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PCR-detection of *Vibrio cholerae* in ballast water

Master's thesis

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Declaration

I herby declare that the work presented in this thesis has been conducted independently and in full accordance with the rules and regulations for the integrated Master's degree in Industrial Chemistry and Biotechnology at the Norwegian University of Science and Technology.

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Acknowledgment

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Abstract

Toxicogenic *Vibrio cholerae* is the cause of the epidemic disease cholera. The bacterium can be transported by ship's ballast water and infect new areas. The international maritime organization (IMO) introduced regulations for discharging of ballast water containing more than 1 colony forming unit (CFU) of *V. cholerae* per 100 ml of ballast water. *V. cholerae* is detected by conventional growth methods that have many drawbacks. The aim of this project was to develop a nucleic acid amplification method for detection of *V. cholerae* in ballast water.

To address this problem an optimized PCR protocol for detection of *V. cholerae* was established, including selection of two efficient primer pairs targeting specific genes that can be used for separation of toxicogenic and non-toxicogenic strains of *V. cholerae*.

The main challenge was to find a method for concentrating and extracting nucleic acids from environmental water samples with a quality sufficient for PCR amplification. Two types of environmental water samples imitating the properties of ballast water were spiked with *V. cholera* and used in experiments on extracting pure DNA to be analyzed using PCR.

An extraction method based on enzymatic lysis and DNA precipitation using ethanol was optimized to give a high yield of DNA from large water samples. This method was used in the extraction of DNA from seawater samples. For samples of brackish water enriched with harvested microorganisms and organic matter, the NucliSens extraction kit was used for DNA extraction. Extraction with this kit successfully removed all inhibitors from the enriched brackish water samples.

The PCR detection limit of *V. cholerae* in environmental samples was found to be approximately 2 cells/ml in seawater and 10 cells/ml in enriched brackish water.

The detection of *V. cholerae* in environmental samples by NASBA was evaluated, and compared to the PCR detection. The same gene targets and MB probes were used. The lowest concentration of *V. cholerae* in seawater detected by NASBA was 10^7 CFU/ml.

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1 Introduction

1.1 Ballast water

Water is used as ballast to stabilize transporting ships. More than 80% of all intercontinental cargo transport is conducted by ships(3). This means that a huge volume of ballast water containing different organisms is transported between continents. When discharging the ballast water the organisms living in it will be introduced to a new environment. Most of the organisms will not survive in a new environment, but in some cases these introduced species have become invasive and have had a drastic effect on the ecosystem (5). Two examples of species transported with ballast water that have caused drastic effect on the ecosystem is the European zebra mussel *Dreissena polymorpha* introduced to the Canadian Great Lakes (21) and the North American comb jelly *Mnemiopsis leidyi* introduced to the Black Sea(11). The American comb jelly caused a collapse of commercial anchovy fisheries (16).

The number of macro organisms such as copepods and fish in ballast water is low compared to the abundance of microorganisms. In addition microorganisms are better poised to be invasive species. Many are small, have simpler requirements for survival, reproduce asexually, grow rapid and can enter a resting stage that make them capable to survive periods of unfavourable conditions (8). The first report of a microorganism suspected of being introduced by ballast water was the diatom *Odontella sinensis* from the Indo-Pacific. It had not been reported in European waters until it produced dense plankton blooms in the North Sea in 1903 (11). A more resent example is the probable transport of pathogenic *Vibrio cholerae* from Latin America to the U.S Gulf of Mexico by ballast water under the 7th cholera pandemic. Toxigenic *V. cholerae* was detected in ballast water from cargo ships docked in the port of U.S Gulf of Mexico and in oysters and fish from the same port. After the discovery that cargo ships can be vehicles for the transmission of epidemic cholerae, it was recommended that ships exchange their ballast water while on the high seas (18).

The International Maritime Organization (14) established the ballast water convention at the international conference on ballast water management for ships on February 13th 2004. The convention stated regulations for how to prevent introduction of non-indigenous species by ballast water and sediments, and for where ballast water shall be discharged and exchanged.

They gave also regulations of how many organisms the ballast water can contain upon discharge, and how to monitor the microbial content in the ballast water tanks.

The regulation of microbial content in ballast water as stated by (IMO) is cited below (14):

Regulation D-2 *Ballast Water Performance Standard*

1. Ships conducting Ballast Water Management in accordance with this regulation shall discharge less than 10 viable organisms per cubic metre greater than or equal to 50 micrometres in minimum dimension and less than 10 viable organisms per millilitre less than 50 micrometres in minimum dimension and greater than or equal to 10 micrometres in minimum dimension; and discharge of the indicator microbes shall not exceed the specified concentrations described in paragraph 2.

2. Indicator microbes, as a human health standard, shall include:
    a. Toxicogenic *Vibrio cholerae* (O1 and O139) with less than 1 colony forming unit (cfu) per 100 millilitres or less than 1 cfu per 1 gram (wet weight) zooplankton samples ;
    b. *Escherichia coli* less than 250 cfu per 100 millilitres;
    c. Intestinal Enterococci less than 100 cfu per 100 milliliters.

This thesis will focus on the detection of toxicogenic *V. cholerae*, and try to establish an improved method for molecular detection of *V. cholerae* in ballast water.

1.2 Vibrio cholerae

Vibrio cholerae is a Gram-negative slightly curved bacillus with a single polar flagellum. It can vary in size between 1 and 3 μm in length, and 0.5 and 0.8 μm in diameter. It belongs to the family Vibrionaceae and shares common characteristics with the family Enterobacteriaceae. Pathogenic strains of the bacterium is the cause of the historically feared epidemic diarrheal disease cholera (24).

The organism is divided into serogroups based on the somatic O antigen. Over 200 serogroups of *V. cholerae* have been reported, but only two of them cause epidemic and

pandemic disease. The epidemic serogroups are O1 and O139, and the others are often referred to as non-O1 non-O139 (23). *V. cholerae* O1 can be further divided into three serotypes according to the presence of antigens A, B and C. The serotypes are called Inaba, Ogawa and Hikojima, but no evidence of different clinical spectra among these serotypes has been observed. The two biotypes Classical and El Tor however, differ remarkable in ratio of symptomatic and asymptomatic cases. The classical biotype causes approximately equal numbers of symptomatic and asymptomatic cases, while El Tor biotype causes only one symptomatic case for every 20 to 100 asymptomatic cases (24).

1.2.1 Cholera epidemics and pandemics

V. cholera O1 serogroup has been the cause of seven cholera pandemics since 1817. The seventh pandemic started in Indonesia and spread to India in 1964, Africa in 1970, southern Europe in 1970, and southern America in 1991 (23). The 7th pandemic is still going on in many countries (24).

Africa's worst cholera epidemic in 15 years is currently fading out. It started in august 2008, and the 1st of June 2009 the Ministry of Health and Child Welfare reported a total of 98 441 cumulative cases and 4 277 deaths (19). The case fatality rate was reported by Zimbabwe WASH cluster to be 4.4 (19). The epidemic has also spread to neighbouring countries in Africa. Cholera is mainly transmitted through contaminated water and food. The reasons for the extent of the epidemic in Zimbabwe are the poor water, sanitary and health systems in the country, combined with densely populated areas (28).

1.2.2 Infection

In average half of the affected individuals with severe cholera are killed of this disease if not treated. The infection leads to symptoms as watery diarrhoea (the characteristic rice-water stool) and vomiting after a period of about 18 h to 5 days. In adults with severe diarrhoea as much as 0.5 to 1 litre of body fluid is lost per hour. If not replaced, death due to dehydration can occur within few hours of onset (23).

Individuals are infected by eating or drinking contaminated food or water. The bacteria that pass the acid barrier of the stomach colonize the epithelium small intestine. They attach to

receptors on the mucosa by means of toxin coregulated pilus (TCP) and other factors. The bacteria start to excrete cholera enterotoxin (CT) that bind to epithelial cell receptors and increase the concentration of cyclic adenosine monophospate (cAMP). cAMP activates enzymes connected to ion-transport, leading to the massive outpouring of fluids from the intestine (15).

1.2.3 Habitat and survival of *V. cholerae*

The natural reservoirs of *V. cholerae* are aquatic environments. Both O1 and non-O1 strains coexist in these environments, but the non-O1 is more widely distributed. They live attached to a particular kind of algae, crustacean shells and copepods. In this way *V. cholerae* can multiply and survive for years without interaction with humans (24).

V. cholerae is known to enter a dormant state in which it is not culturable by concentional growth methods, but is still viable. This state is called a viable but nonculturable state (VBNC) and is believed to be a survival strategy of several bacterial species in the natural environment. A study was conducted were nine volunteers ingested VBNC *V. cholerae* O1. Culturable *V. cholerae* was recovered from the stool from two of the nine volunteers, indicating that VBNC *V. cholerae* O1 is still pathogenic (15).

1.3 Detection methods of Vibrio cholerae from the environment

Clinical samples of *V. cholerae* mostly consist of stool with high concentration of *V. cholerae*. In many cases the presence of *V. cholerae* in the stool samples can be confirmed under darkfield examination in microscopes, adding specific antisera against the serotype. A definitive identification of the organism is done by conventional growth methods (24).

Detection of *V. cholerae* in the environment is more challenging because the concentration of *V. cholerae* is often low and the cells can be in a VBNC state. Several methods can be used to detect and identify *V. cholerae*. Some methods detect viable cells, some detect the presence of dead cells, and some detect only culturable cells. Below is a description of three different methods with their advantages and disadvantages when detecting *V. cholerae* in environmental samples.

When conventional growth method is used for *V. cholerae* in environmental water samples a concentration step is often required. The water sample is filtered through a 0.2-µm polycarbonate membrane filter, and the bacteria on the filter are transferred to a container with alkaline peptone water (APW) for enrichment over night. The aerobic bacteria will grow in the surface layer, and surface aliquots are streaked out onto selective bacteriological media (Thiosulfate citrate bile salts sucrose (TCBS) or tellurite taurocholate gelatine agar (TTGA)). The colonies that *V. cholerae* forms on these media can be distinguished by morphology. Presumptive colonies are purified on a non-selective medium to be identified and confirmed by biochemical tests and serotyped using polyclonal and monoclonal antiserum (13).

Immunological detection methods using antibodies are rapid and culture-independent methods that also detect VBNC cells. *V. cholerae* cells can be stained with fluorescently labelled monoclonal antibodies raised against an antigens specific for the O-serotype. The stained cells can then be observed under an epifluorescent microscope. Positive samples must be confirmed by Polymerase Chain Reaction (PCR) because of the possible presence of autofluorescing constituents found in environmental water samples (13).
PCR can be used to indentify specific strains, toxogenic strains or non-toxogenic strains of *V. cholerae* depending on the gene targeted by the primers chosen. A detailed description of the PCR principles is given in the next section. Identification by PCR is rapid, precise, and less labour intensive than the series of biochemical tests needed for identification. If *V. cholerae* is to be detected from environmental samples by PCR the DNA from the sample should be extracted to remove possible PCR inhibitors. PCR only detects DNA and cannot be used to distinguish between viable, VBNC and dead cells.

The conventional growth method is necessary to isolate strains of *V. cholera* for growth in pure cultures. However, only viable cells that are culturable are detected using this method. The enrichment steps and the growing of colonies require several days, and as much as 12 biochemical test should be performed to confirm the presence of *V. cholerae,* which is also labour intensive. The immunological methods are rapid methods for detection of *V. cholerae* in clinical samples, but additional tests must be done to confirm the results when analyzing environmental samples. PCR detection has the advantage of being a direct, precise, and relatively rapid method that also detects VBNC cells. The focus in this thesis is to establish a protocol for direct and specific detection of *V. cholerae* in environmental water samples by PCR.

1.4 Polymerase chain reaction

PCR amplifies a specific double stranded DNA sequence in vitro using two oligonucleotide primers and the enzyme DNA polymerase. The DNA is copied in a cycle with three steps: denaturation of the DNA strands, annealing of the primers to the DNA strands and finally elongation of the primers by DNA polymerase. Each step is preformed at an optimum temperature. The denaturation temperature must be higher than the melting point of the primers, but not be as high as to cause loss of activity to the DNA polymerase. The annealing temperature is dependent on the properties of the primers, and the temperature of the elongation step must be optimum for the polymerase activity. The duration of the elongation step must be adjusted to the length of the target sequence (22).

In addition to the primers and DNA polymerase the reaction mixture contains a mix of mono nucleotides (dNTP), a buffer and $MgCl_2$. The magnesium ions are necessary co-factors for the DNA polymerases and the concentration is critical for the specificity of the PCR. At low concentrations the DNA polymerase will not be sufficiently active, but at too high concentrations non-specific PCR products can be formed(22).

To monitor the PCR in real time different fluorescent mechanisms can be used. The fluorescence signals can either be generated by a dye that binds specifically to double stranded DNA (dsDNA) or by sequence-specific oligonucleotide probes that are coupled to fluorophores. An example of a fluorescent dye that is specific for dsDNA is SYBR green I which emits fluorescence with a wavelength of 530 nm after intercalating into the DNA-helix. Unbound molecules will give rise to low background fluorescence(29). By measuring the fluorescence at the end of the elongation step the increase of double stranded DNA in the reaction mixture can be monitored during the PCR. To prove that only the desired PCR product has been amplified, a melting curve analysis can be preformed after PCR. The fluorescence is continuously monitored as the temperature increases. The derivative of the fluorescence curve will result in a melting peak which represents the characteristic melting temperature (T_m) of a particular DNA product where the DNA is 50% denaturised. The T_m is determined by the length of the dsDNA and the GC content of the fragment. Generally, if more than one melting peak is observed primer-dimers or other non-specific products are present.

Sequence-specific detection of PCR products can be done either by single-labelled probes, hybridization probes or hydrolysis probes. The fluorophore is activated by different mechanisms when the probes hybridize to their complementary sequence in target PCR products. The probes used in these experiments are stem-loop probes named Molecular beacons (MB). They have fluorescence groups connected to the 5' end and quenching groups in the 3' end. The loop sequence consists of 15-30 nucleotides complementary to the target sequence, and the stem sequences consist of 5-8 base pars complementary to each other. In its free form the MB is structured as a hairpin, bringing the quenching and fluorescence groups in close proximity, see Figure 1-1. The fluorescence emitted by the fluorescence group is almost entirely absorbed by the quenching group, thus giving a very low fluorescent background. In the presence of target DNA the probe will hybridize with the target sequence and the stem sequence will be melted, leading to separatian of the groups. The fluorescence is no longer quenched and the fluorescence can be measured quantitatively as a signal of the presence of the target (4, 17).

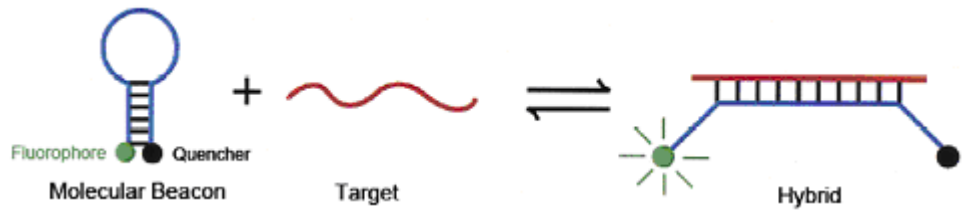

Figure 1-1: A: Molecular Beacon hybridizing with target DNA and emits fluorescence (17).

1.4.1 Quantitative real-time PCR

When performing real-time PCR a crossing point (CP) value can be calculated as the cycle at which the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase. CP is inversely proportional to the log value of the initial concentration of the target. By amplifying several dilutions of a DNA standard, a standard curve can be plotted as CP versus log of the initial concentrations(31). In an ideal PCR amplification two DNA segments are produced from a target segment in each PCR cycle. The number of amplified (N_n) targets after the end of the n^{th} cycle of amplification can therefore be calculated as described in the following:

$N_n = N_0 \times 2^n$,

where N_0 is the initial target concentration. Since the efficiency seldom is 100 % a more general expression of N_n is given by:

$N_n = N_0 \times (1 + \eta/100\%)^n$,
where η is the PCR efficiency. The CP can then be expressed as:

$CP = (\log N_{CP} - \log N_0) / \log(1 + \eta/100\%)^n$

where N_{CP} is the number of amplified target after the crossing point cycle. The slope of the curve plotted as CP versus log N_0 equals to $-1/\log(1 + \eta/100\%)$. An ideal PCR amplification will thus have a slope of $-1/\log 2 = 3.32$. Zhang and Fang (2006) states that a reliable standard curve should have a R^2 value of more than 0.95 and a slope between -3.0 and 3.9, corresponding to PCR efficiencies of 80-115%. The standard curve is only valid in the range in which the target quantity is linear with the CP in the standard curve. This is called the dynamic range.

Factors that affect the precision of quantitative real-time PCR analyses are the potential presence of inhibitors in environmental samples and variations in the DNA extraction efficiency from batch to batch.

1.4.2 Inhibition of PCR

The two most critical factors when performing PCR on environmental samples are the low concentration of the target organism and the presence of PCR inhibitors. Inhibition of PCR generally act at one or more of three essential points in the reaction: i) By interfering with the cell lysis necessary for extraction of DNA, ii) by degrading or capturing nucleic acid, and iii) by inhibiting polymerase activity for amplification of target DNA. The inhibition can be either total, resulting in no amplification of the target, or partly, reducing the detection limit (30).

Some examples of inhibitors in environmental samples are humic compounds, cellulose and nitrocellulose filter particles, non-target DNA, and heavy metals. The inhibition can be overcomed by either extraction of the inhibitor compound, adding a reactant that will bind the inhibitor or diluting the sample (30).

1.5 NASBA principles

Nucleic Acid Sequence Based Amplification (NASBA) is an amplification technique that uses RNA as template. In theory, NASBA can give a lower detection limit compared to PCR because a cell usually contains only one copy of the DNA sequence, but several copies of the same mRNA sequence may be present. The isolation of mRNA is however more challenging than isolation of DNA due to the less stable structure of RNA and the presence of RNases that are difficult to inactivate. On the other hand the easy degradation of RNA means that only viable, mRNA producing cells can be detect by NASBA.

Another advantage of NASBA compared to PCR is that it is an isothermal process which does not need a specialized thermo cycler with rapid temperature changes of the samples. The NASBA process is based on the simultaneous activity of three enzymes: T7 RNA polymerase, RNase H and avian myeloblastosis virus (AMV) reverse transcriptase. The first step in the process is binding of primer 1 to the target RNA. Primer 1 comprises a promoter sequence for T7 RNA polymerase at the 5' end. The primer anneals to the complementary RNA sequence, and AMV reverse transcriptase uses dNTPs to extend the 3' end of primer 1, producing a cDNA copy of the RNA template. RNase H hydrolyses RNA from the RNA:DNA hybrids and the original RNA is destroyed. Primer 2 can then anneal to the single stranded DNA, and AMV reverse transcriptase synthesizes the second DNA strand. This results in dsDNA with a promoter sequence for T7 RNA polymerase. This enzyme transcribes as many as 100 RNA copies from each DNA template molecule. Each new RNA molecule can then be a template for reverse transcriptase and the process repeats it self in cycles (7), see Figure 1-2. The cyclic phase runs continuously at the same temperature. The accumulation of ssRNA can be monitored by adding a fluorescent probe, MB, and measuring the fluorescence level every minute. The amplification usually runs for 90 min.

