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Report on the validation of a method for the determination of bacteria (*Escherichia coli, Enterococci* and *Vibrio cholerae*) in marine water samples based on the VIT® gene probe technology *Fluorescence In Situ Hybridisation* (FISH)

Marine Microbiological Analysis of Ballast Water Samples

Content

Note: see appendix 5.3 for abbreviations used in the text

1 Aim of the Study

Methods for the specific determination of *Enterococcus*, *Escherichia coli* and *Vibrio cholerae* based on FISH shall be used to evaluate the number of bacteria in marine water samples in comparison to the classical microbiological method. The methods were validated regarding:

1. the reliable use of ScanVit Ballast Water Kit *Enterococcus/E.coli* with natural sea water samples.
2. the reliable use of ScanVit Ballast Water Kit *Vibrio cholerae* with natural sea water samples.
3. the robustness of the method.

In addition, the time needed from sample to result and the material needed will be reported.

2 Material and Methods

2.1 Samples

Overnight cultures of *Escherichia coli* and *Enterococcus faecialis* were prepared in TSB12 (Difco) and of *Vibrio cholerae* in Marine Broth (Difco) at 28°C and 120 rpm as standards for the methods. Marine water samples were taken from the Kiel Channel (Holtenau lock) and from the Baltic Sea with sterile flasks submerged to 1 m below the water surface. Sampling took place at three different days in December 2013. All samples were cooled and processed within 4 hours after sampling. All samples were filtered over 1 – 2 µm glass fibre filters into sterile flasks in order to remove all larger organisms (i.e. non-bacterial) from the samples.

2.2 Spiking

Cultures of the target organisms were added to the natural sea water samples under sterile conditions in final concentrations ranging from $1x10^5$ to $1x10^2$ cfu/100 mL. Thus the spiked seawater had a bacterial concentration close to the limit value of the Californian limit value for ballast water quality (i.e. 1000 cfu/100ml, cf. appendix 5.4) and close to the limit values of the IMO Ballast Water Management Convention - BWMC (250 cfu/100 mL for *E. coli; 100 cfu/100 mL forEnterococci* and 1 cfu/100 ML for *Vibrio cholerae*).

2.3 Determination of cell numbers using the FISH method in comparision to the classical method

All samples were processed in parallel; all samples were processed at least in 2 replicats. The number of parallels is indicated in table 1.

a) classical microbiological method: 1, 5, 10 and 100 mL of the samples were filtered (membrane filter discs, nitrocellulose, Ø= 25 mm, pore: 0,45 µm) and the filters placed either on McConkey agar (*E. coli*, *Enterococcus*) or TCBS agar (*Vibrio cholera*) and incubated overnight at 28°C. Colonies were counted; exceeding (high/low) numbers of colonies were ignored. The mean cfu/plate was used to determine the cfu/100ml. In order to discriminate between coliform bacteria and *Enterococci*, colonies were evaluated by microscopy.

b) FISH method using ScanVIT test kits (ScanVIT Ballast Water Enterococcus/E.coli and ScanVIT-Ballast Water Vibrio cholerae): 1, 5, 10 and 100 mL of the samples were filtered (membrane filter discs, nitrocellulose, Ø= 25 mm, pore: 0,45 µm). A rinsing step (50 mL sterile saline) was applied after the filtration of the samples. The filters were placed on M1 agar or TCBS agar and incubated for a minimum of 7.5 h overnight. The test kits were used according to the manufacturer's protocol. The colonies were counted under the fluorescence microscope.

Table 1 (cf. appendix 5.1) summarises the results comprising a variety of samples each of them tested with the classical method against the FISH method:

(a) natural seawater (i.e. natural density of bacteria)
(b) spiked seawater Californian standard (i.e. 1000 cfu/100ml)
(c) spiked seawater BWMC (250 cfu/100 mL for *E. coli*, 100 cfu/100 mL for *Enterococci* and 1 cfu/100 mL for *Vibrio cholerae*)

3 Results

3.1 Reliable use of ScanVit Ballast Water Kit Enterococcus/E.coli with natural sea water samples.

The ScanVIT method was used to measure the bacterial density in different natural sea water samples of different origin sampled at two different days. In parallel, the same samples were analyzed by incubation on McConkey agar. The lower detection limit is given with 1 cfu per 100ml.

The natural density of *Enterococcus* was determined in natural seawater with comparable number in both methods with 10-60 cfu/100 mL. Using spiked seawater close to the limit of the Californian standard and close to the limits of the IMO convention, both cell concentrations have been determined close to the given concentrations of the spiked bacteria.

