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Abstract: Transport of ballast water is one major factor in the transmission of aquatic organisms, including pathogenic bacteria. The IMO-guidelines of the Convention for the Control and Management of Ships' Ballast Water and Sediments, states that ships are to discharge < 1 CFU per 100 ml ballast water of toxigenic Vibrio cholerae, emphasizing the need to establish test methods. To our knowledge, there are no methods sensitive and rapid enough available for cholera surveillance of ballast water. In this study real-time PCR and NASBA methods have been evaluated to specifically detect 1 CFU/100 ml of V. cholerae in ballast water. Ballast water samples spiked with V. cholerae cells were filtered and enriched in alkaline peptone water before PCR or NASBA detection. The entire method, including sample preparation and analysis was performed within 7 h, and has the potential to be used for analysis of ballast water for inspection and enforcement control.

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Kjeller, December 2, 2011

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Title: real-time PCR and NASBA for rapid and sensitive detection of Vibrio cholerae in ballast water.

Responses to Reviewers

Please find enclosed a revised version of the manuscript MPB-D-11-00438. Changes made to the manuscript are labeled with yellow. As suggested by the referee we have included results from testing of sea water from a Norway fjord, ballast water from the Norwegian coastline (Havila Subsea) and sea water from Singapore. These samples were spiked with 1 and 10 CFU/100 ml, and *Vibrio cholerae* DNA was detected using the described presence-absence method including filtration, enrichment in APW, filtration, extraction of DNA and real-time PCR detection. Since real-time PCR turned out to be the most sensitive and reproducible detection method, PCR was chosen for testing of these samples.

Corrections:

Stephanie Delacroix, NIVA, has been included in the author list (instead in the acknowledgement) due to extensive work with the samples.

Minor corrections are made in footnotes in the tables. The inserted text is labeled yellow.

Text describing the new experiments is included in material and methods, results and discussion.

I hope this will deal adequately with the suggestions made by the referee and I am looking forward to hear your decision on this manuscript.

Sincerely yours,

Else Marie Fykse, PhD

Chief scientist

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Highlights

- Ballast water, a factor in the transmission of aquatic organisms, including pathogenic bacteria
- Detection of *V. cholerae* in ballast water
- Detection after enrichment in alkaline peptone water
- Using real-time PCR or NASBA

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Real-time PCR and NASBA for rapid and sensitive detection of *Vibrio cholerae* in ballast water

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Abstract

Transport of ballast water is one major factor in the transmission of aquatic organisms, including pathogenic bacteria. The IMO-guidelines of the Convention for the Control and Management of Ships' Ballast Water and Sediments, states that ships are to discharge < 1 CFU per 100 ml ballast water of toxigenic *Vibrio cholerae*, emphasizing the need to establish test methods. To our knowledge, there are no methods sensitive and rapid enough available for cholera surveillance of ballast water. In this study real-time PCR and NASBA methods have been evaluated to specifically detect 1 CFU/100 ml of *V. cholerae* in ballast water. Ballast water samples spiked with *V. cholerae* cells were filtered and enriched in alkaline peptone water before PCR or NASBA detection. The entire method, including sample preparation and analysis was performed within 7 h, and has the potential to be used for analysis of ballast water for inspection and enforcement control.

Key words: Ship's ballast water, *Vibrio cholerae*, detection, real-time PCR, real-time NASBA

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Introduction

Vibrio cholerae is the aetiological agent of epidemic cholera, which causes watery diarrhoea that can result in rapid dehydration and death of infected persons (Finkelstein, 1996). Coastal waters are an important reservoir of *V. cholerae* and cholera is generally transmitted to humans via contaminated water or seafood (Cholera working group, 1993; Colwell et al., 1977, 1981). The recent outbreak on Haiti emphasizes the severity of this disease (CDC, 2010) as a transnational spread is confirmed, e.g. by travelers to the state of Florida in USA. Genotyping studies indicate that the Haitian epidemic is probably the result of the introduction, through human activity, of a *V. cholerae* strain from a distant geographic source (Chin et al., 2011), emphasizing the epidemic potential of the cholera disease.

Ballast water discharge is one major factor in the transmission of aquatic organisms worldwide (Ruiz et al., 2000; David et al., 2007; Drake et al., 2007), including epidemic cholera. The outbreak of cholera in Latin America, which started in a coastal town of Peru in the early 1990s, was probably caused by an Asian strain of *V. cholerae* transported by ship (CDC, 1991; Wachsmuth et al., 1993). This strain was also found in shellfish beds in Mobile Bay Alabama, USA, suggesting a ballast water transmission (dePaola et al., 1992; Fields et al., 1992; McCarthy et al., 1994; Motes et al., 1994). The International Maritime Organization (IMO) Convention on ballast water management has therefore established requirements for pathogenic indicator bacteria in the ballast water performance standard (IMO-2008). The guidelines state that ships are to discharge < 1 colony forming unit (CFU), < 250 CFU and < 100 CFU per 100 ml ballast water containing toxigenic *V. cholerae* (i.e. O1 and O139), *Escherichia coli*, and intestinal *Enterococci*, respectively. The low limit for the presence of toxigenic *V. cholerae* is due to its toxigenicity and epidemic nature and its ability to adapt and grow in a new environment.