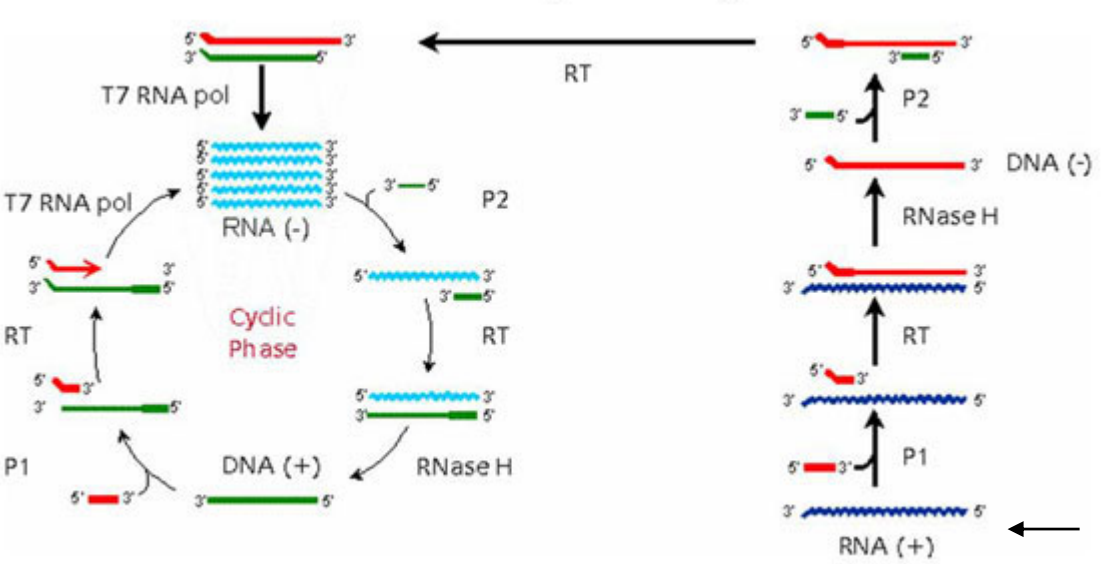

Figure 1-2: NASBA principle (26)

1.6 Aim of this project

The Norwegian Defence Research Establishment (FFI) has established real-time PCR methods for analyzing several bacterial pathogens in environmental samples such as soil, air and water samples. The aim of this project is to develop a nucleic acid amplification method for detection of *V. cholerae* in ballast water to meet the regulation of the international convention for the control and management of ship's ballast water and sediment, 2004. The convention states that ships conducting ballast water management in accordance with that regulation shall discharge less than 1 colony forming unit (cfu) of toxicogenic *Vibrio cholerae* (O1 and O139) per 100 millilitres of ballast water.

To address this problem an optimized PCR protocol for detection of *V. cholerae* must be established, including selecting two efficient primer pairs targeting specific genes that can be used for separation of toxicogenic and non-toxicogenic strains of *V- cholerae*.

The main challenge will be to find a method for concentrating and extracting nucleic acids from environmental water samples with a quality sufficient for PCR amplification. The water samples analyzed will have different properties and thus different challenges regarding PCR inhibitors.

Environmental water samples imitating the properties of ballast water will be spiked with *V. cholera* and used in experiments on extracting a sufficiently pure and concentrated DNA sample to be analyzed using PCR. The PCR detection limit of *V. cholerae* in environmental samples will be investigated.

FFI has previously shown that NASBA is a sensitive method for detection of *V. cholerae* in water (10). The detection of *V. cholerae* in environmental samples by NASBA will be evaluated, and compared to the PCR detection. The same gene targets and MB probes will be used.

2 Materials and methods

2.1 Materials

To find a molecular method of detecting *V. cholerae* in ballast water a toxic strain of *V. cholerae* was used as target. The target needed to be spiked in water samples with properties resembling those of ballast water.

2.1.1 Organisms

The organism used in these experiments is *Vibrio cholerae* strain CIP 106855 O1 Inaba El Tor (American Type Culture Collection). This strain is positive for all the genes groEl, tcpA, ctxA and toxR.

The bacterium was grown in Thryptone Soya Broth (TSB) (20) or on agar (TSA) at 37˚C. Mediums and agars are described in Appendix A. To determine the numbers of CFU per ml in the exponential growth phase, 0.5 ml of an overnight culture was transferred to TSB (50 ml) and incubated 3 to 5 hours at 200 rpm at 37˚C. The optical density (OD) of the culture was measured in a spectrophotometer (UV-1201 UV-Vis, SHIMADZU) at 600 nm. The culture was centrifuged down at 3 000x g for 10 min and resuspended in an equivalent volume of Peptone Buffered Saline (PBS) to stop further multiplying. The concentration of cells in the culture was determined after counting cells on a graticule slide using a phase contrast microscope (Axioshop 2 plus, Zess). The culture was diluted 10^5, 10^6 and 10^7 times, and 100 µl was plated on TSA and incubated at 37˚C over night before CFUs were counted. Three parallels where made from each dilution.

A growth curve was made by measuring the OD for a culture grown at 37˚C with 200 rpm in TSB at regular intervals during a period of 6 hours. The concentration of cells in the culture and the numbers of CFUs was also determined six and three times respectively during the growth period.

2.1.2 Environmental water samples

Water samples were provided by the Norwegian Institute for Water Research (NIVA) in Oslo.

Four types of water samples were used: high and low salinity seawater and enrichments of these samples that meet the international maritime organization's requirements of test water for ballast water treatment (see Appendix B, section B.1). A summary of average values of the most important parameters of the different test waters are given in Table 2-1. For a complete description of biological and chemical parameters of the samples used in the experiments see Appendix B.

Table 2-1: Test waters with average values of salinity, bacteria concentration, turbidity and total organic carbon.

Test water	Salinity [g/l]	Average marine [CFU/ml]	Turbidity [FNU]	Total organic carbon [mg C/l]
Seawater (SW)	33	10^2	0.6	1.4
Enrich seawater (ESW)	33	10^4	6.4	4.4
Brackish water (BW)	21	10^2	0.7	1.4
Enriched brackish water (EBW)	21	10^4	30	14

2.2 Determination of bacterial concentration in environmental samples

Properties of the water samples used in the experiments were investigated to see if the environmental bacteria in the water could affect the results of the detection experiments.

2.2.1 Counting colony forming bacteria

As a general procedure, NIVA tested bacterial content of the test water by plating on Marin Agar and Thiosulfate Citrate Bile Sucrose (TCBS) agar. In some cases the water samples provided by NIVA were kept for several days at 4˚C before used in the experiments. The bacteria content in the samples after storage was controlled with the same plate tests:

Dilutions of the samples were plated on Marine Agar (Conda) by spreading out 100µl sample and incubated at room temperature for two days before counting. When incubating more than two days more colonies are formed, but the fast growing colonies will cover most of the plates.

Samples were also plated on TCBS agar (Oxoid) after concentration 10 to 100 times by centrifugation of the samples at 3 000 x g for 10 minutes. The agar plates were incubated at 37˚C for 24 hours before counting[a]. TCBS is a selective medium for *Vibrio* species, but other species may also grow on this medium. *Vibrio cholerae* colonies are seen as yellow and flat, with a diameter of 2-3 mm on TCBS agar (20).

2.2.2 Identification of presumptive *Vibrio* species

Since other bacteria than *Vibrio sp.* also may form yellow colonies on TCBS, some of the yellow colonies on the TCBS plates where isolated, and the 16S rRNA gene was amplified using PCR. The 16S rRNA PCR product was purified using ExoSAP-IT before sequencing. Sequencing was performed at Eurofins. An online Nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed on the sequences. A detailed description of 16S rRNA amplification and ExoSAP-IT treatment is given in Appendix C.

2.3 Primers and beacons used in PCR

The PCR methods used in these experiments are real-time PCR with SYBR Green I or molecular beacons (MB). A PCR reaction mixture(20 µl) for SYBR Green I was made by mixing LightCycler® 480 SYBR Green I Master(10 µl), a 5 µM solution of primer mix(4 µl), PCR-grade water(1-4 µl) and DNA template (2-5 µl). The PCR reaction mixture for MB was made by mixing LightCycler® 480 Probes Master (10 µl), a 5 µM solution of primer mix (4 µl), a 5 µM concentration of MB (1 µl), DNA template (2-5 µl) and PCR-grade water (0-3 µl). The primer mix consisted of a 5 µM concentration of a forward and a reverse primer from Table 2-2. PCR was performed either on LightCycler® 480 (Roche) or SmartCycler II (Ceoheid™).

Four different genomic regions of *V. cholerae* were used as targets in these experiments: *gro*El coding for the 60 kDa chaperonin product, *tcp*A coding for the toxin-coregulated philus, *ctx*A coding for cholera toxin and *tox*R coding for a transcription factor that regulates

[a] NIVA did not concentrate the samples, but filtrated 100 ml samples on a membrane filter and place the filter on a TCBS agar plate.

the expression of cholera toxin. The primers and probes used are listed in Table 2-2. Primers are synthesized by Invitrogen and MB by Eurogentec.

Table 2-2 also show the primers used when amplifying the 16S ribosomal RNA gene used to identify unknown bacteria, and primers for the amplifying of lamda phage used as internal marker in PCR.

Table 2-2: Primers and molecular beacons used in PCR

Primers	**Nucleotide sequence 5'-> 3'**
groEl –r	ATG ATG TTG CCC ACG CTA GA
groEl –f	GGT TAT CGC TGC GGT AGA AG
tcpA –r	CGC TGA GAC CAC ACC CAT A
tcpA –f	GAA GAA GTT TGT AAA AGA AGA ACA CG
ctxA –r	TGG GTG CAG TGG CTA TAA CA
ctxA –f	TGA TCA TGC AAG AGG AAC TCA
toxR –r	CGG AAC CGT TTT GAC GTA TT
toxR –f	CTC GCA ATG ATT TGC ATG AC
Eub 933f (16S)	GCACAAGCGGTGGAGCATGTGG
Eub 1387r (16S)	GCCCGGGAACGTATTCACCG
Lamda r	GCATAAACGAAGCAGTCGAGT
Lamda f	ATGCCACGTAAGCGAAACA
Molecular beacons	
MBvc13-groEl	ccgatcCTGTCTGTACCTTGTGCCGAgatcgg
MBvc11-tcpA	ccgatcAGAAAACCGGTCAAGAGGGTgatcgg
MBvc12 ctxA	ccgatcTTGTTAGGCACGATGATGGAgatcgg
MBvc14-toxR	ccgatcTTAACCCAAGCCATTTCGACgatcgg

The 5' ends of the molecular beacons were labelled with 6-carboxyfluorescein as fluorescence group, and the 3'ends were labelled with DABCYL as quencher.

2.4 Optimization of PCR protocol for detection of V. cholerae

The PCR protocols were optimized with regard to run-time and fluorescence signal. It was assumed that PCR amplification of *V. cholerae* genes using MB or SYBR Green I needed different protocols because of the different binding strategy and detection points. Therefore the same tests were run on each.

The tests were performed by amplifying all four primer sets targeting *V. cholerae* genes with two parallels of a negative control, a high concentration, and a low concentration of DNA template made of cell extracts (see section 2.8 for description of cell extract). The fluorescent signal was compared for each experiment.

The protocol includes the following steps:

1. Denaturing all DNA before the amplification cycles can start, and activating the hot start reaction mix in both LightCycler® 480 Probes Master mix and SYBR Green I Master mix. The temperature was 95˚C, and a duration time of 5-10 min was tested.
2. Amplification cycle:
    a. Melting at 95˚C, duration of 5 and 30 sec. tested
    b. Annealing temperatures of 56, 58 and 60˚C tested, and duration of 10, 30 and 60 sec tested.
    c. Amplification at 72˚C, duration of 15s and 30 sec. tested.
3. Melting curve (only for SYBRgreen)

Some tests were made both on the LightCycler® 480 (Roche) and SmartCycler II (Ceoheid™) with the same results. SmartCycler II has the possibility to run several samples with different protocols at the same time, and was thus used for the optimizing experiments.

2.5 Standard curves and detection limit of PCR

PCR detection was first investigated in pure culture of *V. cholerae.* PCR detection was tested with different concentrations of target with primers targeting four different genes using MB and SYBR greenI. For comparison, standard curves and detection limits were worked out for the different gene targets.

2.5.1 PCR standard curves for pure cell culture

PCR standard curves were made for all four gene targets with both MB and SYBR Green I. Ten folds dilutions of cell extracts and purified DNA where used.

Cell extracts were diluted to 10^6 to10^2 cells/ml. The concentration of the extracted DNA was measured spectrophotometric to 4.5ng/µl using the Nano Drop ND-1000 Spectrophotometer (Saveen Werner). Since 2 µl of samples was used in each test well, a concentration gradient was made of $5x10^{-10}$ to $5x10^{15}$ g/ml to give the final content of DNA in the PCR-samples of 1ng to10fg.

Three parallels of each sample were analyzed. PCR using MB where run 45 cycles, and 40 cycles where run using SYBR Green I.

The amplification curves were analyzed in Light cycler® software, giving the 2nd derivative maximum (CP value) of the amplification curves, and a calculated standard curve relating CP and concentration of template in the samples.

2.5.2 Detection limit of V. cholerae PCR using pure cell culture

The detection limit of *V. cholerae* PCR were evaluated using six parallels of the two concentrations assumed to be close to the PCR detection limit. All four target genes in *V. cholerae* were amplified from cell extract as well as DNA extract. Both SYBR green I and MB where used.

The PCR products were analyzed on agarose gel or on the Agilent 2100 Bioanalyzer (Caliper LabChip®) to verify the presence of the PCR product. See section 2.7 for description of the method.

In the further experiments the primers targeting the *tcp*A and *gro*El genes were used, and MB was used to monitor the amplification. An additional test was run to determine the number of

V. cholerae cells needed as template to give a positive amplification of these gene sequences. A dilution series was made with 28, 14, 9, 7, 6, 4 and 2 cells as template in each PCR reaction.

2.6 PCR on environmental samples

The environmental samples introduce a new challenge to PCR detection: possible PCR inhibitors. The presence of inhibitors in the PCR template must be documented to understand what affect the detection limit in environmental samples.

2.6.1 Demonstration of PCR inhibitors

An initial test was performed to investigate the possible influence of environmental water samples on PCR. SYBR green I and the primers targeting *tox*R were used in the PCR reaction. As template 2 µl *V. cholerae* cell extract or DNA extract was added to the reaction wells. The environmental samples were added as either extracted cells, extracted DNA or 10 times concentrated DNA extract from BW or EBW. DNA was extracted with the DNeasy blood and tissue kit as described in section 2.8. Three concentrations of *V. cholerae* were used: 10^4, 10^6 and 10^7 CFU/ml. The water samples had been stored 4 days at 4˚C.

Systematic tests of how environmental water samples could affect the amplification of the four gene targets using SYBR green I and MB were conducted. Cell and DNA extract from *V. cholerae* (2µl) were combined with cell or DNA extract from environmental water samples (2µl) and added to the PCR reaction mixture as templates. This time BW and EBW were taken from the freezer, thawed and washed once with PBS before cell extraction by heating. DNA extract of the samples from the previous experiment were used. Amplification of each of the gene targets *gro*El, *tcp*A, *ctx*A and *tox*R were performed using MB and SYBR green I. Two concentrations of *V. cholerae* were used (10^4 and10^7 CFU/ml) and all PCR was run with two parallels. Pure water, BW and EBW were run as negative controls. The amplification curves were compared to PCR amplifications of pure cell culture samples added PCR grade water in stead of environmental water.

To compare the different DNA extraction methods described in section 2.8.1 to 2.8.3, the degree of inhibition was measured in two ways; using high concentration *V. cholerae* cells or

using lamda phage as internal markers. When using *V. cholerae* cells as internal marker, cell extract (2µl) with several order of magnitude higher concentration of *V. cholerae* cells than the spiked environmental samples were added to the PCR reaction, together with DNA extract (2µl) of the environmental sample. PCR was run amplifying the *gro*El gene using MB. Two samples with three parallels of each were run for each extraction method, and the amplification curves were compared with a positive control of *V. cholerae.*

Lamda phage DNA was added to the PCR reaction together with environmental DNA as described for *V. cholerae* cell extract. Lamda DNA where amplified using SYBR Green I for fluorescents measurements. Lamda primers are described in Table 2-2. PCR was initiated with 10 min. at 95˚C followed by an amplification cycle of 5 sec. at 95˚C, 10 sec. at 62˚C and 20 sec. at 72˚C.

2.6.2 Removal of PCR inhibitors

The inhibitors from EBW were probably related to the high concentration of algae in those samples. Therefore an experiment was conducted to try to separate the algae from the EBW samples using an 8.0 µm pore diameter isopore® filter (Millipore). EBW was spiked with 200 *V. cholerae* cells per ml and 200 ml of the spiked sample was filtrated through an 8.0 µm isopor® filter. The filtration used two hours using vacuum. The filtrate was then filtrated on 0.22 µm nitrocellulose filter (Millipore). Both filters where extracted with optimized Boström's method as described in Figure 2-1. A second portion of 200 ml spiked EBW was filtrated through an 8.0 µm isopor® filter over night, only applying vacuum the next morning to filtrate the last 50-100 ml. The filtrate was filtrated on 0.22 µm nitrocellulose filter and both filters where extracted with NucliSens® isolation reagents.

A PCR amplification of *gro*El was performed on extracts from all filters, with and without added *V. cholerae* as an internal marker.

2.6.3 Detection limit of *V. cholerae* in environmental samples

Seawater (SW) and enriched brackish water (EBW) were spiked with *V. cholerae* cells and the DNA extracted. SW represents water with high salinity and low microbial activity, while EBW represents low salinity, high turbidity and high microbial activity. These samples

represented different challenges when it came to DNA extraction. Three DNA extraction methods described in section 2.8.2 to 2.8.4 were used on both samples. The method giving the highest yield of DNA and lowest inhibition were used in further experiments.

The environmental samples where spiked with *V. cholerae* cells yielding the concentrations shown in Table 2-3.

Table 2-3: Approximate concentration of *V. cholerae* cells in environmental samples before extraction and the volume of sample filtrated.

Concentration of *V. cholerae* in samples [cells/ 100ml]	Filtration volume [ml]
20	400
20	200
50	200
100	200
200	200
1000	200

Two parallels of each concentration were filtered. The filters were stored on ice before filtration. DNA extracts from each filter was analyzed on PCR in three parallels, amplifying the *gro*El and *tcp*A genes using MB. The PCR products where confirmed on agarose gels, see next section.

2.7 Confirmation of PCR products

Dependent on the number of samples to be analyzed either agarose gels or Bioanalyzer was used to confirm that a PCR product of the correct size was formed. Agarose gels could be used for up to 29 samples each time, whereas 12 samples could be analyzed in the Bioanalyzer.

2.7.1 Agarose gel

A 1% agarose gel was made by dissolving 1.0 g of LE agarose (Seakem) in 100 ml Tris-Boro acid-EDTA (10:10:1 mM) (TBE) buffer. The solution was heated at full effect in a

microwave oven for 2 minutes to make a homogeneous solution and cooled to approximately 60˚C before adding one droplet of ethidiumbromide (2mg/ml). The mixed solution was poured out in a frame. PCR product (4 µl) and loading buffer (4 µl) were mixed before applied to the agarose gel. A 100 bp DNA ladder (Invitrogen) was added to one agarose well.

The gel was run in TBE buffer at 0.88V for approximately 40 minutes. The DNA was visualized using UV-light and photographed.

2.7.2 Bioanalyzer

Another method for visualizing the PCR products was using the Agilent 2100 Bioanalyzer (Caliper Labchip®). The samples were analyzed using the Agilent DNA 1000 Reagents and DNA chip according to the handbook (Agilent Technologies). Only 1 µl sample is required for analyzing, but only12 samples can be ran at each chip. The principle of the chip is the use of electrophoreses to drive the DNA through interconnected micro channels to separate the DNA fragments based on their size. The size of the fragment is measured and compared with the size of a known DNA ladder. The data are analyzed in the software 2100 expert (Agilent Technologies).

2.8 Nucleic acid extraction

Preparation of PCR template from pure cell cultures were done either by making cell extracts or extract DNA with the DNeasy™ Tissue Kit (Qiagen) as described for Gram-negative bacteria in the DNeasy™ Tissue Kit handbook. Extracts were made from an overnight cell culture of *V. cholerae*. The culture was washed once in PBS. The cell extract was made by disrupting 1 ml of washed cells by heating to 96 ˚C for 10 minutes. The concentrations of CFU per ml and cells per ml were determined for the samples.

Two different methods where used for nucleic acid extraction from the environmental samples. The first was based on an article by Boström *et al.* (2004). It was chosen because of the potential high yield. The second was NucliSens® Isolation Reagents (Biomérieux). The later has earlier been used successfully to extract RNA for NASBA (10). Both methods can be used to extract DNA and RNA from bacteria.

2.8.1 Boström's extraction method

Boström *et al*. (2004) optimized a DNA extraction protocol based on the studies of Fuhrman *et al*. (1988) to isolate DNA for bacterial community analysis in aquatic microbial ecology. Their goal was to isolate DNA from a small volume of marine water with a high yield of DNA (92-96%) (2).

An attempt was made to reproduce the results in the article following the same protocol with only minor changes due to availability of equipment. The solution used in the protocol is described in Appendix D. In this experiment BW and EBW were used. The environmental samples were spiked with a *V. cholerae* culture to a final concentration of $3x10^4$ cells/ml.

