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Ballast water compliance monitoring: A new application for ATP

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ABSTRACT

The coming into force of the USCG ballast water regulations and the IMO ballast water management convention resulted in the development of several technologies approved for the treatment of ballast water. To ensure compliance of these technologies, the development of rapid and robust analysis methods was necessary.

In collaboration with the SGS Group (Switzerland) and LuminUltra (Canada), Aqua-tools (France) has developed an innovative Ballast Water Treatment Monitoring (BWTM) kit for rapid onboard testing. The affordable kit provides results in less than 1 h, is easy to use and durable ensuring that the ballast water treatment system on the ship is fully compliant with the discharge standards upon arrival in port.

The core of this method is a combination of high-quality reagents (lysis solution and ATP 2G Luminase™ enzyme) not inhibited by salinity and a patented fast homogenizing method for ATP extraction developed for a higher ATP recovery from zooplankton and phytoplankton. Compared to traditional analysis methods, the BWTM Kit provides fast and accurate results for all three fractions of microorganisms (≥ 50 µm, $\geq 10 \div < 50$ µm and bacteria). Preliminary tests carried out in cooperation with SGS showed that the proposed method was able to detect onboard the efficiency of the treatment systems used. Compliance limits were established for all size fractions and a correlation between the standard methods (microscopy, plate count, MPN) and ATP was evaluated. The BWTM kit can provide a fast indication of compliance or gross exceedance. The rare borderline cases, when encountered, of course require additional confirmation.

1. Introduction

To ensure optimal seaworthiness, most commercial vessels are equipped with high capacity tanks in their hull (or double hull) for taking on large volumes of sea water. This water, known as ballast water, can be pumped in or out in relationship with the load weight and distribution. Ballast water enhances both the stability and the balance of a ship, whether it is empty, fully loaded or partially loaded. Using ballast water is essential because it maximizes the amount of cargo to be transported while maintaining optimal buoyancy and seaworthiness, therefore optimizing both speed and fuel consumption.

The spread of organisms through ballast water discharge is a well-known ecological problem. Essentially ballast water, along with all the organisms living in it, is discharged in geographical zones far from the uptake sites. It is estimated that cargo ships actually transport a total of between 3 and 5 billion tons of ballast water each year, transferring aquatic life from one part of the world to another (GEF-UNDP-IMO GloBallast, 2009). The aquatic organisms taken up with the water are so introduced into ecosystems very different from their original environment. Some species may find the new conditions favorable for their growth (temperature, nutrients, absence of predators, etc. (Steichen et al., 2014)). The possible proliferation of these species in their new environment can have a deep impact on the native flora and fauna. In many cases, irreversible damage to the environment (decreased biodiversity, disappearance of vulnerable species, introduction of an invasive species to an ecosystem, etc.) was observed (Casas-Monroy et al., 2015). The introduction of foreign species can also have an economic impact, such as the loss of aquaculture or fisheries productivity, or even a health impact due to the consumption of contaminated fish or seafood (infection with *Dinophyceae* protozoa Chang et al., 1997; cholera epidemics caused by *Vibrio cholerae* bacteria, Dobbs et al., 2013; McCarthy and Khambaty, 1994).

Because of the problems caused by invasive species, the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (2004). This convention contains several regulations aimed at reducing the spread of harmful aquatic organisms and pathogens. The regulations include the D-2 Ballast Water Performance Standard which states the discharge limits for viable organisms in ballast water. These limits are: for the organism class ≥ 50 µm, < 10 viable organ-

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isms per cubic meter. For the organism class ≥ 10 μm, < 50 μm, < 10 viable organisms per 1 mL. For *Escherichia coli*, < 250 CFU (Colony Forming Units) per 100 mL. For *Enterococci*, < 100 CFU per 100 mL. For the indicator microbe *Vibrio cholerae*, < 1 CFU per 100 mL. The United States Coast Guard adopted discharge standards almost identical to those of IMO, except that their limits state 'living' instead of 'viable' organisms.

These discharge standards encouraged the development of so-called Ballast Water Treatment Systems (BWTSs), designed to remove or kill the organisms in sea water. BWTSs usually use a combination of filtration and disinfection (Gregg et al., 2009; Tsolaki and Diamadopoulos, 2010). The most common disinfection methods are Ultra-Violet (UV) radiation or chlorine (David and Gollasch, 2012).

To test BWTSs suitability for the discharge standards, rapid methods testing the compliance of samples onboard are required. One of the common methods shows the total Adenosine Triphosphate (ATP). Compared to other methods such as Pulse Amplitude Modulated fluorometry, ATP is able to detect all living organisms in the sample and not just a sub-fraction.

ATP is characteristic of all living organisms, like DNA (deoxyribonucleic acid). Essentially, ATP acts a key element in the energy metabolism by serving as an energy transporter. The amount of this molecule allows the quantification of all living organisms in a liquid environment: bacteria, fungi, protozoa, algae, diatoms and other planktonic organisms.

ATP detection involves enzymatic measurements using an enzyme produced by biotechnological methods: luciferase, copied from a well-known luminescent insect: the firefly *Photinus pyralis*.

Luciferase produces light in the presence of luciferin and ATP. The amount of light released is directly proportional to the ATP present and thus the volume of organisms in the sample (Deininger and Lee, 2001).

After sea water sampling ATP is extracted from cells in the sample via cell lysis (by enzymatic cell walls digestion of organisms). The cell extract is then combined with a solution containing luciferase and luciferin. The bioluminescence intensity is then measured using a luminometer.

The first ATP-metry tools appeared in the 1970s (Conn et al., 1975). They became more widely available during the 1980s and 1990s and were relatively successful despite limited applications and practical issues.

Differentiating between intra-cellular ATP and extra-cellular ATP is necessary not only to get an accurate measurement of microbial activity, but also to assess microbial health in certain applications. Most of the 1st Generation ATP tests measure the entire ATP content in a sample via Total ATP, thus providing users with an inflated estimate of the living population. Some ATP test kits provide users with protocols to measure extra-cellular ATP, although extra-cellular ATP can exist in many forms, most of which are able to react with Luciferase only in their 'free' form.

Extra-cellular ATP is often bound to cellular debris or to other components in the sample, such as heavy metals, cationic treatment polymers, and other inert substances. These bound ATP molecules are unavailable to react with luciferase, so are not included in an extra-cellular ATP measurement. Therefore, to get a true measurement of extra-cellular ATP, it is important to stabilize all extra-cellular ATP – free and bound – so that it becomes available to react with luciferase.

It is commonly known that the total dissolved solids (TDS) concentration in an assay solution can give rise to problems for enzymatic reactions and other microbiological quantification methods. The ATP assay is severely impacted in terms of measurable signal. Once you get into the 20,000 ppm of TDS range and above, at least 50% loss of light output can be expected, which creates a severe false negative.

Due to key advances in biotechnology, the second generation of ATP-metry is much more powerful, overcoming the limitations of its predecessors:

- Microfiltration: inserting a microporous membrane allows viable cells to be retained, while residual compounds which could interfere with the luminescent signal are eliminated. This minimizes interference and background noise. Microfiltration provides two additional major advantages:

1. concentration of the ATP content from a large sample (up to 100 mL);
2. separation of free extracellular ATP (released by dead cells) from intracellular ATP (contained in living cells).

- Salinity: high dissolved solids issues are minimized. Test results show that ATP spiked into the diluted extracts of the high salinity samples was entirely recovered and results were similar to ATP recovery from the control sample. Salinity shows no negative effects on Luminase™ (Technical Whitepaper LuminUltra, 2016).

These features greatly increase both the sensitivity and the accuracy of the measurement. The level of stress and cell death in a microbial population within a water network, for instance after a disinfection procedure, can also be characterized.

