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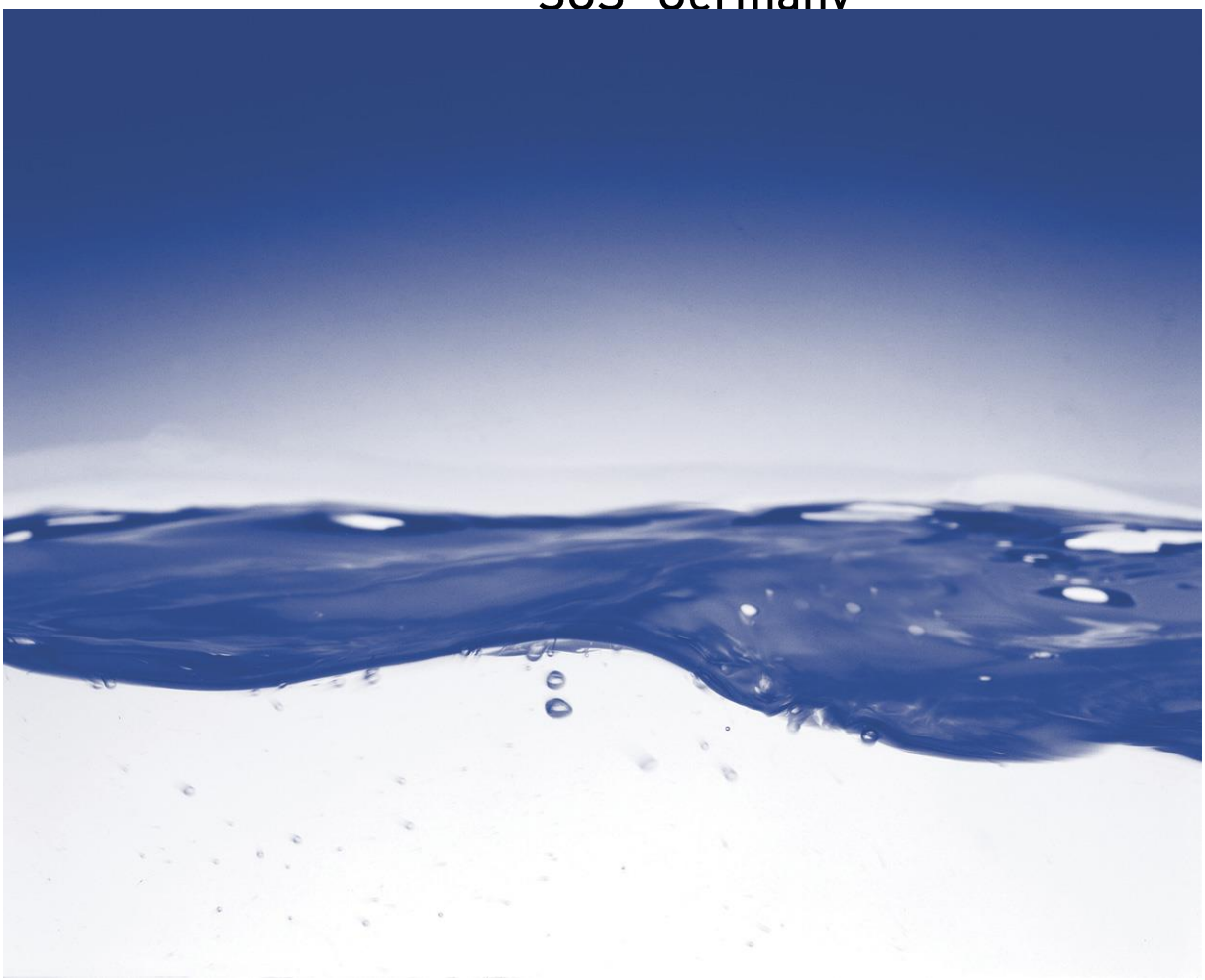
Preliminary validation of an ATP analytical procedure for ballast water