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A lot of indigenous organisms carried in ballast tanks are killed during voyages as a result of temperature changes, reduction in the concentration of oxygen and lack of nutrients. However, it is known that organisms still survive in water and sediments and are able to invade local marine and estuarine ecosystems (Aridgides et al., 2004; Gollasch et al., 2000; Mimura et al., 2005). During such unfavorable conditions *V. cholerae* may enter a viable but nonculturable (VBNC) state (Chaiyanan et al., 2001; Huq and Colwell, 1996; Pruzzo et al., 2003) and it is therefore likely to assume that *V. cholerae* cells are partly VBNC, viable and dead in ballast tanks. The microbial content of ballast water may vary widely. The number of viable *Vibrio* spp. in U.S. military ships varied from <1 to 10^6 CFU per 100 ml. However, toxigenic strains of *V. cholerae* were not detected, but other pathogenic bacteria were detected in about 50 % of the ballast tanks (Burkholder et al., 2007). A mean abundance of $8.3x10^8$ bacterial cells per 100 ml in ballast water vessels is also reported (Ruiz et al., 2000). If the purpose is to detect toxigenic *V. cholerae*, the growth of other bacterial species may totally overgrow on the isolation media, thus making the confirmation of < 1 CFU per 100 ml of *V. cholerae* (O1 and O139) time-consuming and difficult to obtain. Real-time PCR is a widely used molecular method for direct detection of low levels of pathogenic microbes in environmental samples and for specific detection and monitoring of *V. cholerae* (Blackstone et al., 2007; Fields et al., 1992; Goel et al., 2005; Gubala, 2006; Gubala and Proll, 2006; Koskela et al., 2009; Lipp et al., 2003; Lyon, 2001). Toxigenic *V. cholerae* may also be detected by a multi-target real-time NASBA (nucleic acid sequence based amplification) assay amplifying RNA specifically even in the presence of DNA (Fykse et al., 2007).

To our knowledge, there are currently no rapid methods meeting the IMO regulations in order to detect 1 CFU/100 ml in ballast water. Norway has acceded to the International Convention

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for the Control and Management of Ships' Ballast Water and Sediments. This requires tools for disinfection, monitoring and testing of the ballast water for pathogenic indicator bacteria, including *V. cholerae.* Ballast water treatment technologies are mostly still in the developmental phase (Tsolaki and Diamadopoulos, 2010). Experiments show that disinfection of sea water reduces the number of bacteria to less than 1 % of the initial level. However, after three to five days of storage under condition mimicking ballast tanks, the total CFU was back to the starting point including growth of *Vibrio* species (Hess-Erga et al., 2010: Tryland et al., 2010). This supports the finding that bacteria, including *Vibrio*, are able to survive and grow in conditions similar to ballast tanks, indicating a need for rapid screening methods to identify indicator bacteria in ships' ballast water prior to port entry. Even if ballast water treatment systems become widely adopted, detection tools are needed for rapid inspection and enforcement controls.

The objective of this work was to develop highly specific and sensitive molecular methods based on real-time PCR or NASBA for rapid monitoring and identification of *V. cholerae* in ballast water according to the detection levels set by the IMO guidelines. Ballast water was spiked with *V. cholerae* cells at different concentrations per 100 ml test water and the detection level of *V. cholerae* in conjunction with high levels of interfering bacteria/*Vibrio* spp. were examined. We show that the IMO requirements can be obtained by using the presence-absence method described here to successfully and specifically detect 1 CFU/100 ml toxigenic *V. cholerae* within 7 h including 4 h enrichment. To our knowledge, this is the first report describing rapid detection of such extremely low levels of *V. cholerae* in ballast water. The novel method established in this work is an important step toward developing detection tools for pathogenic indicator bacteria and has the potential to be used as a generic method for

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

surveillance of waterborne microorganisms important for public health issues to initiate medical or other preventive measures.

Material and Methods

Ballast water, bacterial strains, media and cultures

The ballast water used in this work was produced at the BallastTech-NIVA AS land-based test centre at Solbergstrand, at the eastern coast of Norway (N 59° 37.282', E 10° 38.843'). Enriched brackish water was used as ballast water, which represents the most challenging water with respect to PCR and NASBA inhibitors (unpublished results at FFI). Biological additives (algae and zooplankton) and chemical additives were supplemented to brackish water (according to IMO guidelines) to mimic real ballast water. Enriched brackish water with supplements are in general characterized by; salinity: 21 PSU, pH: 8.0, dissolved oxygen: 8 mg/L, turbidity: 40, NTU (Total Suspended Solids): 60 mg/L, Particulate Organic Carbon: 7 mg/L, organisms ≥50 µm: 10^5/ m^3, organisms 10-50 µm: 10^3/ ml, heterotrophic bacteria: 10^4-10^5 CFU/ ml, total *Vibrio* spp. (non-*V. cholerae*): 10^3-10^5 CFU/100 ml. No heterotrophic bacteria were added since the IMO-requirements of > 10,000 CFU/ml was met without additives.

The bacterial content of the ballast water was determined by serial dilutions plated on Marine Agar (Conda) and incubated at room temperature (48-75 h). The total number of *Vibrio* spp. in ballast water was determined by plating on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (Oxid), and incubated at 37°C for 24-48 h (Tryland et al., 2010).