- Cell lysis step: the enzyme cocktail (UltraLyse™) has a wide range of action and enhanced activity, and can extract up to 99.99% of the cellular material so the ATP measurement is more representative of the actual biological content (Technical Whitepaper LuminUltra, 2016).
- Standardization step: the standard curve is established using a standard range of increasing amounts of ATP (UltraCheck™). Calibration of the device allows to draw a conversion scale between a physical measurement (RLU - Relative Light Units) and a biological measurement (pg ATP/mL or Microbial Equivalents (ME)/mL). The results are therefore more accurate. Moreover, this conversion into biological units enables comparisons to be made between measurements taken by different luminometers at different sites or different time points (while data in RLU can be compared only within a single system).

Although ATP is generally used as a method for detecting bacteria in water, the ballast water regulations also apply to larger organisms. These larger organisms provide a challenge to the ATP extraction method since their complex structures are more difficult to break down than bacteria. From 2012 to 2016 aqua-tools, in collaboration with SGS and LuminUltra, developed an innovative Ballast Water Treatment Monitoring kit for rapid onboard test using ATP technology. This paper describes the development process of this method to make it applicable to organisms larger than bacteria.

2. Materials and methods

2.1. Method development

Starting from the commercially available ATP kit from LuminUltra (Quench Gone Aqueous, QGA), several experiments were carried out in order to adapt this kit (originally used for bacteria in water) to larger size planktonic organisms (≥ 50 μm and ≥ 10 ÷ < 50 μm).

The QGA kit measures the intra-cellular ATP, quantifying the living microorganisms in water in 3 min. This value can be obtained by sample filtration followed by lysis of the microorganisms retained on the filter and release of their intracellular ATP. ATP reacts with Luciferin and Luciferase releasing photons measured by a luminometer.

Several tests were performed using pure cultures of an aquatic crustacean (*Artemia salina)* and of two microalgae (*Tetraselmis* sp., *Phaeodactylum* sp.) in order to set up the best extraction conditions. Two homogenization systems were evaluated for extraction efficiency (Ultra Turaxx T25 and TD300), using different homogenization times

Table 1
Set-up of experiment.

Dispersing system	Tubes	Intensity	Exposure Time	Volume	N. of crustaceans
TD300	Disposable bead-beating tubes (rotating axis and 10 stainless steel beads)	6000 rpm	1, 2, 3 and 5 min	5 mL sample + 5 mL of UltraLyse™ 7	10/sample (different sizes of *Artemia* adults, no nauplii
Ultra Turaxx T25	Plastic round bottom, graduated volume 12 mL tubes	9000 rpm	1, 3 and 5 min	5 mL sample + 5 mL of UltraLyse™ 7	10/sample (different sizes of *Artemia* adults, no nauplii)

test were improved by different combination of beads (sizes, quantity and types of material) as well as by different extraction solutions (UltraLyse™ 7, UltraLyse™ 30 and UltraLyse™ 30 modified). The resulting analysis protocols were tested on different natural planktonic communities.

2.2. Homogenization

The first tests aimed to choose the most suitable device for sample homogenization. Two homogenizers, an Ultra Turaxx and a TD300 (bead-beating tubes) were compared. The visual results, i.e. the foam produced, and the ATP released were compared. The homogenizers were run at their highest rpm value for different times (Table 1).

Artemia salina adults were collected on a 50 μm nylon mesh filter and re-suspended in artificial seawater both to ensure the right concentration of organisms and to eliminate free ATP. A dissection microscope (Zeiss stemi 2000 C) at 50 × magnification equipped with a Bogorov counting chamber was used to evaluate the homogenization of each sample.

2.3. Foam formation

Since foam occurring during the tests underlines a remarkable difference between the two homogenizers, an additional test was carried out to determine the effect of foam formation on ATP measurements. In these two tests only 1 mL of sample instead of 5 mL was used. Based on the previous test the TD300 was run for 5 min and the Ultra Turaxx T25 for 3 min. *A. salina* samples were prepared in the same way as in the previous tests, using 10 organisms/sample.

2.4. Lysing agent selection.

Foam formation, most likely caused by the surfactant in the UltraLyse™, did not significantly impact the ATP results, but some difficulties were encountered in sample handling. However, the surfactant helps the lysing agent in breaking down cell walls. Because of this, tests with different formulations of UltraLyse™ were carried out. Two new UltraLyse™ formulas were used: UltraLyse™ 30, which has a higher concentration of lysing agent than the previous UltraLyse™ 7, and UltraLyse™ 30 without surfactant. It was also tested if UltraLyse™ 30 was concentrated enough to be able to extract ATP from organisms directly without homogenization.

Conditions were the same as in the previous tests: 5 min exposure time, 1 mL of sample with 10 adult *A. salina* individuals and 5 mL of lysing agent.

2.5. Effect of different organism concentrations

To improve the homogenization protocol settled in the previous experiments, further tests were carried out by using different *A. salina* concentrations with the aim of verifying the relationship between ATP concentration and amount of organisms. The methods were the same as previously: with the TD300, 5 min exposure time, 5 mL of UltraLyse™ 30 without surfactant and 1 mL of sample. *A. salina* concentrations used were 1, 2, 4, 8, 10, 16, 22 and 32 per mL. Organism concentrations were verified by using microscopy. The results are a mean of tests carried out on four separate days.

2.6. Effect of treatment methods on ATP concentration

Since the goal is to develop a method to detect organism numbers in treated ballast water, the effect of treatment methods on the ATP analysis was tested. To this aim, samples were exposed to microwave radiation for 30 s at 1250 W. After 5, 10, 30 and 60 min the ATP concentration in the sample was measured. All samples contained 10 *A. salina* adults per mL and ATP analysis was carried out following the previously established methods.

2.7. Detection of different size classes in a natural sample

In addition to pure cultures of organisms, our ATP protocol was tested also on dilutions of a natural sample collected from the North Sea. The analysis protocol was kept identical to the previous tests, with a single change. The exposure time for the homogenization device was split into two periods of 2 min and 30 s. In between the bead-beating tube was inverted several times to prevent organism entrapping in the drops on the tube lid and so escaping the homogenization step.

The size fractions were collected on nylon filter meshes with a diagonal of 50 μm and 10 μm. Organisms were flushed off the filter using artificial seawater isotonic with the natural seawater. All organisms trapped on the 50 μm filter were considered a part of the ≥ 50 μm fraction; all organisms trapped on the 10 μm filter after being filtered over the 50 μm filter were considered part of the ≥ 10 ÷ < 50 μm size fraction. Organism counts were established by microscopy. For the ≥ 50 μm fraction the sample was concentrated and 20 mL were counted in a Bogorov chamber using a dissection microscope (Zeiss stemi 2000 C). For the ≥ 10 ÷ < 50 μm fraction 1 mL was counted in a Sedgewick Rafter counting chamber. Viability was assessed based on species-specific movement and visible damage.

2.8. Selection of appropriate beads mixture

Homogenization looks effective on adult *A. salina*, its efficiency on smaller organisms is however also relevant. For this purpose, the homogenization steps were tested also on *Tetraselmis* sp. (algae) and *A. salina* sub-adults (copepodites). In addition to the normal 5 mm steel beads the experiment was also carried out with 3 mm steel beads to verify if smaller size beads resulted in a better homogenization of smaller organisms. A microscopic analysis, as previously described was used to observe the effectiveness of the homogenization.

