

Standard Operating Procedure

Procedure for the Analysis of Ballast Water to determine the Concentration of Plankton organisms >50µm using the Adenosin-Triphosphate Method

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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	
<i>Escherichia coli</i>	<250 cfu per 100ml
Intestinal Enterococci	<100 cfu per 100ml
<i>Vibrio cholerae</i> (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 others, the methods require the incubation of human pathogen bacteria.

Therefore the BSH project aimed to identify technologies and methods, 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 cytoplasm. 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 significantly modified to detect the ATP from plankton organisms in natural seawater and ballast water analysis. These modifications mainly address the filtration of the seawater/ballast water sample to concentrate the target organisms in small volumes and the mobilization of the ATP from within the cells of living plankton organisms.

Several test series executed within the frame of the BSH project finally identified the most adequate technique for the disruption of the cells to free the cellular ATP for further analysis steps: a mixture of beads of different size in a small (<100ml) container agitated for a few minutes at a defined input of beating energy yielded the best cell disruption results, with the cellular ATP being mobilized to 100% from various groups of marine plankton organisms: unicellular microalgae, cell aggregates of marine microalgae, diatoms, crustaceans (also Ostracoda), larvae of other marine taxa (e.g. Mollusca, Cnidaria).

As the adequate cell disruption technique has been identified, additional test series with natural plankton from various marine climates were executed to assess the correlation between the concentration of cellular ATP in a seawater/ballast water sample and the density of the target organisms in the sample and to investigate the suitability of this method for compliance tests of ballast water on board ships. Additional test were executed with single plankton organisms >50µm both from natural plankton samples and from crustacean populations cultured in artificial seawater.

The obtained results established a reliable correlation between the concentration of ATP in the seawater/ballast water sample and the containing plankton organisms >50µm.

In addition the test series reveal the feasibility to determine the quantitative concentration of plankton organisms >50µm in ballast water samples on board ships

The time needed from sample to result is 12 minutes.

The method is able to detect one single plankton organism >50µm in a seawater/ballast water sample.

The method has been validated by an external, independent laboratory specialized in marine ecology and seawater analysis.

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

3 EQUIPMENT

- LB 9509 luminometer
- Ultra Turrax Grinder
- 100-1000 µm pipette
- 0.5-5 ml pipette

4 SUPPLIES

- *Luminase*
- *Ultracheck 1*
- 100 ml beaker
- Grinding tube
- Modified *Ultralyse 30*
- Dilution 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

The ballast water sample to be analyzed by the ATP method is generated through the sampling points on board the ships and additional, adequate sampling systems and procedures (cf. para 8 for SOP xx).

Sample Analysis

1. Mix the sample in the small beaker, add 1 ml of well-mixed suspension to a grinding tube.
2. Add 5 ml of modified *Ultralyse 30* to the grinding tube.
3. Place the grinding tube in the grinder, run for 2.5 minutes at 6000 rpm.
4. Remove the tube from the grinder, turn it over twice and place it back on the grinder.
5. Run the grinder for 2.5 minutes at 6000 rpm.
6. Remove the tube from the grinder, leave the tube for 5 minutes to let the solids settle.
7. Transfer 100 µl of supernatant to the dilution tube.
8. Close the dilution tube and mix it.
9. Take 100 µl of diluted extract and add it to a luminometer tube.
10. Add 100 µl of *Luminase* to luminometer tube, gently swirl tube 5 times.
11. Insert luminometer tube into Luminometer, record RLU \Rightarrow RLU_{CATP} after 10 seconds.

Final Calculations

1. The obtained RLU values are converted into ATP concentrations using the *Calculate Software*, which is part of the test kit according to the equation cellular ATP (cATP) (ng/ml) = (RLU_{CATP} / RLU_{UC1}) x 306.
2. Since ATP concentrations are proportional to the number of plankton organisms per m³, the cATP sample concentrations are converted into the organism density as follows:
 $cATP / (SVBW \times 1000) = \text{Number of organisms} / m^3$, where SVBW is the sampled volume of ballast water.

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

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