Development and testing of a rapid, sensitive ATP assay to detect living organisms in ballast water

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Abstract

To reduce the spread of aquatic invasive species, the discharge of ballast water by ships will soon be compulsorily regulated by the International Maritime Organization (IMO) and the United States Coast Guard (USCG). Compliance with their regulations will have to be achieved by onboard ballast water management systems. To monitor the treatment system performance, rapid and easy compliance techniques are required. This paper reports on the suitability of Adenosine Triphosphate (ATP) to quantify living 10 to 50 µm organisms at <10 cells mL^{-1}, which is the upper limit of the IMO D-2 and USCG regulations. Initial tests revealed that commercially available ATP assays lacked sufficient sensitivity to monitor ATP in treated ballast water. A rapid and easy concentration method was developed to increase sensitivity and remove interfering salts, non-target organisms (*Micromonas pusilla*) and dissolved ATP. Laboratory experiments revealed that salinity was reduced 33 times and concentration efficiencies reached 85%. The ATP assay was tested in a UV-based full-scale ballast water management system, treating seawater and fresh water. ATP levels were compared with two alternative compliance tools: FDA and Photosystem II efficiency. Results showed a 10-fold decrease in ATP levels after treatment compared to a 5-fold decrease in alternative compliance techniques. Following refinements, the ATP assay's detection limit reached 2.5 ± 0.5 cells mL^{-1}, using a *Thalassiosira rotula* monoculture. Initial estimates of the pass and fail level were 50 and 6,000 relative luminescence units, respectively. Further validation is recommended. The ATP assay is a promising tool for ballast water compliance testing.

Keywords: CME; ATP; ballast water; IMO D-2; PSII efficiency; FDA

1. Introduction

Ballast water plays an essential function in a ship's stability, trim, draft and structural integrity. Thus, ballast water is critical to enable safe shipping. However, through ballast water transport, huge quantities of viable (able to reproduce) organisms are transported around the world and discharged into to foreign ecosystems (Drake and Lodge 2007). These newly introduced species may become invasive and outcompete local species for habitat and food availability. The ongoing spread of aquatic invasive species can lead to major damage to biodiversity and economic losses (Molnar et al. 2008). To prevent the dispersal of aquatic invasive species through ballast water, the International Maritime Organization (IMO) and United States Coast Guard (USCG) have enacted legislation which limits the number of viable organisms that are allowed to be discharged through ballast water (Anonymous 2004; Anonymous 2012). Both IMO's D-2 regulation and the USCG regulation limit, among others, the discharge of viable 10 to 50 µm organisms to <10 mL^{-1} and the discharge of viable >50 µm organisms to <10 m^{-3}.

To comply with the upcoming discharge regulations, most ships will have to be fitted with ballast water management systems (BWMSs), to disinfect ballast water before discharge. After acquisition and implementation of a BWMS, ship owners may want to monitor the biological efficacy of their BWMS over time and in various water types and qualities. In addition, Port State Control (PSC) officers are obliged to monitor the compliance of ships to the ballast water convention. In accordance with the recommendations outlined in the IMO ballast water sampling guidelines (G2), a quick screening method to identify ships that are potentially in violation of the D-2 standard is needed (Anonymous 2008b). Sampling and monitoring obligations require that ballast water discharge should be analyzed for the presence of viable organisms. Due to their low abundance, accurate zooplankton (>50 µm) estimates require cubic meters of water to be sampled and analyzed microscopically. For the smaller phytoplankton and micro-zooplankton organisms (10 to 50 µm), analysis often requires expensive and complicated equipment such as flow cytometry. All of these analyses require trained personnel to produce reliable results. In practice therefore, detailed quantitative biological analysis of ballast water is time-consuming, tedious and expensive.

Commonly, ship owners and PSC will not have the capabilities to carry out specialistic quantitative biological analyses. Although they are authorized to sample ballast water, PSC inspectors will mainly focus on checking the presence of a treatment system, the availability of qualified personnel to run the system and whether the system has reported any errors in its mechanical or chemical operation specifications (personal communication K. Hak, inspector of the Ministry of Infrastructure and the Environment, The Netherlands). To

improve the capabilities of ship owners and PSC to monitor the biological efficacy of BWMS, tools are needed that can estimate the concentrations of viable organisms. In addition, these so-called Compliance, Monitoring and Enforcement (CME) techniques will have to be reliable, yet quick and simple enough to be used by minimally trained crew on board ships. In recent years several CME techniques have been developed to monitor viable organisms in discharged ballast water (Anonymous 2014; Delacroix and Liltved 2013; Welschmeyer and Maurer 2011). Usually, sexually reproducing large zooplankton are excluded from CME techniques, since sampling cubic meters of seawater would be too time-consuming and logistically challenging in a ship's engine room. The development of the ATP assay presented here, solely focused on the 10-50 µm size fraction of the IMO and USCG discharge standards.

Whenever a chemical reaction inside a living organism is carried out that requires energy, this energy is provided by ATP (Lipmann 1939a; Lipmann 1939b; Lipmann 1940; Lipmann 1941). For decades, the presence of ATP has been considered a good indicator for the presence of metabolically active organisms (Karl 1993). Although metabolic activity does not guarantee viability it is considered to be a good viability indicator for unicellular organisms since they usually reproduce asexually. ATP quantification is usually based on bioluminescence derived from firefly (*Photinus pyralis*) luciferin/luciferase complexes. Several ATP assays are globally available such as the ENLITEN® ATP assay (Promega, Wisconsin, USA), Molecular Probes® ATP Determination Kit (Invitrogen, California, USA) and the Clean-Trace™ system (3M, Minnesota, USA). These commercial ATP assays require less than $5,000 to acquire and cost no more than $10 per analysis. In seawater however, the large amount of metal ions interfere with the luciferin/luciferase reaction which inhibits the light production (Sudhaharan and Reddy 2000). To solve this, elaborate pre-treatment steps were developed involving ATP extraction using boiling Tromethamine (Tris), H_2SO_4 or activated carbon (Hodson et al. 1976), which are still in use to date (Maurer 2013). Using these extractions techniques, much research has been devoted to correlate ATP to marine microbial biomass (Novitsky 1987), phytoplankton biomass (Hunter and Laws 1981) and zooplankton biomass (Maranda and Lacroix 1983). Though proven effective, these extraction techniques are too complicated and time consuming to be used by PSC officers and ship's personnel.

In the present study, Clean Trace™ ATP assay (3M, Minnesota, USA) was applied. To remove metal ions, concentrate and extract ATP from relevant organisms, a simple and straightforward concentration method was developed. Ships sail in polar as well as tropical regions and both fresh water and seawater are used as ballast. Therefore, the ATP assay was tested at various ambient temperatures and salinities. Chlorine-disinfection is commonly used in BWMSs, therefore the effect of chlorine on the ATP assay was also examined.

Early on in the development of the ATP-based CME technique, the opportunity arose to test the assay on a full-scale UV-based BWMS. The performance of the ATP assay was compared with three additional CME techniques. Firstly, esterase activity using bulk fluorescein-diacetate (FDA) fluorescence was determined using a proprietary system provided by Hach (Colorado, USA). Secondly, photosystem II (PSII) efficiency was estimated using [3-(3,4-dichlorophenyl)-1, 1-dimethylurea] (DCMU), also provided by Hach. Thirdly, PSII efficiency was determined using Pulse Amplitude Modulation (PAM) fluorometry (Walz 2000).

Esterase enzymes are exclusively produced by living organisms and thus considered a proxy for the presence of living organisms (Rotman and Papermaster 1966). Before the development of PAM fluorometry, the PSII efficiency of active chlorophyll was estimated using the photosynthetic inhibitor DCMU (Cullen and Renger 1979). Results of the tests using a full-scale BWMS are presented early on, to reflect the chronology of the development process. Following these tests, modifications to the concentration method were made to increase the usability, precision and sensitivity of the ATP assay. The practical use of the concentration method in combination with ATP analysis in ballast water compliance testing will be discussed.