2.9. Beads mixture test on Tetraselmis sp.

The previous experiment showed that smaller beads performed better, so for this experiment various combinations of smaller beads were used to determine an optimal mixture. The bead mixtures used were: steel beads 2 mm mixed with glass beads 0.1 mm, glass beads 0.1 mm alone, glass beads 0.5 mm alone, granite beads 0.7 mm alone and steel beads 3 mm alone. The tests were performed on samples containing *Tetraselmis* sp.

Table 2
Overview of ATP compliance limit experiments for the ≥ 50 μm and ≥ 10 ÷ < 50 μm and bacteria fractions.

TEST	Sample Treatment	Sample Dilution	Volume tested
1	UV, 10–20-30 min exposure Chlorine, 2 h contact time	1/10, 1/100, 1/1000	Bacteria: 60 mL for undiluted and UV treated, 120 mL for dilutions and chlorine treated
2	UV, 30 min exposure Chlorine, 2 h contact time	1/10, 1/100, 1/1000	Bacteria: 120 mL for all
3	UV, 60 min exposure Chlorine, 2 h contact time	1/5, 1/20, 1/50	Bacteria: 120 mL for all
4	UV, 60 min exposure Chlorine, 2 h contact time	1/5, 1/20, 1/50	Bacteria: 120 mL for all ≥ 50 μm fraction: untreated 10 L ≥ 10 ÷ < 50 μm fraction: untreated 1000 mL
5	UV, 60 min exposure Chlorine, 2 h contact time	None	Bacteria: 120 mL for all ≥ 50 μm fraction: untreated 9,5 L, UV 1 L, chlorine 8,2 L ≥ 10 ÷ < 50 μm fraction: untreated 500 mL, UV 1000 mL, chlorine 700 mL
6	UV, 60 min exposure Chlorine, 2 h contact time	None	Bacteria: 120 mL for all ≥ 50 μm fraction: untreated 10 L, UV 1 L, chlorine 8,4 L ≥ 10 ÷ < 50 μm fraction: untreated 550 mL, UV 500 mL, chlorine 500 mL
7	UV, 60 min exposure Chlorine, 3 h contact time	None	Bacteria: 120 mL for all ≥ 50 μm fraction: untreated 10 L, UV 1 L, chlorine 9,3 L ≥ 10 ÷ < 50 μm fraction: untreated 500 mL, UV 1000 mL, chlorine 550 mL

2.10. Bead mixture test on Phaeodactylum sp. and Artemia salina

To test the beads mixture chosen in the previous experiment (2 mm stainless steel beads/0,1 mm glass beads), a more challenging experiment was set up using the algae *Phaeodactylum* sp. This alga has an extracellular silica-based skeleton, which should make it more resistant to mechanical damage. Moreover, the mixture was also tested on newly hatched *A. salina* (nauplia) to observe its effectiveness on larger organisms.

2.11. Bead mixture test on a natural sample

A selected beads mixture (2 mm stainless steel beads/0,1 mm glass beads) was tested on a sample of Baltic Sea water, to observe its performance on a natural sample. Samples were diluted to achieve different amounts of organisms per milliliter, which were further distributed in size classes as previously described. The amount of organisms was determined using microscopy as previously described.

2.12. Verification tests

To further verify the previous results and test the handling of the proposed method, the ATP protocol was tested by an SGS team, who had no previous experience with it and repeated the previous experiments, testing the ATP method on dilution series of *Tetraselmis* sp., *A. salina* nauplia and copepods.

Tetraselmis sp. amounts were calculated referring to the counts of the original sample provided by the supplier. *A. salina* nauplia and copepods were counted by microscope as previously described.

2.13. Filtration of a whole sample

In order to separate the different fractions in the ballast water samples, filtration is needed. In the preliminary tests, reusable nylon mesh membranes to separate the different fractions were used. The organisms were then re-suspended in artificial sea water, as previously described. There were several issues with this method: i.e. the risk of part of the sample being lost during the procedure, its resulting larger sample volume when for analysis only 1 mL is used, the time-consuming mesh cleaning between two samplings slowing down the procedure and since the filtration relies on gravity, this can also slow the process down further. Even with regular cleaning the mesh would lose in performance after several samplings and need to be replaced.

Different kinds of disposable membranes were tested together with a filtration system operated by hand-powered vacuum pump to speed up the process in order to eliminate the risk of errors caused by the filtration process.

2.14. Determining ATP compliance limits.

The final development step was to determine a correlation between the IMO and USCG compliance limits for the different ballast water fractions and ATP concentrations. Preliminary tests on sea water samples (sampled from the Venice lagoon in Italy) were carried out in November 2015 just before the winter season to be sure to catch different types of microorganisms (zooplankton, phytoplankton and bacteria). The analysis protocol used in these tests is reported in Appendix 1.

Several comparative tests between the classical methods (microscopy count for ≥ 10 ÷ < 50 μm and ≥ 50 μm, most probable number and heterotrophic plate count for total heterotrophic bacteria) and ATP concentrations were carried out. Different water treatments (chlorination and Ultra Violet) were also evaluated.

The bacteria fraction was analyzed in seven different experiments to observe the most suitable treatment and dilution conditions to compare the ATP results with the classic methods results. For the last four experiments, the ≥ 50 μm and ≥ 10 ÷ < 50 μm size fractions were also included. All tests were performed in triplicate and the mean value is reported. (See Table 2.)

3. Results

3.1. Homogenization

The TD300 achieved a good homogenization score after only 5 min (Table 3), so it appears suitable for use with ATP analysis. A positive aspect of the TD300 is the use of disposable tubes, which eliminates the

Table 3
Results of the TD300 homogenizer.

Exposure Time (min)	Observations	Comments
1	Presence of small and large carapace fragments. Homogenization score +	Not suitable
2	Presence of small and medium carapace fragments. Homogenization score + +	Not suitable
3	Remnants of medium carapace fragments. Homogenization score + + +	Possibly suitable
5	No fragments visible. Homogenization score + + + +	Suitable

Table 4
Results of the Ultra Turaxx T25 homogenizer.

Exposure Time (min)	Observations	Comments
1	Presence of small and medium carapace fragments. Homogenization score + +	Not suitable
3	Homogenization score + + + +	Suitable
5	Homogenization score + + + +	Suitable

wash step of the equipment in between samples. The main negative aspect concerns the persistent foaming of the UltraLyse™.

The Ultra Turaxx T25 achieved a good homogenization score already after 3 min of exposure time (Table 4). A positive aspect of the Ultra Turaxx T25 is the lack of foam development while the negative is the time loss due to the equipment washing in between samples.

3.2. Homogenization and foam formation

The results show that foaming does not negatively affect the luminescence reaction. In fact, the TD300 samples which showed persistent foaming had a slightly higher tATP (total ATP) measurements (Fig. 1), indicating that the device homogenized the sample more effectively.

3.3. Lysing agent selection

The degree of homogenization was similar to the previous experiments. The UltraLyse™ 30 samples showed abundant and persistent foaming. Slight and transient foaming was observed in the UltraLyse™ 30 without surfactant samples. Use of UltraLyse™ 30 without surfactant resulted in a slightly higher ATP levels than using normal UltraLyse™ 30.

UltraLyse™ 30 without homogenization showed very low ATP levels compared with the homogenized samples (Fig. 2).

3.4. Detection of different organism concentrations

ATP concentrations show a good correlation ($R^2 = 0.90$) with *A. salina* numbers (Fig. 3). The higher the organism concentration, the larger the variation between replicate measurements.

3.5. Effect of disinfection methods on ATP concentration

The base line for this experiment is about 20,000 ng/mL tATP, based on the experiment carried out in the same day (Fig. 3). ATP concentrations dropped after microwave treatment (Fig. 4). After 60 min only 10% of the original ATP level was left, indicating that the ATP quickly degrades in the organism debris.