SGS- Germany



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1.1 Introduction

A research project have been conducted by the Federal German Hydrographic and Maritime Agency - BSH, Hamburg, Germany, the SGS Institut Fresenius GmbH, Taunusstein, Germany to develop an analytical method for treated ballast water based on ATP measurements. The intention is to use the the test kit for a rapid onboard analysis to indicate compliance with the D-2 regulation from International Maritime Organisation (IMO). According to the IMO Ballast Water Convention, all ships have to disinfect their ballast water to ensure a maximum viable organism density for ≥50µm organisms, ≥10-50µm organisms and bacteria during discharge (**Table 1**).

As an independent third part, Norwegian Institute for Water Research (NIVA) has tested the ATP instrument developed by SGS in the laboratory. NIVA has 7 years of experience in ballast water treatment system testing at its full scale test facility and laboratories located in Norway.

The results of the tests of the ATP instrument are presented in this report.

Table 1. Required biological water quality in influent water and treated water during discharge according to IMO regulation D-2 (BWM Convention).

Organism group	Influent water	In treated on discharge (Regulation D-2)
≥50 µm min. dimension	Pref. 10^6 m^{-3}, ≥10^5 m^{-3} Min. 5 species from 3 diff. phyla/divisions	<10 viable organisms per m^3
≥10-50 µm min. dimension	10^4 ml^{-1}, ≥10^3 ml^{-1} Min. 5 species from 3 diff. phyla/divisions	<10 viable organisms per ml
Heterotrophic bacteria	≥10^4 cfu ml^{-1}	-
Escherichia coli	-	< 250cfu/100 ml
Intestinal *enterococci*	-	<100 cfu/100 ml
Vibrio Cholerae	-	<1 cfu/100 ml

1.2 Objectives

The main objective of this laboratory test was to do a preliminary validation of the ATP analytical procedure developed by SGS. The test was done by comparing the organism count results obtained by the ATP instrument to the results obtained by NIVA's standard methods applied for Ballast Water Treatment System testing according to IMO guidelines. Cultivated organisms and indigenous organisms from the Oslo fjord were used. The ATP instrument from SGS was tested in laboratory by generating ATP calibration curves for ≥10-50µm organism (*Tetraselmis suecica*) and for ≥50µm organisms (*Artemia franciscana* and rotatoria).

1.3 Material and methods

1.3.1 Description of the instrument to be evaluated

The ATP instrument submitted by SGS was developed by Aquatools (France) for determining the number of viable organisms in ballast water sample based on quantification of the total adenosine triphosphate (tATP) present in the sample. ATP is a coenzyme used as an energy carrier in the cells of all known organisms. Therefore ATP is used as an indicator of metabolically active cells. ATP is

detected with a luminometer by measuring the luminescence produced by the firefly enzyme luciferase when it reacts with ATP.

The ATP analysis procedure is based on the extraction of the intracellular ATP by destruction of the cell wall and lysis of the cell content. The luminescent signal is generated by reaction of the enzyme with the extracted ATP. The luminescent signal is proportional to the amount of ATP present, which is proportional to the number of organisms in the sample. However, the cellular ATP content might vary considerably from species to species and from life stage to life stage within a same species.
The test kit is presented in **Figure 1** and is constituted of:

- Luminase®, UltraCheck®, UltraLyse®, UltraLute® droppers
- Disposable grinding tubes with bead mixture
- Grinding device for disposable sample tubes
- Luminometer tube
- Luminometer with calculation software

