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diluted DNA-extract, and by inhibition of the PCR-amplification of purified DNA in the presence of the DNA-extract (unpublished results FFI).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

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

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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

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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.

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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.

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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

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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,

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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.

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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.

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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.

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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

Conc. CFU/100ml	PCR, % positive identification (No. of specific targets detected / no. of total reactions)		
	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.

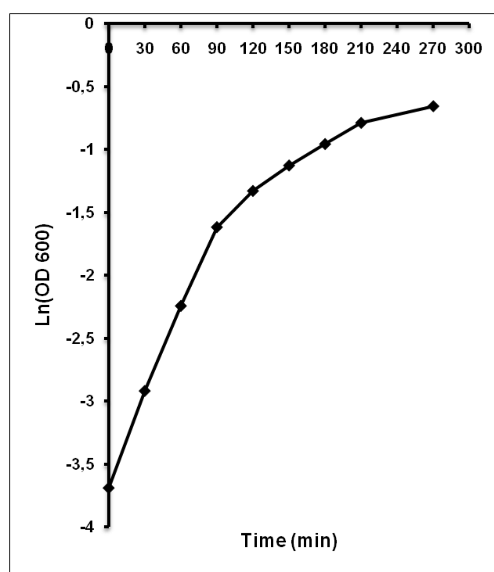

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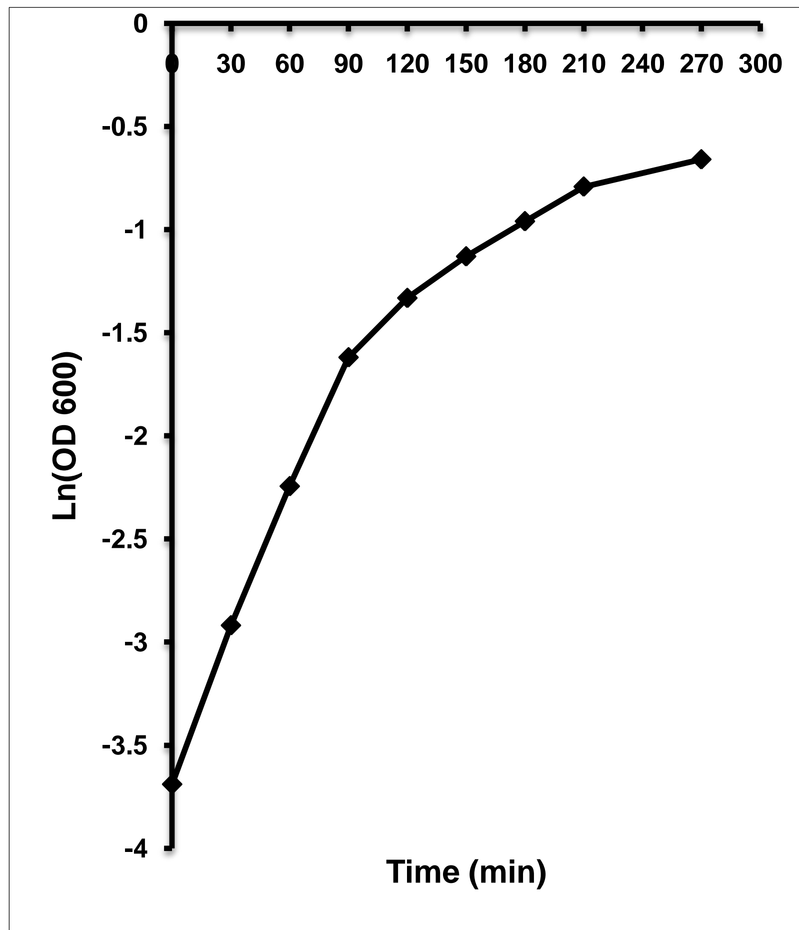
Figure 1



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