2. Methods

Firstly, all analytical methods applied in the research are explained. In order to comprehend the development process, a separate section was devoted to explaining all concentration methods applied during the research (see also Table 1). Finally, the experiments carried out are explained in detail (see also Table 2).

2.1. Analytical methods

The 3M Clean-Trace™ NG luminometer was used in combination with either the 3M Clean-Trace™ Biomass Detection Kit (BDK), or the 3M Clean-Trace™ Water Total ATP swabs (ATP swabs). The BDK was considered more appropriate in a laboratory setting and resulted in more accurate results, however due to the need for pipetting small volumes it was not deemed suitable for use by untrained crewmembers. The ATP swabs required immersing a dip-stick in the sample, which was considered more user-friendly. The methods were used as according to the manufacturers prescription:

BDK: Firstly, 100 µL sample was pipetted into a cuvette. Secondly, 100 µL of proprietary cell lysing extractant was added and incubated for one minute. Finally, 100 µL of 3M luciferin/luciferase reagent was added to the cuvette and mixed. The resulting luminescence was immediately determined using a luminometer and recorded as Relative Luminescence Units (RLU).

ATP swabs: The swabs arrived pre-moistened with extractant on delivery. A swap was dipped into a water sample and inserted into a tube containing the luciferin/luciferase reagents. The sample volume was 157 ± 3 µL (average ± 95% CI). The sample was mixed with the reagents by pressing the dip-stick through two membranes and the RLU was immediately measured using the 3M luminometer .

FDA analysis: A 200 mL sample was filtered over a nylon screen filter (10 µm pore size, 25 mm diameter). The filter was transferred to a 4 ml polyethylene cuvette and immersed in 2 mL proprietary buffer. One drop of FDA was added to the cuvette and incubated for 30 minutes. During incubation, FDA was cleaved by intracellular esterase enzymes thereby producing green fluorescent fluorescein. After a vigorous shake, the filter was removed from the cuvette. The fluorescence in the cuvette was measured (495/517 nm, excitation/emission) using a proprietary Hach fluorometer (Welschmeyer and Maurer 2011).

The terminology for PSII efficiency analyses was adopted from Kromkamp and Forster (Kromkamp and Forster 2003). The Hach DCMU-based method was applied as follows. Initially, the fluorescence (F_0) of a 2 minutes dark-adapted sample was measured, with a proprietary Hach fluorometer using a single turnover (ST) light pulse. Subsequently, the chlorophyll was inactivated by adding DCMU and fluorescence was measured again after 2 minutes dark incubation (F_{DCMU}). From the difference in fluorescence the PSII efficiency was

138 calculated: $(F_{DCMU}–F_0)/F_{DCMU} = F_v/F_{DCMU}$.

139 PAM fluorometry (Water-PAM, Walz, Bavaria, Germany), using a multiple turnover (MT) light pulse,
140 was used to measure the PSII efficiency of active chlorophyll and expressed as: $(F_0\text{-}F_m)/F_m = F_v/F_m$. Samples
141 were dark acclimatized for 30 minutes.

142 To enumerate phytoplankton cells in laboratory trials, a BD Accuri™ C6 flow cytometer (Becton
143 Dickinson, New Jersey, USA) was used. Particles were detected using a 488 nm laser. Phytoplankton cells were
144 discriminated from other particles based on red auto fluorescence of the chlorophyll detected by the FL3 channel
145 (670 nm long pass filter).

146 For a live/dead determination of phytoplankton 0.5 µM SYTOX® Green nucleic acid stain (Invitrogen,
147 California, USA) was used. This stain enters permeable cells where it causes green fluorescence when bound to
148 DNA. The method is based on the assumption that permeable, stained cells are dead and non-stained cells are
149 alive. Stained cells were discriminated from other cells using the FL1 channel (530 ± 30 nm band pass filter).

150 ***2.2. Developing the concentration method***

151 Concentration method 1 (CM1), was based on a traditional flask-filter-beaker assembly. A sample of 200 mL
152 was filtered (nylon screen; 10 µm pore size, 25 mm diameter) (Millipore, Massachusetts, USA) using a 1 L flask
153 with filter beaker on top. After filtration the filter was placed in a 4 mL polyethylene cuvette with 2 mL of sterile
154 milli-Q™ (Millipore), resulting in a 100 times concentration of >10 µm particles. After a vigorous shake the
155 RLU was determined using ATP swaps.

156 To simplify the filtration procedure, concentration method 2 (CM2) was developed. A 100 mL sample
157 was taken up using a 100 mL syringe (Plastipak™, Becton Dickinson). The sample was gently filtered over a
158 nylon screen filter (10 µm pore size, 25 mm diameter, Millipore), contained in a stainless steel reusable filter
159 holder (Millipore). Particles retained in the filter were flushed out with a 5 mL syringe (Terumo, Tokyo, Japan)
160 containing 5 mL milli-Q™ into a 15 mL polypropylene tube (Greiner Bio-One, North Carolina, USA). The
161 concentrate was analyzed for the RLU either with ATP swabs or the BDK.

162 To further simplify the procedure for onboard use, concentration method 3 (CM3) was developed. The
163 stainless steel filter capsule of CM2 was replaced with a custom made polypropylene disposable filter capsule,
164 containing a non-replaceable nylon screen filter (10 µm pore size, 25 mm diameter (Sterlitech, Washington,
165 USA).

166 It was suspected that the concentrate was not extracted sufficiently by the single rinse of 5 mL milli-
167 Q™. To improve the extraction efficiency, concentration method 4 (CM4) was developed. Instead of directly

168 removing the 100 mL syringe after filtration, the 5 mL milli-Q™ was flushed back and forth into the 100 mL
169 syringe five times, to release particles from the filter more effectively.
170 It was noted that in turbid water, 100 mL sample could easily clog the filter. Also, residual salinity
171 could be substantial in concentrated samples. To avoid clogging and increase the salinity removal, concentration
172 method 5 (CM5) was developed. The sample volume was reduced to 50 mL using 50 mL syringe (Terumo).
173 After filtration, a 5 mL syringe containing 5 mL milli-Q™ was connected to the outlet side of the filter. The 50
174 mL filter, contaminated with salts, was removed and on the inlet fitting of the filter a sterile 5 mL syringe
175 (Terumo) was attached. The concentrate was flushed back and forth five times so that the concentrate ended up
176 in the syringe connected to the inlet side of the filter. After removal of the piston the concentrate was sampled
177 directly from the syringe using the ATP swabs.
178 Because various concentration factors among experiments were used it was deemed inappropriate to
179 convert RLU values to absolute ATP concentrations. In addition, due to inherent uncertainties in concentration
180 efficiencies, presenting absolute ATP levels would give a false impression of comparability among different
181 experiments. To evaluate ATP analysis, it was considered most important that <10 cells mL^{-1} were above the
182 detection limit of the device, and that substantial differences were observed between disinfected water (D-2
183 compliant) and control water. For both objectives, reporting results in RLU was considered sufficient.

184 ***2.3. Experimental design***

185 *2.3.1. Linearity and abiotic influences on the ATP assay*

186 Many BWMS use electro-chlorination to produce hypochlorite (ClO^-) as an active substance, to achieve
187 disinfection of ballast water (Anonymous 2013). Therefore, the effect of hypochlorite on a standard solution of
188 ATP was tested. Test solutions were made by diluting a 10-15% sodium hypochlorite solution (Sigma-Aldrich,
189 Missouri, USA) in milli-Q™. Concentrations were determined using DPD Chlorine Total powder pillows for
190 analysis in a Hach DR/890 Colorimeter (Anonymous 2009). As test concentrations 0, 0.25, 5 and 10 mg L^{-1} Cl_2
191 were used. The ATP concentration in all four test solutions was 0.6 ng mL^{-1} by adding an ATP standard
192 (contained in bovine serum albumin, 3M). Test solutions were analyzed in triplicate using the BDK.
193 To verify the linearity between ATP concentration and RLU signal, a test solution was made using
194 milli-Q™ water and an ATP standard. (contained in bovine serum albumin, 3M). A calibration series was
195 prepared by dissolving the ATP standard with milli-Q™ water to reach a concentration of 0, 0.12, 0.6, 1.5, 3,
196 7.5, 15, 30, 45 and 60 ng mL^{-1} ATP. The RLU signals were determined in triplicate for each of the dilutions. To
197 investigate the effect of temperature, all equipment and test solutions were acclimated for one hour in climate

198 rooms at 4°C, 15°C and 26°C prior to analysis.