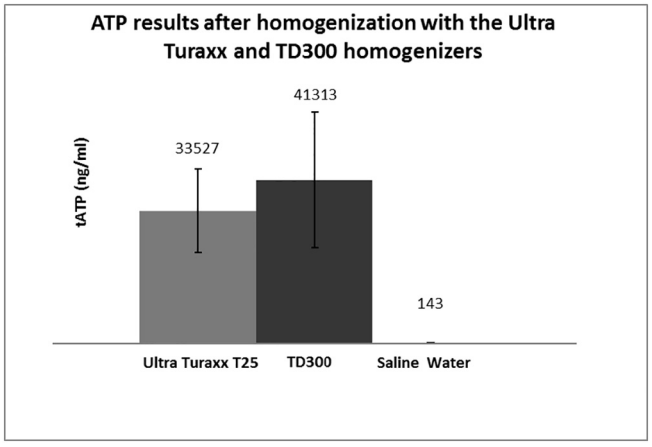

Fig. 1. ATP amounts in samples homogenized by the TD300 and Ultra Turaxx T25 and free ATP levels in the artificial seawater. All ATP values in ng/mL.

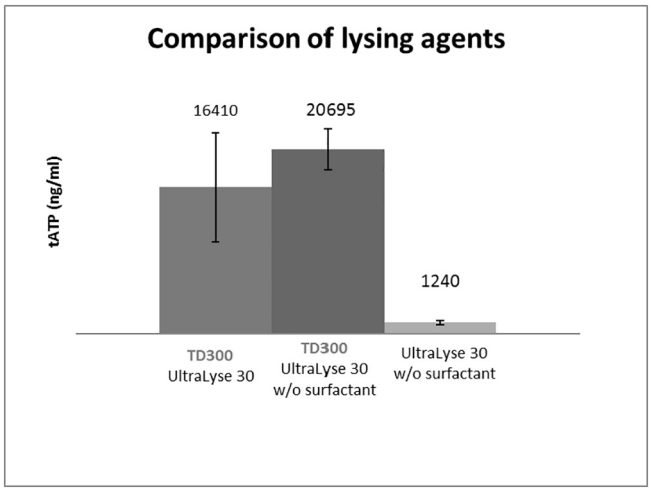

Fig. 2. ATP levels (ng/mL) of samples processed with UltraLyse™ 30 and UltraLyse™ 30 without surfactant and homogenized with the TD300.

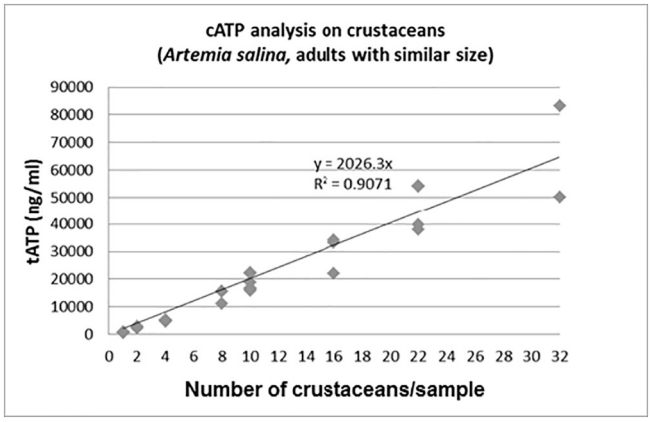

Fig. 3. Concentration of total ATP (tATP) in samples with different concentrations of *Artemia salina*.

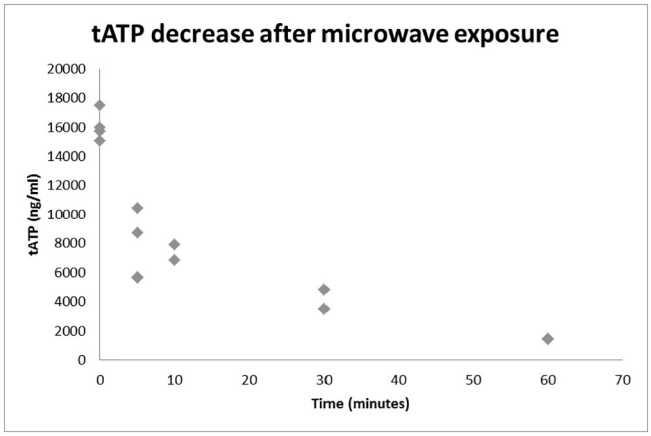

Fig. 4. Total ATP (tATP) concentration after sample exposure to microwave radiation.

3.6. Detection of different size classes in a natural sample

ATP results show a good correlation to organism numbers for the ≥ 50 µm and ≥ 10 ÷ < 50 µm size fractions (Fig. 5).

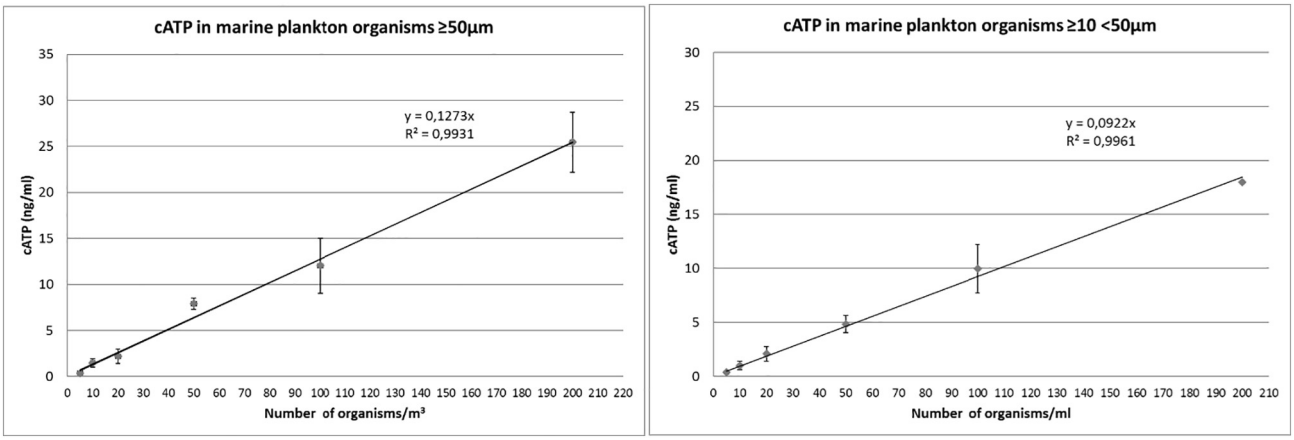

Fig. 5. Dilutions made using the sample of North Sea water. The analysis was split into two size fractions: ≥ 50 µm and ≥ 10 ÷ < 50 µm.

3.7. Selection of appropriate beads mixture

Microscopic analysis of *Tetraselmis* sp. samples showed that intact cells were present after homogenization both with 3 mm and 5 mm steel beads. For *A. salina*, two out of 39 individuals survived the homogenization with 5 mm steel beads. tATP levels for both organisms were also higher with 3 mm beads than with 5 mm beads.

The experiment showed that the 3 mm steel beads performed better than the 5 mm steel beads on *A. salina* sub-adults (Fig. 6). After homogenization, both with 5 mm and 3 mm beads still some intact *Tetraselmis* sp. cells were found in the sample. Although the 3 mm beads performed better than the 5 mm, the beads size needed further optimization.

3.8. Beads mixture test: Tetraselmis sp.

Most bead mixtures release ATP in amounts similar to the simple addition of UltraLyse™ 30 without homogenization. One bead mixture, the 0.7 mm granite beads, actually showed lower ATP levels than the UltraLyse™ addition without grinding. The only bead mixture increasing the amount of ATP released is the mixture of 2 mm steel beads together with 0.1 mm glass beads (Fig. 7).

