Speedy Breedy - Lab Memo 34

Evaluation of *M-Enterococcus medium* for Ballast Water testing in Speedy Breedy

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Aim

The aim of this study was to assess M-Enterococcus Medium (also known as M Azide Medium or Slanetz & Bartley medium) as a suitable substrate for the culture, selective identification and enumeration of Enterococci contaminating ballast water samples.
Ballast water regulation standards (US & IMO) are expected to require ballast water treatment systems to reduce contamination of Enterococci to < 100 cfu per 100 ml. Speedy Breedy protocols must therefore allow enumeration of Enterococci in the range 0 – 250 cfu / 100 ml (0-125 cfu per 50 ml in Speedy Breedy).

Background

The Environment Agency 'Microbiology of Drinking Water 2002'[6] recommend the use of Slanetz and Bartley medium for the enumeration of Enterococci in water supplies, as do ISO in the standard for water quality[7] using a filtration then plating method.

The use of M-Enterococcus medium in Marine Water testing

A number of studies have compared different culture media for testing Enterococcus contamination of marine water. They were compared on the basis of their accuracy, specificity, selectivity, precision and relative recovery efficiency characteristics.

The studies concluded that M-Enterococcus medium showed the best performance characteristics of the seven commonly used enumeration media. Further, since the recovery rate in M-Enterococcus medium is high, this suggests that this medium is less aggressive and detrimental to stressed organisms than others tested.
This Bactest study takes on board these findings and extends the use of M-Enterococcus medium into a Speedy Breedy test format using direct sampling of ballast water.

Technical assessment of suitability of M-Enterococcus Medium in Speedy Breedy

1. Generation of a Pressure Signal:

Of primary importance in selecting a medium for use in Speedy Breedy is the generation of a strong pressure signal by the target organisms. The time to detection of this pressure signal indicates the cfu content of the sample: a shorter Time to Detection indicates a higher cfu and a longer Time to Detection indicates a lower cfu.

Three species of Enterococci were tested to determine whether the M Enterococcus medium produces a significant pressure transient (signal) indicating that Speedy Breedy will easily detect contamination by a range of Enterococci.

Selectivity

Experiments were set up to show selectivity of M-Enterococcus Medium for Enterococci while suppressing other related (but non-enteric) organisms and likely contaminants in water samples. The experimental protocol was performed at 36 ^{0}C – protocol below:

Method

Sterile broth was prepared largely according to the manufacturer's instructions and after cooling, transferred aseptically as 50ml volumes into sterile empty Speedy Breedy culture vessels.

Speedy Breedy culture vessels were inoculated in duplicate with a range of organisms to determine growth or inhibition. Each sample was cultured in parallel on broad-spectrum growth agar plates (Columbia Agar with Horse Blood) to confirm purity and viability and incubated at 36°C for 48 hours before reading of plates. This generated a cfu value for each organism inoculated into the test.

Results

Time to Detection (TTD below) represents the time it took Speedy Breedy to indicate a positive detection result.

Positive Controls:

Species	Enterococcus faecalis NCTC 12697 / ATCC 29212	Enterococcus faecalis NCTC 775 / ATCC 19433	Enterococcus hirae NCTC 13383 /ATCC 10541
CFU in Vessel	2.8 x 10^2	1.0 x 10^2	2.4 x 10^2
TTD (Minutes)	705	810	898
TTD (Hours)	11.45	13.30	14.58
Turbidity	Turbid Growth	Turbid Growth	Turbid Growth