199 Salinity test solutions (30 mL) were prepared in 60 mL glass bottles with aluminum caps using mixtures
200 of milli-Q™ and seawater (0.2 µm filtered and autoclaved) to reach the desired salinities of 0, 4.5, 9, 18, 27, 31.5
201 and 36 g kg^{-1}. Temperatures were set at 4°C, 15°C or 26°C by acclimating all test solutions and equipment into
202 climate chambers at least one hour before starting the analyses. The test solutions were spiked with 6 ng mL^{-1} of
203 ATP analyzed in triplicate using the BDK.

204 To test the effect of 0-2 g kg^{-1} salinity on ATP analysis, sterile seawater (0.2 µm filtered and
205 autoclaved) was added to milli-Q™, to reach salinities of 0, 0.5, 1 and 2 g kg^{-1}. Two salinity dilution series were
206 prepared, containing 0.3 ng mL^{-1} and 3 ng mL^{-1} ATP respectively. The series were analyzed in triplicate using
207 the BDK.

208 *2.3.2. UV-C treatment of Thalassiosira rotula*

209 The marine diatom *Thalassiosira rotula* (CCMP 1018) was obtained from the National Center for Marine Algae
210 and Microbiota (NCMA). To investigate the effect of UV-C radiation on the survival of *T. rotula* and on ATP
211 levels, a laboratory experiment was carried out. *T. rotula* is a chain forming species of approximately 15 µm in
212 minimum dimension. *T. rotula* was cultured in 0.2 µm filtered and autoclaved seawater (salinity: 28 g kg^{-1}) with
213 excess nutrients at 15°C under a 16:8 light:dark regime (50 µmol photons m^{-2} s^{-1}). When the culture was in the
214 exponential growth phase, it was diluted with 0.2 µm filtered and autoclaved seawater to a final density of 1,000
215 cells mL^{-1} (source culture: 94,970 cells mL^{-1}). The dilution was pumped (Aqua-Flow 50 pump, Aquadistri,
216 Klundert, The Netherlands) at 20 mL s^{-1} through a low pressure UV-C reactor (Van Gerven, Son, The
217 Netherlands). The culture was treated with a dose of 139 mJ cm^{-2} of monochromatic UV-C light (254 nm). As a
218 control the culture was pumped through the UV-C reactor with the lamps turned off to compensate for the effects
219 of the pump. Subsequently the cultures were incubated in the dark at 15°C for five days. On day 5, a second UV-
220 C treatment was given to one part of the treated culture, simulating the usual UV treatment at ballast water
221 discharge. The other half was pumped through the UV-C reactor with the lamps off serving as a secondary
222 control. After five days the cultures, including the original control, were placed into a 15°C climate room under a
223 16:8 hour light:dark cycle (50 µmol photons m^{-2} s^{-1}). All cultures were sampled on day 0, day 5 and day 12. The
224 cultures with the second UV treatment and second pump were also sampled on day 6. Samples were taken in
225 triplicate for phytoplankton abundance, PSII efficiency (Walz PAM), FDA and ATP using CM2 and the BDK.

226 *2.3.3. Test CME techniques during IMO G8 land-based verification testing*

227 In the spring of 2012 land-based ballast water tests were performed using natural seawater and fresh water

according to the IMO G8 guidelines (Anonymous 2005; Anonymous 2008a). At uptake, the 200 m^3 h^{-1} treatment system utilized 40 µm filtration and polychromatic UV radiation of 200-400 nm using two medium pressure UV lamps. After 5 days the water was discharged, during which a second UV dose was delivered.

Many biotic and abiotic characteristics of the water were monitored during uptake and discharge of the water (Peperzak 2013). ATP, FDA and DCMU analyses were carried out in triplicate using the same samples that were used for 10 to 50 µm organism abundance and PAM fluorometry analyses. ATP was analyzed using CM1 and ATP swabs. In total, 2 seawater control tanks, 4 freshwater control tanks, 3 seawater UV-treated tanks and 7 freshwater UV-treated tanks were included in the comparison.

2.3.4. Detection limit, concentration efficiency and salinity reduction of the concentration method

To investigate the lower limit of CM3 *T. rotula* was cultured at 15°C under a16:8 light:dark regime (50 µmol photons m^{-2} s^{-1}) in f/2 medium with silicate. When the culture was in the exponential growth phase a dilution series was made using sterile seawater as diluent. Concentrations of 10, 20, 50 and 100 cells mL^{-1} of the culture were made and verified using flow cytometry. The cell dilutions were concentrated in triplicate using CM3 and analyzed for ATP content using the BDK and the ATP swabs.

To increase the flushing efficiency of the filter, CM4 was developed. Fresh water from lake NIOZ, adjacent to the institute, was collected and pre-filtered over a 50 µm screen filter to remove large particles. A fractionation was made using subsequent filtration steps of 0.2 µm and 10 µm to determine the ATP content of the organisms in the 10-50 µm fraction. A freshwater sample of 3 L was placed in a polypropylene beaker and stirred using a magnetic stirrer at 160 rotations per minute (rpm). ATP measurements were made in 7-fold using either CM3 or CM4 and ATP swabs. The RLU level corresponding with 100% concentration efficiency was determined by multiplying the RLU in the 10-50 µm size fraction 20 times, since concentrating 100 mL of sample into 5 mL of milli-Q™ should ideally result in a 20-fold concentration.

To improve the salinity reduction factor, CM5 was developed. Natural seawater (salinity: 27,4 g kg^{-1}) was used for a salinity reduction comparison between CM4 and CM5 in 10-fold.

To test the precision of CM3 and CM5, seawater (salinity: 27 g kg^{-1}) from the Marsdiep inlet was collected at high tide, transferred to a 3 L polyethylene beaker and stirred using a magnetic stirrer at 160 rpm. ATP content was concentrated in 12-fold using CM3 or CM5 and analyzed with ATP swabs.

To investigate the lower limit of CM5 and possible interference of <10 µm cells with the concentration method, *T. rotula* and the prasinophyte *Micromonas pusilla* (CCMP 1545, NCMA) with a 2 µm diameter were cultured at 15°C under a 16:8 hour light:dark regime (50 µmol photons m^{-2} s^{-1}) in f/2 medium with silicate.

When the cultures reached the exponential growth phase, a dilution series was made using 0.2 µm filtered sterile seawater as diluent. A 1 L stock solution of ~160 cells mL^{-1} was quantified in 5-fold using flow cytometry. Subsequently, six consecutive *T. rotula* dilutions of 500 mL with sterile seawater were made using a glass cylinder (500 mL ± 0.5%, DURAN, Germany), resulting in solutions of 80, 40, 20, 10, 5 and 2.5 cells mL^{-1}. In addition, three *T.rotula/M. pusilla* mixtures were made containing 20/20,000; 10/10,000 and 5/5,000 cells mL^{-1} respectively. The respective CI's of cell concentrations were calculated using the confidence interval (CI) of the initial analysis of the ~160 cells mL dilution. For each dilution step 1% error was added since the glass cylinder was used twice per dilution. Cell dilutions/mixtures of 40 *T. rotula* cells mL^{-1} or lower, were concentrated in 5-fold using CM5 and analyzed for ATP content using ATP swabs. Following Box-Plot analysis, single outliers, exceeding 1.5x the interquartile range of the first or third quartile, were excluded from further analysis.