Two ml of these samples were centrifuged at 17 000 x g for 20 min. at room temperature. The pellet was dissolved in lysis buffer (see Appendix D for composition) (175 µl) and added lysozyme solution (3.5 µl 50 mg/ml conc.) (Sigma-Aldrich). The solution was incubated at 37˚C for 30 min. before adding Sodium dodezyl sulphate (SDS) (20 µl, 10% conc.)(Bio-Rad) and proteinase K (1 µl 20 µg/µl conc.) (Quiagen). Half of the samples were incubated at 55˚C for 2 hours and the other half over night (16 hours). The lysate was transferred to a centrifuge tube and added yeast tRNA (5 ml 10 mg/ml conc.) (Roche Applied Science), NaAc (0.5 ml 3M conc.)(Fluka) and 99.6% ethanol (14 ml). After Incubation at - 20˚C for one hour, the tubes were centrifuged at 20 000 x g at 4˚C for 20 min. The pellet was washed with 70% ethanol before the centrifugation was repeated. The supernantant was decanted and the pellet air dried to evaporate residual ethanol. The pellet was eluted in TE buffer (200 µl).

Two hundred ml of water samples were filtered on a 47 mm diameter Durapore membrane filter (0.22 µm pore, Millipore), and the filter was cut in four equal parts. The filter pieces were folded to cones and placed in 2 ml microtubes with the point of the cone turned upwards. Lysis buffer (525 µl) and lysozyme (11 µl) were added, and the mixture was incubated at 37˚C for 30 min. Then, 10% SDS (60 µl), and proteinase K (3 µl) were added before incubating at 55˚C for either 2 or 16 hours. The lysate was transferred to a centrifuge tube and the filter washed with 500µl TE buffer that was also added to the centrifuge tube. Then yeast tRNA (5 ml), 3M NaAc (0.6 ml) and 99.6% ethanol (17 ml) were added before

incubating at -20˚C for one hour. The tubes were centrifuged and washed with ethanol as described for the other samples. After drying the DNA was eluted in TE buffer (500 µl).

The isolated DNA (5µl) was used as template in PCR, and the success of the extraction methods were measured comparing the CP value of the PCR amplification curves.

2.8.2 Optimizing the Boström's method

The method described above was optimized for small water samples. To detect low concentrations of *V. cholerae* in environmental samples it was desirable to optimize a method to concentrate a larger volume of water (200ml). It was also necessary to adapt the method to the available equipment.

Centrifugation of the samples before lysis was also tested on a larger volume. Two times 40 ml samples were centrifuged at 6 000 x g at room temperature for 20 min. The pellets were dissolved in 1 ml water, and the pellet from both centrifugation tubes were pooled together into one 2 ml tube. The tube was centrifuged at 13 000 x g at room temperature for 20 min. before lysis was preformed as described above.

In the further experiments 200 ml of water samples were used in filtration on 47 mm diameter filters. The whole filters were folded into a cone and put in 2 ml tubes. The lysis was preformed as described previous. The first centrifugation was performed in the same centrifugation tubes, but then the pellet was dissolved in 500 µl TE buffer and transferred over to 2 ml tubes. A new precipitation was performed by adding 50 µl 3 M NaAc (1/10 vol.) and 1380 µl 99.6% ethanol (2.5 vol.). The tubes were incubated at - 20˚C for one hour before centrifuged at 10 000 x g for 20 min. at 4˚C. The pellet was washed in 500 µl 70% ethanol before repeating the centrifugation. After drying the pellet was dissolved in 50 µl TE buffer.

An experiment was conducted to compare the precipitation with and without tRNA as a DNA co-precipitant. The same concentration of *V. cholerae* spiked water samples were processed in exactly the same manner. The PCR amplification of *gro*El was used as a measure of DNA extraction efficiency. A summary of the optimized Boström's method without addition of tRNA is shown in Figure 2-1.

Since the polyethersulfone membrane filter used by Boström *et al*.(2004) was not available, both nitrocellulose membrane filter and Durapore® membrane filter (both Millipore, 0.22 μm) were tested.

To investigate if this method was able to distinguished between intact cells and dissolved DNA, environmental samples were spiked with cells heated at 96˚C for 10 min. The samples were filtered through a nitrocellulose membrane filter and processed as described previous.

Modified Boström's DNA extraction method

Filter 200 ml water sample onto 0.22 µm nitrocellulose filter (47 mm diameter)

⇩

Add 535 µl lysis buffer and lysozyme (1mg/ml) to the filter. Incubate at 37˚C for 30 min.

⇩

Add SDS (1%) and proteinase K (100 µg/ml) and incubate at 55˚C for 2 hours.

⇩

Transfer lysate to a centrifuge tube. Wash filter with 500 ml TE and pool with lysate.

⇩

Add 1/10 vol. 3M NaAc and 2.5 vol. 99.6% ethanol. Incubate at - 20˚C for 1 hour.

⇩ Centrifuge at 20 000g (4˚C, 20 min)

Dissolve pellet in 500 µl TE and transfer to 2ml centrifuge tube. Add 1/10 vol. 3M NaAc and 2.5 vol. 99.6% ethanol. Incubate at - 20˚C for 1 hour.

⇩ Centrifuge at 10 000g (4˚C, 20 min)

Wash pellet with 500 µl 70% ethanol

⇩ Centrifuge at 10 000g (4˚C, 20 min)

Dry pellet in a heat block at 56˚C. Dissolve in 50 µl TE.

Figure 2-1: Optimized Boström's method for extracting DNA from large environmental samples

2.8.3 NucliSens® lysis and extraction

NucliSens® nucleic acid isolation is based on the method described by Boom et al. (1990) which involve chemical lysis of the cells, binding of the nucleic acid to silica particles, several washing steps before eluting the nucleic acid in a low salt buffer.

One part sample was added nine parts NucliSens® Lysis Buffer and NucliSens® suspended silica particles (50 µl) and incubated for 10 min with periodic mixing. If the sample to be lysed was a filter, the filter was added one part water and nine parts NucliSens® Lysis Buffer and incubated 10 min before the solution was transferred to a new tube and the filter was washed once with the same amount of lysis buffer and water. That solution was also transferred to the same tube and added suspended silica particles (50 µl) and incubated for 10 min as described. The tubes were centrifuged one min at 13 000g and the supernatant was decanted. The silica particles were washed twice with NucliSens® Wash Buffer, twice with 70% ethanol and once with acetone. The tubes where centrifuged one min at 13 000g for each washing step. After decanting the acetone supernatant the tubes where left open in a heat block at 56˚C for 10 min to dry. NucliSens® Elution Buffer (50 µl) was added and the tubes where sealed and incubated 10 min at 56˚C before centrifugation for two min at 13 000g. The supernatant contained a mixture of RNA and DNA. The elution was repeated using 30 µl elution buffer to increase the yield of DNA.

2.8.4 NucliSens® lysis and Boström's extraction

The elution step in the NucliSens® extraction might result in loss of nucleic acid. By combining the lysis with NucliSens® Lysis Buffer and the nucleic acid precipitation in the Boström's method a fast lysis and high yield DNA isolation procedure might be obtained.

The samples where filtered as described earlier and added NucliSens® Lysis Buffer as described. The lysis solution was transferred to a centrifuge tube and precipitated with ethanol as described in the modified Boström's method.

2.9 NASBA

NASBA was run using reagents from the NucliSens EasyQ® Basic Kit V2 (Biomérieux). A primer/MB mix was made of 2 µM of a primer pair from Table 2-4 and 2.5 µM of MB from

Table 2-2 targeting the same gene. A reaction mixture (20 µl) was made by adding reagents mix (6.7 µl) (NucliSens EasyQ®), primer/MB mix (2.0 µl), KCl (1.3 µl) (NucliSens EasyQ®) and template (5 µl). RNA template for the NASBA reaction was extracted from pure culture *V. cholerae* with NucliSense® as described previous. The reaction tubes were incubated 10 min at 65˚C in SmartCycler II before the enzyme mix (5 µl)(NucliSens EasyQ®) where added. The reaction was carried out at 41˚C for 90 min in the SmartCycler II.

Table 2-4: Sequences of primers used in NASBA. T7-RNA polymerase template sequence is shown in lower case letters.

Primer	Sequence
P65 VC*gro*EL	aattctaatacgactcactatagggATGATGTTGCCCACGCTAGA
P66 VC*gro*EL	GGTTATCGCTGCGGTAGAAG
Pvc62 *tcp*A	aattctaatacgactcactatagggCGCTGAGACCACACCCATA
Pvc60-103*tcp*A	GAAGAAGTTTGTAAAAGAAGAACACG

The amplification was monitored in real-time by measure the fluorescent from the MB every minute. The time point when the fluorescent value was 10 times the standard deviation of the baseline intensity was noted as the t value.

Pure culture of *V. cholerae* in exponential growth phase was harvested with centrifugation, washed in PBS and extracted with NucliSens® Isolation reagents. The extracted nucleic acids were diluted to the following concentrations before *gro*El and *tcp*A were amplified using the NASBA protocol: 10^8, 10^7, 10^5, 10^3 and 10^2 CFU/ml.

Two hundred ml SW was spiked with 10^9 CFU *V. cholerae*. The sample was filtrated on a 0.22 µm nitrocellulose filter and RNA was extracted using the NucliSens® Isolation reagents. The extract was used in NASBA reaction directly and 10^2, 10^4 and 10^5 times diluted. Both *gro*El and *tcp*A were amplified.

To improve the yield of RNA from nucleic acid extractions the cell pellet or Durapore filter was treated with RNAprotect™ (Qiagen) according to the handbook before extraction.

Nucleic acids amplified with optimized Boström's method were also used as template in the NASBA analysis.

3 Results

3.1 Determination of CFU/cells

The concentration of *V. cholerae* allowed in ballast water by IMO is stated as 1 CFU per 100 ml. PCR, however, detects cells regardless of their viability. It was thus required to determine the ratio between CFU and number of cells in *V. cholerae* cultures.

For each experiment the concentration of cells per ml and CFU per ml for the *V. cholerae* culture used were determined as described in section 2.1.1. The ratio between the numbers of cells counted in a microscope and the number of colonies formed on TSA-plates varied for each sample. The average percentage cells forming colonies after 14 measurements were 7±2 %, calculated using a linear regression of the plotted counts, see Figure 3-1. Cells from over night cultures as well as cultures in the exponential growth phase were counted.

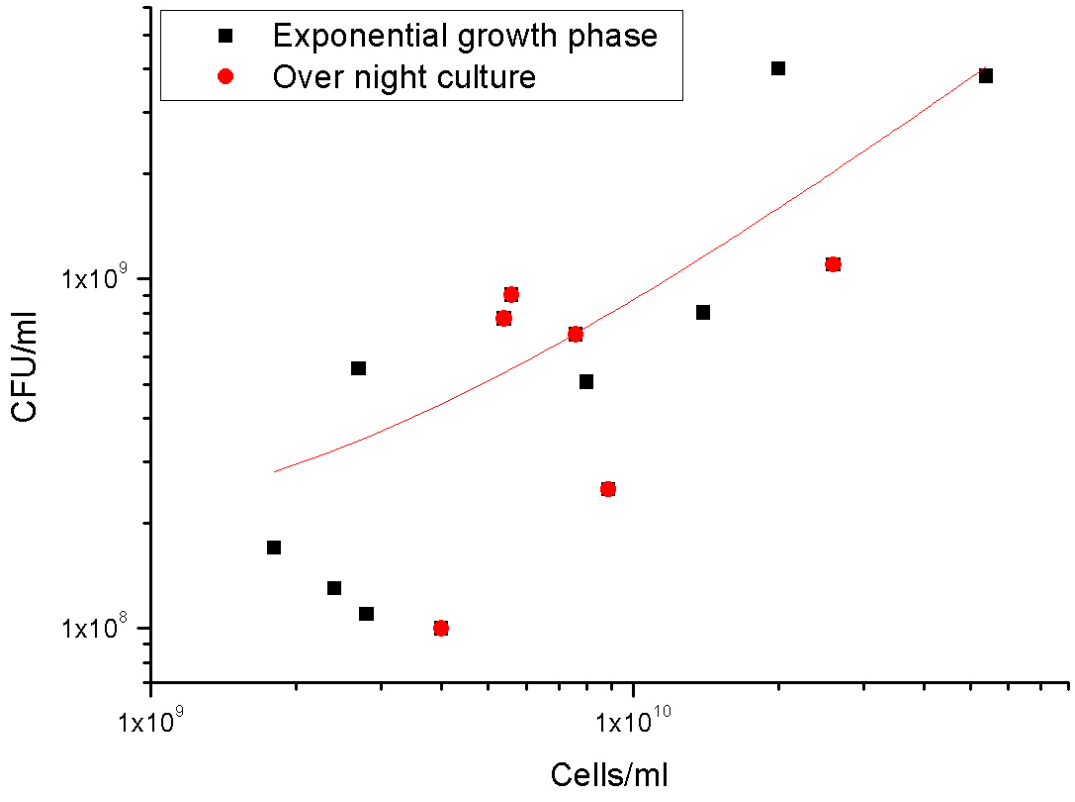

Figure 3-1: Number of cells plotted as a function of colony forming units in *V. cholerae* cultures. The red line is a linear regression of the plot.

To confirm that the cells harvested 3-5 hours after inoculating the culture was in an exponential growth phase a growth curve was made: A culture was inoculated with *V.*

cholerae as described and the growth of the culture was followed by measuring the optical density (OD_{600}), see Figure 3-2. Between 120 and 300 minutes the curve took an exponential form. The cells varied in size during the growth phase. During the first two hours very small and larger bacteria were seen in the microscope. However, the small bacteria disappeared after some time. After 6-7 hours of growth the average size of the bacteria had decreased significantly again. The difference in cell size might have affected the microscope counting due to difficulties in distinguishing the smallest bacteria.

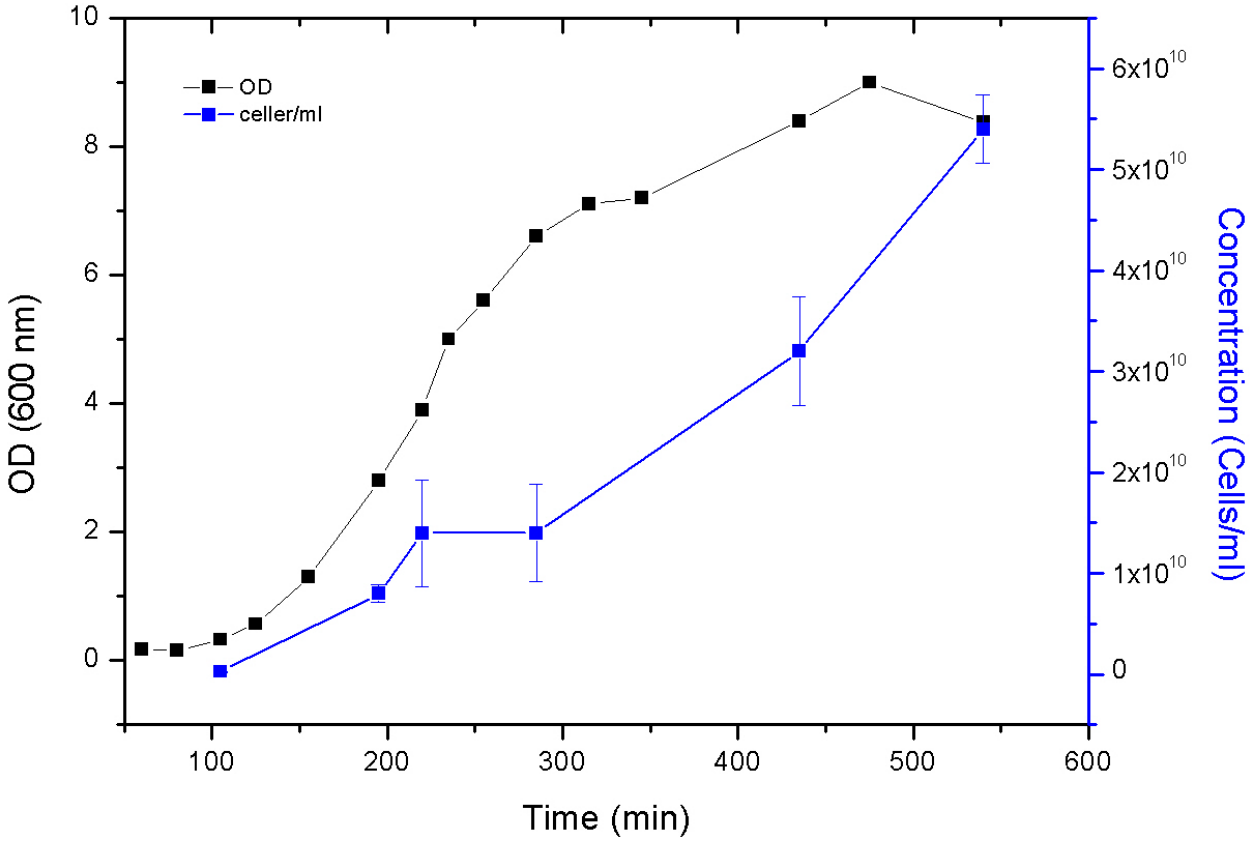

Figure 3-2: Growth curve for *V. cholerae* grown at 37˚C with 200 rpm measured as OD at 600 nm and as counted cells with error bars calculated as standard deviation of the parallels counted.

3.2 Microbial content of environmental water samples

The microbial content in the water samples might affect the detection of spiked *V. cholerae.* It was thus required to survey the differences in bacterial content in the different types of environmental samples. The bacterial content in samples stored before analyzing must also be surveyed.

The enriched sea and brackish water showed larger colonies with less variation of morphology than the normal sea and brackish water when plated on marine agar. Figure 3-3 shows bacteria from brackish water. The number of CFUs where fairly stable and varied no more than a factor 10 during storage of test water up to a month at 4˚C. The zooplankton and the algae on the other hand died after about one week.

Samples were plated on TCBS to investigate the presence of *Vibrio sp.* Only bacteria from the enriched environmental samples (EBW and ESW) grew on TCBS and they were all yellow, see Figure 3-4. No colonies appeared when plating 100 times concentrated samples of SW and BW.

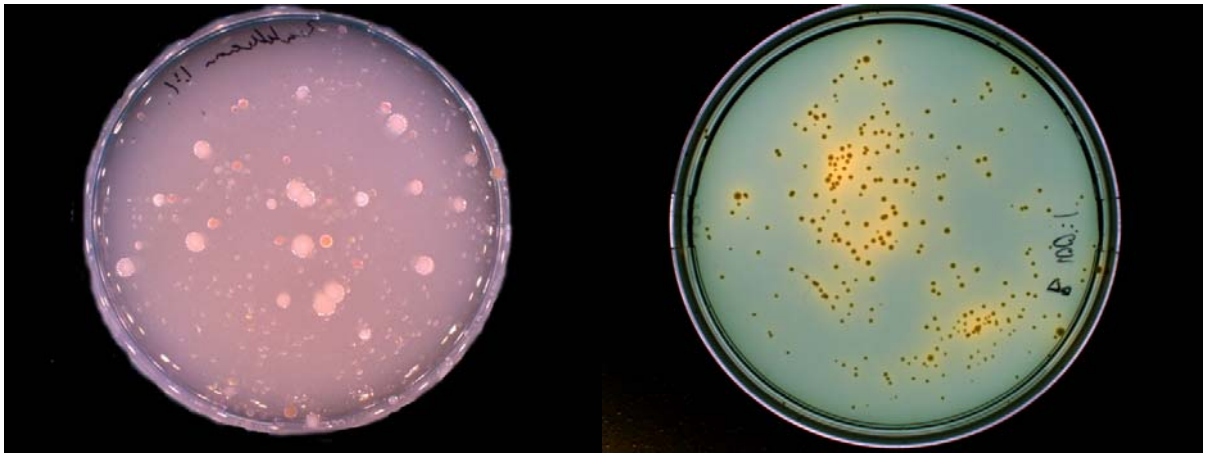

Figure 3-3: Environmental bacteria on marine agar. Figure 3-4: Presumptive *Vibrio* on TCBS agar.