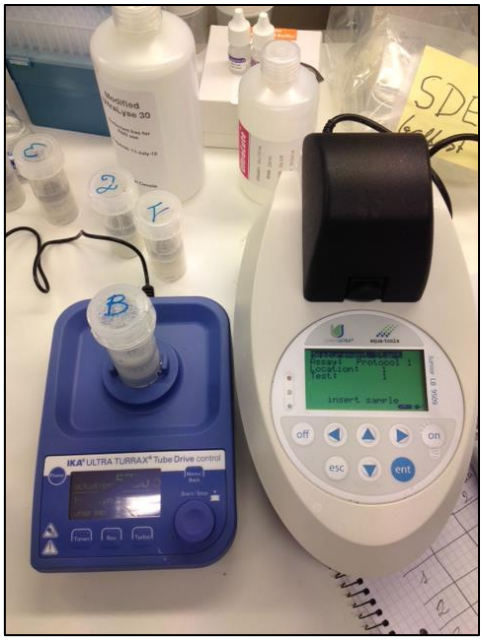

Figure 1 Picture of the luminometer (on right) for ATP measurement developed by Aquatools with the grinding device (on left) to mix the sample in the grinding tube containing beads for ATP extraction.

1.3.2 Test protocol

Test organisms used were collected from NIVA's cultures (*Artemia franciscana* for ≥50µm organism and *Tetraselmis sueccica* for ≥10-50µm organism). Samples with indigenous organisms ≥50µm were collected from the surface water of Oslofjord. The initial organism concentrations were determined by microscopy for ≥50µm or by coulter counter for *Tetraselmis suecica*, as described in section 1.3.3. From these initial samples, serial dilutions were generated.

The initial algal concentration required by IMO in challenge test water for ballast water treatment system testing is 1,000 cell/mL. Therefore, five dilution series were prepared in sterilised aged filtered 60m deep seawater from Oslofjord to cover the organism density range of approximately 5,000-10 cell/mL for ≥10-50µm organism. To determine the appropriate dilution factor according to the initial

algal concentration, the initial algal concentration was estimated by using a coulter counter, as described in section 1.3.3.

For ≥50µm organism, *Artemia franciscana* or indigenous organism were carefully collected individually with a pipette under microscope and transferred to 1mL of sterilised aged filtered 60m deep seawater from Oslofjord. In this way, 7 samples were prepared with ≥50µm organism densities from 1 to 15 organisms/mL. 1mL samples were prepared as 1mL volume is required for the ATP analysis.

Each sample was analyzed in duplicate or triplicate with the ATP instrument from SGS to generate an ATP calibration curve for each organism size group. The initial organism densities in the undiluted samples were confirmed by using microscopy for ≥50µm organism and by the plate count method for *Tetraselmis suecica*.

1.3.3 Analytical methods

ATP method: the measurement was performed according to the supplier's procedure provided by SGS. The analytical method is detailed in Appendix 1. The results were given as Relative Light Unit (RLU).

Determination of the initial algal density: The initial algal concentration was measured with a coulter counter (Beckman Multisizer 3, Beckman-Coulter, USA) with a 100 µm aperture. Particles within 5-15µm in spherical diameter were counted in 300 size intervals. A sample was checked under microscope to assess the condition of the culture to assume that most of the particles counted were viable cells. The algal culture was in the exponential growth phase.

Culture method for counting viable cells of Tetraselmis suecica: A cultivation method for enumeration of viable and reproducible *Tetraselmis suecica* was used by plating the samples on agar plates. 100 µl of samples was spread on a seawater agar growth medium and incubated in constant light for 72 hours at 20˚C. Colonies of *Tetraselmis sp.* were observed by viewing agar plates in stereo microscope at 16x magnification. The procedure has a detection limit of 10 cells/ml.

Identification and quantification of organisms ≥50 µm: Samples for organisms ≥50 µm were inspected using a stereo loupe at 10-40x magnification. Viable organisms were counted and identified based on motility and integrity according to OECD (1985): OECD Test Guideline for Testing of Chemicals 202, "*Daphnia* sp. acute immobilisation test and reproduction test".