2.4. Statistical analysis

For all statistical test the null hypothesis was that there was no significant difference between treatment and control. As confidence level for statistical tests and CI's 95% was chosen ($\alpha = 0.05$).

When samples were analyzed in duplicate or more CI was calculated based on a Student's t-distribution using the MS Excel 2010 function CONFIDENCE.T. The Student's t-distribution was deemed more appropriate for small sample sizes than a normal distribution.

Least-squares linear regression models, Analyses of Variance (ANOVA) and Box-Plot analyses were calculated in SYSTAT 13 (SYSTAT Software Inc. California, USA).

3. Results

3.1. Linearity and abiotic influences on the ATP assay

A regression analysis was made where the RLU signal was plotted against the chlorine concentration (data not shown). The slope of the model was not significantly different from zero (ANOVA: $P > 0.05$), indicating that chlorine levels of ≤10 mg L^{-1} did not significantly affect ATP measurements.

The least squares regression models of RLU as function of ATP concentration were: $y = 1{,}081x + 211$ (4°C); $y = 2{,}080x + 347$ (15°C) and $y = 2{,}104x + 150$ (26°C) (Figure 1a). The intercepts were not significantly different from zero which means that no blank subtraction was needed. However, at 4°C the RLU signal decreased 50% compared to the measurements at 15°C and 26°C.

Increasing salinity caused the RLU signal to decline logarithmically (Figure 1b). At a salinity of 5 g kg^{-1} already 50% of the original RLU signal was lost. At the average salinity of seawater (35 g kg^{-1}) more than 90% of the original RLU signal was lost. The relative RLU decrease was similar for all three temperatures tested.

When the various types of concentration methods were applied, a residual level of salinity remained. The salinity usually ranged between 0.5-1.5 g kg^{-1} which had a significant effect on the resulting RLU signal. In Figure 1c the relative effect of the decrease in RLU signal resulting from a salinity of 0-2 g kg^{-1} is depicted.

When the measurements of 3 ng mL^{-1} ATP were divided by the measurements observed at 0.3 ng mL^{-1} a factor of ±10 was observed. To investigate whether this factor (y) was constant at all salinities tested (x) a least squares linear regression was carried out resulting in the model: $y = 0.18x + 9.6$. The slope had a P-value of 0.171, which exceeds α, so the salinity effect was similar at 0.3 and 3 ng mL^{-1} ATP for salinities of 0-2 g kg^{-1}. To correct for the percentage RLU loss (y) due to residual salinity in g kg^{-1} (x) the model: $y = -12.7x$ was used in further experiments. This model was derived from the observed RLU losses at 3 ng mL^{-1} ATP (Figure 1c).

3.2. UV-C treatment of T. rotula

None of the compliance methods showed a significant change directly after UV treatment. (Figure 2). The abundance of UV-treated cells increased significantly after five days ($P < 0.05$; Figure 2a). ATP levels decreased significantly after five days ($P < 0.05$; Figure 2b), but FDA levels remained unchanged in the UV-treated incubation (Figure 2c). ATP levels were unchanged in the control incubation, but FDA levels in the control almost doubled. The PSII efficiency was strongly reduced, but still detectable in the UV-treated culture (Figure 2d). After the second UV treatment only the PSII efficiency was significantly lower than the pre-treatment value ($P < 0.005$). The other three compliance methods did not detect a significant change directly after the second UV treatment.

306 Both ATP levels ($P < 0.05$) and PSII efficiency ($P < 0.05$) were significantly reduced one day after the
307 second UV treatment. Also the PSII efficiency of the double pumped UV-treated culture showed a significant
308 decline ($P < 0.01$) and was similar to the second UV-treated culture on day 6. The cell abundance and FDA
309 fluorescence appeared unaffected by the second UV treatment.
310 On day 12, following 7 days of light incubation, the cell abundance in the control incubation increased
311 to >45,000 cells mL^{-1}. The cell abundance of the single and double UV-treated culture were significantly lower
312 ($P < 0.005$; $P < 0.05$ respectively), but still well above 500 cells mL^{-1}. ATP levels decreased to 100-250 RLU,
313 which represents 1-2% of the original RLU level. PSII efficiency was below the detection limit for all UV-
314 treated cultures and remained at very high levels in the control. FDA levels did not significantly decrease
315 between day 5 and day 12 in UV-treated incubations. In the control FDA and ATP levels increased 8-fold and
316 25-fold respectively between day 5 and day 12 coinciding with the increase in cell density.
317 At day 5 numbers of living cells were 100-200 cells mL^{-1} in the various UV-treated incubations, which
318 was 10-20 times exceeding the D-2 standard (Anonymous 2004). At day 12 no living cells were detected in all
319 UV-treated cultures. ATP showed a good correlation between living *T. rotula* cells and RLU levels with $R^2 =$
320 0.73 (Figure 3a). However, at cell numbers above 50 cells mL^{-1} a plateau appeared. FDA levels showed no
321 correlation with the number of living cells (Figure 3b). Although PSII efficiency is not a quantitative indicator it
322 showed the best correlation with living cells indicated by $R^2 = 0.87$ (Figure 3c).

323 ***3.3. Test CME techniques during IMO G8 land-based verification testing***

324 The full-scale land-based test were successfully carried out according to the IMO G8 test guidelines using
325 seawater and fresh water (Anonymous 2005; Anonymous 2008a; Peperzak 2013). All three compliance tools
326 showed a significant reduction in their respective signals between samples from the uptake before treatment and
327 discharge after treatment (Figure 4). The largest reduction was recorded for ATP analysis (91%) between
328 untreated uptake samples and treated discharge samples (Figure 4a). FDA fluorescence showed a decrease of
329 82% (Figure 4b). PSII efficiency levels derived from DCMU analysis resulted in decreases of 83% (Figure 4c).
330 All compliance tools showed significant differences between untreated and treated water at uptake.
331 Official data for the G8 test protocol (10-50 µm cells mL^{-1} and PAM fluorometry derived PSII
332 efficiencies) were compared with the three compliance tools (Figure 5). DCMU derived PSII efficiency data
333 showed the highest correlation with cell concentrations ($R^2 = 0.72$; Figure 5c), followed by ATP ($R^2 = 0.62$;
334 Figure 5a) and FDA ($R^2 = 0.43$; Figure 5e). DCMU derived PSII efficiency data showed the highest correlation

with PAM fluorometry derived PSII efficiency data (R^2 = 0.75; Figure 5h), followed by FDA and ATP analysis (R^2 = 0.64 and 0.47, respectively).

3.4. Detection limit, concentration efficiency and salinity reduction of the concentration method

During the detection limit test of CM3, the BDK produced statistically different RLU values between all dilutions except between 10 and 20 cells mL^{-1}. When the ATP swabs were used no significant difference was observed between 20 and 50 cells mL^{-1} (Data not shown). So, CM3 in combination with ATP swabs was not sensitive enough to distinguish *T. rotula* concentrations <50 cells mL^{-1}.

The concentration efficiency of CM3 and CM4 was 63% ± 12% and 85% ± 25%, respectively (average ± CI). Due to variability in the measurements the difference was not statistically significant (p = 0.15). However, CM4 was not statistically different from 100% concentration efficiency.

CM4 was detrimental to the salinity reduction factor due to mixing the milli-Q™ water with the residual sample in the 100 mL syringe. Using CM5 the salinity reduction factor was increased significantly from 17 to 33 times (P = <0.001). This meant that a seawater sample containing 35 g kg^{-1} salts, after concentration typically contained 1.1 g kg^{-1} salts (35/33 = 1.1). This salinity reduction was deemed sufficient for typical seawater samples, since RLU signal loss is likely to be ~15% or less, at a residual salinity of 1.1 g kg^{-1}.

Results of the precision test showed that at two replicates the 95% CI was larger than the average RLU signal observed for both concentration methods (Figure 6a). At five replicates, the average RLU levels of CM5 stabilized and the CI was 24%, while the CI of CM3 still was 38% of the average. The average RLU values obtained using CM5 were not significantly different from CM3, whilst the concentration factor was 10 instead of 20 which illustrated the improved flushing efficiency of CM5. The variability among measurements using CM5 appeared to be lower than using CM3, which might be attributed to the improved resuspension efficiency of five times back and forth flushing.