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

The enriched brackish water used as ballast water in spiking experiments was collected at Solbergstrand. In some cases the water was immediately used for spiking experiments and in other cases the water was stored at -20 °C until used in spiking experiments, and similar PCR and NASBA results were obtained using either water treatment. After thawing, the water samples were stored at 4 °C for 1-2 weeks. When ballast water samples were stored at 4 ^{o}C for a month a 10-fold variation of the total CFU numbers were observed.

Sea water sampled in Norway (Rakkevik, Stokke, Norway (N 59° 15.262', E 10° 22.442') surface) and Singapore (5600 Merban N 01° 15.801', E 103° 43.790', 11 meter) and ballast water from Havila Subsea (taken on at the Norwegian coast between Stavanger (N 58° 58.485', E 05° 45.046') and Kristiansund (N 63° 6.796', E 07° 44.148')) were also used in spiking experiments. The number of heterotrophic bacteria and total *Vibrio* spp. was $5x10^3$ CFU/ml and $3x10^3$ CFU/100 ml, $6.7x10^4$ CFU/ml and $1.4x10^4$ CFU/100 ml, and $7x10^2$ CFU/ml and 14 CFU/100 ml in sea water from Norway, Singapore and ballast water from Havila Subsea, respectively.

The *V. cholerae* strains non-O1/non-O139 VC 021 or Cip 106855 O1 Inaba El Tor were used for spiking experiments, and these strains were grown aerobically in Tryptic Soy Broth (TSB) at 37°C for 24 h. The number of cells was counted in a counting chamber (Toma) using a phase-contrast microscope (Zeiss, Germany). The CFU numbers were enumerated by plating 100 µl of serially diluted cells onto Tryptic Soy Agar (TSA) and incubated at 37ºC for 24 h.

To mimic the conditions in ballast tanks, *V. cholerae* VC 021cells were added to pre-sterilized enriched brackish water samples and stored in a closed bottle in the dark at 4 °C, and the

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

number of viable VC 021 cells gradually decreased from $2x10^8$ to less $7x10^4$ CFU/ml during 8 weeks. These cells were used in spiking experiments with enriched brackish water when the CFU/ml was less than 1% of the initial concentration. For each experiments the number of culturable *V. cholerae* cells was determined by growth on TSA

Detection of *V. cholerae* in ballast water

Ballast water samples (200 ml) (enriched brackish water) was supplemented with 1-100 CFU/100 ml of *V. cholera* VC 021 cells or *V. cholera* Cip 106855 O1 Inaba El Tor cells. The cells were collected by filtration (0.22 µm Polyethersulfone filters from MOBIO Laboratories Inc.) and RNA or DNA was extracted from the filters as described below. A presence-absence method for detection of *V. cholerae* based on membrane filtration of a 200 ml spiked ballast water sample and enrichment of filter with bacteria was also performed. In those experiments the filters with the trapped cells were aseptically transferred to 10 ml alkaline peptone water (APW), pH 8.6, vortexed for 5 to 10 s to release the cells and incubated aerobically at 37 °C for various times (as indicated in the results). After incubation the bacterial cells were collected by filtration of the entire volume of the APW growth medium, followed by washing of the filters by APW and DNA or RNA extraction. The presence of *V. cholerae* DNA and RNA was examined by real-time PCR and NASBA amplification, respectively. In some experiments, real-time PCR was performed on a crude DNA extract isolated from one ml of the APW growth medium. The growth of *V. cholerae* on the filters after 18 h incubation in APW was also examined (Huq et al., 2006; Tryland et al., 2010). Briefly, the surface growth on the filters (0.2 µm nitrocellulose filters from Sartorius was used in these experiments) enriched in APW was cultured on TCBS agar, and the growth of *Vibrio*

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spp. was further cultured on nutrient agar without NaCl followed by oxidase test and API 20E (BioMèrieux) for confirmation of the *V. cholerae* colonies.

Each spiking experiment was repeated at least three times if not otherwise indicated. Ballast water samples not spiked with *V. cholerae* was used as negative control samples and neither of the gene targets was consistently amplified.

DNA and RNA extraction

If not otherwise stated DNA was isolated from ballast water samples using the MOBIO PowerWater® DNA Isolation Kit (MOBIO Laboratories, Inc.) and a mixture of DNA and RNA was isolated using the MOBIO PowerWater® RNA Isolation Kit (MOBIO Laboratories, Inc). DNA and RNA were isolated according to the manufacturer's instructions and DNA or RNA was eluted in 100 µl of elution buffer. RNA was stored at -80 °C and DNA at -20 °C until used. In some experiments DNA was isolated using a modified procedure described by Boström et al., 2004 or the MOBIO UltraWater® DNA Isolation Kit (MOBIO Laboratories, Inc.), which elutes DNA in 3 ml volume. A crude DNA extract was also isolated from one ml of the APW growth medium or from a cell culture. Cells were heated to 95 ^{o}C for 10 min, centrifuged and the supernatant containing a crude DNA extract was used in real-time PCR when indicated in the results.