3.9. Bead mixture test: Phaeodactylum sp. and Artemia salina

The bead mixture extracted ATP at the same level as exposure to just UltraLyse™ 30 without homogenization (Fig. 8). Even without addition of UltraLyse™ high levels of ATP were measured. For this particular alga no homogenization is needed.

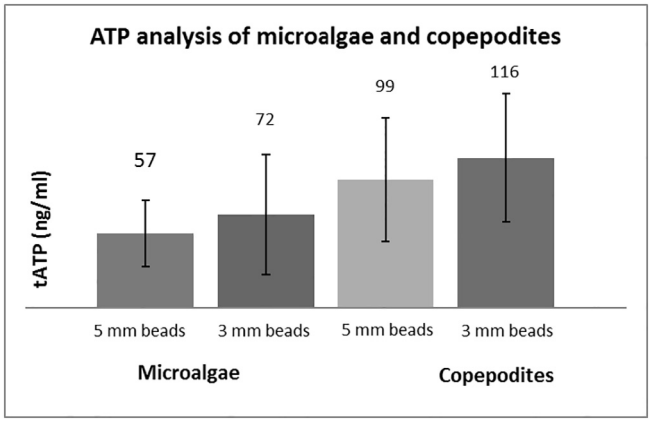

Fig. 6. ATP values in samples containing microalgae (*Tetraselmis* sp.) and copepodites (*A. salina*) after homogenization with 5 mm steel beads and 3 mm steel beads.

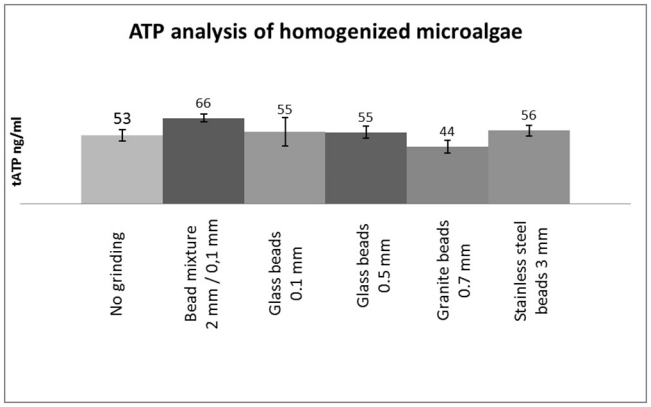

Fig. 7. ATP analysis of *Tetraselmis* sp. samples homogenized using different bead mixtures.

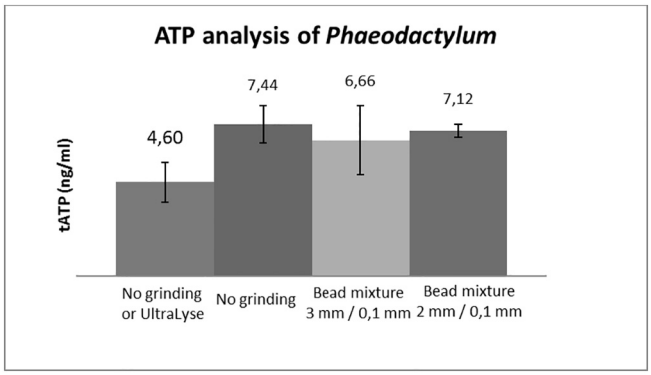

Fig. 8. ATP concentrations of samples containing Phaeodactylum sp. after exposure to different homogenization methods.

Newly hatched (nauplia) *A. salina* were effectively homogenized by the beads mixture. Without homogenization but just with UltraLyse™ 30 w/o surfactant ATP levels in the sample were very low (Fig. 9), showing that even for small *A. salina* individuals homogenization of the sample is essential.

3.10. Bead mixture test: natural sample

The ATP values measured show a good correlation with the number of organisms in the samples (Fig. 10). It shows the potential for ATP to be used to assess organism concentrations in ballast water.

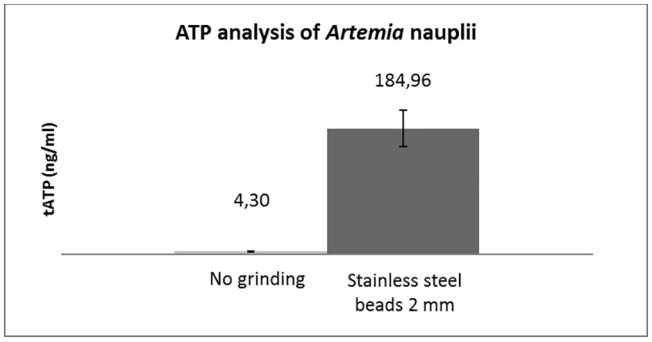

Fig. 9. ATP concentrations in samples containing *A. salina* nauplii, with or without homogenization.

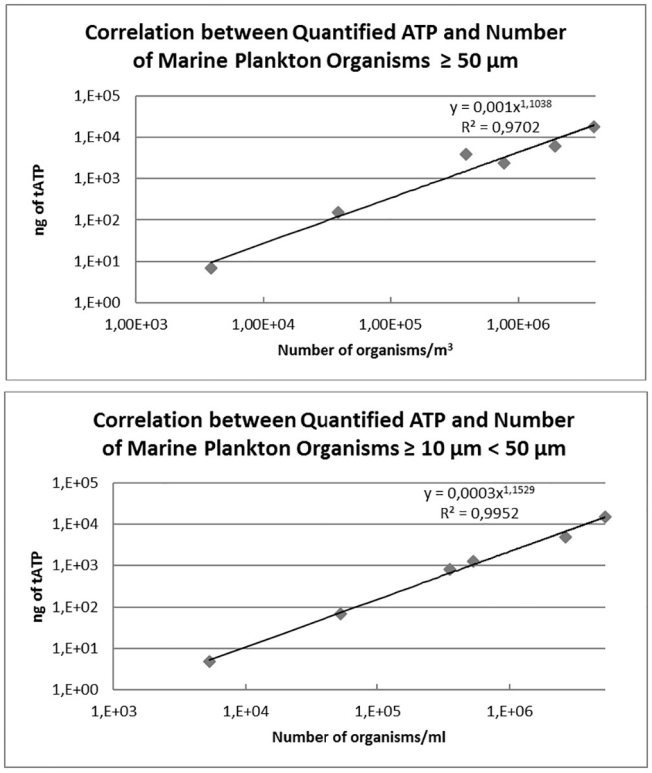

Fig. 10. Correlation between ATP concentration and number of organisms in samples of Baltic Sea water.

3.11. Validation experiments

These tests, carried out both on laboratory grown plankton and on natural plankton show a good correlation between ATP concentrations and organism numbers (Figs. 11, 12 and 13).

3.12. Filtration of whole sample

50 µm and 10 µm filtration membranes were chosen based on their retention ability, compatibility with the homogenization system and non-interference with the lysing agent and Luminase™. Because these filtration membranes are transferred straight from the filtration system to the homogenization system, assures a 100% sample extraction.

All these tests allowed the creation of protocols for ballast water analysis of all three fractions (≥ 50 µm, ≥ 10 ÷ < 50 µm and bacteria), included in Appendix 1.