The colonies on the TCBS showed similar morphology (see Figure 3-4). Sixteen colonies were picked randomly from both plates with EBW and ESW. They where restreaked on TSA plates forming colonies on TSA had different morphologies. Some were large, some small, some had a dark core, some had a halo, but all were different shades of white. PCR amplification of the 16s rRNA gene was performed as described in Appendix C, and the melting peaks of the PCR products were all around 88˚C. Since the melting peaks were similar, only eight of the PCR products where sequenced. The sequences where checked in a Nucleotide BLAST database search. Seven of the sequences matched with a sequence in the organism Exiguobacterium sp. of the phylum Firimicutes and one sequence matched with a sequence in Pseudomonas sp of the phylum Proteobacteria. The amplified 16s rRNA sequences are given in Appendix C.

3.3 PCR on pure culture V. cholerae

Several experiments were conducted to find the optimal PCR protocol for detection of *V. cholerae* with the most efficient primers. MB and SYBR green I were also compared.

3.3.1 Optimized PCR protocol

PCR amplification of *V. cholerae* genes were optimized by variations in time and temperatures in the PCR protocol. There was no difference in the optimum protocol for the use of SYBR green I and MB in PCR. There was no significant difference in the fluorescence when the initial denaturing time varied between 5 and 10 minutes. An annealing temperature on 60˚C resulted in a lower fluorescence level than with 56˚C or 58˚C. The shortest melting and amplification times that were tested were sufficient. The annealing time which gave the best result was 30 seconds.

The optimized PCR protocol is:

Initial denaturing:	95˚C for 5 min.
Amplification cycle:	95˚C for 5 sec.
	58˚C for 30 sec.
	72˚C for 15 sec.

3.3.2 PCR detection limit

To decide which of the four target gens should be used in the further experiments standard curves for all target genes were made with SYBR green I and MB. Extracted cells and DNA were used as template, and three parallels of each concentration were analyzed on PCR.

Typical amplification curves from amplification of *gro*El using different concentrations of DNA extracts with SYBR green I and MB are shown in Figure 3-5.

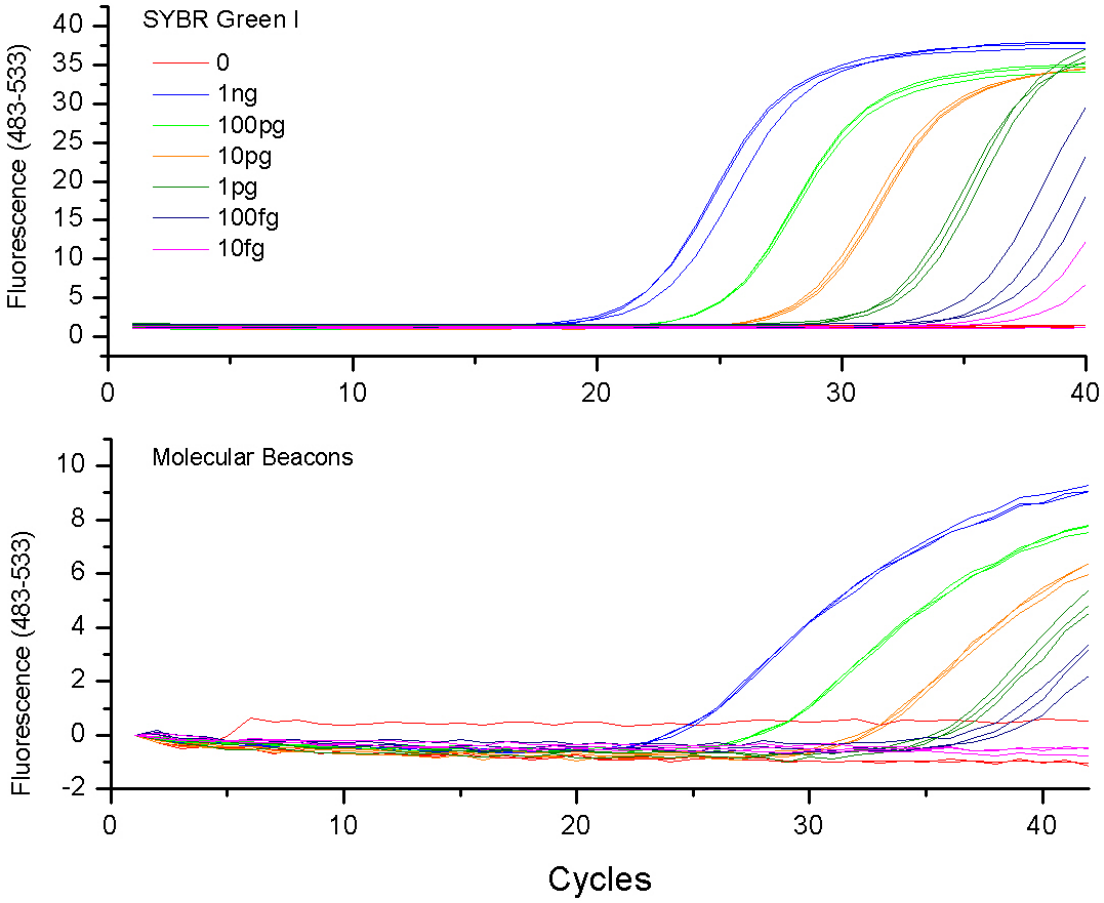

Figure 3-5: PCR amplification of *gro*El with SYBR green I and Molecular Beacons.

The CP values were plotted against log initial concentration to create standard curves. The standard curves are shown in Appendix E, and the properties for the different targets are listed in Table 3-1.

Table 3-1: Properties of standard curves of PCR amplification of *V. cholerae* genes with 2µl template.

Target gene	Extract in template	Fluorescence marker	Area of application	Slope	Error	R^2
*gro*El	Cell	MB	$3x10^4$ – $3x10^8$ cells/ml	-3.51	0.017	0.97
		SYBR	$3x10^4$ – $3x10^8$ cells/ml	-3.49	0.016	0.97
	DNA	MB	10^{-13} – 10^{-9} g DNA	-3.77	0.003	0.99
		SYBR	10^{-13} – 10^{-9} g DNA	-3.48	0.023	0.95
*tcp*A	Cell	MB	$3x10^4$ – $3x10^8$ cells/ml	-3.43	0.021	0.96
		SYBR	$3x10^4$ – $3x10^8$ cells/ml	-3.59	0.008	0.98
	DNA	MB	10^{-11} – 10^{-9} g DNA	-3.40	0.035	0.93
		SYBR	10^{-13} – 10^{-9} g DNA	-3.68	0.029	0.94
*ctx*A	Cell	MB	$3x10^5$ – $3x10^8$ cells/ml	-3.72	0.028	0.94
		SYBR	$3x10^4$ – $3x10^8$ cells/ml	-3.62	0.015	0.97
	DNA	MB	10^{-12} – 10^{-9} g DNA	-3.87	0.018	0.96
		SYBR	10^{-12} – 10^{-9} g DNA	-3.33	0.073	0.86
*tox*R	Cell	MB	$3x10^3$ – $3x10^8$ cells/ml	-3.32	0.016	0.97
		SYBR	$3x10^4$ – $3x10^8$ cells/ml	-3.49	0.015	0.97
	DNA	MB	10^{-13} – 10^{-9} g DNA	-3.80	0.023	0.95
		SYBR	10^{-12} – 10^{-9} g DNA	-3.61	0.001	1

The area of application of the standard curves was narrowed until the R^2 value was 0.95 or higher. Amplification of *tcp*A from DNA extract, *ctx*A from cell extract with MB and DNA extract with SYBR green I did not give standard curves with R^2 of 0.95 or above. All the standard curves had slopes between -3.32 and -3.87 which is within the range that Zhang and Fang (2006) stated as reliable standard curves.

The two lowest detected concentrations of template were used as template in a new PCR experiment with six parallels using all four gene targets to determine the detection limits. See Figure 3-6 for statistics of the number of successful PCR amplifications of the target genes.

In the experiments conducted with extracted DNA all primers had 100% detection of 1 pg DNA, while only *tcp*A amplification was successful in all 9 parallels using 100 fg DNA as template and detected with SYBR green I. Amplification of *ctx*A resulted in a higher detection limit compared to the other gene targets using cell extracts.

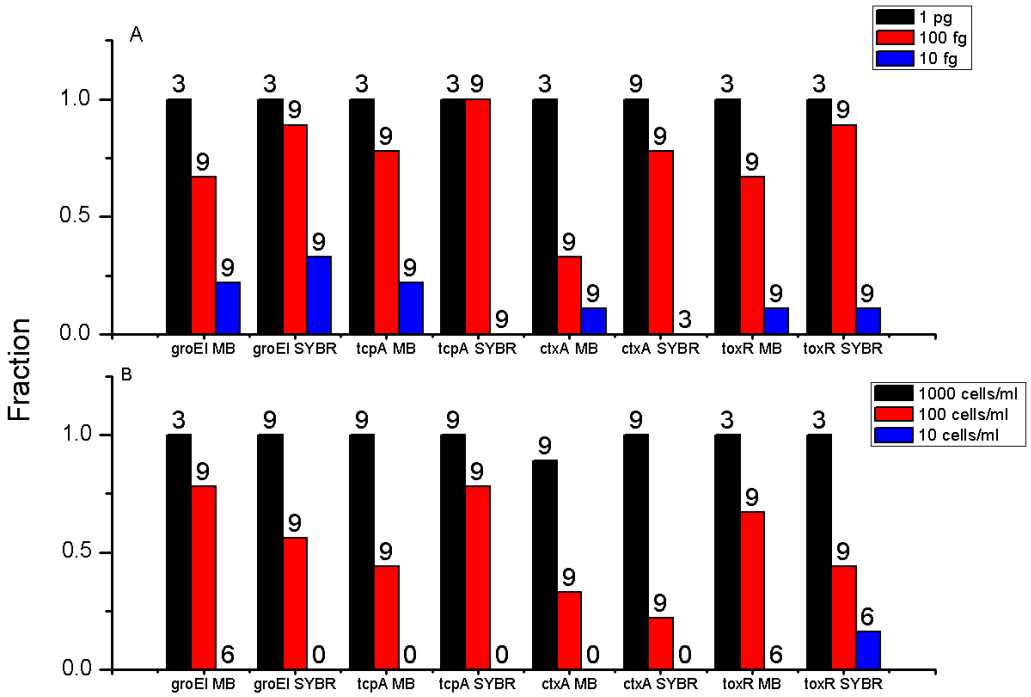

Figure 3-6: PCR detection of different target genes of *V. cholerae* using DNA extract (1 pg, 100 fg, and 10 fg) and cell extract (1000, 100 and 10 cells/ml) as template. Both SYBR green I and MB were used. The numbers on each column represent the number of PCR trials used to calculate the fraction of successful PCR amplifications.

3.3.3 Effect of potential PCR-inhibitors in the environmental samples

Another factor was considered before the primers and detection methods were chosen: the effect of possible inhibitors in the environmental samples on PCR. An initial test was conducted using *tox*R amplification with SYBR green I to investigate if addition of heat treated or DNA extracted environmental water samples inhibited PCR amplification.

DNA extracts from BW and EBW did not give any sign of inhibition of the *tox*R amplification. BW and EBW with no other preparation than heating before applied to the PCR reaction mixture seamed to partly inhibit the PCR amplification, see Figure 3-7. PCR reactions where BW and EBW were added had amplification curves with higher CP-values

than the control without added environmental samples, and did not reach the same fluorescence level as the control either.

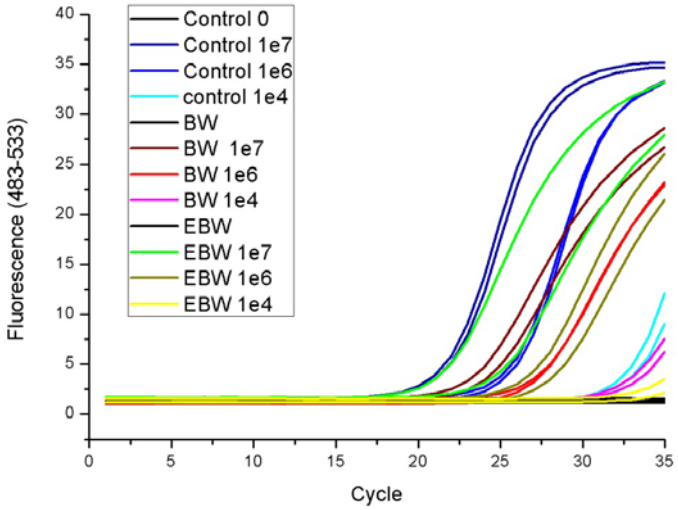

Figure 3-7: PCR amplification of *tox*R with SYBR green I, with and without addition of heat treated BW and EBW.

After confirming that environmental samples contain PCR inhibitors, all the primers were tested for the effect of the inhibitors. DNA and cells were extracted from BW and EBW as described for *V. cholerae* in 2.8. All target genes were amplified in PCR with both added DNA extract and cell extract from environmental samples using SYBR green I and MB.

No inhibition of PCR amplification was observed when MB was used, and no inhibition was observed when extracted DNA from environmental samples were added to reactions with SYBR green I. Addition of cell extract from BW and EBW to PCR using SYBR green I affected PCR amplification slightly for all targets expect *ctx*A which was clearly inhibited, see Figure 3-8. The inhibitors had most effect at low concentrations of target DNA.

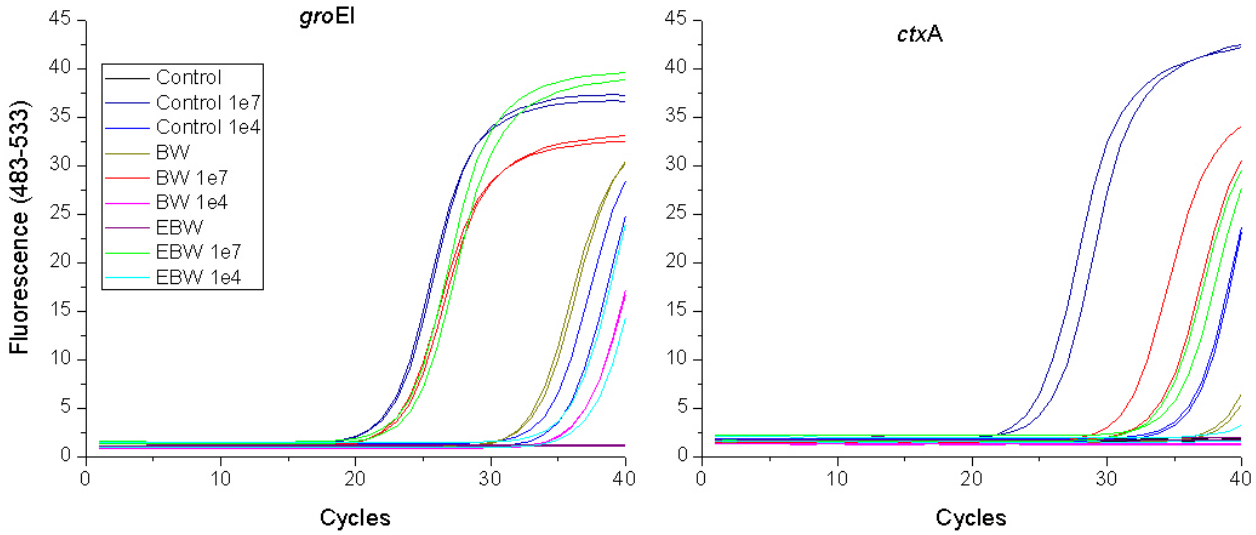

Figure 3-8: Influence of cell extract from BW and EBW on PCR amplification of *gro*El and *ctx*A with SYBR green I. Two parallels of each reaction added 0, 10^4 and10^7 cells/ml of *V. cholerae* as template was run.

In Figure 3-8 the negative control with BW showed positive signal. The sample was rerun on PCR together with a new extract from the same BW sample. None of the *V. cholerae* genes were amplified from the new BW sample.

3.3.4 Choice of PCR target genes

Due to findings stated previous the primers targeting the *gro*El and t*cp*A genes were chosen as the primers to be used in the further experiments. Primers amplifying the *gro*El gene detect all *V. cholerae* strains and primers targeting the *tcp*A gene detect only the toxic strains.

MB was chosen as the preferred fluorescent marker because it is more specific and was less affected by inhibitors.

PCR experiments were conducted to investigate the PCR amplification of the *gro*El and *tcp*A genes with low concentrations of *V. cholerae* as template. Three parallels were run and the CP values were plotted against number of cells in template, see Figure 3-9. Amplification of *gro*El and *tcp*A of all tree parallels with only two cells as template were successful. The CP value of the parallels differed at low concentrations of template.

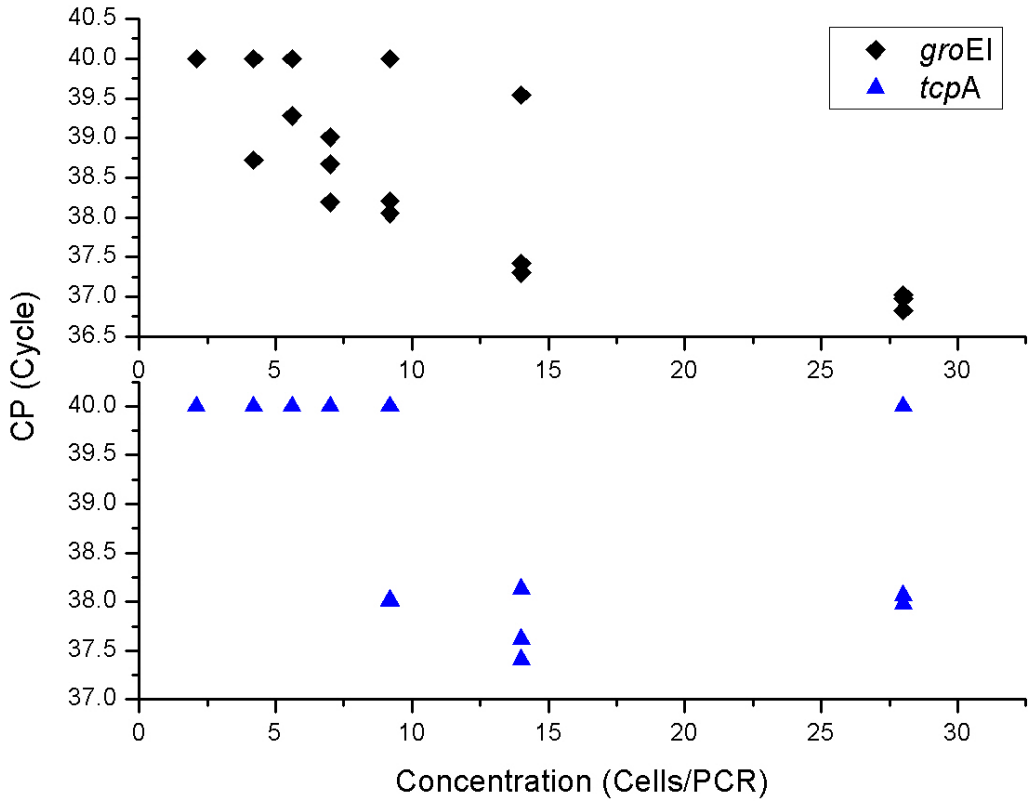

Figure 3-9: PCR amplification of *gro*El and *tcp*A using 2 to 28 *V. cholerae* cells as template. Each concentration has tree parallels, plotted as the template concentration as a function of the respective CP-values of the amplifications.

3.4 Optimization of nucleic acid extraction

An optimal DNA extraction method for extraction of high yield DNA and removal of PCR inhibitors from environmental samples was requested. DNA extraction was adapted to the different challenges in different water samples.

3.4.1 Adapted Boström's method

DNA was isolated from spiked environmental samples according to Boström *et al*. (2004) using centrifugation and filtration with Durapore membrane filters, see section 2.8.1. DNA from two ml sample spiked with $3x10^4$ *V. cholerae* cells per ml was extracted after centrifugation and two parallels analyzed on PCR using amplification of *gro*El. Eight of 16 PCR parallels were positive with a CP-value of 40 cycles. DNA extracted from 50 ml filtrated samples spiked with $3x10^4$ *V. cholerae* cells per ml gave all positive amplification of *gro*El. DNA from eight filters was extracted, and two parallels analyzed on PCR yielding an average CP-value between 35 and 36 cycles.

Since *V. cholerae* are gram negative cells a lysis time of 12 hours might not be necessary. Thus half of the samples where incubated with proteinase K and SDS for only 2 hours, and half for 16 hours. No difference in PCR amplification where observed from the samples with different incubation times.