The initial 1 L *T. rotula* solution for the detection limit test of CM5, contained 176 ± 15% cells mL^{-1} (average ± %CI). Following 6 dilutions steps the error had increased to 21% (15+6). So the final dilution had a concentration of 2.5 ± 0.5 cells mL^{-1} (average ± CI). Significantly different RLU signals were observed for all *T. rotula* dilutions tested using CM5 and ATP swabs (Figure 6b). This indicated that the detection limit of CM5 is at least 2.5 ± 0.5 cells mL^{-1}. The improvement of the detection limit compared to CM3 was mainly attributed to a reduction in variability among the replicates presumably due to the improved flushing of the filter. Adding *M. pusilla* cells to the dilutions did not result in significantly different RLU levels. This was a strong indication that the concentration method was highly effective in disregarding cells <10 µm whilst concentrating cells >10 µm.

Using the regression model from Figure 6b, it is possible to estimate pass/fail levels for the ATP assay using CM5 and ATP swabs. According to the regression model (RLU = 23.1 * cell concentration + 10.6), the RLU level of 10 *T. rotula* cells is 241.6 RLU. *T. rotula* is a cylindrical cell of 15 µm in diameter and height. So, the volume of 10 cells is 26.507 μL^3 (10 * Volume = π * 7.5^2 * 15). Assuming that ATP levels remain constant among organism species and sizes, this translates to 0.009 RLU μL^{-1} cell volume (241.6 / 26.507). Using this value, it is possible to estimate the lower and upper limit of ATP assay at which ballast water is either D-2 compliant or likely non-compliant. In further calculations, cells are assumed to be spherical. A spherical cell of 10 µm would have a volume of 524 µL (Volume = 4/3 * π * 7.5^3). So, 10 cells of 10 µm would result in 48 RLU (10 * 524 * 0.009), which is significantly higher than the blank measurement: 11 ± 6 RLU (average ± CI). The upper limit would be when 10 cells of 50µm are present in the sample. This would result in a RLU level of 5,951 RLU (10 * 65,450 µL * 0.009). So, assuming constant ATP levels per cell volume, if the ATP assay yields a result of less than ~50 RLU, the ballast water sample is most likely D-2 compliant. If the ATP assay yields result of more than ~6,000 RLU the ballast water sample is most likely non-compliant. RLU levels between these two numbers are ambiguous, because a high abundance of small cells can give the same RLU signal as a few large cells.

4. Discussion

4.1. Data quality

Several aspects have been considered to assess the data quality obtained from the compliance tools. Firstly, it was shown that the ATP assay was not affected by chlorine conditions typically encountered in chlorine-treated ballast water. Moreover, the incorporation of the pre-concentration procedure tackled three major challenges at once. First, salinity interference was sufficiently eliminated by reducing the salinity 33 times. Second, non-target dissolved ATP and ATP derived from <10 µm organisms were effectively removed from the concentrate, shown by the lack of RLU signal increase after the addition of *M. pusilla*. Third, the detection limit was decreased to 2.5 ± 0.5 cells mL^{-1}. These developments contribute to ATP having a high potential to become a viable ballast water compliance tool. It should be noted that the ATP assay is affected by ambient temperature. So in order to obtain reliable results, all analyses should be carried out at room temperature. In Arctic regions, where ballast water temperatures are around freezing point, no problems are expected as long as the ATP-free water to flush the filter and other reagents and equipment are kept at room temperature.

In laboratory tests, of the three compliance techniques tested, ATP and PAM fluorometry showed the most promising results, since both demonstrated a reasonable to good correlation with the amount of living *T. rotula* cells ($R^2 = 0.73$ and 0.87, respectively). The correlation of PSII efficiency and cell concentration was considered to be indirect because water disinfection both decreased cell densities as well as PSII efficiency simultaneously. In principle, high PSII efficiency can be detected both at low and high cell densities since it is a relative measurement. However, due to the high correlations observed between PSII efficiency and cell density it can be of value for indicative testing.

The absence of a correlation ($R^2 = 0.03$) between FDA and living cells could be caused by intact enzymes still residing in the permeable cells. FDA fluorescence was based on esterase activity. However, UV-treatment of *T. rotula* did not appear to inhibit esterase enzymes. The concentration method used for ATP analysis appeared to effectively discard the ATP content of permeable and dead cells, indicated by the relatively high correlation with living cells and RLU signal ($R^2 = 0.73$). The living cells on day 5 in the UV-treated incubations were no longer viable, indicated by the absence of living cells on day 12 after 7 days of light incubation. The detection of living cells at day 5 clearly demonstrated the delayed effect of UV disinfection often observed after UV treatment (Stehouwer et al. 2010). Most compliance tools are designed to detect living cells instead of viable cells, whereas viability is the variable that is needed to establish whether ballast water discharge is in compliance with IMO and USCG regulations (Anonymous 2004; Anonymous 2012).

In full-scale tests, major ATP differences between treated and untreated water were observed, both in seawater and fresh water. Correlation plots revealed that ATP correlates well with the concentration of 10-50 µm organisms. The strong correlation between DCMU and PAM fluorometry derived PSII efficiencies was expected, since both methods essentially aim to measure the same variable. It was surprising that DCMU showed a higher correlation with cell concentration than ATP or FDA. The latter two methods aim to quantify total metabolic activity and enzymatic activity, which is presumably a good indication for cell concentration. In contrast, DCMU aims to measure PSII efficiency which is independent of concentration. Previous studies however, have indicated that PSII efficiency was a poor predictor for phytoplankton regrowth potential (Van Slooten et al. 2014). Of the two quantitative methods, ATP was considered superior to FDA since ATP results correlated better with cell concentrations.

A major limitation of relying on the presence of PSII efficiency as compliance tool is that it only targets autotrophic organisms. Heterotrophic organisms such as ciliates, protozoa and many dinoflagellates cannot be detected using DCMU, Walz PAM or any other PSII-based method. Coastal ecosystems can rapidly shift from phytoplankton dominated to zooplankton dominated states in a matter of weeks (Peperzak et al. 1998) so the need for a compliance tool capable of detecting all types or organisms is evident. Both ATP and FDA are capable of detecting all types of organisms, however ATP analysis is much less time-consuming than FDA analysis.

Differences in delayed disinfection effect between laboratory studies and full-scale land-based studies could be caused by the use of different UV technologies. In the laboratory, a low pressure UV-C reactor was used which produced monochromatic UV-C radiation at 254 nm. The medium pressure UV reactor in the full-scale land-based test produced a broad range of UV-C and UV-B radiation, ranging from 200-400 nm (personal communication M. Voigt, Cathelco, UK). Although disinfection efficiency is highest at a radiation of 254 nm, this wavelength is often quickly absorbed in natural freshwater due to dissolved organic matter. Each wavelength exhibits its own absorption rate which also tends to vary with various water qualities (Carter et al. 2012). Thus, it could be preferable to apply medium pressure UV systems to account for varying water qualities a ship encounters at different moments and locations.

4.2. Sources of false-positive and -negative results

Leaking filters might produce false-negative results. However, the five-fold replicates should ensure the detection of such events. Risks of damaged filters are relatively small, since the filters used in the ATP assay are contained in sturdy plastic capsules and are intended for single use only. As mentioned earlier, the risk of filter

damage or leakage is considerable using the FDA method. Moreover, similar risks for false-positive and -negative results are present when using the FDA method. Using DCMU however, no risk of bacteria induced false-positives are present, since DCMU specifically target PSII efficiency which is exclusively present in phototrophic organisms. On the other hand, DCMU can lead to false-positive results when the phytoplankton present in a sample comprises mainly of <10 µm cells since no separation between large and small cells was made beforehand. In addition, false-negative results are also possible when using DCMU, since the absence PSII activity does not guarantee phytoplankton's loss of regrowth potential. Moreover, even when phytoplankton is totally absent, micro-zooplankton might still be present in the ballast water, undetected, leading to false-negative results.