Real-time PCR

Real-time PCR was performed in a LightCycler® 480 instrument (Roche, Germany) using the LightCycler® Probe Masters kit (2x) containing 10 µl of the LightCycler® Probe Masters

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

master mix, 1 µM primers, 0.2 µM TaqMan probe, 2 µl template and water until a total volume of 20 µl. The reaction mixture was denatured at 95 °C for 5 min followed by a 40-45 cycle PCR profile; denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s and extension at 72 °C for 15 s. Specific amplification was indicated by the threshold-cycle (Ct) value representing the cycle number at which the fluorescence intensity crossed a fixed threshold ten times the standard deviation of the baseline intensity. Deionized water was used as a negative PCR-control. *V. cholerae* VC 242, Ogawa El Tor DNA was used as a positive control and six real-time PCR parallels were run for each sample. Primers and probes targeting the *groEL* general marker and the t*cpA* toxin gene marker were used (Table 1). The specificity of these primers was investigated in Fykse et al., (2007). The size of the PCR-product was routinely verified by gel-electrophoresis (BioAnalyzer; Agilent technology). The detection range, evaluated by real-time PCR of a crude DNA extract from 10-fold serially diluted *V. cholerae* cells, was between $2x10^6$ DNA copies/PCR and 2 DNA copies/PCR (unpublished results FFI).

NASBA

The NASBA reaction was carried out according to the manufacturer's instructions (NucliSens EasyQ Basic kit) (BioMèrieux Ltd., Boxtel, The Netherlands) and mainly as described in Fykse et al. (2007). In these experiments a KCl concentration of 80 mM and 1- 3 µl template were used. Primers and probes targeting the *groEL* gene were applied (Fykse et al. , 2007). Amplification of a specific NASBA product was indicated by the detection time (T value in min). Deionized water and *V. cholerae* VC 021 RNA was used as a negative and positive control, respectively. The RNA-amplicons were verified by gel-electrophoresis (Bioanalyzer. Two to four NASBA parallels of each individual sample were performed.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

Results

Real-time PCR detection of *V. cholerae* in ballast water

A real-time PCR method for detection of *V. cholerae* in ballast water was developed. A volume of 200 ml of ballast water was chosen since IMO guidelines states that < 1 CFU/100 ml of *V. cholerae* can be released from ballast water. The ballast water was spiked with 1, 10 or 100 CFU of *V. cholerae* VC 021 or cip 106855 cells per 100 ml test water. Cells were collected by filtration and DNA was isolated using tree different methods. An evaluation of the three methods based on limit of detection (LOD) and the level of inhibition of PCR-amplification is presented in Table 2. Consistent detection using the method based on Boström et al., (2004) of $1x10^4$ CFU/100 ml was obtained. However, extracted DNA contained substantial amount of inhibitors, indicated by decreasing Ct-values of amplification of serial diluted DNA-extract, and by inhibition of the PCR-amplification of purified DNA in the presence of the DNA-extract (unpublished results FFI). The detection level was decreased 10-100 times using the Gene Clean Turbo kit (BioRad) to remove inhibitors from DNA extracted from enriched brackish water. However, more efficient DNA extraction was obtained by the MOBIO kits and no PCR-inhibitors were detected in the DNA- extract (Table 3). Therefore, the MOBIO PowerWater® DNA Isolation Kit was chosen for further experiments based on a consistent detection of 100 CFU/100 ml, compared to $1x10^3$ CFU/100 ml for the MOBIO UltraWater® DNA Isolation Kit (Table 2 and 4). These results indicated that *V. cholerae* in ballast water can be detected using real-time PCR amplification in a complex background of other microorganisms (shown by plating on Marine and TCSB agar) in which their DNA is also concentrated during the filtration step of the DNA extraction method used. Extracting DNA from spiked sea water indicated less inhibition and a higher sensitivity of the detection

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

Non-spiked ballast water samples treated similarly were used as negative controls, and neither of the PCR target genes *V. cholerae groEL* and *tcpA* was consistently detected. However, occasionally some PCR parallels were positive for the *groEL* target, but this amplification was late (Ct-value > 40) indicating that no *V. cholerae* DNA was consistently detected.

Enrichment in alkaline peptone water

An enrichment step in APW was included to increase the chance of detecting low levels of *V. cholerae* in ballast water. The maximum doubling time of *V. cholerae* VC 021 cells in APW was determined to approximately 30 min by growth experiments (Fig. 1), and since real-time PCR was able to consistently detect 100 CFU/100 ml, 3-6 h enrichment in APW should be sufficient to detect a spiking concentration of 1 CFU/100 ml. Thus, ballast water samples were spiked with *V. cholerae* VC 021 cells in the range of 1 to $1x10^4$ CFU/100ml and the bacterial cells were collected on filters and enriched in APW. Using direct real-time PCR (i.e. using a crude DNA extract from one ml of APW) *V. cholerae groEL* DNA was consistently detected from samples spiked with 1 CFU/100 ml after 18 h enrichment, in samples spiked with 10 CFU/100 ml after 6 h enrichment, and in samples spiked with 100 CFU/100 ml after 3 h enrichment (Table 5). Growth analysis on TCBS and nutrient agar of the filters enriched in APW for 18 h showed that *V. cholerae* cells were isolated from spiked samples containing > 6 CFU/100 ml.