The results for the centrifugal samples were not satisfying, and a new experiment was conducted with larger sample volumes to compare centrifugation and filtration. SW and ESW was spiked with *V. cholerae* to a final concentration of 0.36, 36 and $3.6x10^4$ cells/ml. DNA was extracted as described in section 2.8.2. The volumes were adjusted to give the same theoretical concentration of DNA in the extracts, making it possible to compare the extraction methods. DNA extracts from two samples of each spiked concentration of *V. cholerae* where analyzed with three parallels on PCR for each extraction method. The result is shown in Figure 3-10.

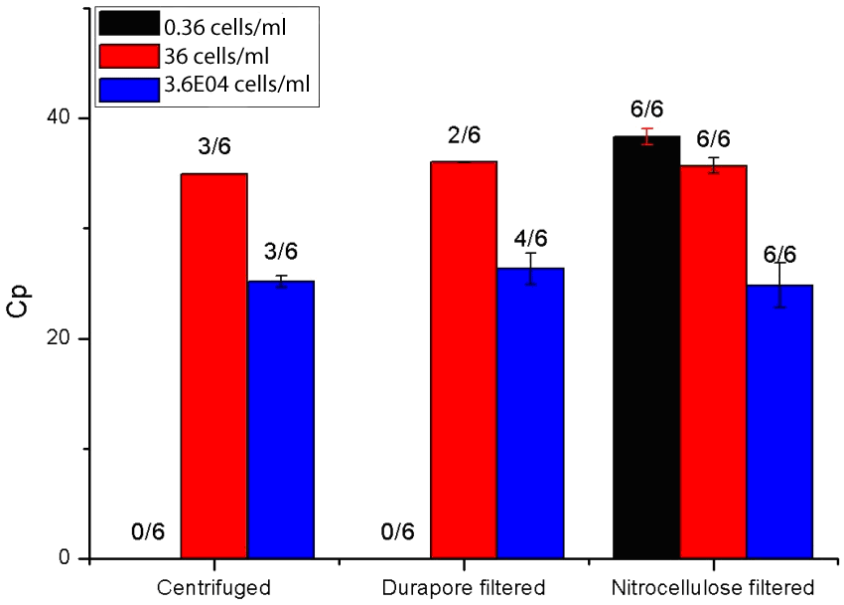

Figure 3-10: Comparison of PCR amplification of *gro*El from spiked environmental samples extracted from filtered or centrifuged samples. Numbers above columns represent number of successful PCR amplifications and heights of the columns represent the CP values with calculated standard deviations.

Some of the DNA extracts where diluted and analyzed once more on PCR. That resulted in better results for these samples, see Table 3-2. Nevertheless filtration on nitrocellulose filters gave the lowest CP values and lowest detection.

Table 3-2: PCR amplification of the *gro*El target from spiked environmental samples extracted from filtered or centrifuged samples. The extracts were diluted two times with water before applied in PCR.

Extraction method	Concentration (cells/ml)	Cp	Specific PCR products detected/ total number of reactions
Centrifuged	36	40	2/3
Centrifuged	$3.6x10^4$	31	3/3
Durapore filtrated	36	38	3/3
Durapore filtrated	$3.6x10^4$	31	3/3

DNA extracts from the filters were added lamda phage DNA as an internal marker and analyzed on PCR with lamda primers to check for inhibition, see Figure 3-11. Samples extracted from Durapore filters totally inhibited the lamda amplifications, while samples extracted from nitrocellulose filters only partly inhibited the amplification. DNA extracted from EBW samples were more inhibitory than DNA extracted from the SW samples.

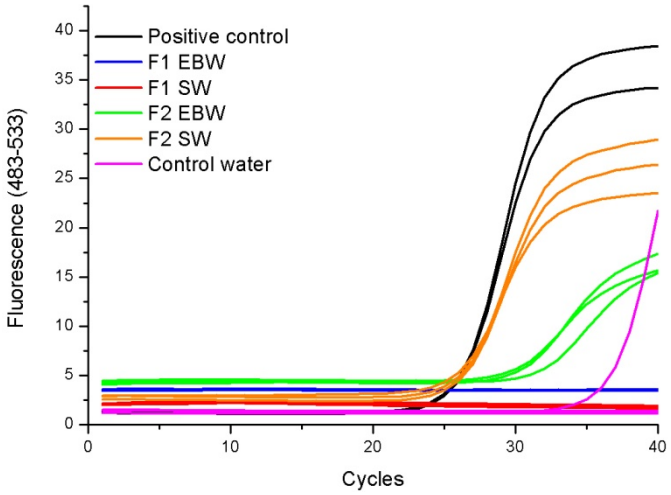

Figure 3-11: Three parallels of DNA extracts from Durapore filters (F1) and Nitrocellulose filters (F2) with EBW and SW added lamda phage and analyzed in PCR using lamda primers. Positive control is lamda amplification without added DNA extracts from environmental samples.

It was investigated if addition of yeast tRNA was necessary to obtain a high yield of DNA in these experiments. The DNA extracts were analyzed by amplification of the *gro*El target using PCR. No improvements of CP values were observed for the samples added tRNA.

Based on these results it was decided to use filtration with nitrocellulose filters in the further experiments. The protocol shown in Figure 2-1 with incubation time of 2 hours for lysis and no tRNA as co precipitant was used as the optimized Boström's method.

It was also tested if DNA from lysed cells were absorbed to the nitrocellulose filters. Environmental water was spiked with heat killed cells and filtrated. DNA was extracted from the filter according to Figure 2-1. PCR amplification of the *gro*El target resulted in a low CP value, indicating that DNA from dead cells was captured in the filter.

3.4.2 Extraction of DNA from seawater

Three DNA extraction methods were tested on SW to find the method giving the highest yield. Extraction using the optimized Boström's method (B) (Figure 2-1), combination of Nucliscens lysis and Boström's extraction (N/B) and Nuclisenc lysis and extraction kit (N) was tested on spiked seawater samples. The experiment was conducted three times with slightly different concentrations of *V. cholerae* cells added to the samples, see Figure 3-12. The results indicate that the optimized Boström's method resulted in a more effective DNA-extraction. The CP values were lower for the samples extracted with optimized Boström's method than the other methods.

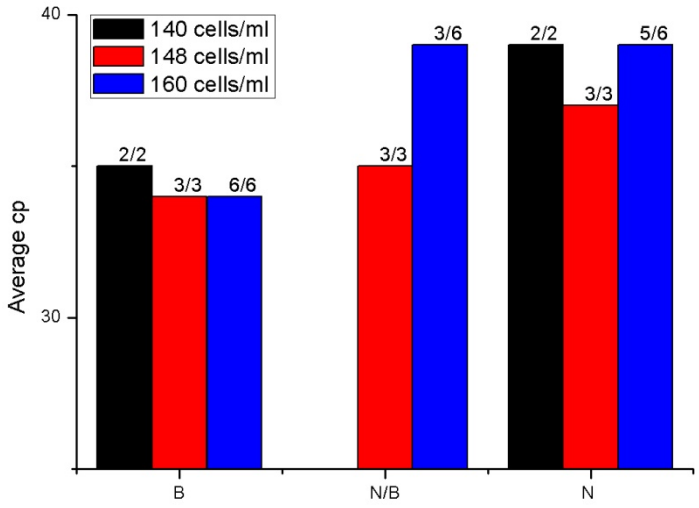

Figure 3-12: Spiked seawater samples extracted with adapted Boström's method (B), NucliSens lysis and Boström's extraction (N/B) and NucliSens extraction kit (N). Numbers above columns represent number of successful PCR trials.

The extracted DNA samples were also subjected to inhibitor testing by adding a highly concentrated *V. cholerae* cell extract or lamda phage DNA as internal markers. No PCR inhibition was observed for any of the samples.

3.4.3 Extraction of DNA from enriched brackish water

EBW had a higher turbidity and a higher content of microorganisms than SW, and this resulted in more challenges for the DNA extraction. The water samples were spiked with *V. cholerae* and DNA was extracted using the same methods that where tried on SW. Two experiments were conducted with a small variation of *V. cholerae* cell concentration, see Figure 3-13.

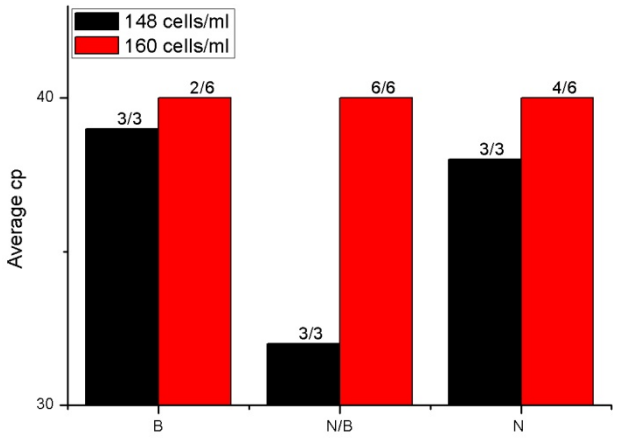

Figure 3-13: Spiked enriched brackish water samples extracted with adapted Boström's method (B), NucliSens lysis and Boström's extraction (N/B) and NucliSens extraction kit (N). Numbers above columns represent number of successful PCR trials.

Inhibitor tests of all extraction methods were also conducted. When using lamda phage as internal marker the samples extracted with B and N/B fully inhibited the PCR amplification, while samples extracted with N did not affect the result of the PCR amplification. PCR with a highly concentrated *V. cholerae* cell extract and extracted EBW samples are shown in Figure 3-14. The NucliSens extraction removed all PCR inhibitors. DNA extracted using the optimized Boström's method inhibited the PCR amplification completely, while samples extracted using a combination of NucliSens lysis and Boström's extraction only partly inhibited PCR. Some amplification was observed in the negative control containing water, but the possible contamination causing this amplification was of so low concentration that it would not affect the results. Based on these results the best method for DNA extraction from EBW was extraction with NucliSens extraction kit.

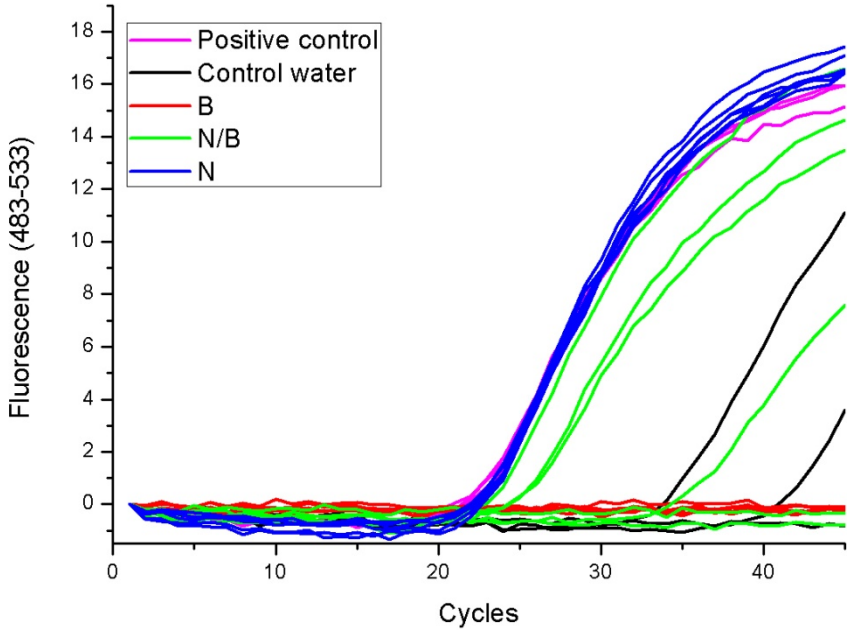

Figure 3-14: Influence of PCR inhibitors from EBW samples extracted with Boström's method (B), NucliSens lysis and Boström's extraction (N/B) and NucliSens extraction kit (N) of PCR amplification of *gro*El from *V. cholerae.*

The EBW samples had a high concentration of algae which probably caused some of the PCR inhibition. In order to remove the algae a pre filtration through a polycarbonate filter Isopore® 8.0 µm pore size was conducted before the filtrate was filtered through a 0.22 µm nitrocellulose filter. Figure 3-15 shows clearly that a pre filtration of EBW remove most of the algae indicated by the green colour.

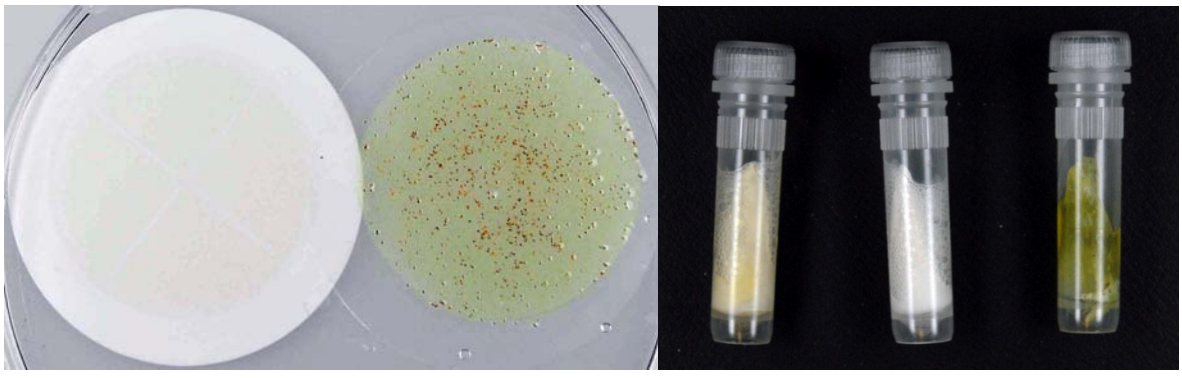

Figure 3-15: Left: 0.22 µm filter after pre filtration (white) and 8.0 µm pre filter (transparent with plankton).

Right: Filters folded, placed in tubes and added lysis buffer. From left: 0.22 µm filter without pre filtration, 0.22 µm filter after pre filtration, and 8.0 µm pre filter.

EBW was spiked with 200 *V. cholerae* cells/ml and 200 ml was filtrated on an 8.0 µm isopore filter. The filtrate was filtered on a 0.22 µm nitrocellulose filter. The filtrations were conducted with two samples. One set of isopore filter and nitrocellulose filter was extracted with NucliSens, and one set with optimized Boström's method. All DNA extracts where analyzed using PCR amplification of the *gro*El gene. None of the samples extracted using the Boström's method gave amplification products. However, both the DNA extract from the isopore filter and the nitrocellulose filter extracted using the NucliSens extraction kit gave amplification of the *gro*El gene. The isopore filter sample had a lower CP value than the nitrocellulose filter, indication that most of the *V. cholerae* cells were trapped in the isopore filter. The samples extracted with the Boström's method was diluted 1:1 in TE buffer and analyzed again, resulting in amplification product from the nitrocellulose filter sample in all three PCR parallels, and one of 3 parallels for the isopore filter.

An inhibitor test was performed by adding high concentrated *V. cholerae* cell extract to the samples and analyzing by PCR amplification of the *gro*El gene. Neither the DNA extract from the isopore filter nor the nitrocellulose filter extracted with Nuclisens showed any inhibition; while the samples extracted with Boström's method showed some degree of PCR inhibition, see Figure 3-16.

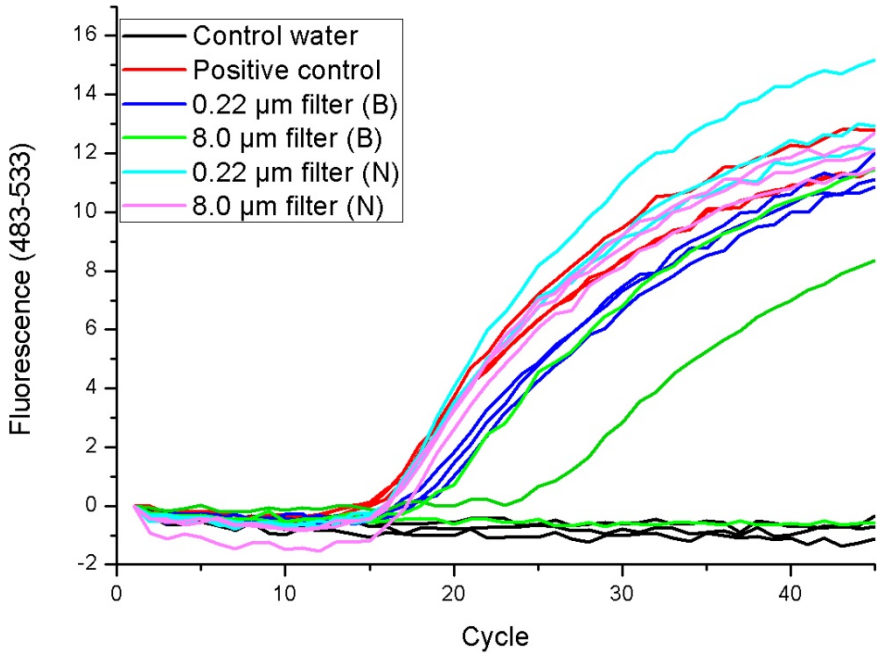

Figure 3-16: Inhibition test on DNA extracts from 8.0 µm isopor filters and 0.22 µm nitrocellulose filters extracted with Boström's method (B) and NucliSens extraction kit (N).

3.5 PCR detection limits of V. cholerae in environmental samples

The sensitivity of the detection of *V. cholerae* in SW and EBW were investigated using the most optimal extraction method for DNA based on the previous results. The optimized Boström's method was used for DNA extraction from SW, whereas the NucliSens extraction kit was used for DNA extraction from EBW.

3.5.1 PCR detection of *V. cholerae* in seawater samples

Two separate experiments were conducted to investigate the PCR detection limit of *V. cholerae* in SW. All samples were filtered on nitrocellulose filters and DNA extracted with optimized Boström's method according to Figure 2-1. In the first experiment SW was spiked with 18± 2, 88± 10, 180± 22 and 880± 110 *V. cholerae* cells per 100 ml. The detection limit was estimated to be approximate 90 cells per 100ml, hence a new experiment was conducted using 20 ± 4, 50 ± 11 and 90 ± 20 *V. cholerae* cells per 100 ml SW. In the second experiment the concentration of 90 ± 20 cells per 100 ml was only detected in half of the experiments with amplification of the *gro*El gene. The detection limit was thus increased to 180± 22 *V. colerae* cells per 100 ml. All samples had a volume of 200 ml, except for one extra sample of 400 ml of water containing 20 cells per 100ml. The results of the two experiments are shown in Table 3-3. The PCR products where confirmed on agarose gel. Some of the samples that did not get a CP value on the PCR software were also confirmed on agarose gel. See Appendix F for picture of gels.

Table 3-3: PCR amplification of the *gro*El and *tcp*A genes from seawater samples spiked with *V. cholerae.* Positive results have been confirmed on agarose gels, also on some samples where no CP value was registered. The numbers of specific PCR products are compared to the total numbers of parallels analyzed.

V. cholerae	*gro*El		*tcp*A	
Cells/100 ml	Positive result	Average cp	Positive result	Average cp
18 ± 2	3/6	>40	4/6	-
20 ± 4	2/6	-	1/6	40
20 ± 4 *	5/6	39	4/6	39
50 ± 11	3/6	40	6/6	40
88 ± 10	6/6	38	5/6	>40
90 ± 20	3/6	40	1/6	40
180 ± 22	6/6	36	6/6	37
880 ± 110	6/6	34	6/6	36

* 400 ml sample was filtrated

3.5.2 PCR detection of *V. cholerae* in enriched brackish water

The PCR detection limit of *V. cholerae* in EBW was also investigated. EBW was spiked with five different concentrations of *V. cholerae* (20 ± 6, 50 ± 14, 100 ± 28, 200 ± 56, and 1000 ± 280 cells/100ml), filtered on nitrocellulose filters and extracted using NucliSens extraction kit. None of the concentrations gave PCR amplification in all six parallels. The most successful PCR detection was by amplifying of the *gro*El and *tcp*A genes from the sample with the highest concentration of *V. cholerae* cells. The detection limit was thus 1000 ± 280 cells per 100 ml. See Table 3-4 for results. All PCR products where confirmed on agarose gels, see Appendix F.