4.3. Validation recommendations

Despite a first attempt to calculate pass/fail levels for the ATP assay, several factors could pose additional challenges. During the growth cycle of phytoplankton, cellular ATP concentrations may vary (Holm-Hansen 1970). During the exponential growth phase, ATP levels are expected to be elevated compared to phases where cells are no longer dividing e.g. under nutrient limited conditions. Also, different species can exhibit different ATP levels depending on size and species-related metabolic states. However, a decrease of ATP during 5 day dark incubations was not observed in full-scale tests (Figure 4a). So, it is recommended to measure the ATP levels of a wide variety of 10-50 µm organisms in various stages of their growth cycle between 5 and 50 cells mL^{-1} to obtain an expected ATP level of D-2 compliant ballast water. In addition, to corroborate the excellent separation capacity of the filtration method in *T. rotula* and *M. pusilla* culture mixes, species of more similar cell sizes could be tested as well. ATP measurements should be carried out alongside full-scale land-based and shipboard trials of various BWMS techniques to examine the typical ATP concentration of D-2 compliant test water. It can be expected that chlorine-treated ballast water contains different ATP concentrations than UV-treated ballast water due to inherently different disinfection mechanisms. It is recommended that the ATP assay is tested using a representative number of available ballast water treatment techniques to investigate expected differences in typical ATP concentrations of D-2 compliant discharge water.

When ballast water with a high sediment load is taken up, bacteria adhered to the surface of sediment particles could end up in the concentrate and interfere with the ATP analysis, leading to false-positive results (First and Drake 2013). Sediment interference is only expected after short voyages since the larger particles will quickly sink out to the bottom of ballast tanks and typically will remain in the tank during ballast water

discharge. It is recommended to investigate the effect of high sediment loads with and without bacteria on the performance of the ATP assay.

4.4. Comparison with previous ballast water-ATP studies

Quantifying ATP to estimate living biomass after ballast water treatment has been attempted before. In all studies a pre-filtration procedure was performed using 10 µm or 0.2-0.7 µm filters to differentiate between microbial and >10 µm organisms. In congruence with the current findings, all studies reported a strong (-90% to -99%) decline in ATP content after ballast water disinfection using full-scale systems applying peracetic acid, peroxide and electro-chlorination (de Lafontaine et al. 2008; Welschmeyer and Davidson 2011). A delay in ATP degradation was observed in a laboratory study using UV radiation (First and Drake 2013), which was also observed in the current UV-based laboratory study. The delay was most likely caused by the delayed cell death caused by UV disinfection. Cells do not die right after treatment, but DNA damage inflicted by the radiation eventually leads to cell death. However, in the current research, the full-scale UV-based treatment test, ATP levels had strongly declined, leading to the suspicion that differences between low pressure and medium pressure UV systems could be of more significance than earlier expected.

4.5. Usability and time

The DCMU-based method was the most easy to use since the procedure involved very little equipment and sample handling which ensures an analysis time of <5 minutes. In stark contrast, the FDA-based method required at least 40 minutes to acquire a single measurement. During field tests, triplicates usually took one hour to obtain, since incubations could be run in parallel. Clogging of filters was a common issue with the FDA method, due to the large volume required to filter (200 mL) relative to the filter diameter (25 mm). The provided manifold required manual replacement of individual filters from the manifold, creating many opportunities for contamination and damaging of the filter before and after the filtration process.

Concerning the ATP assay, the concentration procedure to remove dissolved ATP and <10 µm organisms from the sample proved straightforward and easy to use. Syringes and filters were provided in sealed packages which proved clean due to consistently low blank measurements. It is of importance that a blank measurement is made using only ATP-free elution water to ensure cleanliness of the procedure. Contamination is unlikely if the operator uses a clean beaker to acquire the sample and any contact with the sample is limited to the syringes and filters. Variation among measurements can be considerable though, so it is advisable that at least five replicates are made for each ballast water sample. All equipment needed to use the ATP compliance tool can be transported in a lightweight briefcase. Setting up the equipment and carrying out the concentration

and analysis steps is done in a matter of minutes. In practice, the most time-consuming aspect of the procedure most likely will be the proper collection of a ballast water sample.

5. Conclusions

- The concentration procedure solved three problems: Interference of high salinity. Interference of dissolved ATP and <10 µm organisms. The detection limit was sufficiently decreased.
- Reagents for ATP analysis should be kept at room temperature.
- ATP and DCMU results correlate well with living *T. rotula* cells (R^2 = 0.73 and 0.87, respectively) but fail to predict viability.
- ATP and DCMU analysis exhibited reasonable correlations with 10-50 µm cells mL^{-1} (R^2 = 0.64 and 0.73, respectively).
- FDA analysis was considered too time-consuming (>40 minutes per analysis) to be an effective compliance method.
- When assessing ballast water for D-2 compliance, the estimated pass level of the ATP assay using concentration method 5 is ~50 RLU and the estimated fail level is ~6,000 RLU.
- Additional lab- and field-tests, incorporating phytoplankton monocultures, high sediment load and different treatment methods, are required to validate the ATP assay.

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Table 1
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Table 1 Overview of all experiments conducted. The number of independent experiments is denoted as 'n'. During each experiment, multiple replicated were analysed. The null-hypothesis describes the result if no effect was found

Experiment	n	Null-hypothesis (H_0)
The influence of hypochlorite on ATP detection.	1	Hypochlorite up to 10 mg L^{-1} does not influence the light output of the 3M Clean Trace™ ATP assay[a] using the BDK[b].
The relationship between the ATP concentration and the resulting RLU signal.	1	There is no linear correlation between the ATP concentration and light produced during ATP analysis using the BDK.
The influence of salinity on ATP detection at 4°C, 15°C and 26°C.	1	1. Salts have no effect on the light production of the ATP assay using the BDK. 2. Temperatures of 4°C, 15°C and 26°C have no relative effect on the light production of the ATP assay using the BDK.
UV-C treatment of *T. rotula.*	1	1. A dose of 139 mJ cm^{-2} UV-C (254 nm) has no effect on the viability of *T. rotula* cells. 2. The effect of UV-C treatment on *T. rotula* cannot be effectively monitored using: a. Flow cytometry b. Variable fluorescence c. FDA analysis d. ATP analysis 3. Data resulting from flow cytometry, variable fluorescence, FDA analysis and ATP analysis are not correlated.
Test compliance kits during IMO G8 land-based testing.	6[c]/10[d]	Organism concentrations derived from flow cytometry and microscopy (the official land based test data) cannot be correlated with the indicative compliance tools: a. DCMU b. FDA c. ATP
Detection limit of ATP analysis using CM3.	1	1. ATP analysis using the ATP assay with either the ATP swabs or the BDK following CM3 is not linearly correlated with the concentration of *T. rotula*. 2. ATP analysis using either the ATP swabs or the BDK following CM3 is not able to detect <10 *T. rotula* cells mL^{-1}.
Improving the concentration efficiency and salinity reduction of the CM.	1	1. Flushing 5 mL milli-Q™ back and forth five times instead of one flush does not improve the collection of particles from the concentration filter. 2. Replacing the salt-contaminated 50 mL syringe with a sterile 5 mL syringe when back flushing, does not improve the removal of salts in the concentrate.
Comparing the precision of CM3 and CM5.	1	Changes to the back flush procedure do not lead to less variation among replicate measurements of natural seawater.
Detection limit of ATP analysis using CM5.	1	1. ATP analysis using the ATP swabs following CM5 is not linearly correlated with the concentration of *T. rotula*. 2. The ATP assay using the ATP swabs following CM5 is not able to detect <10 *T. rotula* cells mL^{-1}.