Since a spiking concentration of 1 CFU/100 ml enriched for 3 and 6 h was not consistently detected using the direct PCR assay, DNA was extracted from the entire APW enrichment culture, and with the purpose to shorten the analysis time an enrichment of 2 and 4 h was tested. In these experiments *V. cholerae* VC 021 DNA was consistently detected by real-time PCR of the *groEL* gene after 4 h enrichment when spiked with 1 CFU/100 ml. A spiking

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

concentration of 10 CFU/100 ml was detected in all experiments after 2 and 4 h enrichment (Table 4). In spiking experiments using the toxigenic *V. cholerae* Cip 106855 O1 Inaba El Tor strain similar results were obtained by amplification of the toxin gene *tcpA* in addition to the *groEL* gene target (Table 4).

To investigate the impact of any PCR inhibitors on this detection assay purified *V. cholerae* DNA was added to the mixture of DNA extracted from non-spiked ballast water enriched in APW for 4 h. No inhibition of real-time PCR amplification was detected (Table 3), indicating that the PCR inhibitors were removed during the DNA extraction protocol.

Detection of DNA from 1 CFU/100 ml of toxigenic *V. choleae* was obtained using the presence-absence method based on enrichment in APW, filtration of the enrichment broth and isolation of DNA from the filter followed by real-time PCR. This was found to be a useful method for detection of *V. cholerae* DNA in a complex background of DNA isolated simultaneously from other microorganisms in the ballast water. The entire assay was completed within 7 h in which 4 h were dedicated to the APW enrichment step.

Detection of *V. cholerae* by NASBA

A real-time NASBA method for detection of *V. cholerae* in ballast water was also tested and compared to the real-time PCR method. Similar spiking experiments were carried out as for the real-time PCR analysis. Spiking concentrations of 1 and 10 CFU/100 ml of *V. cholerae* VC 021 cells was consistently detected by NASBA amplification of *groEL* after 6 h enrichment. A 4 h enrichment step resulted in a positive detection of approximately 50 % of the samples spiked with 1 CFU/100 ml (Table 4). However, a late amplification (T-value >40) with low fluorescence values were observed. RNA extracted from ballast water spiked with

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

100 CFU/100 ml was always detected after 4 and 6 h enrichment (Table 4), while RNA extracted from pure ballast water was never detected and was thus referred to as negative control samples. Detection of *V. cholerae groEL* RNA without the enrichment step resulted in a detection limit of only $5x10^3$ CFU/100 ml.

The presence of potential NASBA-inhibitors were examined by amplifying *V. choleae* RNA in the presence of and absence of a RNA-extract from non-spiked ballast water. The presence of this complex RNA extract mixture caused an inhibition of the NASBA reaction as indicated by the 10 min increase of the amplification time (T-value) and a lowering of the fluorescence value. The inhibition was most likely due to salt, non-target RNA or other compounds in the isolated RNA.

In conclusion, after 6 h enrichment real-time NASBA amplification was able to detect *V. cholerae* RNA from ballast water spiked with 1 CFU/100ml of *V. cholerae* cells in a background of RNA/ DNA from other bacterial species present in the ballast water. This entire assay was completed within 9 h.

Detection of *V. cholerae* cells stored in "artificial" ballast tank environments

V. cholerae VC 021 cells (< 1% culturability) were stored in conditions mimicking ballast tanks and then used for spiking experiments with ballast water. *V. cholerae groEL* DNA was consistently detected by real-time PCR after 4 h enrichment of samples spiked with 1 and 10 CFU/100ml. However, real-time PCR of *V. cholerae groEL* DNA extracted directly from cells collected by filtration without enrichment was also positive (Table 4), indicating that DNA extracted from potential VBNC cells and/or dead cells were detected as well.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

Similar experiments showed positive detection of *V. cholerae* VC 021 *groEL* RNA only in samples spiked with 10 and 100 CFU/100 ml after 6 h enrichment using NASBA amplification. In ballast water samples spiked with 1 CFU/100 ml and enriched for 6 h, only 75 % of the samples detected positive for *V. cholerae groEL* RNA (Table 4).

Detection of *V. cholerae* from genuine ballast water and sea water

Genuine ballast water from Havila Subsea and sea water from Norway and Singapore spiked with 1 and 10 CFU/100 ml of *V. cholerae* VC 021 cells detected positive for *V. cholerae groEL* DNA. The presence-absence method based on filtration, enrichment in APW for 4 h, filtration, DNA isolation and real-time PCR detection was used. Representative Ct values for PCR detection of a spiking concentration of 1 CFU/100 ml were 30 and 30.4 for sea water from Norway and Singapore, respectively, and 31.5 for ballast water from Havila Subsea. Two separate experiments were performed.