1.4 Results and discussion

The laboratory test was performed on the 24th and 25th of September 2013. The calibration curve of the ATP instrument was generated for ≥10-50µm organism (*Tetraselmis suecica* culture) as presented in Figure 1 and for ≥50µm organism (*Artemia franciscana* culture and indigenous copepods from Oslofjord) as presented in Figure 2 and Figure 3. All samples were collected, diluted and analysed the same day than sampling day.

ATP calibration curve for *Tetraselmis suecica*

The initial concentration of the undiluted algal culture sample was analysed by plate count method in triplicate. After the incubation period, there were observed 2840, 2850 and 3070 cells/mL in each of the triplicate plates, giving an initial average algal concentration of 2920 cell/mL. To cover an algal concentration range from approximately 5,000 to 10 cell/mL, a serial dilution was prepared stepwise with a dilution factor of 2 as indicated in the **Table 2**. The ATP was measured in duplicate only for each dilution step due to limited available material for ATP analysis. The ATP average results are presented for all samples in the **Table 2**. The ATP results varied from 40 RLU to 139 RLU, with a

relative high ATP average in the sterile seawater of 28 RLU. The calibration curve on logarithmic scale (**Figure 1a)** shows a correlation between the algal concentration and the ATP results with a correlation factor R^2 of 0.77. However, by excluding the deviating results from the samples diluted 4 times (730 cell/mL), the correlation factor was very good with R^2 of 0.98 (**Figure 1b**). The reason of the deviating results is difficult to explain, but highly diluted samples will tend to be more inaccurate than samples with lower dilution factors. According to the supplier, the luminase solution is approved for luminescent signal above 5,000 RLU. It was measured 10,253 RLU in the luminase solution used for this test.

In this test, the ATP instrument could not be used to measure the ATP in samples with lower algal densities than 183 cell/mL, due to the relatively high ATP signal in sterile seawater used as blank.

Table 2. ATP measurement and dilution operations for *Tetraselmis suecica* samples. SW: sterile seawater.

Algal concentration	Dilution factor	Dilution operation	ATP Measurements (RLU)		
cell/mL		-	1	2	Average
2920	-	-	140	138	**139**
1460	2	2 mL culture in 2 mL SW	84	88	**86**
730	4	2 mL of 2xdilution in 2 mL SW	107	100	**104**
365	8	2mL of 4x dilution in 2 mL SW	39	61	**50**
183	16	2 mL of 8x dilution in 2 mL SW	41	38	**40**
Blank	-	-	28	27	**28**