[a]All ATP analyses were performed using the 3M Clean Trace™ ATP assay. [b]BDK: Biomass Detection Kit. [c]Control tanks. [d]Treated tanks

Table 2
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Table 2 Overview of the development process of the concentration method, compared with the FDA- and DCMU-based methods

	Concentration Method (CM)						
Feature	CM1	CM2	CM3	CM4	CM5	FDA	DCMU
Sample volume (mL)	200	100	100	100	50	200	3
Extractant volume (mL)	2	5	5	5	5	2	
Concentration factor	100x	20x	20x	20x	10x	100x	
Salinity reduction factor	nd[b]	nd	17x	nd	33x	nd	
Concentration efficiency	nd	nd	63%	85%	85%[c]	nd	
Detection limit (cells mL^{-1}; average ± CI)[a]	nd	nd	>50	nd	2.5 ± 0.5	nd	nd
Time required (minutes)	~5	~3	~3	~3	~3	~40	~5
Usability at dock	-	-	+	+	+	-	++
10 µm pore size / 25 mm Ø nylon screen filter	X	X	X	X	X	X	
Beaker-flask-cuvette filtration manifold	X					X	
Syringe filtration system		X	X	X	X		
Reusable stainless steel syringe filter capsule		X					
Disposable polypropylene filter capsule			X	X	X		
Pipettes and tweezers needed	X					X	
Five times back flush			X	X	X		

[a]Using ATP swabs. [b]not determined. [c]derived from CM4

Captions

Fig. 1 (a) ATP standard dilutions analyzed in triplicate with the biomass detection kit at 4°C, 15°C and 26°C. (b) ATP standard (6 ng mL^{-1}) analyzed in triplicate with the biomass detection kit at 4°C, 15°C and 26°C. (c) ATP standard (6 ng mL^{-1}) analyzed with the biomass detection kit. Error bars depict the 95% confidence interval

Fig. 2 *Thalassiosira rotula* cells analyzed with (a) Flow cytometry and SYTOX® Green. Living cells were not fluorescent after SYTOX® Green staining. (b) ATP assay using concentration method 2 and the biomass detection kit. (c) FDA and (d) PAM fluorometry. The black and white bars between the graphs indicate the dark (black) an illuminated (white) periods during the incubation. Error bars depict the 95% confidence interval of triplicate measurements

Fig. 3 Correlation plots comparing living *Thalassiosira rotula* cells to (a) ATP analysis using concentration method 2 and the biomass detection kit. (b) FDA and (c) PAM fluorometry. Error bars depict the 95% confidence interval of triplicate measurements

Fig. 4 Three compliance tools used during the testing of a full-scale UV-based ballast water management system. (a) ATP analysis using concentration method 1 and ATP swabs, (b) FDA and (c) DCMU. Values represent the average of all tests carried out. Control: n=6. Treated: n=10. Error bars depict the 95% confidence interval

Fig. 5 Correlation plots between the official IMO G8 test results and CME techniques. 10-50µm organism concentrations are based on phytoplankton and micro-zooplankton enumerations, obtained from the Cathelco test report (Peperzak 2013). Relative Luminescence Units (RLU) depict the results of the ATP assay using concentration method 1 and ATP swabs. F_v/F_{DCMU} indicates the PSII efficiency estimation based on DCMU. F_v/F_m indicates the PSII determination based on PAM fluorometry

Fig. 6 (a) Precision test comparing concentration method 3 and 5 using ATP swabs and natural seawater. RLU: relative luminescence units. (b) RLU: relative luminesce units resulting from concentration method 5 using ATP swabs. Closed circles indicate results of only *T. rotula* cells. Open circles represent solutions containing *T. rotula* and *M. pusilla* in a 1:1000 ratio. Open circles were moved to the right by 0.8 cells mL^{-1} to enhance visibility. Error bars depict the 95% confidence interval

Figure 1
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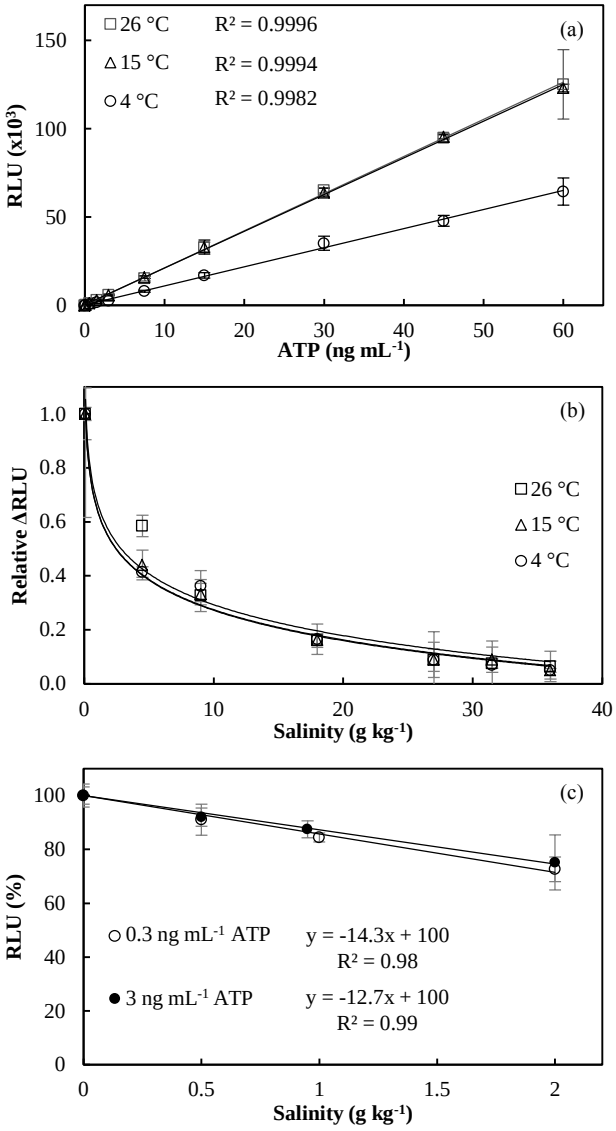

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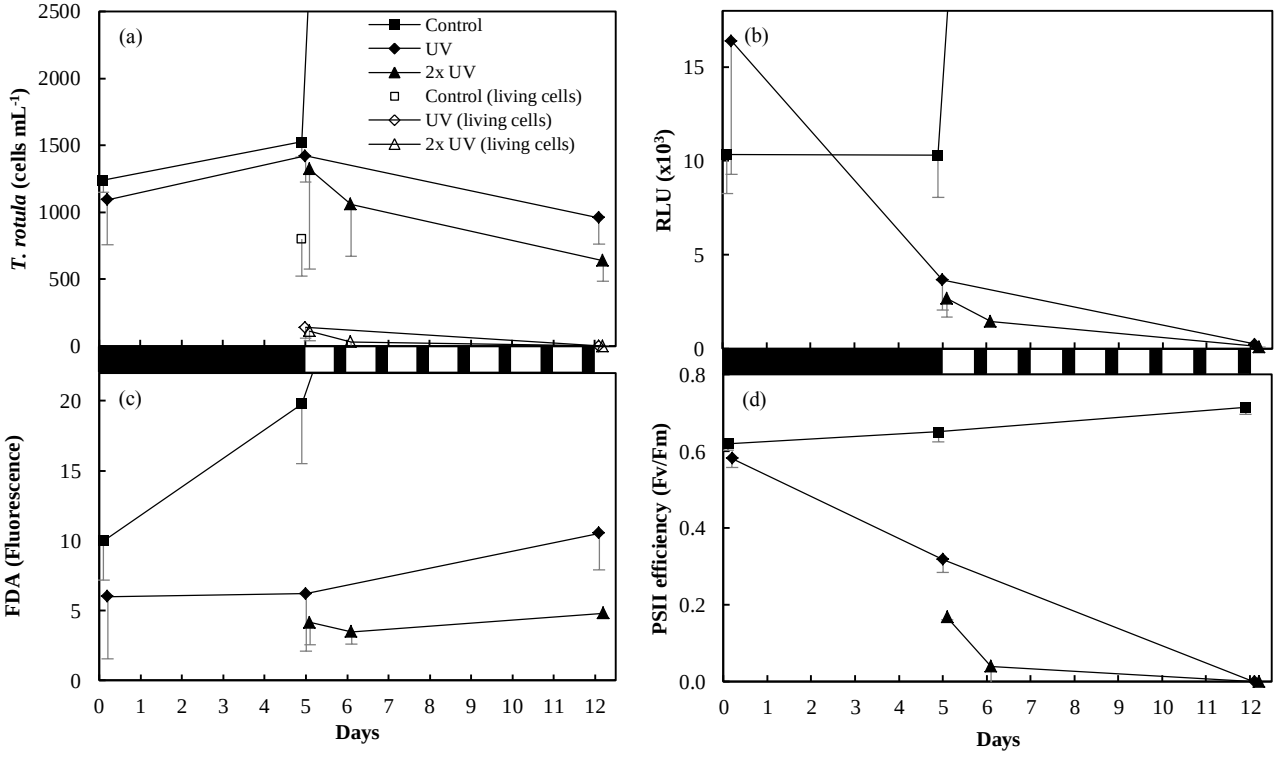