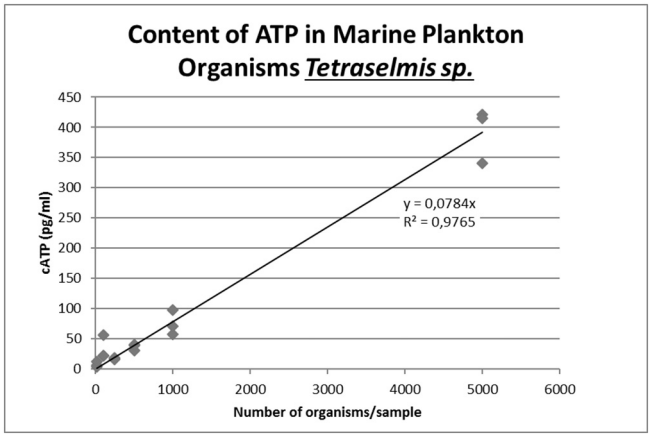

Fig. 11. Dilution experiment using *Tetraselmis* sp.

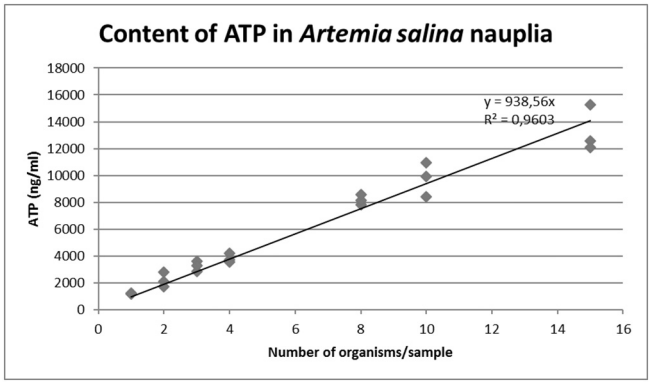

Fig. 12. Dilution experiment using *A. salina* nauplia (larvae).

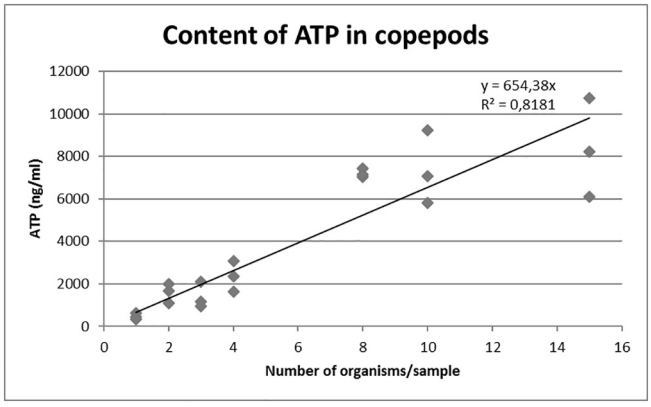

Fig. 13. Dilution experiment using copepods collected from natural sea water.

3.13. Determining ATP compliance limits

For the ≥ 50 µm fraction comparative data analysis was performed between the ATP results and microscopy counts. The sample largely consisted of needle-shaped diatoms and dinoflagellates which, due to their shape, have low cellular volumes and thus low ATP content. The microorganisms found in the samples, different in size and species, show a very good linearity (Fig. 14) between the results of different methods used to define the new ATP values and the establishing of compliance limits for this fraction.

For the ≥ 10 ÷ < 50 µm fraction comparative data analysis was performed between the 2G ATP vs. microscopy count. The different populations of microorganisms observed before and after treatment had the same interpretation issues as the ≥ 50 µm fraction. Despite the variety in size and species of microorganisms a very good linearity

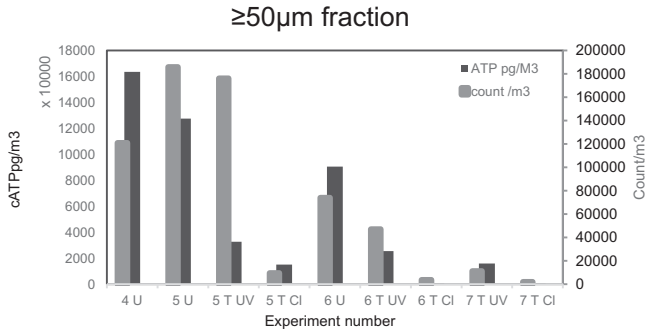

Fig. 14. Comparison between ATP 2G and microscopy counts for the ≥ 50 µm fraction. U = untreated, T = treated, UV = UV-radiation, Cl = chlorine.

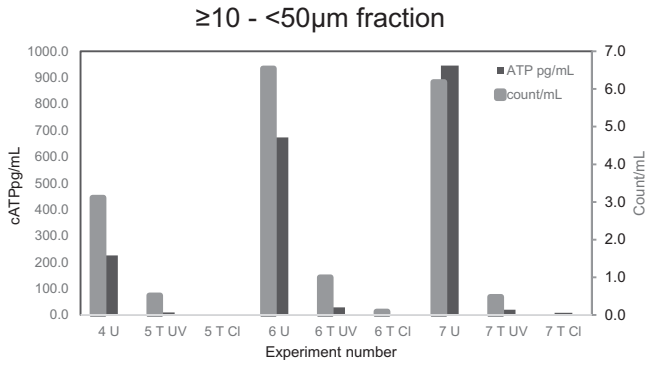

Fig. 15. Comparison between ATP 2G and microscopy counts for the ≥ 10 ÷ < 50 µm fraction. U = untreated, T = treated, UV = UV-radiation, Cl = chlorine.

(Fig. 15) between the results of different methods confirmed the ATP values with respect to compliance limits for this fraction.

For the bacteria fraction, comparative data analysis was performed between ATP 2G vs plate count and ATP 2G vs SimPlate® to evaluate the correlation between the methods. Comparative tests with Colilert® and Enterolert® methods were also carried out. Correlation between the methods was good for UV treated and undiluted or low dilution samples, chlorine treated and high dilution samples showed a rather low correlation (Fig. 16). This difference is probably due to the lower detection limits of the methods.

4. Discussion

Several years of tests were necessary to develop a robust method for ballast water application based on ATP in living organisms. Each step

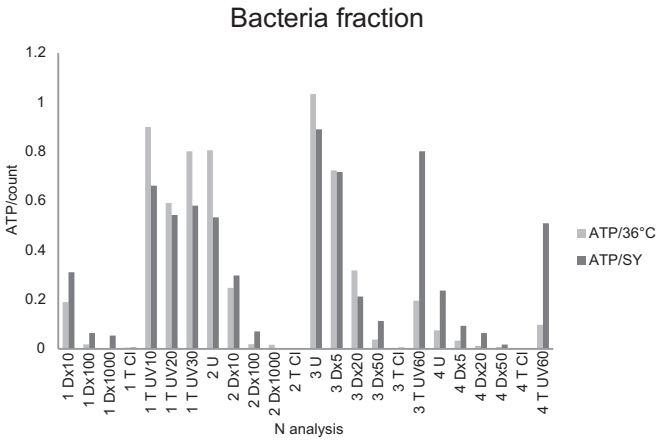

Fig. 16. Correlation between ATP 2G/plate count and ATP 2G/mpn (SY). U = untreated, D = dilution, T = treated, UV = UV-radiation, Cl = chlorine.

was deeply studied in order to reach accurate results.

The homogenizer selection was the first step: one of the major issues was the foaming using the UltraLyse™ 7 but the results showed that it did not significantly affect the results. The disposable tube of the TD300 was considered more practical compared with the Ultra Turaxx T25, thus TD300 was chosen for all further experiments and was always run at its standard intensity (6000 rpm) in all further experiments.

The need for a complete breakdown of the cell walls and shells of marine organisms addressed our research towards a stronger lysing agent compared with the standard one (UltraLyse™ 7) used in the original QGA kit. A more powerful lysing agent (UltraLyse™ 30) was developed in two different versions: both with and without surfactant agent. The results showed that, even at the higher concentrations of lysing agent the homogenization step is necessary.

