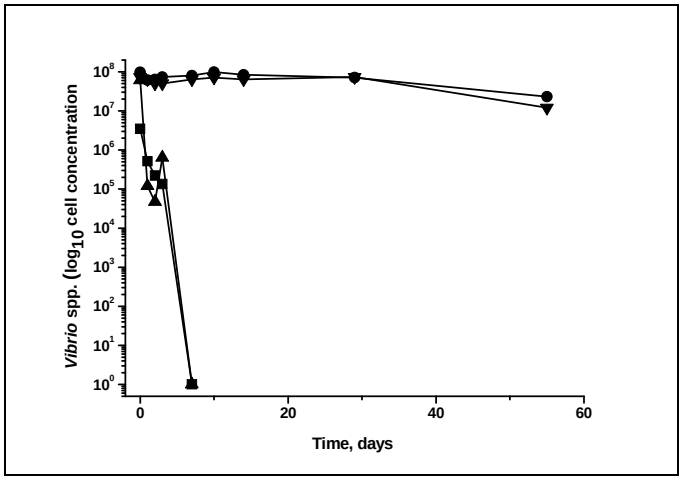

Figure 4 *Changes in the number of V. cholera (CIP 106855) and V. cholera (VC503) cells in drinking water at 20 ºC as a function of time. -■- V. cholera (CIP 106855) CFU ml^{-1}; -●- V. cholera (CIP 106855) cells ml^{-1}; -▲- V. cholera (VC503) CFU ml^{-1}; -▼- V. cholera (VC503) cells ml^{-1}. Initial cell concentrations of V. cholerae cells were 4x10^{6} CFU ml^{-1} (2x10^{8} cells ml^{-1}) and 6 x10^{7} CFU ml^{-1} (1x10^{8} cells ml^{-1}) of the two strains, respectively. TSB medium was used for positive controls.*

3.4 Survival of *B. cereus* cells

It is well known that spores can survive for decades in the environment (Sinclair *et al.* 2008). In this study a comparison of the fate of a spore forming Gram-positive bacterium with *Salmonella* and other Gram-negative bacteria was performed (Table 2). *B. cereus* cells were used as a model organism for the pathogenic bioterror agent *B. anthracis*. *B. cereus* cells inoculated into drinking water started to sporulate within one day, and within two days all cells had sporulated, (not surprisingly). The *B. cereus* cells in raw water were sporulated within three days (Figure 5). Real-time PCR analysis of samples from different time points showed a constant level of DNA throughout the experiment (not shown), indicating that the cells survived by sporulation.

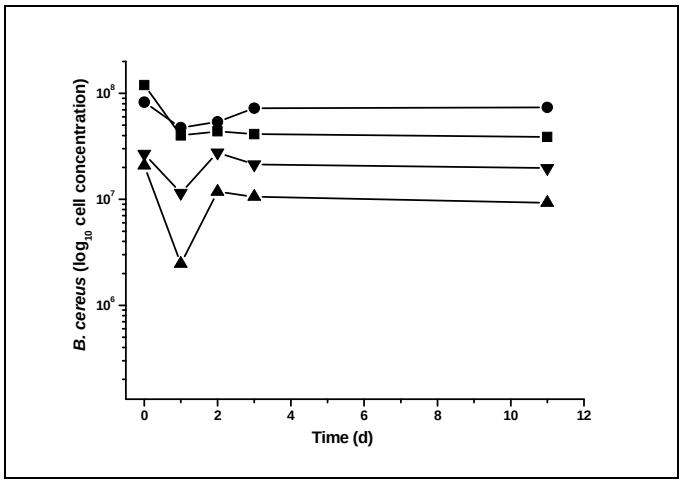

Figure 5 *The fate of B.cereus cells in drinking water and raw water at 20ºC . -■- B. cereus cells ml^{-1} in drinking water; -●- B. cereus cells ml^{-1} in raw water; -▲-B. cereus CFU ml^{-1} in drinking; -▼- B. cereus CFU ml^{-1} in raw water. The initial cell concentration was 2x10^{7}CFU ml^{-1} (1.0x10^{8} cells ml^{-1}) in raw water and drinking water. BHI medium was used for positive controls.*

4 Discussion

Waterborne dispersion of infectious agents can have a major impact on public health, and a deliberate biological attack or even a threat of an attack on the drinking water supply system or smaller utilities including bottled water are of important concern. However, the effect of an intentional contamination of the water system on the public health depends on the survival of the pathogens used. In the present study the fate of the several bacterial species *S. typhimurium*, *S. bongori*, *E. coli*, *V. cholerae* and *B. cereus* in water was addressed and compared. To our knowledge this is the first fate study addressing survival of *S. bongori*. The *S. bongori* cells were culurable for more than three months in filtered raw water and even able to grow for a short time period, suggesting that the filtered raw water contained sufficient nutrient (organic and inorganic) to support survival. However, the culturability in the non-filtered raw water was approximately seven fold shorter. The natural micro-flora was probably competing for the nutrient in the water explaining the shorter survival time. This is supported by the observation that other bacterial species were growing on the non-selective R2 A agar plates when the *S. bongori* cells had lost the culturability on the selective agar. Previously, it has also been reported that *E. coli* and *Campylobacter jejuni* survived better in filtered than in untreated water (Korhonen *et al*. 1991).

