Standard Operating Procedure

Procedure for the Analysis of Ballast Water to determine the Concentration of Bacteria using the Adenosin-Triphosphate Method

Compiled by :

Name: Peter Paul Stehouwer

Title / Institution, Company : Marine Biologist, MSc, SGS Germany GmbH, Hamburg, Germany

Name: Lothar Schillak

Title / Institution, Company : Marine Biologist, PhD, SGS S.A. Environmental Services, Geneva, Switzerland, SGS Institut Fresenius GmbH, Taunusstein, Germany

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Content

1 BACKGROUND

In 2004 the International Maritime Organization of the United Nations set up the '*International Convention for the Control and Management of Ships' Ballast Water and Sediments*'. According to article 18 of this convention the regulations and requirements therein come into force 12 months after 30 states ratified the convention representing 35% gross tonnage of the world's merchant shipping.

In view of the fact that organism transported with ballast water of ships and released into the sea including estuaries and into fresh water courses may cause severe and irreversible ecological damages, impair biological diversity and create hazards to human health, property or resources, this convention regulates that all ships to which this convention applies shall treat the ballast water by adequate on-board technologies to achieve a quality, which is defined by limit values for the density of organisms in the treated ballast water to be re-discharged to the sea, estuaries or fresh water courses.

The annex to the convention, Section D, '*Standards for Ballast Water Management*', Regulation D-2 '*Ballast Water Performance Standard*' defines these limit values for the density of organisms in ballast water to be re-discharged to the sea, estuaries or freshwater courses as :

Organism / Organism Group	Limit Value
Plankton Organisms >50µm	<10 viable organisms per m^3
Plankton Organisms >10µm<50µm	<10 viable organisms per ml
Bacteria	
Escherichia coli	<250 cfu per 100ml
Intestinal Enterococci	<100 cfu per 100ml
Vibrio cholerae (O1 and O139)	<1 cfu per 100ml
(cfu : colony forming unit)	

To control the compliance of this regulation adequate technologies and methods to generate ballast water samples on board ships and to execute the analysis of the ballast water have to be defined.

In 2011 the *Federal German Hydrographic and Maritime Agency (BSH)*, Hamburg, Germany, launched the research and development project '*Effective New Technologies for the Assessment of Compliance with the Ballast Water Management Convention*', which aimed at the development of technologies and methods to rapidly sample and assess the ballast water quality on board ships.

Within the frame of this project a new, innovative sampling system as well as several analytical methods have been developed which allow for the rapid assessment of the ballast water on board ships.

The project was managed, conducted and executed by *SGS S.A., Environmental Services, Geneva, Switzerland* and *SGS Institut Fresenius GmbH, Taunusstein, Germany* in cooperation with international scientific institutions and companies.

On board technologies and methods to sample ballast water and assess its quality should, above all, generate reliable data within a minimal time, since these compliance tests can, at present, only be executed during unload and load procedures while the ships stay in the harbor.

The classical methods (visual counts of plankton organisms under the microscope; 24/48 hour incubation of ballast water sample on species specific agars) are not suitable to be executed on board ships, especially since, among other, the methods require the incubation of human pathogen bacteria.

Therefore the BSH project aimed to indentify technologies and method, which can be executed on board ships even if these methods could only generated indicative results.

2 INTRODUCTION

The approach to identify an analytical method, which allows for the rapid, indicative assessment of the ballast water quality on board ships, focused on measuring the concentration of a chemical substance in a ballast water sample and correlate this concentration to the number of the target organisms in the ballast water sample.

Once the correlation is established the second aspect of indicative compliance testing on board ships was to determine the lower sensitivity range of the method, i.e. the minimal number of target organisms in a ballast water sample, which can still be detected by the method.

Adenosin-Triphosphate (ATP, $C_{10}H_{16}N_5O_{13}P_3$) is a biochemical compound, which is found in living cells of all organisms: in eukaryote organisms ATP is found in the mitochondria, in prokaryote organisms ATP is found in the cytoplasma. ATP provides the energy necessary for most of the biochemical, physiological processes within living cells. ATP breaks down quickly after the organism dies, therefore in dead organisms ATP is not detectable.

The ATP method presented in this document is based on rapid assessment ATP test kits for the detection of bacteria in water samples already available on the market. The test kits represent the second generation of the relevant product line, which excludes interferences with ATP from other sources, e.g. free ATP.

Within the BSH project mentioned above (cf. para 1) the original protocol for the determination of bacterial ATP in a water sample has been slightly modified for adaptation to natural seawater and ballast water analysis. This modification mainly addresses the filtration of the seawater/ballast water sample to eliminate other marine organisms, which could lead to false positives.

Several test series executed within the frame of the BSH project established a reliable correlation between the concentration of ATP in the seawater/ballast water sample and the containing bacteria and investigated the suitability of this method for compliance tests of ballast water on board ships.