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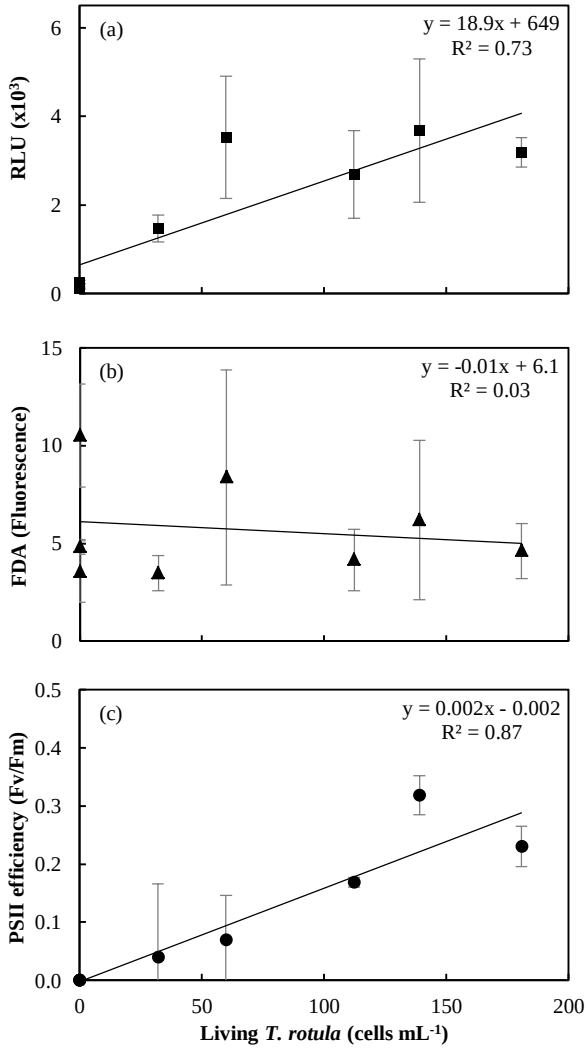

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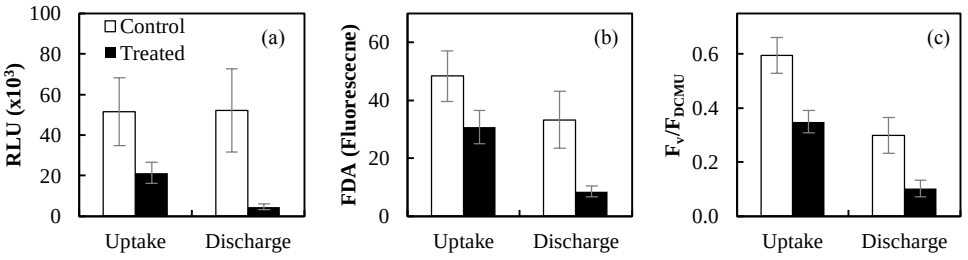

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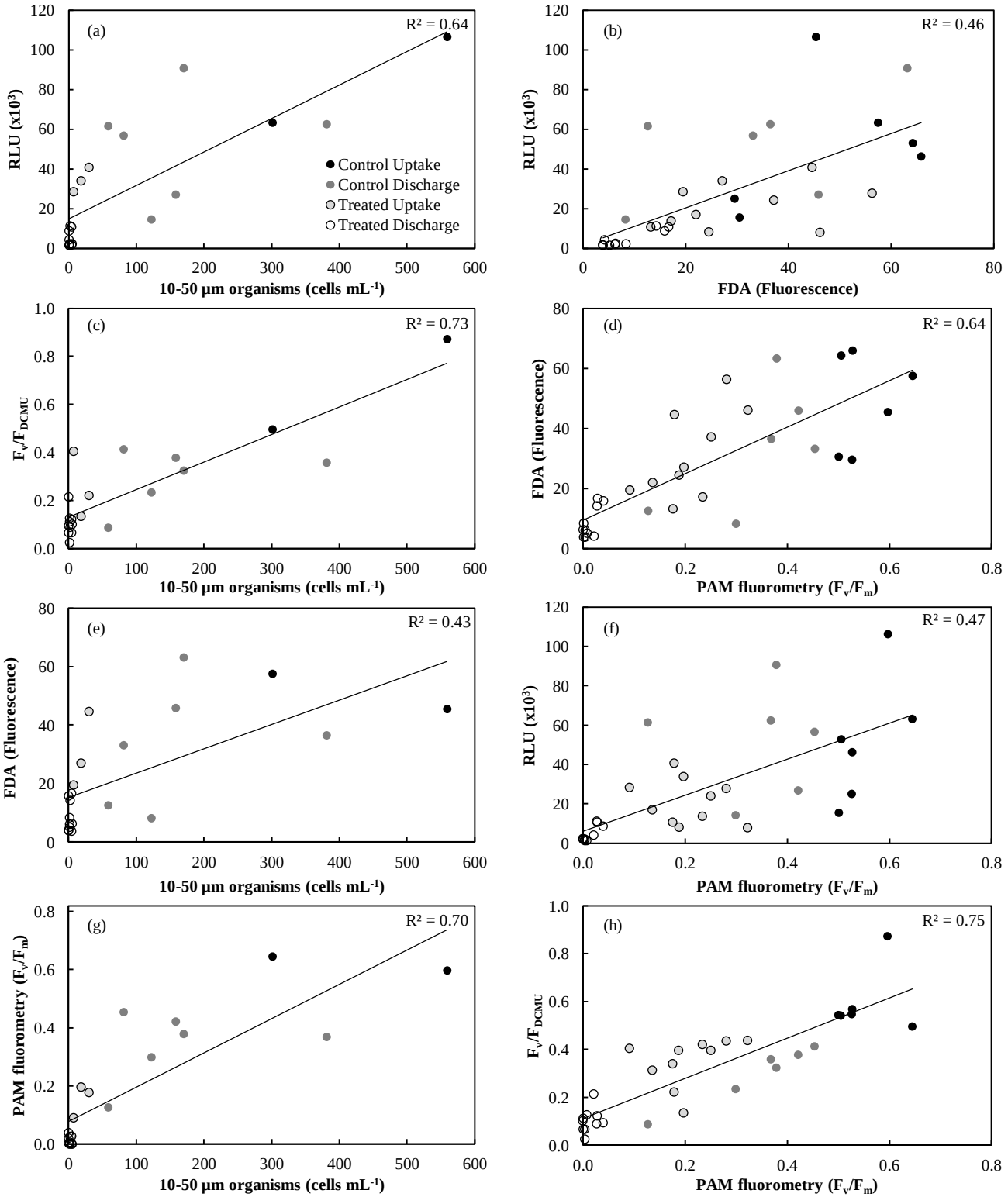

Figure 6

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