The natural density of *Escherichia coli* was determined in natural seawater with comparable number in both methods with 35-75 cfu/100 mL. Using spiked seawater close to the limit of the Californian standard and close to the limits of the BWMC, both cell concentrations have been determined close to the given cincentrations of the spiked bacteria. The cell counts using the classical method are generally a bit higher compared to the ScanVit method, as the classical method does not allow to discriminate between different coliform bacteria.

Both organism groups, E.coli and Enterococci, could be detected in one analytical procedure by the comprehensive ScanVit Ballast Water Kit Enterococcus/E.coli. Both experiments, the single spiking and the combined spiking, yielded values close to the predefined organism densities (i.e. 10-10000 cfu/100 mL).

3.2 Reliable use of ScanVit Ballast Water Kit Vibrio cholerae with natural sea water samples.

The ScanVIT method was used to measure the bacterial density in different natural sea water samples of different origin sampled at two different days. In parallel, of the same samples were analyzed by incubation on TCBS agar. The detection limit is given with 1 cfu per 100ml.

The natural density of *Vibrio cholera* was determined in natural seawater to be below 1 cfu/100 mL, even when filtering approximately 5 L of sea water. Therefore, only spiking experiments were used to evaluate the ScanVit Ballast Water Kit Vibrio cholerae. Using

spiked seawater close to the limit of the Californian standard and close to the limits of the BWMC (250 cfu/100 mL)., Both cell concentrations have been determined asclose to the given concentrations of the spiked bacteria.

3.3 Robustness of the methods

Both methods gave reliable results and were robust against deviations, e.g. day of sampling, processing time, day of processing, executing personnel etc. However, it has to be kept in mind that ballast water is quite different compared to natural seawater. This is especially true for the methodological extension of the protocol needed for the processing of sea water samples. The use of a glas fibre filter (1-2µm) as pre-filtration step considerably reduced the cell number (cfu) of cultured *Escherichia coli* in spiked samples by the factor of 100.
It has to be assumed that this reduction by pre-filtration also happens when analyzing natural seawater samples in contrast to ballast water samples.
A reason for this phenomenon may be the elimination of those bacteria aggregated to larger pellets in a solution (i.e. the culture of *E. coli*) or the attachment of bacteria to particles. Both, particles and pellets, would be removed by a filtration step. However, the applied filtration step and the included rinsing (after the initial filtration) were necessary to remove a strong background fluorescence that occurred when using non diluted sea water samples.
Also, since filtration is an integrated treatment step of ballast water treatment systems on board ships steps, it has to be explicitly noted that the bacterial density in ballast water is rather low compared to the bacterial density of natural seawater.
Both methods are sensitive and robust enough to detect the required species with regard to the limit of the Californian standard and the limits of the IMO convention.

3.4 Time requirements

Including all preparatory steps, such as dilution, filtration, etc. the FISH method required less than 12 hours from sample to result.
For the classical method using selective media, a minimum processing time of 8 h was reached. However, this classical approach results in sum parameters of quantitative yields only (number of coliform bacteria). Specific yields, i.e. cfu of *Escherichia coli* and discrimination between *E. coli* and Enterococci need longer incubation periods when applying classical methods.
For the FISH method an intermediate pre-cultivation step is needed. In total, including a pre-cultivation time of 8 hours (according to the protocol), the time from sample to result sums up to a maximum of 12 hours with 3-4 hours analysis step.
A direct processing of membrane filters is only possible for seawater samples containing a high number of e.g. *E.coli* cells with a bacterial density of >105 cfu/ml.

The FISH method gives very fast answers concerning the presence or absence of specific bacteria (at species level) in relation to limit values within the frame of water quality regulations.

4 Conclusion

The classical cultivation approach on selective media and the approach using detection by FISH (after cultivation) gain comparable results in sea water samples. Only the latter allows for the detection on species level.
In general, two critical aspects have to be considered:

(1) An adjusted volume must be filtered. Since the cell number is unknown in the most cases, a variety of volumes has to be applied (or a pre-experiment is necessary)
(2) Samples alter due to storage. Immediate processing is necessary.

4.1 Suitability of the ScanVIT Ballast water kits for fast detection of *E. coli*, *Enterococcus* and *Vibrio cholerae* in sea water samples

The FISH kit for Enterococcus/*Escherichia coli* worked properly with cultures of both *E. coli* and *Enterococcus*, the same was true for the kit for *Vibrio cholerae*. However, using non-diluted natural sea water samples, very high background fluorescence was obtained. An optimisation could be achieved by an additional rinsing steps and the application of a glass fibre filter as initial processing step.
By this optimisation, the results of the FISH analyses are comparable to the classical method and are therefore suitable for the analyses of sea water samples for the fast and specific detection of the respective bacteria.
Both ScanVIT Ballast Water test kits are sensitive and robust enough to detect the required species with regard to the limit values of the Californian standard and the limit value of the BWMC.