Table 3-4: PCR amplification of the *gro*El and *tcp*A gene from enriched brackish water spiked with *V. cholerae.* Positive results have been confirmed on agarose gels, also on some samples where no CP value was registered. The numbers of specific PCR products are compared to the total numbers of parallels analyzed.

V. cholerae	*gro*El		*tcp*A	
Cells/100 ml	Positive result	Average cp	Positive result	Average cp
20 ± 6	1/6	40	1/6	40
20 ± 6*	1/6	-	2/6	40
50 ± 14	2/6	40	2/6	40
100 ± 28	1/6	40	2/6	40
200 ± 56	3/6	40	3/6	40
1000 ± 280	5/6	39	5/6	38

* 400 ml sample was filtrated

3.6 NASBA on V. cholerae

Previous experiments with detection of *V. cholerae* using NASBA reported a detection limit of 50 CFU/ml (10). Experiments were conducted recreate this detection and to investigate if NASBA could be used in the detection of *V. cholerae* in environmental samples.

Several dilutions of a pure culture of *V. cholerae* cells extracted with NucliSens extraction kit were analyzed with NASBA amplifying the *gro*El and *tcp*A genes. The effect of nucleic acids extracted from seawater was also tested by isolating DNA and RNA from SW spiked with high concentration of *V. cholerae* cells. See Figure 3-17 for amplification curves. Concentrations of *V. cholerae* cells lower than 10^5 CFU per ml for the *tcp*A gene amplification and 10^3 CFU per ml for the *gro*El gene amplification were not amplified using NASBA. Amplification of RNA extracted from spiked SW samples had lower detection limits.

Attempts were done to amplify *gro*El from nucleic acid extracted with optimized Boström's method using NASBA, but with negative results. No improvements were obtained with samples pre treated with RNA-protect.

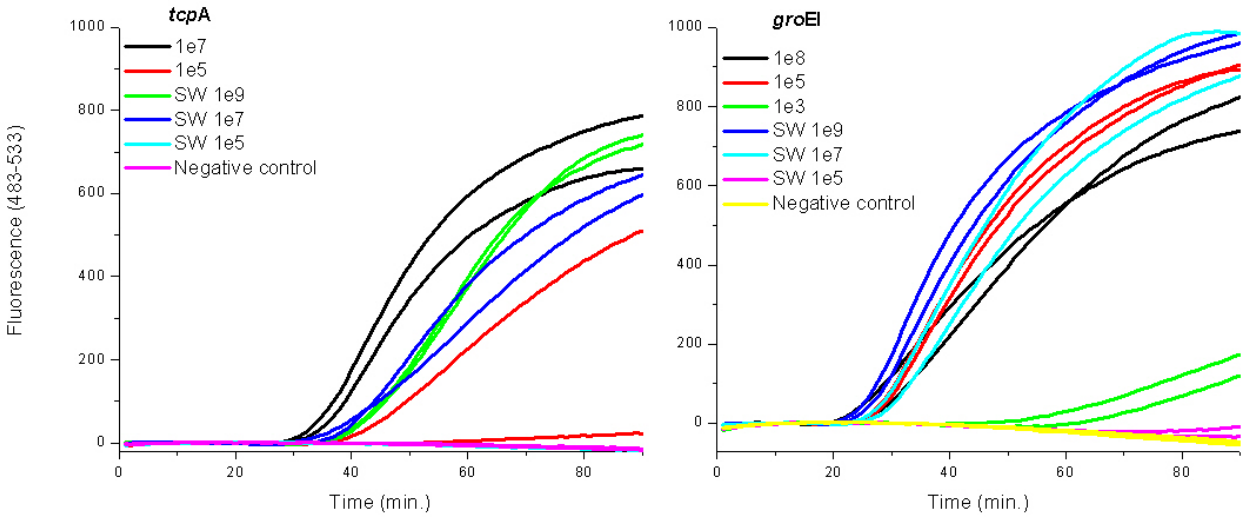

Figure 3-17: NASBA of the *gro*El and *tcp*A gens from DNA/RNA extracts of pure culture *V. cholerae* and seawater (SW) spiked with *V. cholerae.*

4 Discussion

Experiments were conducted to establish a PCR protocol for detection of *V. cholerae*. The efficiency of four different target genes in *V. cholerae* were investigated to choose the two most efficient.

Two different methods for DNA extraction from environmental water samples were optimized and compared for SW and EBW samples. The presence of PCR inhibitors was examined. The NucliSens extraction kit was proved to be the best extraction method for EBW, and the optimized Boström's method was the best extraction method for SW, giving the best PCR detection limit for *V. cholerae.* The PCR detection limit for *V. cholerae* in SW and EBW was found.

NASBA was investigated as a possible alternative to PCR detection. The same target genes and probes were used. NASBA proved not to be as efficient as PCR in the detection of *V. cholerae.*

4.1 Choice of PCR primers

The *V. cholerae* target genes *gro*El and *tcp*A were chosen based on the results of the PCR standard curves, PCR detection limits and the primers sensitivity towards PCR inhibitors. However, the difference in efficiency for the primers targeting the *gro*El, *tcp*A, *ctx*A and *tox*R genes were not prominent, see Table 3-5 and Figure 3-6. The only target gene standing out as less efficient than the other is *ctx*A, because of the sensitivity towards inhibitors in environmental water samples (Figure 3-8). The *tox*R gene could probably be used as target with almost similar results as *gro*El, but only two target genes were chosen to detect and distinguish between toxicogenic and non toxicogenic *V. cholerae*.

4.2 Challenges with DNA extraction

The two extraction methods tested in these experiments were chosen because they were not limited by the sample size and had no obvious extraction steps that would result in loss of DNA. The DNeasy Blod and Tissue Kit used in the preliminary experiments were ruled out as a possible method for extracting DNA from environmental samples because of the limited

membrane area for DNA adsorption. Several of the other kits considered also had limitations of sample size, or included extraction with organic solvents resulting in loss of DNA.

Upon extraction of DNA from SW, samples extracted with the optimized Boström's method showed more efficient PCR amplifications than samples extracted with the NucliSens extraction kit (Figure 3-12). Since no PCR inhibitors were found in either of these samples one can conclude that the optimized Boström's method gave a higher yield of DNA than the Nuclisens extraction kit. However, when EBW was analyzed the samples extracted with NucliSens had the best result (Figure 3-13). This can be explained by the evident presence of PCR inhibitors in the samples extracted using the optimized Boström's method (Figure 3-14). Based on these findings, it was decided to use the NucliSense extraction kit for EBW samples and the optimized Boström's method for SW samples. The combined NucliSens lysis and Boström's extraction showed variable results in both experiments (Figure 3-12 and 3-13) and was therefore not investigated further.

Ballast water from different ships will have different concentrations of PCR inhibitors, and one optimal DNA extraction method for all water types would be preferred. If the NucliSens extraction kit is to be used, a larger sample volume could be applied to increase the yield of DNA and thus lower the PCR detection limit. Another option is to investigate the effect of using larger elution volumes when eluting DNA from the silica particles and concentrating the DNA by ethanol precipitation.

If the optimized Boström's method should be used to extract DNA from environmental water samples, a PCR inhibitor test should also be performed. In general, a PCR inhibitor control (internal control) should be included in PCR analysis of environmental samples.

4.3 Characterization and managing of the PCR inhibitors

In the experiments using unwashed cells from environmental samples added to the PCR reaction mixture, samples added BW and EBW were equally inhibited (Figure 3-7). This indicates that salts or other dissolved chemicals were the cause of inhibition of PCR amplification, and not the organic matter and organisms added to BW to give EBW. In the further experiments the environmental samples were either washed in PBS or filtrated, removing the dissolved chemicals.

The preliminary inhibitor tests for all gene targets showed no inhibition except for PCR amplification of the *ctx*A gene (Figure 3-8). However, concentrated environmental samples were not applied in these experiments. When the samples were concentrated by filtration, the PCR inhibitors from the enriched samples became a problem (see Figure 3-11, 3-14 and 3-16). PCR amplification of DNA extracted from an 8.0 µm isopore filter showed more inhibition than DNA extracted from the filtrate from the 8.0 µm filter filtered on a 0.22 µm nitrocellulose filter (Figure 3-16). Thus, the particles with size range over 8.0 µm seem to be responsible for most of the PCR inhibition in the EBW samples. Nevertheless, a pre filtration is not to be recommended because V. cholerae is reported to attach to plankton (24).

The PCR inhibition tests conducted on EBW samples extracted using optimized Boström's method showed total inhibition of the PCR amplifications (Figure 3-14). Nevertheless, two of six parallels were positive for amplification of the groEl gene (Figure 3-13). Samples extracted using NucliSens lysis and Boström's extraction were all positive for amplification of the *gro*El gene (Figure 3-13), but totally inhibited the PCR amplification of lamda in the inhibitor test. Both inhibitor tests are conducted by adding a high concentration of target DNA to the PCR reaction mix, and it seems that a high concentration of nucleic acids enhances the PCR inhibition. Non-target DNA has been reported to inhibit PCR amplification (25), hence it is probable that the PCR inhibitors in the samples are DNA and other polymers with properties resembling those of DNA. On the other hand, samples extracted using the NucliSens extraction kit showed no sign of inhibitors. Thus, non-target DNA is probably not the only inhibitor. The fact that the inhibitors loose their effect upon dilution of the DNA samples supports the theory that the inhibition is due to polymerase inhibitors, and not failure of cell lysis or degradation of DNA.

Several methods can be explored for removing the inhibitors by new purifications steps, including solvent extraction or ion-exchange chromatography (30). This, however, will result in loss of some target DNA. Another option is to add components that can inactivate the inhibitors, such as bovine serum albumin (BSA) or polyvinylpolypyrrolidone (PVPP) (30). The last option is to dilute the samples. Dilutions of the samples reduce the amount of DNA in the samples; hence an addition of components that can inactivate the inhibitors is preferred. Due to time restrains the effect of such components has not been investigated.

4.4 Variations in detection limits

When the detection limit of *V. cholerae* in SW was investigated, the results of the separate experiments varied. Table 3-3 shows variation of success of detection for samples with approximately the same concentration: 88 ± 10 and 90 ± 20 cells/100ml. The same variation is not seen in Table 3-4 where all samples are from the same experiment. The variations may be due to inexact counting of cells used to calculate the cell concentrations, sampling errors, errors from serial dilutions or errors in the extraction procedures. Samples from the same experiments show a correlation between success of detection and concentration of *V. cholerae* (Table 3-3 and 3-4), so the error is probably linked to the *V. cholerae* cell concentrations prior to spiking the environmental samples.

Another factor that affects the variability of the detection limits are whether the concentration of V. cholerae is reported in CFU or cells. The requirements of *V. cholerae* concentration in ballast water is stated as less than 1 CFU per 100 ml, thus a comparison of detected cells and CFU should be performed. Because PCR detects DNA regardless of whether it comes from viable or dead cells, all the results have been related to the cell concentration of *V. cholerae* and not CFU.

Huge variation in CFU/cell ratio for *V. cholerae* cultures was observed. The CFU/cell ratios plotted for each experiment are distributed randomly around the average ratio of 7±2 % represented by the red line in Figure 3-1. This makes it difficult to relate the detection limit of the spiked environmental samples given in cells/ml to a concentration in CFU/ml. However, the ratio between CFU and cells in a ballast water tank might have larger variations than those experienced in a pure lab culture, grown at optimal growth conditions for V. cholerae.
On the other hand, the need for knowing the CFU concentration might be questioned. The probable reason why the legal concentration limits of organisms are given as CFU is that the conventional method of detecting bacteria is growth methods. The conventional growth methods, however, have their limitations: they only detect bacteria able to grow under the conditions in the laboratory. Cells not able to grow under these conditions are either dead or in a state called viable but non-culturable(VBNC) (12). Experiments conducted have shown that culturable *V. cholerae* can be detected in the stool of volunteers after ingesting VBNC *V. cholerae* cells (6). It is thus essential to also detect these cells.

4.5 Failure of NASBA on V. cholerae

NASBA has the potential of distinguishing between viable and dead cells and thus has an advantage compared to PCR. However, the detection limit of NASBA experienced in these trials were much higher than those reported by Fykse *et al* (2007) using the same protocol and primers on pure cultures of V. cholerae.

SW had a negative effect on the NASBA-analysis even if no inhibitors were detected using PCR on the same samples. One probable reason for the unsatisfying NASBA detection is loss of RNA during extraction of RNA from filters. However, this does not explain the poor detection limit of V. cholerae in pure culture obtained in these experiments. The only obvious difference from the procedures described by Fykse *et al.* (2007) is a small modification of the NASBA kit from Biomérieux: A preservative was added to the reagents in the kit, which makes it able to be stored in a refrigerator in stead of a freezer. This might have affected the optimal KCl concentration for the NASBA reaction, and there was no time to do preliminary experiments to optimize this concentration.

To investigate if the lack of RNA or failure in NASBA was the main problem, a Reverse Transcriptase-PCR could be performed.

4.6 Can a detection limit of 1 CFU per 100 ml be obtained by a nucleic acid amplification method?

A detection limit of 10 cells/ml (1000 ± 280 cells/100ml) in EBW and 2 cells/ml (180 ± 22 cells/100ml) for SW was demonstrated in these experiments. When using an average CFU/cell ratio of 7%, the estimated detection limits would be 70±20 CFU per 100ml EBW and 13±2 CFU per 100ml SW. However, in the experiments with different DNA extraction methods (Figure 3-10), 36 cells/100 ml (2.5 CFU/100 ml) in SW and ESW were amplified using PCR in all six parallels. The CFU/cell ratio in ballast water is believed to be lower than in pure culture because *V. cholerae* often enters a VBNC state in environmental waters. Hence a concentration of 1 CFU of *V. cholerae* per 100 ml real ballast water could be detected using the methods described in this thesis. A reliable method is however needed.

The intention of using nucleic acid amplification for detection of *V. cholerae* was, however, to establish a detection method superior to the conventional growth methods. The method should detect all viable cells, including VBNC cells. The current problem with the PCR methods described here is that DNA from dead cell might be detectable by PCR several days after lysis. Dead cells can, however, be separated from viable cells using ethidium bromide monoazide (EMA) by cross-linking with DNA (27). Another challenge is to improve the DNA extraction methods to give a lower detection limit. Since two cells per reaction was amplified in all parallels in the preliminary experiments (Figure 3-9), the efficiency of the PCR reaction was not the limiting factor when finding the detection limit in environmental water samples. Extracts of all the spiked environmental samples tested could in theory contain DNA from more than two cells per reaction if the extraction method was without loss.

A better detection limit than 1 CFU per 100 ml is feasible with nucleic acid amplification methods by improving some of the factors mentioned above.

5 Conclusion

In the preliminary studies, a PCR protocol for amplification of *V. cholerae* genes was established. Two DNA extraction methods were investigated. One method was optimized for large water samples, resulting in a high yield of DNA. This method (the optimized Boström's method) was preferred when extracting DNA from seawater. The other method (the NucliSens extraction kit) did not give the same high yield of DNA, but was more efficient for removing PCR inhibitors from enriched brackish water.

Based on these findings, procedures for detecting *V. cholerae* in two different environmental samples were established. The detection limit of *V. cholerae* found in seawater was approximately 2 cells/ml (180 ± 22 cells/100ml), and approximately 10 cells/ml (1000 ± 280 cells/100ml) in enriched brackish water.

The procedures established have the potential to be further improved regarding detection limit and application area.

Reference list

1. **Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J.Clin.Microbiol. **28:**495-503.
2. **Boström, K. H., Hagström, L. Riemann, and K. Simu.** 2004. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Limnology and oceanography:methods **2:**365-378.
3. **Botnen, H., and A. Jelmert.** 2002. Ballastvann-en fare for det norske havmilj›et?, p. 78-81, Havets milj› 2002.
4. **Broude, N. E.** 2002. Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology. Trends in Biotechnology **20:**249-256.
5. **Burkholder, J. M., G. M. Hallegraeff, G. Melia, A. Cohen, H. A. Bowers, D. W. Oldach, M. W. Parrow, M. J. Sullivan, P. V. Zimba, E. H. Allen, C. A. Kinder, and M. A. Mallin.** 2007. Phytoplankton and bacterial assemblages in ballast water of US military ships as a function of port of origin, voyage time, and ocean exchange practices. Harmful Algae **6:**486-518.
6. **Colwell, R. R., P. Brayton, D. Herrington, B. Tall, A. Huq, and M. M. Levine.** 1996. Viable but non culturable Vibrio cholerae 01 revert to a cultivable state in the human intestine. World Journal of Microbiology & Biotechnology **12:**28-31.
7. **Compton, J.** 1991. Nucleic-Acid Sequence-Based Amplification. Nature **350:**91-92.
8. **Drake, L. A., M. A. Doblin, and F. C. Dobbs.** 2007. Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm. Marine Pollution Bulletin **55:**333-341.
9. **Fuhrman, J. A., D. E. Comeau, Hagstr”m, and A. M. Chan.** 1988. Exctraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Applied and Environmental Microbiology **54:**1426-1429.
10. **Fykse, E. M., G. Skogan, W. Davies, J. S. Olsen, and J. M. Blatny.** 2007. Detection of Vibrio cholerae by real-time nucleic acid sequence-based amplification. Applied and Environmental Microbiology **73:**1457-1466.
11. **Hallegraeff, G. M.** 2007. Ballast water - Foreword. Harmful Algae **6:**461-464.
12. **Huq, A., and R. R. Colwell.** 1996. A microbiological paradox: Viable but nonculturable bacteria with special reference to vibrio cholerae. Journal of Food Protection **59:**96-101.
13. **Huq, A. G., C.; Colwell, R. R.; Nair, G. B.** 2006. Detection, Isloation, and Identification of *Vibrio cholerae* from the environment, Current protocols in microbiology. John Whiley & Sons.
14. **IMO.** 2004. International conventrion for the control and managment for ship's ballast water and sediments, 2004, International conference on ballast water management for ships.
15. **Kaper, J. B., J. G. Morris, and M. M. Levine.** 1995. CHOLERA. Clinical Microbiology Reviews **8:**48-86.
16. **Knowler, D.** 2005. Reassessing the costs of biological invasion: Mnemiopsis leidyi in the Black sea. Ecological Economics **52:**187-199.
17. **Li, Y. S., X. Y. Zhou, and D. Y. Ye.** 2008. Molecular beacons: An optimal multifunctional biological probe. Biochemical and Biophysical Research Communications **373:**457-461.
18. **McCarthy, S. A., and F. M. Khambaty.** 1994. International Dissemination of Epidemic Vibrio-Cholerae by Cargo Ship Ballast and Other Nonpotable Waters. Applied and Environmental Microbiology **60:**2597-2601.

19. **OCHA.** 2009. Cholerae situation in Zimbabwe, http://ochaonline.un.org/CholeraSituation/tabid/5147/language/en-US/Default.aspx 01.06.2009 [online]
20. **Oxoid.** 2001. Cholera medium TCBS. http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0333&org=146&sec=2&c=UK&lang=EN 10.05.2009 [online]
21. **Padilla, D. K., M. A. Chotkowski, and L. A. J. Buchan.** 1996. Predicting the spread of zebra mussels (Dreissena polymorpha) to inland waters using boater movement patterns. Global Ecology and Biogeography Letters **5:**353-359.
22. **Reece, R. J.** 2004. Analysis of Genes and Genomes. Wiley, West Sussex.
23. **Sack, D. A., R. B. Sack, G. B. Nair, and A. K. Siddique.** 2004. Cholera. The Lanchet **363:**223-233.
24. **Seas, C., E. Gotuzzo, G. L. Mandell, J. E. Bennett, and R. Dolin.** 2005. Vibrio cholerae, p. 2536-2544, Principles and practice of infectious diseases, vol. 6th. Elsevier, Philadelphia.
25. **Tebbe, C. C., and W. Vahjen.** 1993. Interference of hunic acids and DNA extracted directly from soil in detection and transformation of recombinant-DNA from bacteria and a yeast. Applied and Environmental Microbiology **59:**2657-2665.
26. **Wagningen, U. R.** 2009, posting date. NASBA Principle. [Online.]
27. **Wang, S. S., and R. E. Levin.** 2006. Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. Journal of Microbiological Methods **64:**1-8.
28. **WHO.** 2008. Epidemic and Pandemic Alert and Response, http://www.who.int/csr/don/2008_12_02/en/index.html 02.12.2008 ed. World Health Organization. [online]
29. **Wilhelm, J., and A. Pingoud.** 2003. Real-time polymerase chain reaction. Chembiochem **4:**1120-1128.
30. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology **63:**3741-3751.
31. **Zhang, T., and H. H. P. Fang.** 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology **70:**281-289.