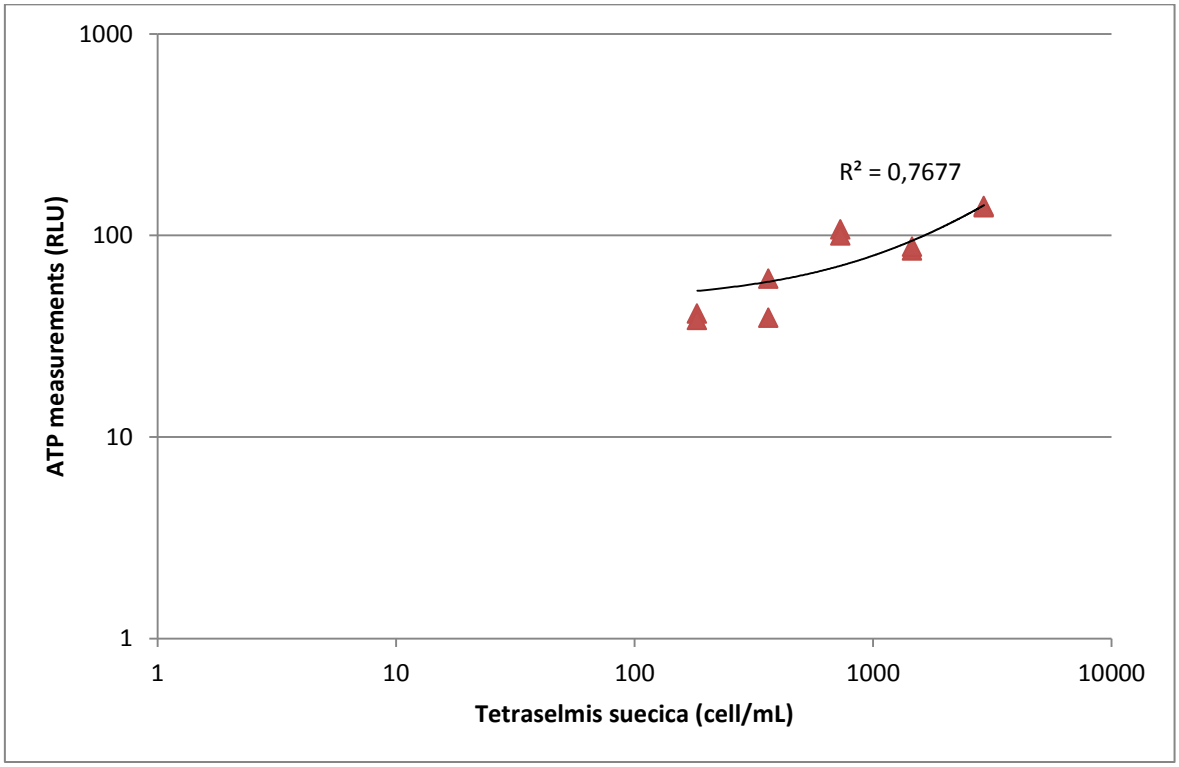

Figure 1a. ATP calibration curve for *Tetraselmis suecica* monoculture for all samples.

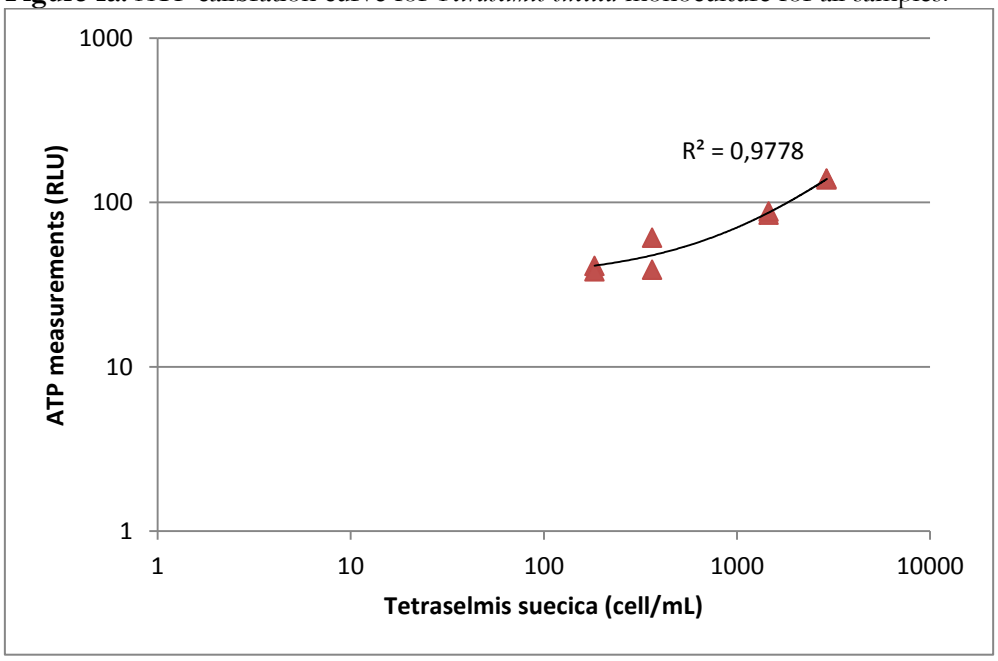

Figure 1b. ATP calibration curve for *Tetraselmis suecica* monoculture, excluding the samples diluted four times.