Discussion

The described detection method of *V. cholerae* in ballast water is an important step towards developing tools for inspection of ships for compliance to the IMO Convention for the Control and Management of Ships' Ballast Water and Sediments which states that < 1 CFU/100 ml of toxigenic *V. cholerae* (i.e. O1 or O139) cells can be released during ballast water discharge. To our knowledge, this is the first report with a strategy to detect 1 CFU/100 ml of *V. cholerae* in ballast water within one working day. We succeeded in obtaining a same-day detection assay of *V. cholerae* by using a combination of membrane filtration, 4 h enrichment and real-time PCR.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

In general, it is difficult to detect of a few viable cholera bacteria in ballast water containing a background of 10^3-10^5 *Vibrio* spp/100 ml and 10^4-10^5 heterotrophic bacteria/ml. It is known that the genus *Vibrio* is widespread in coastal waters (Thompson et al., 2004), although, it tends to be more common in warm water (Kaspar and Tamplin, 1993). *Vibrio* spp. has been detected in blue mussels along the coast of Norway (Bauer et al., 2006), and a *V.cholerae*/*Vibrio mimicus* population is also detected along the entire Swedish coastline (Eiler et al., 2006). However, highly sensitive PCR-detection in environmental samples is also challenging and depends on efficient extraction of DNA and removal of potential inhibitors. The MOBIO PowerWater®DNA isolation kit chosen in these experiments (in contrast to the Boström method) efficiently removed PCR inhibitors present in the ballast water. In the present investigation, real-time PCR turned out to be more sensitive than NASBA since a spiking concentration of 1 CFU/100 ml was consistently detected with real-time PCR using the 4 h enrichment. This was not the case with real-time NASBA, which can be due to different yield of DNA and RNA (not tested in this study due to the mixed population of DNA and RNA isolated from ballast water). Another explanation is the presence of NASBA inhibitors such that more efficient methods for RNA extraction are required. The NASBA amplification process involves three different enzymes which could be more sensitive to inhibitors compared to PCR amplification (Compton, 1991). Previously, *V. cholerae* RNA was detected by NASBA amplification in a spiked sea water sample, and in that case, no inhibition was observed (Fykse et al., 2007), supporting the initial PCR results that enriched brackish water is a more challenging environmental sample compared to sea water.

Our hypothesis was that, in addition to speed up the entire detection process by using PCR or NASBA, an introduction of an APW enrichment step would increase the possibility to detect

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

low amounts of *V. cholerae* cells in ballast water samples. This study demonstrated that this was possible and the entire analysis could be completed within 7 h. In contrast, conventional culturing methods and subsequent bacterial identification are time-consuming (days) (Huq et al., 2006). A disadvantage of real-time PCR is that the method does not differentiate between viable and non-viable cells. However, by introducing the short enrichment step in APW viable cells are detected and in 4 h a100-fold increase of the amount of DNA template present were obtained. In principle, the presence of RNA in bacterial cells may serve as an indicator for viable cells (Keer and Birch, 2003). Unfortunately, in this study the NASBA amplification was not sufficiently sensitive to detect RNA extracted from 1 CFU/100 ml.

V. cholerae DNA has also been detected after enrichment in drinking water samples, treated effluent and surface water seeded with *V. cholerae* cells at 15 CFU/100ml, 3 CFU/100 ml and 1 CFU/100 ml, respectively (du Preez et al., 2003). In that study a combination of filtration, 6 h enrichment and a pit-stop seminested PCR using gel-electrophoresis for confirmation of the PCR amplicons was used. Our study, using real-time PCR provided a similar and even an improved sensitivity level in a complex background, which was also collected and concentrated during filtration and 4 h enrichment. Furthermore, in this study, *V. cholerae groEL* DNA was detected at a sensitivity of 1 CFU/100 ml without enrichment as well, using *V. cholerae* cells that were stored in the dark in a closed bottle at 4 °C to mimic the conditions in ballast tanks. Thus, it is likely to assume that DNA from non-viable cells, VBNC and viable cells were detected. Rivera et al. (2001) reported a detection limit of 100 *V. cholerae* cells per 250 ml of sea water. Recently, a PCR method with an enrichment step for 6 h detected 4-10 CFU/100 ml of *V. cholerae* spiked into river water, and 40-100 CFU/100 ml was detected without enrichment (Ntema et al., 2010). However, this river water contains most likely less PCR inhibitors compared to enriched brackish water used in this study,

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

supported by PCR and NASBA amplification of DNA and RNA extracted from sea water, lakes and tap-water (Fykse et al., 2007; unpublished results FFI). Aridgides et al. (2004) also showed that ballast water itself is inhibitory to PCR.

A molecular detection system based on microfluidic carbonnano tubes for detection of invasive species in ballast water is described (Mahon et al., 2011). However, to our knowledge there are no such screening methods available for cholera surveillance of ballast water. The method presented in this study has the potential to be used for sensitive testing for toxigenic *V. cholerae*. For public health preventive issues the method might be useful for monitoring of environmental water samples for *V. cholerae.* The infectious dose of *V. cholerae* (O1) is approximately 10^4 to 10^6 organisms (Cash et al., 1974) and our method is useful for detecting such doses even in a large volume (litres) of water. In general, the method has potential to be used as a generic method detecting other microorganisms in water as well by using species specific primers and probes.

Our results indicated that the presence of *V. cholerae* in a sample can be underestimated if only one method is used. A combination of a short enrichment step followed by real-time PCR turned out to be the most sensitive method for detection of toxigenic *V. cholerae* in ballast water. The culturing step ensures detection of viable cells as required in the IMO guidelines and using rapid real-time PCR ensures detection of DNA from VBNC cells and shortens the time of analysis. The described presence-absence method including real-time PCR was also used to detect 1 CFU of *V. cholerae* cells per 100 ml of genuine ballast water and sea water from Norway and Singapore. The *Vibrio* spp. content of the sea water from Singapore was similar to the *Vibrio* spp. content in the enriched brackish water used in this work, which is higher than the average concentration in water from the Norwegian coast.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

Performing automatic sampling and filtration during ballast water discharge would simplify processing of a large volume of water and the screening of ballast water for indicator bacteria, which also increases the sensitivity of the method.