In order to evaluate the ATP accuracy on microorganism detection several tests with different numbers of crustaceans were carried out. These tests showed that the ATP method accurately detects changes in organism numbers.

Disinfection methods were also evaluated in order to simulate the ballast water treatment system. Microwave radiation is not a common ballast water treatment but it showed the capability of ATP to detect reductions in biomass. Further tests were also carried out with common ballast water treatments such as UV radiation and chlorine, which also showed a strong reduction of ATP after exposure of the sample to these treatments. van Slooten et al. (2015) observed a similar decrease in ATP levels after treatment in their experiments. Many ballast water treatment systems, such as chlorine and ozone, treat the ballast water when pumped into the ballast water tank and measurements are made only on discharge, thus giving the ATP in the organism sufficient time to degrade. However, UV based ballast water treatment systems treat both on intake and discharge, which can potentially mean some organisms are treated only seconds before sampling. These experiments showed that the ATP levels of these organisms will degrade sufficiently in the time between sampling and analysis, as in practical situations the analysis equipment will never be set up directly by the sampling point.

Natural samples from the North Sea were analyzed to proof that the ATP method is suitable for use with samples containing different species of microorganisms. The results showed a good correlation between the numbers of organisms and ATP for both ≥ 50 µm size classes (R^2 = 0.97) and for ≥ 10 ÷ < 50 µm size classes (R^2 = 0.99).

A selection of beads mixtures was tested in a natural sample to choose the most effective mixture for disintegrating cell walls and shells. After several tests the beads mixture of 2 mm stainless steel/0.1 mm glass beads was chosen because it showed the best ATP recovery rate.

Several tests on natural and artificial samples were carried out to confirm that with the changes to the protocol ATP is still a suitable tool to determine organism concentrations in sea water. As part of a project commissioned by the German Federal Maritime and Hydrographic Agency (Bundesamt für Seeschifffahrt und Hydrographie, BSH) these tests were repeated independently by the Norwegian institute NIVA as a verification test. The report on these tests was submitted to IMO by the BSH (Delacroix and Liltved, 2013). This report confirmed the good correlation of ATP with *A. salina* numbers (R^2 = 0.96), copepods (R^2 = 0.82) and *Tetraselmis* sp. (R^2 = 0.98).

The comparative data analysis for the different fractions (≥ 50 µm; ≥ 10 ÷ < 50 µm and bacteria) between microscopy count, plate count and ATP, allowed the determination of different thresholds for ATP compliance limits for ballast water discharge.

Based on the maximum, minimum and average ATP values per organism, the compliance limit value established for the ≥ 50 µm fraction was < 10,000 pg/m^3 (equivalent to < 10 organisms/m^3). The non-compliance limit value for the ≥ 50 µm fraction was 750,000 pg/m^3 and the range in between no certain compliance statement was made.

Based on the maximum, minimum and average ATP concentrations

per organism, the compliance limit value established for the ≥ 10 ÷ < 50 μm fraction was < 500 pg/mL (equivalent to < 10 organisms/mL). The non-compliance limit value for the ≥ 10 ÷ < 50 μm fraction was > 1500 pg/mL. For the range in between no certain compliance statement can be made.

Based on the maximum, minimum and average ATP per CFU values, the compliance limit value established for the bacteria fraction was < 1000 pg/100 mL, equivalent to the California ballast water discharge standard of < 1000 CFU/100 mL of heterotrophic bacteria 36 °C. Below this level of bacteria, the ballast water indicator microbes (< 250 CFU/100 mL *E. coli*, < 100 CFU/100 mL Enterococci and < 1 CFU/100 mL of *Vibrio cholerae*) are not likely to exceed their limit values. The non-compliance limit value was > 5000 pg/100 mL and for the range in between no certain compliance statement can be made.

These compliance limits are based only on information from plankton in one location and season. Information from other locations is still being gathered to increase the accuracy of these limits.

4.1. Practical experiences

The ATP kit described in this paper is already in use as a so-called compliance monitoring technique during on board tests of vessels. A report on the effectiveness of this technique has been issued to IMO by the government of Singapore (Jow and Chakravarty, 2015). It is also already in regular use by SGS as a ballast water compliance monitoring technique (pers comm. P. Stehouwer).

5. Conclusions

A second generation ATP method designed for detecting bacterial ATP was successfully adapted to measure samples of larger planktonic organisms. The adaptations consist of:

- Homogenization of the sample to eliminate complex structures that the lysing agent cannot break down.
- Use of a stronger lysing agent to deal with the more complex cell walls found in phytoplankton and zooplankton.
- Use of a mixture of large and small beads for homogenization so that both ≥ 50 μm and ≥ 10 but < 50 μm organisms can be homogenized.
- Total sample concentration on a filter, thus increasing both method sensitivity and eliminating the risk of losing organisms in resuspension steps.

These adaptations resulted in an ATP method which is able to detect organism down to below the D-2 limit values.

The test is moreover capable, < 1 h, to produce results that give a strong indication of the performance of the ballast water treatment system on the ship and, when used on a representative sample, give a clear response in terms of gross exceedance of the discharge standards upon arrival in the harbor.

The method was developed to work on ≥ 50 μm plankton, ≥ 10 ÷ < 50 μm plankton as well as on bacteria, since all three classes provide important information on the functioning of the ballast water treatment system. Non-compliance of the ≥ 50 μm fraction indicates problems arising from the filtration unit of the treatment system, while non-compliance of the ≥ 10 ÷ < 50 μm and bacteria fractions indicate problems with the disinfection unit.

Preliminary compliance limits for each of the three methods were established, keeping in mind to minimize the false compliance or non-compliance signals. These compliance limits may be adjusted in the future based on new data.

Acknowledgments

We would like to thank everyone at Aqua-tools, LuminUltra and SGS who contributed to the work in this paper.

Appendix 1. ATP analysis protocol

The protocol below includes the steps for all three size fractions (≥ 50 μm, ≥ 10 ÷ < 50 μm and bacteria) Full protocols for all three size fractions are also available on the aqua-tools website or by contacting the authors.

Filtration step

Place a 50 μm membrane on a vacuum filtration system and tighten the top funnel to lock the system.

Add the whole ≥ 50 μm sample of ballast water fraction concentrated from the initial 1 m^3 (please note the filtrated volume) collected from the 50 μm filter integrated sampler (SGS BWS1).

Use the manual vacuum pump to speed up the filtration step.

ATP extraction – bead beating step

Add 9 mL of UltraLyse™ 30 and 1 mL of UltraLute™ buffer (dilution buffer to ensure sufficient volume) into AT*Prep* tube.

Unscrew the top funnel to release the system.

Take the 50 μm membrane from the vacuum filtration system support and put it in to the AT*Prep* tube.

Vigorously shake the AT*Prep* tube for at least 10 s.

Run the dispersing system 3 times for 2 min each.

Between each step vigorously shake the AT*Prep* tube for at least 10 s.

ATP dilution step

Wait at least 5 min to allow sedimentation - Don't move the AT*Prep* tube after the last shaking step.

Transfer 100 μL of supernatant from AT*Prep* tube to 5 mL UltraLute™ tube.

The sample is stable up to 2 h (verified by manufacturer LuminUltra) and ready for the analysis.

The ≥ 10 ÷ < 50 μm faction protocol includes the same steps as the ≥ 50 μm fraction protocol, with minor differences:

Filtration step

Place a 10 μm membrane on the vacuum filtration system support and screw the top funnel to lock the system.