ATP calibration curve for *Artemia franciscana* culture

A total of seven 1mL samples were prepared containing a concentration of *Artemia sp.* between 1 and 15 per mL. For each sample, 3 measurements of ATP were performed. The average and standard deviation of the ATP measurements are presented in the **Table 2**. The ATP results varied from 549 RLU to 9075 RLU for densities from 1 to 15 organism/mL, respectively. The ATP average in the

sterile seawater used as blank was relatively low with 40 RLU, as average value of triplicates. The ATP calibration curve for *Artemia sp.* is presented in the **Figure 2** in logarithmic scale. The correlation between the Artemia concentration and the ATP results was very good with a correlation factor R^2 of 0.98. The results show that the ATP instrument would be able to detect 1 Artemia in 1 mL of sample. IMO regulation requires to collect $1m^3$ of ballast water for each sample by sieving through a 35µm plankton net. If only 1 Artemia is present in the $1m^3$ of ballast water, the ATP instrument will be able to detect it after filtration of the sieved sample to 1 mL necessary for ATP analysis.

Table 2. ATP values for single analyses and averages, including standard deviations, for Artemia samples. .

Number of Artemia/mL	ATP measurements (RLU)				
	1	2	3	Average	Stdev
1	544	538	564	**549**	14
2	956	890	1143	**996**	131
3	1157	1157	1171	**1162**	8
4	1608	1695	1725	**1676**	61
8	5334	5092	5178	**5201**	123
10	7227	6912	6916	**7018**	181
15	9229	8819	9176	**9075**	223
Blank	36	43	42	**40**	4
Luminase	18500			-	-

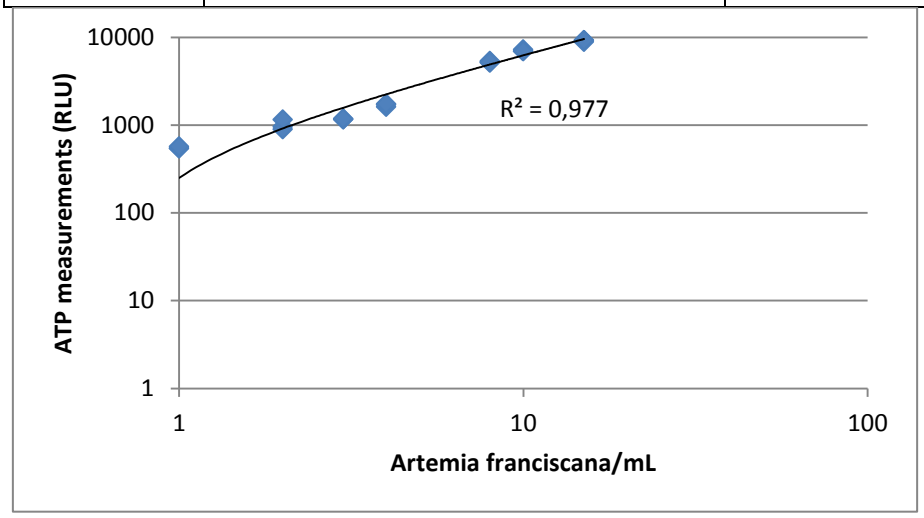

Figure 2. ATP calibration curve for Artemia samples.