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TABLE 1. Primers and probes used in real-time PCR detection of DNA from *V. cholerae*

Primer or TM[a]	**Nucleotide sequence 5' → 3'**	**Size of amplicon (bp)**
[b] Pvc-f *groEL*	GGT TAT CGC TGC GGT AGA AG	116
[b] Pvc-r *groEL*	ATG ATG TTG CCC ACG CTA GA	
TMvc*groEL*	FAM[c]-CTGTCTGTACCTTGTGCCGATACTAAAGC-BBQ[d]	
[b] Pvc-f *tcpA*	GAA GAA GTT TGT AAA AGA AGA ACA CG	102
[b] Pvc-r *tcpA*	CGC TGA GAC CAC ACC CAT A	
TMvc*tcpA*	FAM-ACTTCGAGTAATGTCATACCCTCTTGACC-BBQ	

[a] TM, TaqMan probe.
[b] Forward primer in PCR corresponds to primer 2 in NASBA (Pvc-f *groEL* corresponds to (~) Pvc66-2 *groEL*, Pvc-r *groEL*~Pvc65-1 *groEL*, Pvc-f *tcpA*~Pvc60-2 *tcpA*, Pvc-r *tcpA*~Pvc62-1 *tcpA* (Fykse et al., 2007).
[c] FAM, 6-carboxyfluorescein.
[d] BBQ, Black Berry Quencher 650.

TABLE 2. Evaluation of bacterial DNA extraction methods from ballast water [a] by using real-time PCR

Extraction methods	**PCR (LOD)[b], CFU/100 ml/ PCR inhibition**
Modified method of Bostrøm et al., 2004	$1x10^4$/considerable
MOBIO UltraClean®Water DNA isolation kit	$1x10^3$/no
MOBIO Power water®Water DNA isolation kit	$1x10^2$/no

[a] Ballast water was produced at the Ballast Tech-NIVA AS
[b] LOD: Limit of detection

TABLE 3. Test for inhibition of *V. cholerae* real-time PCR in the presence of DNA extracted from non-spiked ballast water

	Dilution of DNA from non-spiked ballast water					
	CTR	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**
Ct-value	25.12	25.18	25.17	25.09	25.28	25.22
SD	0.07	0.08	0.03	0.07	0.18	0.11

Serially diluted, 1:1 to 1:16, DNA (2 µl) extracted from non-spiked ballast water enriched in APW for 4 h were added to purified *V. cholerae* DNA (1 µl) before the real-time PCR reaction. The CTR (control) sample is PCR with no addition of DNA isolated from ballast water. Ballast water was produced at the Ballast Tech-NIVA AS.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

TABLE 4. PCR and NASBA detection of DNA and RNA extracted from *V. cholerae* cells spiked into ballast water

Strain	**PCR, % positive identification (No. of specific targets detected / no. of total reactions)**			**NASBA, % positive identification (No. of specific targets detected / no. of total reactions)**		
Concn	**Enrichment in APW (h)**			**Enrichment in APW (h)**		
CFU/100ml	0	2	4	4	6	18 [e]
VC 021[a]						
1	10 (2/21)	52 (14/27)	100 (31/31))	55 (11/20)	97 (31/32)	100 (4/4)
10	44 (8/18)	100 (12/12)	100 (18/18)	86 (24/28)	100 (8/8)	ND
100 [e]	100 (18/18)	100 (6/6)	100 (6/6)	ND	ND	ND
CIP 106855[b]						
1	6 (1/18)	39 (7/18)	100 (18/18)	ND	ND	ND
10[e]	17 (1/6)	67 (4/6)	100 (6/6) [e]	ND	ND	ND
100	ND[d]	ND	ND	ND	ND	ND
VC 021 VBNC[c]						
1	100 (18/18)	ND	100 (18/18)	31 (5/16)	75 (12/16)	ND
10	100 (18/18)	ND	100 (18/18)	69 (11/16)	100 (12/12)	ND
100	ND	ND	ND	ND	100 (4/4)	ND

Ballast water was produced at the Ballast Tech-NIVA AS. Non-spiked ballast water was used as negative control samples and no consistent PCR or NASBA amplification was detected. Average Ct values in PCR were: Positive control DNA (2 µl) (*groEL*): 21.5±4.2 (SD, 9 separate experiments (n=9)); spiking concentration of 1 CFU/100 ml enriched for 4 h: VC 021 cells 34.3±1.3 (SD, n=5), VBNC VC 021 35.1±3.4 (SD, n=4), cip 106844 cells 36.1±3.2 (SD; n=4). Average T-values in NASBA were: positive control RNA (2 µl) (*groEL*): 30.8±3.2 (SD; =6); spiking concentration of 1 CFU/100 ml enriched for 6 h: VC 021 cells 33.6±2.3 (SD; n=3), VBNC VC021cells 28.4±2.6 (SD; n=3).

[a] DNA and RNA from *V. cholerae* strain VC 021 was detected by amplification of the *groEL* gene target.