Add at least 1 L of ≥ 10 ÷ < 50 μm sample of ballast water fraction collected from the 10 μm filter integrated sampler (SGS BWS1).

Use manual vacuum pump to speed up the filtration step.

Note that final volume filtrated through 10 μm membrane is at least 1 L (if not, filtrate more volume to reach 1 L)

ATP extraction step – bead beating step

Add 5 mL UltraLyse™ 30 and 1 mL UltraLute™ buffer into AT*Prep* tube.

Unscrew the top funnel to release the system.

Take the 10 μm membrane from the vacuum filtration system support and put it in to the AT*Prep* tube.

Upside down the AT*Prep* tube twice.

Run the dispersing system 3 times for 2 min each.

Between each step upside down the ATPrep tube twice.

ATP dilution step

Wait at least 5 min to allow sedimentation – Don't move the ATPrep tube after the last upside down step.

Transfer 100 µL of supernatant from ATPrep tube to 5 mL UltraLute™ tube.

The sample is stable up to 2 h and ready for the analysis.

The bacteria fraction protocol contains less steps than the ≥ 50 µm and ≥ 10 ÷ < 50 µm fraction protocols:

Filtration step

Prepare the 60 mL syringe for the filtration step (remove the plunger).

Assemble the two filters on series to the syringe the 2.7 µm and 0.7 µm (is the terminal one) filters.

Filter 50 mL of sample from ≥ 10 ÷ < 50 µm fraction.

Unscrew the 2.7 µm + 0.7 µm assembled filter system, remove the plunger.

Screw again to the syringe the 2.7 µm + 0.7 µm assembled filter system.

Filter again 50 mL of sample from ≥ 10 ÷ < 50 µm fraction to reach 100 mL filtered.

Unscrew the 2.7 µm + 0.7 µm assembled filter system, remove the plunger.

Discard the 2.7 µm filter.

ATP extraction step

Screw the 0.7 µm filter to the syringe and add 1 mL UltraLyse™ 7.

Put the plunger back and push it to filter into 9 mL UltraLute™ tube.

The sample is stable up to 4 h and ready for the analysis.

Finally the analysis protocol includes additional steps that are common to all three fractions:

Rehydratation step

Unscrew the cap from buffer and Luminase™ bottles.

Remove red rubber cap and throw it away.

Rehydrate dried Luminase™ by adding buffer.

Screw white cap and upside down the bottle.

Wait at least 15 min before use.

ATP calibration step

Add 100 µL of Luminase™ enzyme + 2 drops of UltraCheck 1 into a luminometer tube.

Insert it into the luminometer and press start.

Note RLU value after 10 s of reading (> 5000 RLU).

Negative controls step

Insert a luminometer tube into the luminometer and press start.

Record the RLU value after 10 s of reading as $RLU_{empty\ tube}$ (< 10 RLU).

Add 100 µL of Luminase™ enzyme into a luminometer tube.

Insert it to the luminometer and press start.

Record the RLU value after 10 s of reading as RLU_{tube} (< 10 RLU).

Add 100 µL of Luminase™ enzyme + 100 µL of UltraLute™ buffer into a luminometer tube.

Insert it to the luminometer and press start.

Record the RLU value after 10 s of reading as RLU_{bn} (< 10 RLU).

ATP extract measurement ≥ 50 µm, ≥ 10 ÷ < 50 µm and bacteria fraction

Add 100 µL of Luminase™ enzyme + 100 µL of ≥ 50 µm ATP extract into a luminometer tube.

Insert it to the luminometer and press start. Note RLU value after 10 s of reading.

Add 100 µL of Luminase™ enzyme + 100 µL of ≥ 10 ÷ < 50 µm ATP extract into a luminometer tube.

Insert it to the luminometer and press start. Note RLU value after 10 s of reading.

Add 100 µL of Luminase™ enzyme + 100 µL of bacteria ATP extract into a luminometer tube.

Insert it to the luminometer and press start. Note RLU value after 10 s of reading.

References

Casas-Monroy, O., Linley, R.D., Adams, J.K., Chan, F.T., Drake, D.A.R., Bailey, S.A., 2015. Relative invasion risk for plankton across marine and freshwater systems: examining efficacy of proposed International ballast water discharge standards. PLoS One 10 (3), e0118267. http://dx.doi.org/10.1371/journal.pone.0118267.

Chang, F.H., Anderson, D.M., Kulis, D.M., Till, D.G., 1997. Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. Toxicon 35, 393–409. http://dx.doi.org/10.1016/S0041-0101(96)00168-7.

Conn, Rex B.M.D., Charache Patricia, M.D., Chappelle Emmett, W.M.S., 1975. Limits of Applicability of the Firefly Luminescence atp Assay for the Detection of Bacteria in Clinical Specimens. http://dx.doi.org/10.1093/ajcp/63.4.493 493-501. (First published online: 1 April 1975).

David, M., Gollasch, S., 2012. Ballast water treatment systems – a summary. In: Werschkun, B., Höfer, T., Greiner, M. (Eds.), Emerging Risks from Ballast Water Treatment, pp. 23–30.

Deininger, R.A., Lee, J., 2001. Rapid determination of bacteria in drinking water using an ATP assay. Field Anal. Chem. Technol. 5 (4), 185–189. http://dx.doi.org/10.1002/fact.1020.

Delacroix, S., Liltved, H., 2013. Preliminary Validation of an ATP Analytical Procedure for Ballast Water. NIVA Journal No.: 1894/13.

Dobbs, F.C., Goodrich, A.L., Thomson III, F.K., Hynes, W., 2013. Pandemic serotypes of *Vibrio cholerae* isolated from ship's ballast tanks and coastal waters: assessment of antibiotic resistance and virulence genes (tcpA and ctxA). Microb. Ecol. 65, 969–974. http://dx.doi.org/10.1007/s00248-013-0182-7.

GEF-UNDP-IMO GloBallast Partnerships and IOI, 2009. Guidelines for National Ballast Water Status Assessments. GloBallast Monographs No. 17. Available: http://globallast.imo.org/wp-content/uploads/2014/11/Mono17_English.pdf.

Gregg, M., Rigby, G., Hallegraeff, G.M., 2009. Review of two decades of progress in the development of management options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water. Aquat. Invasions 4, 521–565. http://dx.doi.org/10.3391/ai.2009.4.3.14.

Jow, T.S., Chakravarty, R., 2015. Report of Voluntarily Trial Study Conducted by Singapore on Onboard Sampling and Analysis of Ballast Water. Maritime and Port Authority of Singapore Shipping Circular No. 5 of 2015.

McCarthy, S.A., Khambaty, F.M., 1994. International dissemination of epidemic *Vibrio cholerae* by cargo ship ballast and other nonpotable waters. Appl. Environ. Microbiol. 60, 2597–2601.

van Slooten, C., Wijers, T., Buma, A.G.J., Peperzak, L., 2015. Development and testing of a rapid, sensitive ATP assay to detect living organisms in ballast water. J. Appl. Phycol. 27, 2299. http://dx.doi.org/10.1007/s10811-014-0518-9.

Steichen, J., Schulze, A., Brinkmeyer, R., Quigg, A., 2014. All aboard! A biological survey of ballast water onboard vessels travelling the North Atlantic Ocean. Mar. Pollut. Bull. 87, 201–210. http://dx.doi.org/10.1016/j.marpolbul.2014.07.058.

Technical Whitepaper Comparing 2nd Generation ATP to 1st Generation ATP Technology, 2016. www.luminultra.com.

Tsolaki, E., Diamadopoulos, E., 2010. Technologies for ballast water treatment: a review. J. Chem. Technol. Biotechnol. 85, 19–32. http://dx.doi.org/10.1002/jctb.2276.