The culturability of *S. bongori* and *S. typhimurium* in drinking water decreased gradually and was lost within approximately two weeks. However, *S. typhimurium* cells in drinking water containing traces of chlorine lost the culturability within one day. NASBA analysis detected mRNA for more than 30 days, implying viable cells. To our knowledge this is the first study combining CFU measurements and NASBA analysis for fate studies of *S. typhimurium* in drinking water. The

present results are consistent with previous studies where *E. coli* and *S. typhimurium* cells entered a VBNC form following chlorination of waste water (Oliver, 2005; Oliver *et al*. 2005). In general, few studies have been performed on the fate of *Salmonella* species in water samples. It is shown that *S. typhimurium* cells incubated at 15 or 21 ºC in waste water remained culturable for 150-200 days. However, a gradually drop in culturability was observed when the cells were incubated at 5 ºC and they entered the VBNC form within 60-80 days (Oliver *et al.* 2005). Another study showed that a strain of *S. typhimurium* in sediment samples remained culture positive for 119 days whereas in the overlaying freshwater they remained culture positive for 54 days (Moore *et al*. 2003). Experiments in autoclaved water supplemented with freshwater sediments showed a survival of *Salmonella* species and *E. coli* cells for at least 28 days. However, in water without sediments the density of *E. coli* cells declined over a two weeks period, which is consistent with the present results (Fish and Pettibone, 1995). In general, enteric bacteria survive better in water and sediment microcosm than in pure water implying that nutrient present in the sediments support survival. Previously it was shown that the survival of *Salmonella* suspended in river samples was dependent on the strain used, and that some strains were able to survive for more than 45 days indicated by culturability. The culturability decreased gradually, however, but the total cell count remained stable for the whole period which indicates that the cells had entered the VBNC form (Santo Domingo *et al*. 2000). In a review discussing deliberately contamination of water, *Salmonella* spp. is supposed to be stable for eight days in drinking water (Khan *et al.* 2001), which is supporting the present results in drinking water. Also in a tropical rain forest watershed *S. typhimurium* cells was shown to survive for approximately five days (Jiménez *et al*. 1989).

In contrast to the *S. bongori* cells, the *E. coli* and *V. cholerae* cells most likely entered the VBNC form after inoculation in water. This is consistent with previous work (Xu *et al.* 1982; Colwell *et al.* 1996; Bjærgbæk and Roslev, 2005; Oliver, 2005). The culturability, as a function of time, did not differ between the two strains of *V. cholerea*; a clinical toxic strain vs. an environmental strain. Similar results were obtained in another study where the growth and survival of a clinical vs. environmental strains of *Aeromonas* in tap water was studied (Mary *et al*. 2001). In contrast, survival of *C. jejuni* in drinking water was dependent on the strain used and the origin of the strain had an effect on the survival (Cools *et al*. 2003). *B.cereus* cells were chosen as a model organism for the similar, but more lethal, *B. anthracis*. As expected, the vegetative cells sporulated within two to three days. *B. cereus* spores have also previously been found in water and rivers (Østenvik *et al.* 2004). *B. anthracis* spores are supposed to be stable and survive for two years in water (Khan *et al.* 2001).

The stability of *Salmonella* species in water (determined by culturability) makes it a potential threat if deliberately released in water. This is exemplified with *S. bongori* cells that were able to survive in raw water for more than three mounts. However, the survival in drinking water was approximately two weeks. In drinking water with traces of chlorine non-culturable *S. typhimurium* cells remained viable for 30 days shown by NASBA analysis. The present study also indicates that the bacterial species *E. coli* and *V. cholerae* would be able to survive in the VBNC form in drinking water after a deliberate attack. These bacterial species have previously been

shown to be infectious in the VBNC state (Colwell *et al*. 1996; Makino *et al*. 2000). The Gram-positive *B. cereus* survived by transformation into spores as expected and would be a potential threat if a pathogenic strain was used in a deliberate attack on water sources. The infectious dose of *S. typhimurim* is approximately 10^6cells, however, an infectious dose of only 10-100 cells in children, elderly or immunosuppressive persons have even been assumed (Blaser and Newman, 1982). If drinking water contains viable infectious cells in a concentration of 10^{3}-10^4 cells ml^{-1} for one week or more, an infectious dose of 10^6 cells could easily be obtained by drinking 0.1 to one liter of water. This could potentially have an impact on the public health and the society if pathogenic bacterial species were used in deliberate contamination water.

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