[b] DNA from *V. cholerae* strain CIP 106855 was detected by amplification of the *groEL* and *tcpA* gene target. Results for *tcpA* amplication presented.

[c] *V. cholerae* VC 021 cells were stored for > 8 weeks in pre-sterilized ballast water at 4 °C in the dark. The cells were used in experiments when the culturability was < 1 % of the initial culturability.

[d] ND: not determined.

[e] Only one experiment performed.

DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

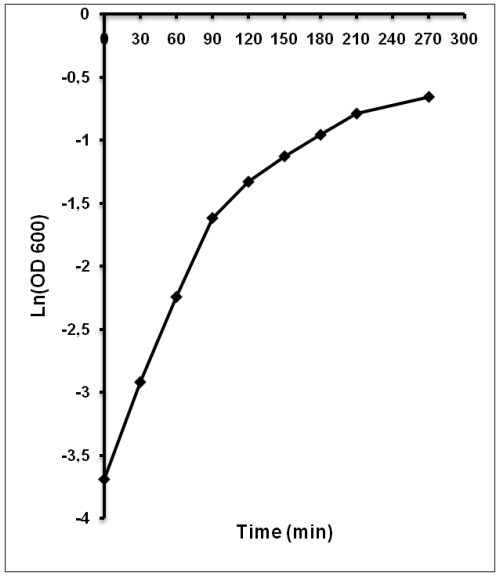
TABLE 5. PCR detection of *V. cholerae* VC 021 DNA extracted from *V. cholerae* cells spiked into ballast water after 3, 6 and 18 hour's enrichment in APW, followed by a direct real-time PCR of heat inactivated cells

	PCR, % positive identification (No. of specific targets detected / no. of total reactions)		
Conc. CFU/100ml	**Enrichment in APW (h)**		
	3	6	18
1	44 (4/9)	78 (7/9)	100 (6/6)
10	67 (6/9)	100 (9/9)	100 (6/6)
100	100 (9/9)	100 (9/9)	100 (6/6)
$1x10^3$	100 (9/9)	100 (9/9)	100 (6/6)
$1x10^4$	100 (9/9)	100 (9/9)	100 (6/6)

The results from 3 and 6 h incubation are based on three individual experiments and three PCR parallels per individual sample. Results from 18 h enrichment are based on two separate experiments and three PCR parallels. Ballast water was produced at the Ballast Tech-NIVA AS.

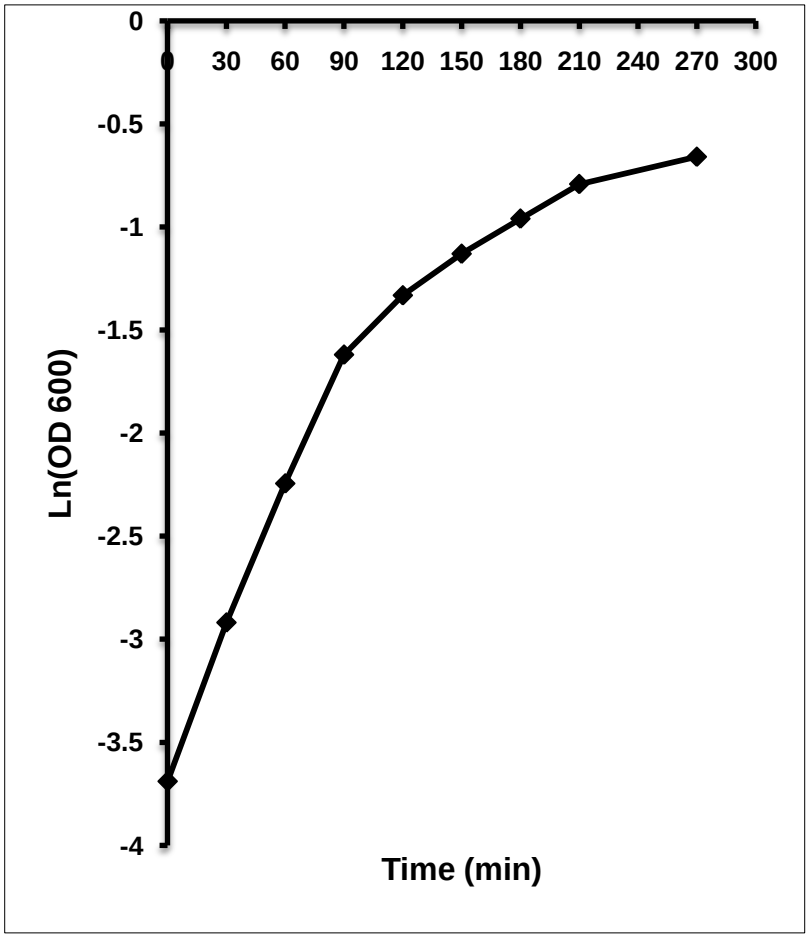
Legend to figure

Fig 1. Growth curve for *V. cholerae* 021cells in APW at 37°C. Generation time in APW is calculated to 32 minutes. Theoretically 6-7 doublings in 3-4 h would give a 100 fold increase of the number of cells.



DOI til publisert versjon/DOI to published version: 10.1016/j.marpolbul.2011.12.007

Figure 1



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