Appendix A Mediums and agar

Liquid cultures of *Vibrio cholerae* were grown in Thryptone Soya Broth (TSB) (Oxoid). The medium was prepared according to the guidelines from the manufacturer: 30 g powder was dispersed in one litre distilled water and autoclaved at 121˚C for 20 min.

V. cholerae was plated on Thryptone Soya Broth Agar (TSA) made by dispersing 30 g of Thryptone Soya Broth (Oxoid) powder and 12-15g Microbiology Agar-Agar (Merck) in one litre of distilled water. The dispersion was autoclaved at 121˚C for 20 min and cooled down to 60˚C before pouring out on plates.

For total marine bacteria count Marine Agar (Conda) was used. The agar plates were prepared according to the manufacturer: 55.1g powder was dispersed in one litre of distilled water and autoclaved at 121˚C for 20 min. The solution was cooled down to 60˚C before pouring out on plates.

To investigate if the samples contained *V. cholerae* the *V. cholerae* specific agar Thiosulfate Citrate Bile Sucrose Agar (TCBS) (Oxoid) was used. The agar plates were prepared according to the manufacturer: 88g of powder was dissolved in one litre of distilled water and heated to boiling. The solution was cooled down to 60˚C before pouring out on plates.

Cell cultures were washed and diluted in Phosphate Buffered Saline (PBS) (Sigma) to protect the cells and prevent multiplying. PBS solution was prepared according to the manufacturer: 1 tablet was dissolved in 200 ml distilled water and autoclaved.

Appendix B Characterization of environmental samples

B.1 Requirements for ballast test water

The environmental water samples provided by the Norwegian Institute for Water Research, NIVA, was prepared to meet the regulations for test waters used in ballast water management tests stated in Annex 3 of Resolution MEPC.125(53), adopted on 22 July 2005: Guidelines for approval of ballast water management systems (G8).

Below is the preparation of test waters preformed at NIVA described by Ingun Tryland (personal communication):

Test waters will be prepared in a 516 m^3 tank (WST) from high salinity sea water from 60 meters depth or from brackish surface water depending on the required salinity (>32 PSU or 3-32 PSU, respectively, with a minimum difference of 10 PSU). In the lower salinity range 22 PSU is envisaged. If necessary, fresh water from a surface water source or groundwater wells will be added to adjust the salinity. The 516 m^3 of test water will be used for both testing and control. A combination of harvested indigenous organisms and cultured surrogate species (>50 µm: *organism A* ; 10-50 µm: *organism B*) will be added to fulfil the biological water quality criteria (see Table B-1), and freshwater, chemical X, chemical Y and chemical Z will be added to adjust the salinity and the contents of dissolved organic carbon (DOC), particulate organic carbon (POC) and total suspended solids (TSS), respectively, to within the limits of chemical water quality criteria (see Table B-2).

Table B-1: Required biological water quality in influent and in control and treated test water after 5 days storage according to G8 (section 2.3.19 and 2.3.35) and regulation D-2 (BWM Convention).

Organism group	Influent water	In control after 5 days storage	In treated on discharge (Regulation D-2)
≥50 µm min. dimension	Pref. 10^6 m^{-3}, ≥10^5 m^{-3} Min. 5 species from 3 diff. phyla/divisions	>10x Regulation D-2	(<10) viable organisms per m^3
≥10-50 µm min. dimension	10^4 ml^{-1}, ≥10^3 ml^{-1} Min. 5 species from 3 diff. phyla/divisions	>10x Regulation D-2	(<10) viable organisms per ml
Heterotrophic bacteria	≥10^4 cfu ml^{-1}	-	-
Escherichia coli	-	-	< 250cfu/100 ml
Intestinal *enterococci*	-	-	<100 cfu/100 ml
Vibrio Cholerae	-	-	<1 cfu/100 ml

Table B-2: Required chemical water quality of test waters before any additional test organisms have been added. Salinities should be separated by at least 10 PSU.

	Salinity	DOC	POC	TSS
Test water 1	>32 PSU	>1 mg/l	>1 mg/l	>1 mg/l
Test water 2	3-32 PSU	>5 mg/l	>5 mg/l	>50 mg/l

B.2 Analysis of water samples

The chemical parameters of the water samples were tested to control that the water fulfilled the regulations. The results of the chemical analysis of some of the samples are given in Table B-3.

Table B-3: Chemical analysis of environmental water samples

Water sample	pH	Turbidity FNU	Suspended dry substance mg/l	Suspended Fixed solids mg/l	UV-transmittance % T	Total organic Carbon mg C/l	Dissolved organic Carbon mg C/l	Salinity g/L (per mill)
Test water 1								
ESW		7,31	12,0	3,4	96,6	4,5	1,9	32,7
ESW	8,02	6,38	13,0	5,0	97,7	4,5	1,8	32,4
ESW	8,02	6,44	14,3	4,3	97,9	4,4	1,8	33,7
SW	7,98	0,61	2,0	<0,8	97,4	1,4	1,2	33,7
Test water 2								
EBW	7,89	30,5	68,4	38,0	92,0	14,6	5,4	21,3
EBW	7,92	23,8	58,8	38,0	92,8	14,9	5,3	21,3
EBW	7,92	32,1	68,8	38,8	94,6	14,2	5,4	21,3
Bw	7,94	0,72	3,4	1,1	94,9	1,4	1,4	21,2

Water samples that were not used immediately were stored at 4°C. The bacterial content was monitored by plating on marine agar and TCBS agar. The colonies on the marine agar were counted after 2 days at room temperature, and the TCBS agars were inspected after 24 hours at 37°C. See Figure B-1 for pictures of marine agars, and Table B-4 for number of colonies registered.

Table B-4: CFU counts of environmental samples stored at 4°C.

	SW			BW			ESW			EBW		
Date	18/2			28/1			18/2			28/1		
Age [days]	0	1	14	0	1	34	0	1	14	0	1	34
Marine [CFU/ml]	238	10^2	$3x10^2$	99	10^4*	$3x10^3$	$2x10^4$	10^4	$1,3x10^5$	$3x10^4$	10^6*	$2x10^2$
TCBS [CFU/100ml]	4			1			$5x10^3$			$4x10^3$		
Date	4/3						4/3			11/3		
Age [days]	0	5					0	5	15	0	1	8
Marine [CFU/ml]	30	$6x10^2$					$2x10^4$	$4x10^4$		$2x10^4$		
TCBS [CFU/100ml]	1						$4x10^4$		10^3-10^4	$5,6x10^3$	$3x10^3$	$2x10^3$

*counted after 5 days instead of 2

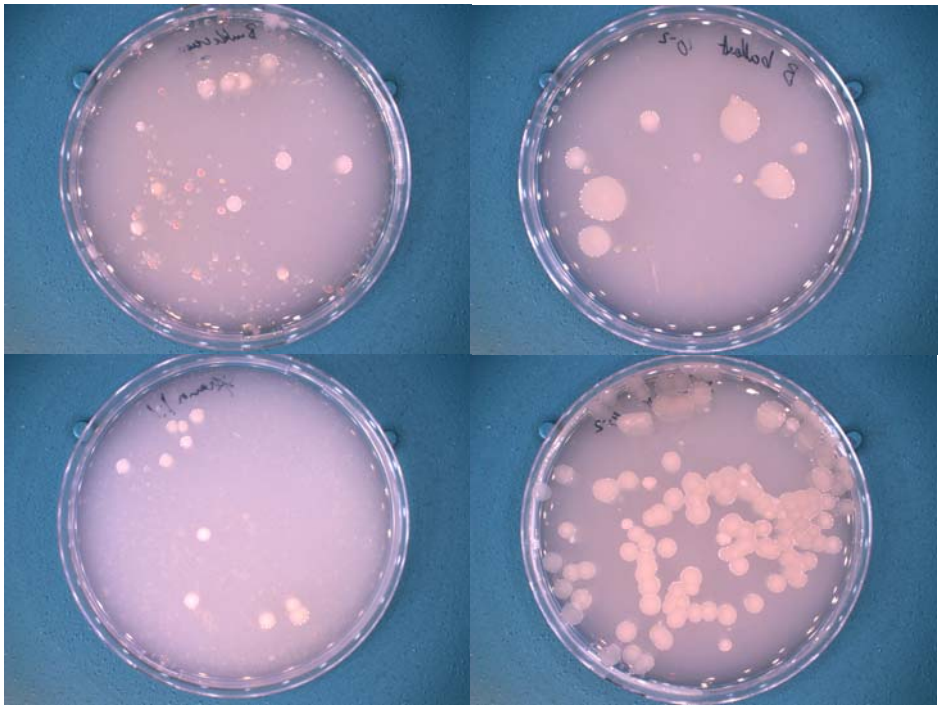

Figure B-1: First row left: BW, first row right: EBW diluted 1:100, second row left: SW, second row right: ESW diluted 1:100.

Appendix C Amplification and sequencing of the 16s ribosomal RNA gene

C.1 16s rRNA amplification

Bacteria from colonies of unknown taxonomy were added to PCR wells containing a PCR reaction mixture of LightCycler® 480 SYBR Green I Master (10 µl), 5 µM primer mix (4µl) and PCR-grade water (4 µl). The primer mix contained the primers Eub 933f and Eub 1387r from Table 2-2 targeting the 16 s ribosomal RNA gene in Eubacteria. PCR was run with the following protocol: Initial denaturising at 95˚C for 5 min, amplification cycles with 5 sec. at 95˚C, 5 sec. at 55˚C and 25 sec. at 72˚C, followed by a melt point analysis.

The PCR products were added 2 µl exoSAP-IT® (usb) per 5µl DNA to remove short DNA fragments and dNTPs. The mixture was incubated at 37˚C for15 min. followed by a inactivation of the enzymes at 80˚C for 15 min. The active enzymes in exoSAP-IT are exonuclease and Shrimp Alkaline Phospatase.

Some amplification was also observed in the controls only containing water. These samples were also prepared and sent to sequencing.

C.2 Sequencing

The purified PCR product was sequenced by Eurofins. The sequences where reviewed using the Staden package. The sequences were clipped, yielding the FASTA sequences listed below. An online Nucleotide BLAST search was performed on the sequences (http://blast.ncbi.nlm.nih.gov/)

The sample #4 and the negative control 2: matched 99-100% with a sequence in the bacteria *Pseudomonas sp.* The lineage of the organism is: Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;Pseudomonadaceae; Pseudomonas.

The samples #1-3, #5-8 and negative control 1 matched 100% with a sequence in the bacteria Exiguobacterium. The lineage of the organism is: Bacteria; Firmicutes; Bacillales; Bacillales Family XII. Incertae Sedis; Exiguobacterium.

C.3 FASTA sequences:

1. acttcatgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgcc ctttgtaccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggttt gtcaccggcagtctccttagagtgcccaactaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaaccca acatctcacgacacgagctgacgacaaccatgcaccacctgtcacccctgcccccgaaggggaaggtacatctctgtacc ggtcagggggatgtcaaga

2. acttcatgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgcc
ctttgtaccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggttt
gtcaccggcagtctccttagagtgcccaactgaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaaccc
aacatctcacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaagtacatctctgtacc
ggtcaggggatgtcaagag

3. tgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgccctttgta
ccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggtttgtcacc
ggcagtctccttagagtgcccaactgaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaacccaacatct
cacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaaggtacatctctgtaccggtcag
ggggatgtcaagag

4. agtcgagttgcagactgcgatccggactacgatcggttttctgggattagctcccctcgcggcttggcaaccctctgtaccga
ccattgtagcacgtgtgtagcccaggccgtaagggccatgatgacttgacgtcatccccaccttcctccggtttgtcaccggc
agtctccttagagtgcccaccattacgtgctggtaactaaggacaagggttgcgctcgttacgggacttaacccaacatctca
cgacacgagctgacgacagccatgcagcacctgtctcaatgttcccgaaggcaccaatctatctctagaaagttc

5. acttcatgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgcc
ctttgtaccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggttt
gtcaccggcagtctccttagagtgcccaactgaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaacccc
aacatctcacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaagtacatctctgtacc
ggtcaggggatgtcaagag

6. acttcatgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgcc
ctttgtaccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggttt
gtcaccggcagtctccttagagtgcccaactgaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaacccc
aacatctcacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaagtacatctctgtacc
ggtcaggggatgtcaagag

7. gacttcatgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgc
cctttgtaccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggtt
tgtcaccggcagtctccttagagtgcccaactaaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaaccc
aacatctcacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaaggtacatctctgtac
cggtcaggggatgtcaagag

8. tgcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgcccttgta
ccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggtttgtcacc
ggcagtctccttagagtgcccaactgaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaacccaacatct
cacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccgaaggggaagtacatctctgtaccggtcagg
gggatgtcaagag

Negative control 1:

gcaggcgagttgcagcctgcaatccgaactgagaacggctttctgggattggctccacctcgcggcttcgctgccctttgta
ccgtccattgtagcacgtgtgtagcccaactcataaggggcatgatgatttgacgtcatccccaccttcctccggtttgtcacc
ggcagtctccttagagtgcccaactaaatgctggcaactaaggacaagggttgcgctcgttgcgggacttaacccaacatct
cacgacacgagctgacgacaaccatgcaccacctgtcacccctgccccccgaaggggaaggtacatctctgtaccggtcag
ggggatgtcaagag

Negative control 2:

tgcagtcgagttgcagactgcaatccggactgacggtttttgggattagctcccctcgcggttggcaccctctgtaccgccatt
gtagcacgtgtgtagcccaggctaagggccatgatgacttgacgtcatccccaccttcctccggtttgtcaccggcagtctcc
ttagagtgcccaacatta

Appendix D Solutions for DNA extraction

- Lysis buffer:
  - 400 mM NaCl (Merck)
  - 750 mM Sucrose (BHD AnalaR®)
  - 20 mM EDTA (EDTA Disodium salt dihydrat, Calbiochem)
  - 50 mM Tris-HCl pH 9,0 (Trizma® base, Sigma. Aadjusted pH with HCl)

- Lysozyme stock: 50 mg/ml Lysozyme from chicken egg white (47700u/mg, L6876, Sigma-Aldrich)

- Sodium dodecyl sulfate (SDS) stock: 10 % SDS (Bio-Rad)

- Proteinase K : 20 µg/µl (Qiagen)

- TE buffer:
  - 10 mM Tris-HCl pH 8,0 (Trizma® base, Sigma)
  - 0,1 mM EDTA (Calbiochem)

- NaAc 3 M (Merck)

- Baker's yeast tRNA (Roche Applied Science, nr 109 509) 50µg/50ml

Appendix E PCR standard curves

PCR amplification of serial dilutions of DNA and cell extracts from *V. cholerae* were performed using SYBR green I and molecular beacons. Standard curves were made for four target genes.

E.2 Molecular Beacons and extracted DNA

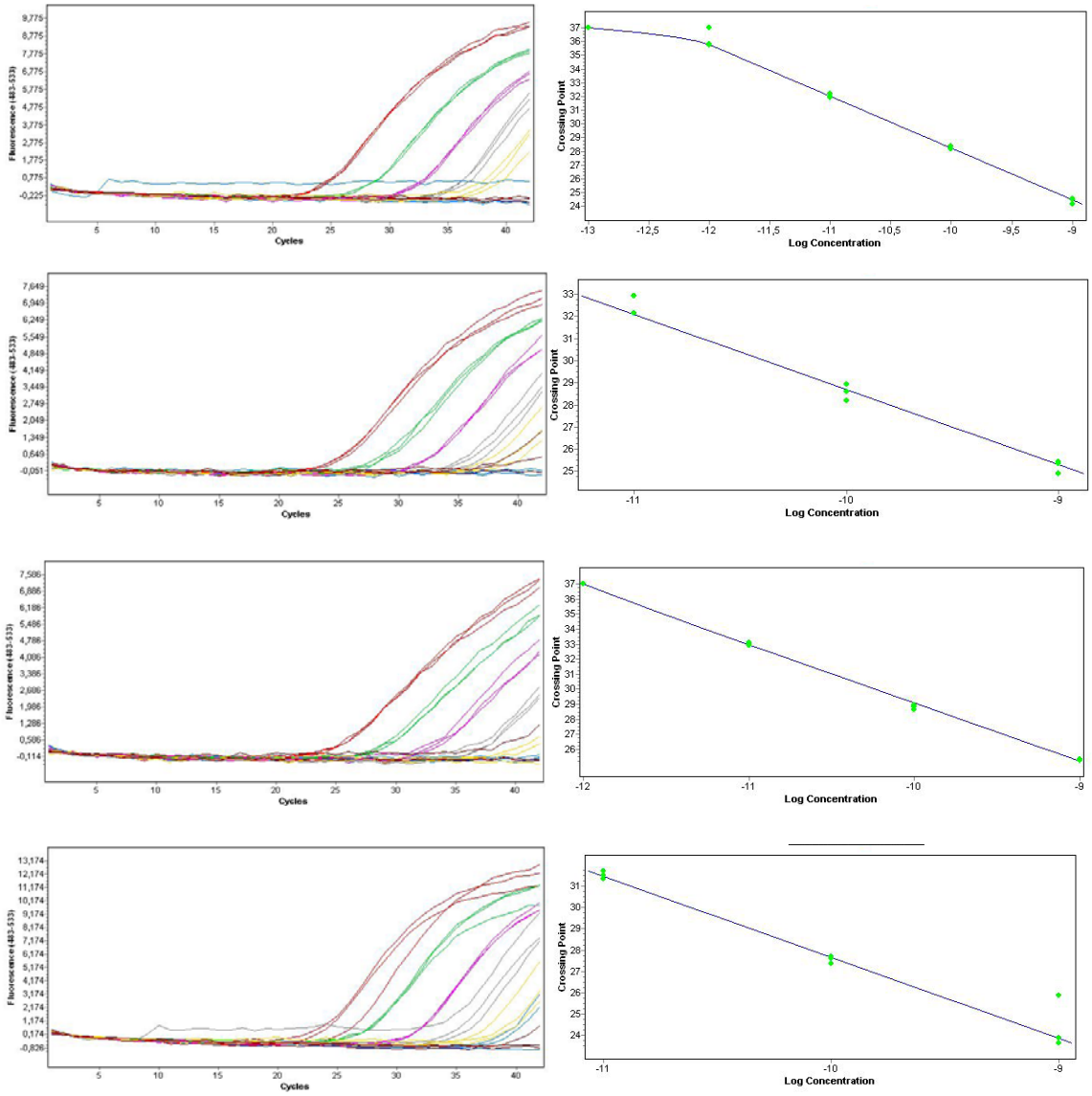

Figure E-1: PCR with molecular beacons run with the primers targeting (from the top) the *gro*El, *tcp*A, *ctx*A and *tox*R genes. The template concentrations are 1 ng (–), 100 pg (–), 10 pg (–), 1 pg (–), 100 fg (–) and 10 fg(–) extracted DNA. The properties of the standard curves are given in Table E.1.

Table E-1: Properties of standard curves for PCR amplification of extracted DNA from *V. cholerae* analyzed using MB.