ATP calibration curve for indigenous copepods from Oslofjord

A total of 6 samples were prepared containing a concentration of indigenous copepods from Oslofjord between 1 and 15 per mL. For each sample, 3 measurements of ATP were performed. The average and standard deviation of the ATP measurements are presented in the **Table 3**. The ATP results varied from 759 RLU to 3427 RLU for densities from 1 to 15 organism/mL, respectively. The ATP average in the sterile seawater used as blank was low, with 12 RLU as average of triplicates. The ATP calibration curve is presented in the **Figure 3** in logarithmic scale. The correlation between the copepod concentration and the ATP results was relatively good with a correlation factor R^2 of 0.84. The reason for lower correlation factor compared to the correlation factor obtained with Artemia is

probably due to analytical challenges. Since the copepods are smaller (not easily visible with bare eyes) than Artemia, it was difficult to ensure the transfer of all of them to the bead beating tube. Even if the emptied tubes were controlled under microscope after each transfer to the bead beating tube. This might explain the deviating results for the samples with 2 and 4 copepods/mL. The copepods should have been collected individually under microscope and transferred directly to a bead beating tube, but due to lack of ATP analysis material, the experiment couldn't be repeated. However, the results indicate that the ATP instrument would be able to detect only 1 copepod present in 1 mL of sample. IMO requires to collect $1m^3$ of ballast water for each sample by sieving through a 35µm plankton net. If only 1 copepod is present in the $1m^3$ of ballast water, the ATP instrument would be able to detect it if it is possible to collect the only one copepod from the sieved sample to the 1 mL necessary for ATP analysis.

Table 3. ATP values for single analyses and averages, including standard deviations, for copepods in samples collected from Oslofjord.

Number of copepods/mL	RLU				
	1	2	3	Average	Stdev
1	831	733	714	**759**	63
2	359	430	626	**472**	138
4	315	210	202	**242**	63
8	1164	1185	1166	**1172**	12
10	1577	2472	2240	**2096**	464
15	3443	3477	3361	**3427**	60
Blank	4	13	19	**12**	8
Luminase	16717				