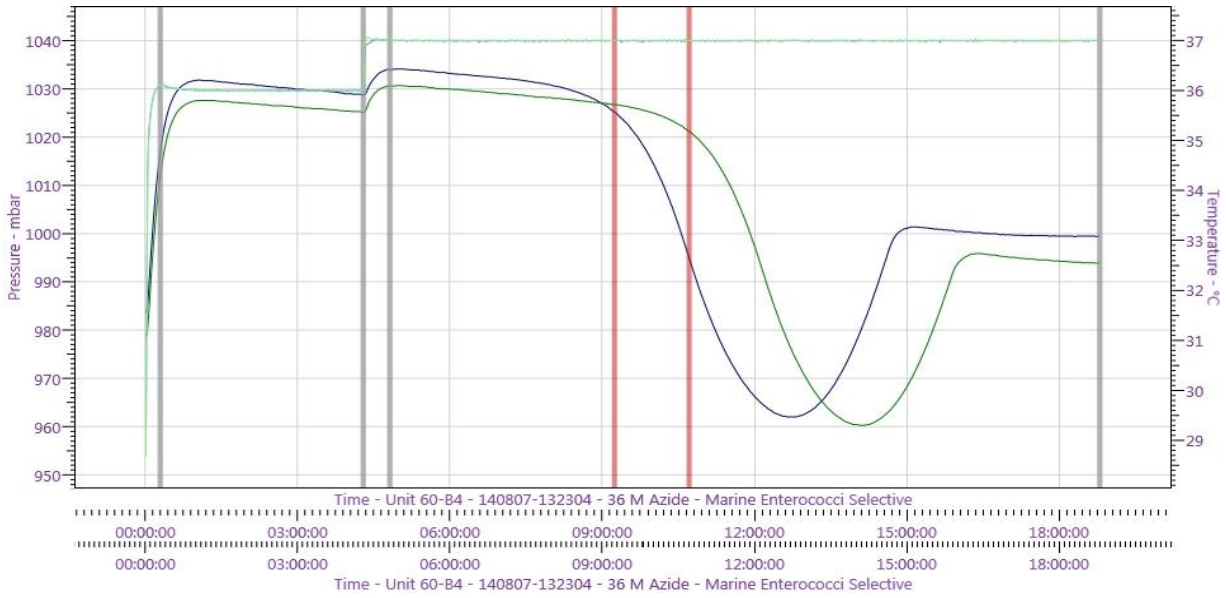
Related species likely to cross react:

Species	Streptococcus pyogenes NCTC 12696 / ATCC 19615	Streptococcus agalactiae NCTC 8181 / ATCC 13813
CFU in Vessel	8.5 x 10^2	7.1 x 10^2
TTD (Hours)	No Detection	No Detection
Turbidity	No Growth	No Growth

Unrelated common species likely to be found in the sample:

Species	E. coli	Staphylococcus aureus NCTC 6571	Raoultella planticola NCTC 9528
CFU in Vessel	4.8 x 10^2	2.8 x 10^2	3.3 x 10^2
TTD (Hours)	No Detection	No Detection	No Detection
Turbidity	No Growth	No Growth	No Growth
Species	Enterobacter aerogenes	Pseudomonas aeruginosa ATCC 9027	
CFU in Vessel	2.3 x 10^2	3.6 x 10^2	
TTD (Hours)	No Detection	No Detection	
Turbidity	No Growth	No Growth	

Enterococcus faecalis NCTC 775 / ATCC 19433



Conclusions

- All Enterococci grew in less than 18 hours for samples of less than 3.0 x 102 suggesting that a local test can be performed within one day.
- All Enterococci gave strong pressure events meaning that Speedy Breedy can readily detect an Enterococcus contaminant.
- Common contaminants that are likely to be found in the sample gave no pressure events or evidence of growth (turbidity) and were therefore suppressed; indicating that the protocol and medium is selective and unrelated organisms will not significantly affect the result.
- Closely related organisms such as Streptococci, when present in small numbers (<103 per sample), gave no pressure signal or evidence of growth (turbidity) in Speedy Breedy.
- The protocol and medium is therefore highly selective.

Calibration of Time to Detection (TTD) vs. number of organisms in sample (cfu)

In order to determine the number of organisms present in a test sample, a calibration curve is essential for Speedy Breedy to calculate the expected CFU value against the Time to Detection using the software's enumeration facility. A calibration curve is produced by testing a number of samples of known bacterial content to determine the Time to Detection (TTD) values associated with these numbers and then plotting these against each other. Calibration is performed across a range of contamination levels, the more data produced, the better the quality of the calibration.

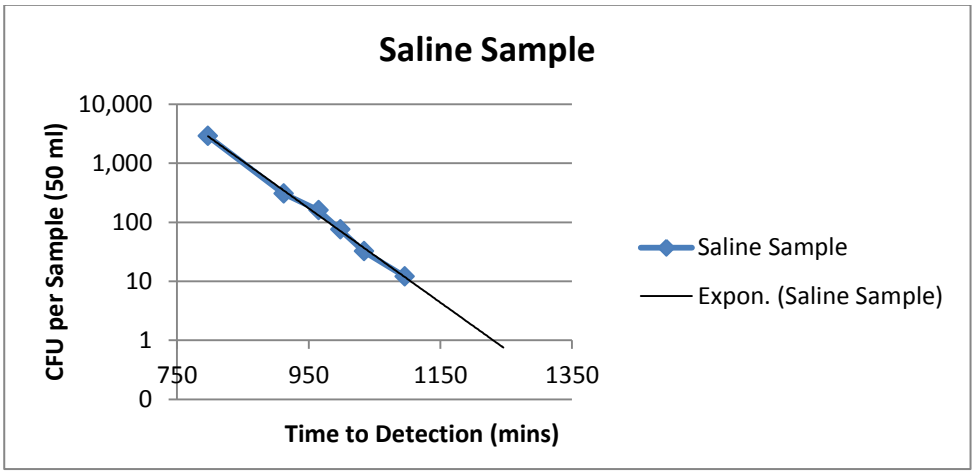
A calibration curve was developed largely in line with Bactest SOP # QP14009 with the exception that samples were processed in duplicate rather than triplicate.