The results of all these test series reveal the feasibility to determine the quantitative concentration of bacteria in ballast water samples on board ships.

The time needed from sample to result is 4 minutes.

The lower sensitivity range is given with 30 cfu per 100ml.

The method has been validated by an external, independent laboratory specialized in marine microbiology.

Reference is made to various documents and other sources listed in para 8 (cf. page 5).

3 EQUIPMENT

- LB 9509 luminometer
- 10 µm nylon filter

4 SUPPLIES

- *Luminase*
- *Ultracheck 1*
- 60 ml syringe
- 2 µm glass fiber filter
- 100 ml beaker
- *QuenchGone* filter
- *Ultralyse 7*
- *Ultralute*

- 12 ml extraction tube
- Luminometer tube

5 PROCEDURE

Calibration

1. Pour 100µl *Luminase* into a luminometer tube
2. Add two drops (=100µl) of *UltraCheck 1*
3. Insert luminometer tube into Luminometer, record RLU $\Rightarrow RLU_{UC1}$
4. If RLU_{UC1}<5000 with an LB 9509, use new bottle of *Luminase*

Sample Preparation

1. Filter ballast water sample through 10 µm nylon filter to keep out large organisms and particles, collect filtrate in small beaker.
2. Remove plunger from 60 ml syringe and attach 2 µm glass fiber filter to the front of syringe.
3. Pour filtrate into syringe, insert plunger.
4. Push sample through 2 µm filter, collect filtrate in small beaker.

Sample Analysis

1. Remove plunger from 60 ml syringe and fix *QuenchGone* filter to syringe.
2. Pour 60 ml of sample (from Sample Preparation step 4) into the syringe with attached filter, insert plunger.
3. Gently push plunger down ensuring that the filter is kept wet, discard filtrate.
4. Detach filter from syringe, remove plunger from syringe, re-attach filter to syringe.
5. Pour 1 ml of *Ultralyse 7* into syringe, insert plunger.
6. Gently pass the liquid through the filter into a 12 ml extraction tube until dryness of filter, discard filter.
7. Pour 9 ml of *Ultralute* into the extraction tube, put cap on tube, invert 3 times.
8. Transfer 100 µl from the extraction tube to a luminometer tube.
9. Add 100 µl of Luminase to luminometer tube, gently swirl tube 5 times.
10. Insert luminometer tube into Luminometer, record RLU $\Rightarrow RLU_{cATP}$

Final Calculations

1. Amount of cellular ATP (cATP) is given as : $cATP\,(pgATP/ml) = \frac{RLUcATP}{RLUUC1} \times \frac{10.000\ (pgATP)}{Vsample\ (ml)}$
2. 1 *Escherichia coli* sized bacterium contains 0,001 pg cATP, i.e. the microbial equivalent (ME)
3. The bacterial concentration (BC) in the ballast water sample is then :

$$BC\ (ME/ml) = \frac{RLUcATP}{RLUUC1} \times \frac{10.000\ (pg\ ATP)}{Vsample\ (ml)} \times 1000$$

6 QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

1. Procedures outlined in this SOP should be followed to the letter. Any deviation should be documented.
2. Conduct all quality assurance and quality control procedures according to relevant QA/QC standards of the executing institution or company.

7 DATA STORAGE AND ARCHIVING

1. Storage and archival storage of data should be executed following relevant guidelines and SOPs of the executing institution or company.

8 REFERENCES AND RELATED DOCUMENTS

SOP '*Procedure to generate representative ballast water samples from ballast water pipe systems onboard ships'*

SOP '*Procedure to produce artificial seawater from prefabricated salt mixtures*'

'The Analysis of the plankton concentration in ballast water samples by the ATP method', Validation Report

'*Effective New Technologies for the Assessment of Compliance with the Ballast Water Management Convention*', project reports

Websites :

International Maritime Organization – IMO : www.imo.org

Luminultra (producer of chemicals for ATP test kits) : www.luminultra.com

Aqua Tools (distributor of ATP test kits) : www.aqua-tools.com

9 APPENDIX

CONTACT

Dr.Lothar Schillak

Marine Biologist PhD; Senior Marine Expert

SGS S.A.
SGS-Environmental Services
1 Place des Alpes
Box Postale 2155
CH-1211 Geneva 1

SGS Institut Fresenius GmbH
Auf dem kleinen Feld 15a
D-65232 Taunusstein/Germany
Phone : +49 (0)6128 748 73 809
Mobile . +49 (0)152 226 186 56
Fax : +49-(0)89 125 040 698 09
Email : lothar.schillak@sgs.com

Peter-Paul Stehouwer

Marine Biologist MSc; Marine Expert

SGS Germany GmbH
SGS-Environmental Services

Rödingsmarkt 16
D-20459 Hamburg/Germany
Phone : +49 (0)40 30101 316
Mobile . +49 (0)152 226 873 14
Email : peter.stehouwer@sgs.com

www.sgsgroup.de

www.sgs.com