Target gene	*gro*El	*tcp*A	*ctx*A	*tox*R
Error	0.00320	0,0355	0,0183	0,0233
Efficiency	1.843	1,970	1,812	1,834
Slope	-3.766	-3,396	-3,874	-3,796
Y Intercept	-4.354	-5,273	-10,11	-10,30

E.2 SYBR green I and extracted DNA

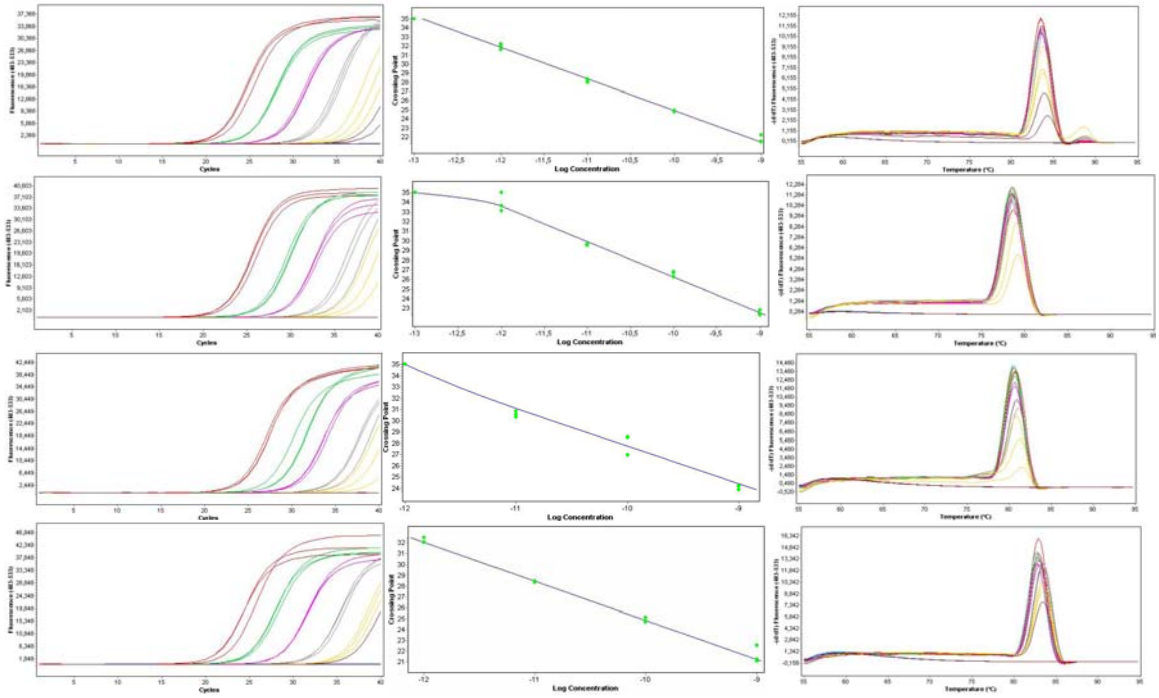

Figure E-2 PCR with SYBR green I run with the primers targeting (from the top) the *gro*El, *tcp*A, *ctx*A and *tox*R genes. The template concentrations are 1 ng (–), 100 pg (–), 10 pg (–), 1 pg (–), 100 fg (–) and 10 fg(–) extracted DNA. The properties of the standard curves are given in Table E-2.

Table E-2: Properties of standard curves for PCR amplification of extracted DNA from *V. cholerae* analyzed using SYBR green I.

Target gene	*gro*El	*tcp*A	*ctx*A	*tox*R
Error	0.0233	0,0288	0,0731	0,000733
Efficiency	1.939	1,871	1,995	1,894
Slope	-3.478	-3,676	-3,333	-3,605
Y Intercept	-9.201	-5,892	-6,726	-11,21

E.3 Molecular Beacons and cell extract

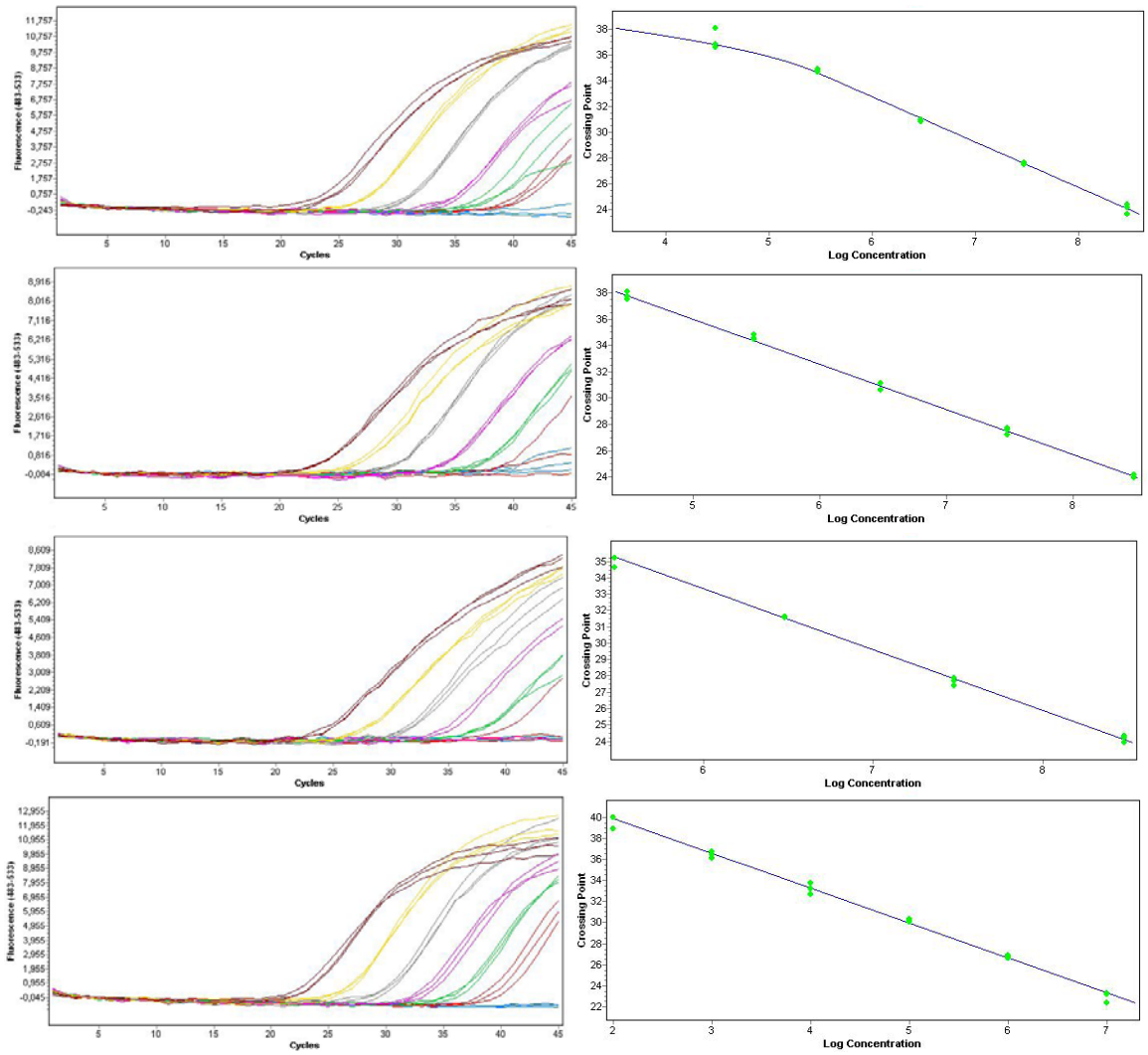

Figure E-3: PCR with molecular beacons run with the primers targeting (from the top) the *gro*El, *tcp*A, *ctx*A and *tox*R genes. The template concentrations are 10^2 (–), 10^3 (–), 10^4 (–), 10^5 (–), 10^6 (–) and 10^7 (–) CFU/ml. The properties of the standard curves are given in Table E-3.

Table E-3: Properties of standard curves for PCR amplification of extracted cells of *V. cholerae* analyzed using MB.

Target gene	*gro*El	*tcp*A	*ctx*A	*tox*R
Error	0,0166	0,0209	0,0279	0,0156
Efficiency	1,928	1,957	1,857	2,003
Slope	-3,508	-3,431	-3,719	-3,315
Y Intercept	51,84	53,02	54,78	46,60

E.4 SYBR green I and cell extract

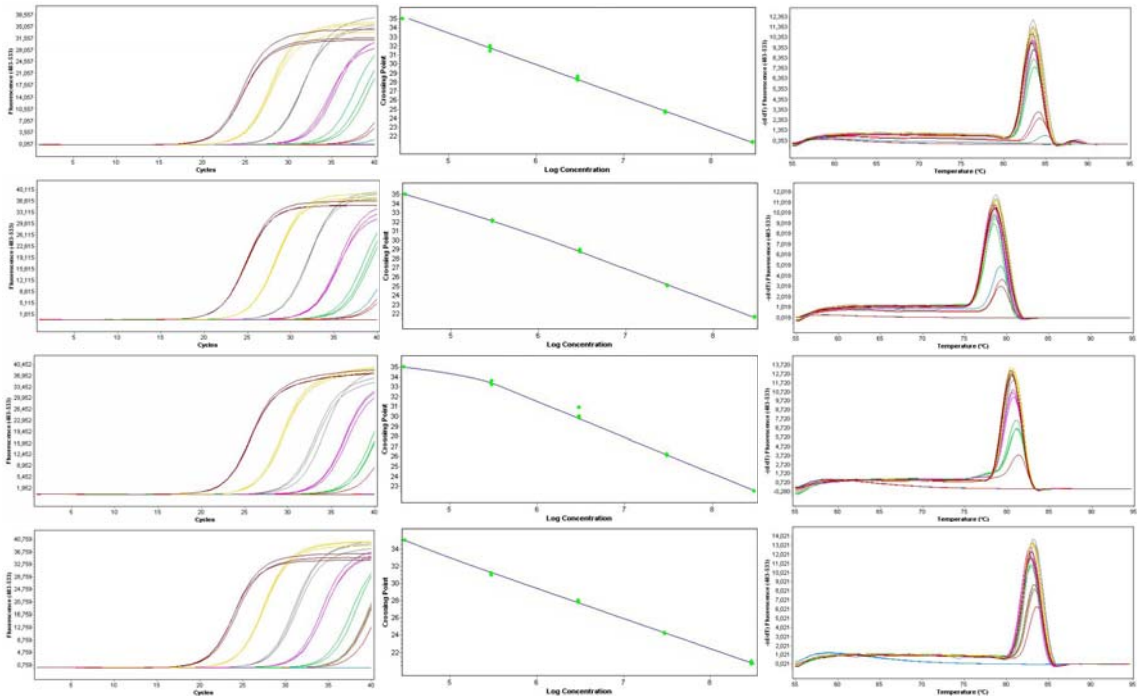

Figure E-4: PCR with SYBR green I run with the primers targeting (from the top) the *gro*El, *tcp*A, *ctx*A and *tox*R genes. The template concentrations are 10^2 (–), 10^3 (–), 10^4 (–), 10^5 (–), 10^6 (–) and 10^7 (–) CFU/ml. The properties of the standard curves are given in Table E-4.

Table E-4: Properties of standard curves for PCR amplification of extracted cells of *V. cholerae* analyzed using SYBR green I.

Target gene	*gro*El	*tcp*A	*ctx*A	*tox*R
Error	0,0162	0,00757	0,0151	0,0145
Efficiency	1,934	1,900	1,890	1,933
Slope	-3,490	-3,587	-3,617	-3,494
Y Intercept	50,49	50,37	50,18	50,76

Appendix F Agarose gels

The PCR products from amplification of the *gro*El and *tcp*A genes from environmental samples spiked with *V. cholerae* were analysed on agarose gels.

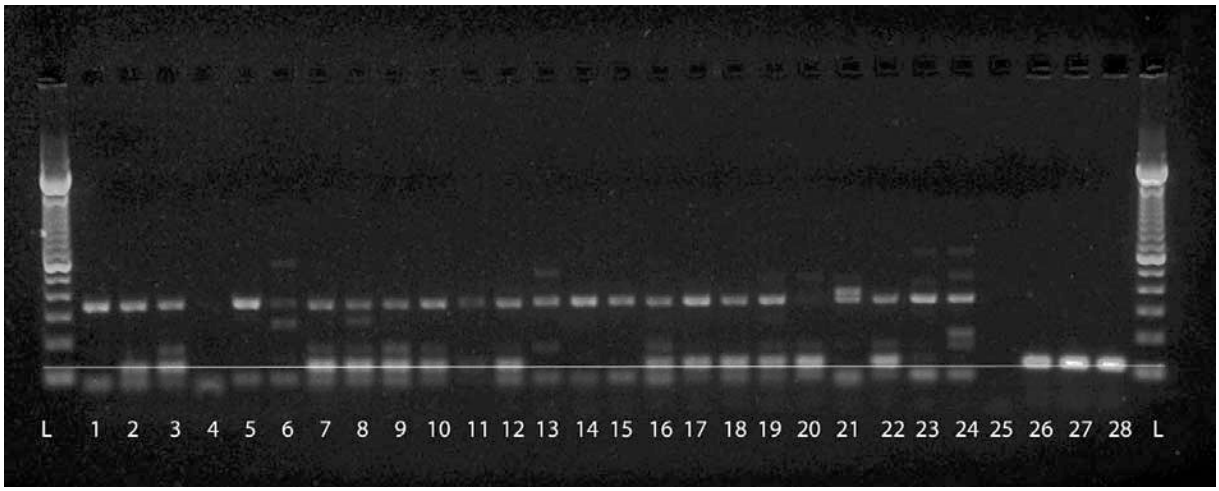

Figure F-1: PCR products of *gro*El amplifications from SW samples. The lanes named L contain a 100 bp DNA ladder. The remaining lanes contain the samples with the following concentration of spiked *V. cholerae* given in cells/100 ml: #1-6: 20, #7-12: 20 (400 ml sample), #13-18: 50, #19-24: 90, #25: negative control, #26-28: positive control

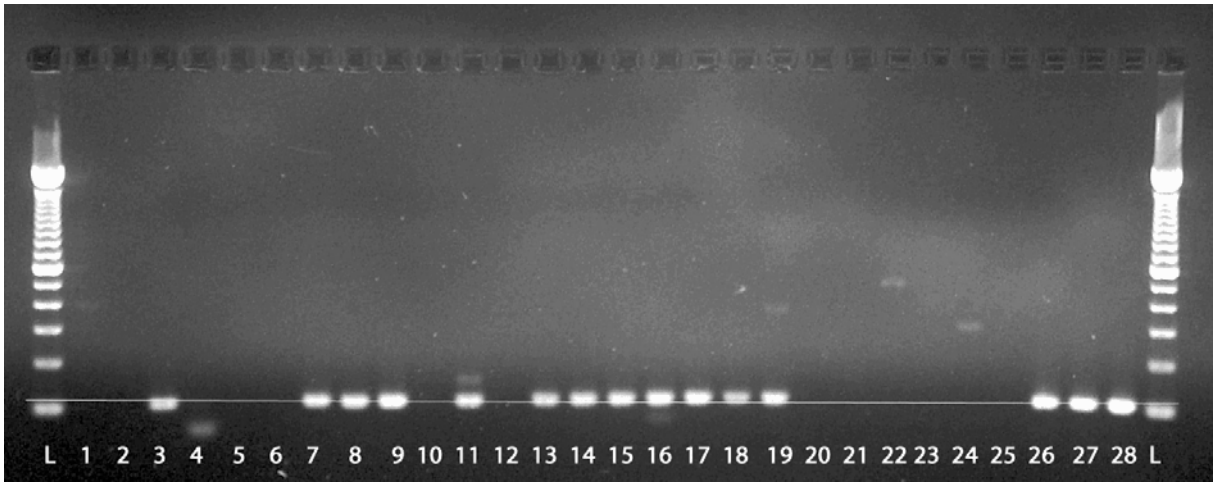

Figure F-2: PCR products of *tcp*A amplifications from SW samples. The lanes named L contain a 100 bp DNA ladder. The remaining lanes contain the samples with the following concentration of spiked *V. cholerae* given in cells/100 ml : #1-6: 20, #7-12: 20 (400 ml sample), #13-18: 50, #19-24: 90, #25: negative control, #26-28: positive control

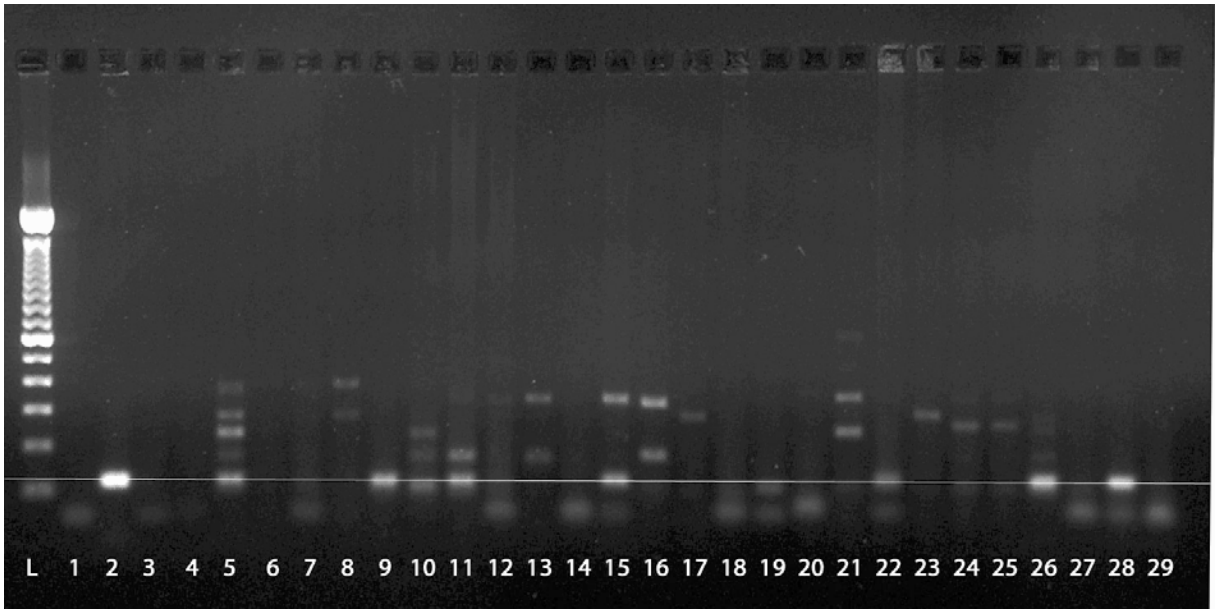

Figure F-3: PCR products of *gro*El amplifications from EBW samples. The lane named L contains a 100 bp DNA ladder. The remaining lanes contain the samples with the following concentration of spiked *V. cholerae* given in cells/100 ml: #1: negative control, #2: positive control, #3-5: 20, #6-9: 20 (400 ml sample), #10-15: 50, #16-21: 100, #22-27: 200, #28- 29: 1000

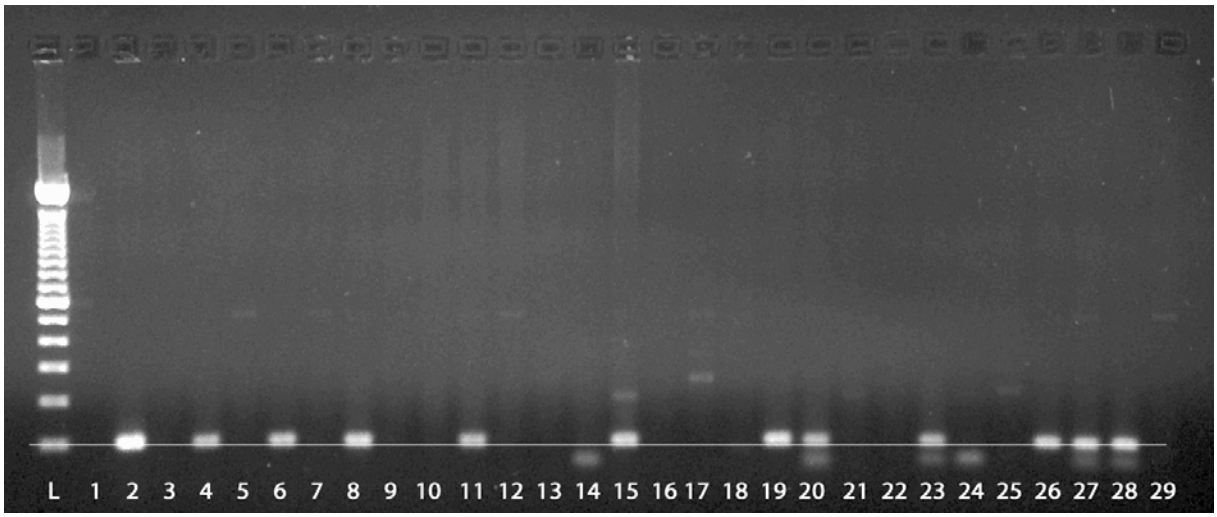

Figure F-4: PCR products of *tcp*A amplifications from EBW samples. The lane named L contains a 100 bp DNA ladder. The remaining lanes contain the samples with the following concentration of spiked *V. cholerae* given in cells/100 ml: #1: negative control, #2: positive control, #3-5: 20, #6-9: 20 (400 ml sample), #10-15: 50, #16-21: 100, #22-27: 200, #28-29: 1000