Reference marine water was prepared with a salinity of 3.5% (Millero et al., 2008) 10 and sterilised by autoclaving.

Samples were prepared by spiking Reference Marine Water with Enterococcus faecalis NCTC 775 at a range of target concentrations by serial dilution and acclimatising them for 24 hours at 8 0C to mimic the situation that might be found in a real ballast water test. Suspensions were prepared from Vitroid discs (Sigma Aldrich), with discs allowed to equilibrate to room temperature before re-suspension in phosphate buffered saline and subsequent dilution into the sample matrix.

Each sample was then cultured in Speedy Breedy in duplicate and plated out on broad-spectrum growth agar plates (Columbia Agar with Horse Blood) to confirm purity and viability and incubated at 36°C for 48 hours before reading of plates. By reading the number of Colony forming units on each plate, we generated a cfu value for each dilution of the organisms.

Calibration of TTD vs. number of organisms in the sample:



The data above can be automatically stored by the Speedy Breedy software feature and the appropriate line equation determined. This is then linked to subsequently tests to determine the numbers of bacteria present in a sample. Extrapolating from the equation of this trend-line, and approximate time for detection for 1 CFU within the vessel can be determined. This provides advice as to the overall time required for a successful test protocol.

Where sample dilution factors are applied, the software feature automatically totals data from multiple tests or multiples data against dilution factors to give an estimated CFU present in the 50ml sample volume.
A Speedy Breedy protocol for Enterococcus testing has now been defined by the calibration data above.

Conclusions

- M-Enterococcus medium has been demonstrated to be a highly effective growth medium which has good specificity, produces strong pressure events in Speedy Breedy with Enterococcus faecalis, the most common target Enterococcus species in ballast water testing, and rapid growth and detection for efficient turn-around of results.
- Speedy Breedy can be used as a highly effective tool to detect and enumerate the numbers of Enterococci contaminating ballast water. Speedy Breedy can calculate the level of contamination required by Ballast water regulation standards.

- Tests can be performed on-site without the need for samples to be sent to an external laboratory. The test time required is only 24 hours for single organism detection although operators may wish to run for 48 hours in some cases.

References

1. Slanetz L. W. and Bartley C. H. (1957) *J. Bact*. 74. 591-595.
2. Burkwall M. K. and Hartman P. A. (1964) *Appl. Microbiol*. 12. 18-23.
3. Nordic Committee on Food Analysis (1968) *Leaflet 68*.
4. Taylor E. W. and Burman N. P. (1964) *J. Appl. Bact*. 27. 294-303.
5. Mead G. C. (1966) *Proc. Soc. Wat. Treat. Exam*. 15. 207-221.
6. Environment Agency *'Microbiology of Drinking Water 2002'. Methods for Examination of Waters and Associated Materials.*
7. ISO Standard for Water Quality - Detection and enumeration of intestinal enterococci - Part 2 : Membrane filtration method.
8. Evaluation of Media for Monitoring Fecal Streptococci in Seawater
YONA YOSHPE-PURER. Tie A. Felix Public Health Laboratory, Ministry of Health, Israel. APPL-IED AND ENVIRONMENTAL MICROBIOILOGY Aug. 1989. p. 2041-2045
9. Evaluation of media for the enumeration of faecal streptococci from natural water samples - Lidia PO Catalao Dionisio", Juan J. Borregob'*- Journal of Microbiological Methods 23 (1995) 183-203
10. Millero, F.J., Feistel, R., Wright, D.G., McDougall, T.J. (2008) The composition of Standard Seawater and the definition of Reference-Composition Salinity Scale. *Deep Sea Research, Part I: Oceanographic Research Papers*, Vol. 55 (1), pp 50-72.
11. Rice, E.W., Baird, R.B., Eaton, A.D. and Clesceri, L.S. (2012) *Standard Methods for the Examination of Water and Wastewater,22nd Edition*. Published by American Pub