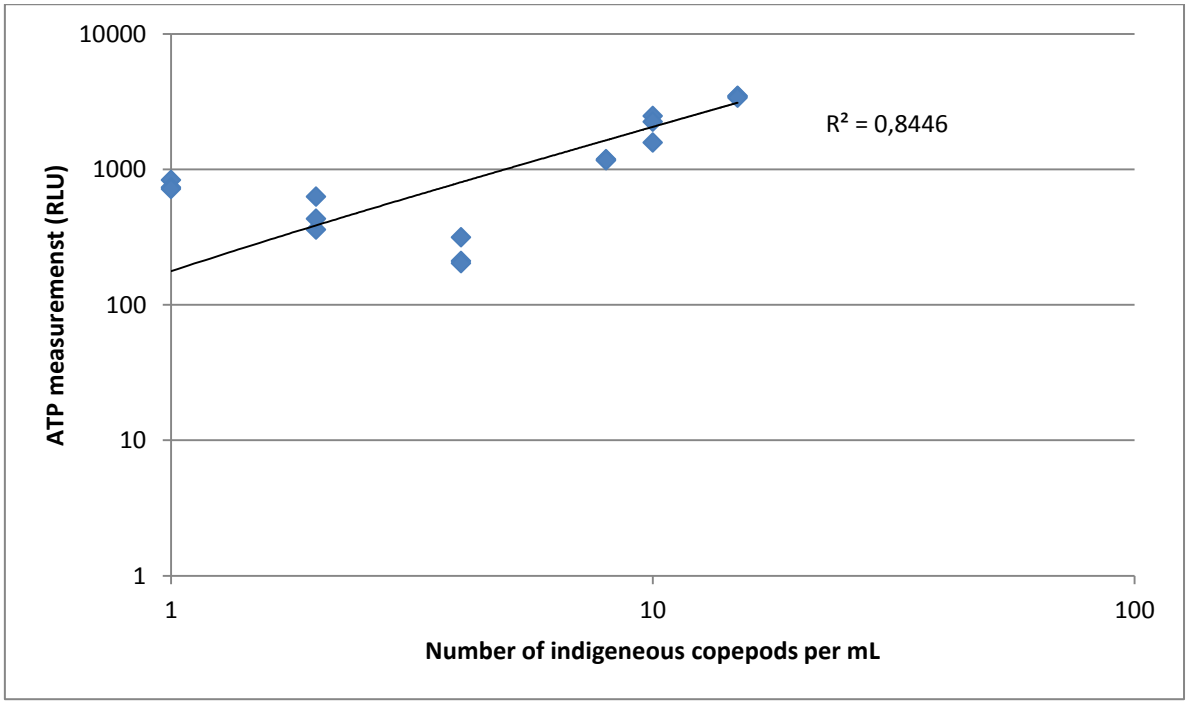

Figure 3. ATP calibration curve for indigeneous copepods from Oslofjord.

1.5 Conclusion

In conclusion, the correlations between the ATP results and the organism concentration based on standard counts were good for ≥50µm organism with both surrogate standard test organism (R2>0.97) and indigenous organisms (R2>0.84). Moreover, the luminescent signal for samples with only 1 organism/mL was significantly higher than the signal detected in the sterile seawater which was used as blank. This indicates that the detection limit might be below the maximum concentration of 10 organism/m3 required by IMO regulation D-2. However, this requires that the few viable organisms present in the ballast water sample can be collected and resuspended in the 1mL artificial seawater solution before the grinding and ATP extraction. The ATP measurements were performed with samples containing only viable organisms in this experiment.

For ≥10-50µm organism, the correlation between the Tetraselmis sp. concentration and the ATP results was relatively good (R2>0.77 or 0.98 when excluding deviating results). However, the luminescence signal measured in the sterile seawater was in the same range as in samples with algal density below 200cell/mL. In the ≥10-50µm organism group, IMO requires a maximum concentration of 10 cell/mL. Solution for resuspension with low luminescence signal should be used to improve the detection limit of the instrument. The use of artificial seawater as solution for resuspension was not investigated in this experiment. In this test, the samples of organisms were not filtered to remove bacteria that followed with the algal culture.

References

OECD (1985). OECD Test Guideline for Testing of Chemicals 202; *Daphnia* sp. Acute immobolisation test and reproduction test.

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Appendix 1: ATP procedure from SGS/Aquatools

BALLAST WATER SAMPLING

Sample a known volume of natural field water (ballast water)

FILTRATION

Sequential filtration of sample:
Using a 50 µm filter → Using a 10 µm filter

RESUSPENSION OF PLANKTONIC ORGANISMS

The organisms which were retained on the filters are suspended in artificial sea water*

** Use filter sterilized artificial sea water considering convenient salinity*

GRINDING AND ATP EXTRACTION

Add 1 ml of well mixed sample suspension and 5 ml of Modified UltraLyse 30 to a "bead-beating" tube

Place the beat-beating tube on the grinding apparatus and run the following program:

2.5 min at 6000 rpm

↓

Remove the tube, turn over twice and replace the tube

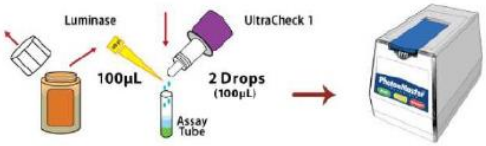
↓

2.5 min at 6000 rpm

INCUBATION

Allow 5 min for solids to settle

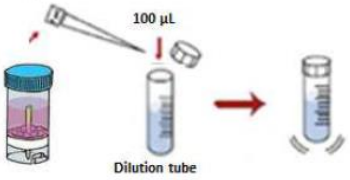
***Note:** Perform UltraCheck 1 calibration during this time*



DILUTION

After incubation transfer 100 µl of extract supernatant to a Dilution Tube containing 5 ml of UltraLute, cap and mix

***Note:** Proceed to assay step immediately upon dilution*



ASSAY

Transfer 100 µl of diluted extract into a Luminometer tube and add 2 drops of Luminase, gently mix. Immediately place the assay tube in Luminometer, hit 'Enter' and record RLU after 10 sec