5 Appendix

5.1 Table 1: Samples and values

NOK – Kiel Channel, Holtenau ferry station

MC – classical method using McConkey agar

TCBS – classical method using TCBS agar

ScanVit – FISH method with the respective specific ScanVit Kit

Sample ID	Method	Species	cfu/100 mL (mean value)	Replicates	Comment
NOK day 1	MC	colifoms	57	3x2	three different volumes filtered
	MC	Enterococcus	16	3x2	three different volumes filtered
	MC	Enterococcus	full recovery of 10^2 and 10^5 cfu/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	TCBS	V. cholerae	<1	3x2	three different volumes filtered
	TCBS	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
NOK day 1	Scan VIT	E. coli	36	3x2	three different volumes filtered
	Scan VIT	Enterococcus	20	3x2	three different volumes filtered
	Scan VIT	Enterococcus	full recovery of 10^2 and 10^5/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	Scan VIT	V. cholerae	<1	3x2	three different volumes filtered
	Scan VIT	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered

Sample ID	Method	Species	cfu/100 mL (mean value)	Replicates	Comment
Baltic Sea, Kiel Bight, Bülk	MC	colifoms	69	3x2	three different volumes filtered
	MC	Enterococcus	42	3x2	three different volumes filtered
	MC	Enterococcus	full recovery of 10^2 and 10^5/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	TCBS	V. cholerae	<1	3x2	three different volumes filtered
	TCBS	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
Baltic Sea, Kiel Bight, Bülk	Scan VIT	E. coli	54	3x2	three different volumes filtered
	Scan VIT	Enterococcus	51	3x2	three different volumes filtered
	Scan VIT	Enterococcus	full recovery of 10^2 and 10^5/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	Scan VIT	V. cholerae	<1	3x2	three different volumes filtered
	Scan VIT	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered

Sample ID	Method	Species	cfu/100 mL (mean value)	Replicates	Comment
NOK day 2	MC	colifoms	74	3x2	three different volumes filtered
	MC	Enterococcus	5	3x2	three different volumes filtered
	MC	Enterococcus	full recovery of 10^2 and 10^5/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	TCBS	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
NOK day 2	Scan VIT	E. coli	61	3x2	three different volumes filtered
	Scan VIT	Enterococcus	8	3x2	three different volumes filtered
	Scan VIT	Enterococcus	full recovery of 10^2 and 10^5/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered
	Scan VIT	V. cholerae	full recovery of 10^2 and 10^4/100 mL	2x2	spiked with 10^6 cells/100 mL, two different volumes filtered

5.2 Material requirements

5.2.1 Classical method

For the examination of 400 ballast water samples approximately 2400 filters have to be used for subsequent cultivation on agar plates (2400). This allows performing at least two replicates per sample. As the amount of bacteria in a given sample is not known, in the initial filtration step at least three different volumes should be filtered in replicates. This applies for all tested organisms.

5.2.2 ScanVIT

For the examination of 400 ballast water samples approximately 8 ScanVit test kits (a 100 samples per kit) are necessary, in order to perform at least two replicates per sample. As the amount of bacteria in a given sample is not known, in the initial filtration step at least four different volumes should be filtered in replicates. In the subsequent analysis, only the filters with an appropriate number of colonies shall be processed.
This applies for both, the ScanVIT Kit Ballast Water Enterococcus/E. coli and for the ScanVIT Kit Ballast water V. cholerae.

All other requirements are listed in the SOP for the ScanVit methods.

5.3 List of abbreviations used

Abbr.	Definition
BWMC	IMO Ballast Water Management Convention
DIFCO	Supplier of culture media for microbiological analysis
M1	Culture medium (agar) for heterotrophic bacteria
MC	McConkey, specific culture medium (agar) for Enterococci and coliform bacteria
NOK	Kiel Channel, international shipping route, which connects the North Sea with the Baltic Sea
TCBS	Thiosulfate Citrate Bile Sucrose, specific culture mediu m (agar) for Vibrio species
TSB 12	Tryptic Soy Broth 12 g/L

5.4 Literature cited

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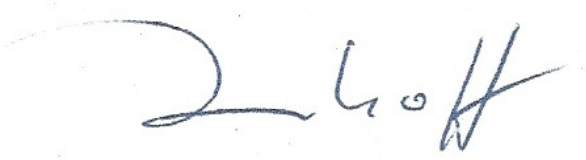

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