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Development of an ATP assay for rapid onboard testing to detect living microorganisms in ballast water

Bonggil Hyun [a], Hyung-Gon Cha [a], Nayoung Lee [b], Seungshic Yum [b], Seung Ho Baek [b], Kyoungsoon Shin [a,*]

[a] Ballast Water Research Center, Korea Institute of Ocean Science & Technology, Geoje 53201, Republic of Korea
[b] South Sea Environment Research Center, Korea Institute of Ocean Science & Technology, Geoje 53201, Republic of Korea

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ABSTRACT

Ballast water is a principal pathway for the introduction of pathogens and non-indigenous species to ports worldwide. The International Maritime Organization (IMO) and the United States Coast Guard (USCG) have adopted ballast water management regulations that require, e.g., the installation of shipboard ballast water management systems (BWMS). Rapid and simple analytical methods are needed to monitor whether ballast water disinfection ensures compliance with the discharge standards. In this study laboratory and full scale land-based testing was used to investigate the suitability of an adenosine triphosphate (ATP) assay for quantifying living organisms (≥10 and <50 μm minimum dimension) in ballast water. In laboratory experiments the ATP assay was highly sensitive, with a detection limit of <5 cells 0.1 mL^{-1}. Diatom species (*Chaetoceros simplex* and *Skeletonema costatum*) had low ATP concentrations compared with dinoflagellate, Raphidophyceae, and Chrysophyceae species. This was because of differences in cell volume, as the ATP concentration increased exponentially with increasing cell volume. Using a regression model between ATP concentration and cell volume, an estimated the pass and fail ATP concentration in this study (788–98,610 pg mL^{-1}) was developed for the discharge of ballast water. In land-based testing the ATP assay also showed a good correlation with the presence of living natural plankton cells in control samples, but the ATP concentration (137 pg mL^{-1}) was much lower than the ATP guideline. The low ATP concentration in natural plankton cells may reflect a decline in their biological activity because of extended exposure to dark conditions. Although our results need further validation, the ATP assay is a suitable tool for monitoring compliance of ballast water treatment.

1. Introduction

Ballast water is essential for safe and efficient modern shipping operations, and approximately 3.5 gigatons of ballast water are discharged annually worldwide (e.g., Roy et al., 2012). Ballast water contains organisms ranging from viruses to large plankton, some of which may be non-native and become nuisances in receiving waters, causing extensive ecological and economic damage (Cha et al., 2015). To prevent the dispersal of harmful aquatic organisms and pathogens (HAOP) via ballast water, the International Maritime Organization (IMO) and United States Coast Guard (USCG) have enacted new regulations that limit the number of living organisms that are allowed to be discharged in ballast water (IMO, 2004; van Slooten et al., 2015). The discharge standards for living plankton organisms in ballast water are based on size, and for ≥50 μm organisms to <10 individuals per m^3 and the discharge of living ≥10 and <50 μm (hereafter 10–50 μm organisms) organisms to <10 individuals per mL (IMO D-2, USCG Phase-I). The discharge standard regulation will enter into force on September 8, 2017.

To achieve the above discharge standard, technology developers and manufactures have developed various ballast water management systems (BWMSs). The treatment options for ballast water include physical (filtration, cavitation, sonication, heating: Cangelosi et al., 2007), chemical (applied or generated biocides including chlorine and peroxide: Gregg and Hallegraeff, 2007; Kuzirian et al., 2001), and biological (microbial deoxygenation: McCollin et al., 2007) processes. Most commercial systems involve two or more stages of treatment, with a physical process stage being followed by a disinfection stage.

Once the IMO and the USCG regulations enter into force, ships may be subject to inspection by Port State Control (PSC) inspectors to ensure they comply with the D-2 standard. Although verification of the biological efficiency of BWMS in relation to the D-2 standard is complex, testing must be conducted rapidly so as not to impede commerce (IMO, 2013). As the IMO recognized the necessity for rapid and reliable methods to analyze ballast water, it established the G2 guidelines for a

* Corresponding author.
E-mail address: ksshin@kiost.ac.kr (K. Shin).

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rapid screening method (indicative analysis method) to identify ships potentially in violation of the D-2 standard (IMO, 2013). An indicative analysis is a compliance test that is a relatively quick indirect or direct measurement of a representative sample of the ballast water at discharge (IMO, 2013). It may be undertaken as an initial step to establish whether a ship is potentially not in compliance with the D-2 standard, prior to detailed analysis (Gollasch et al., 2015).

Several analytical methods for indicative ballast water sample analysis have been developed (Briski et al., 2011, 2013; Welschmeyer and Maurer, 2011; Zetsche and Meysman, 2012; Drake et al., 2014; Gollasch et al., 2015; Wright et al., 2015), but no widely accepted technique to quickly evaluate ballast water for compliance with the discharge standard is in use. As the USCG will enact the more stringent regulation than IMO D2 standard, a highly sensitive and reliable indicative method is need. However, for ≥50 μm organism (mainly zooplankton) no reliable indicative sample analysis method was identified, since sampling cubic meters of seawater would be too time-consuming and require specialized equipment to collect and concentrate organisms (Gollasch et al., 2015; First et al., 2016; van Slooten et al., 2015). Further, no appropriate method and instrument was identified to distinguish between living and dead of ≥50 μm organisms (Gollasch et al., 2015). The development of the adenosine triphosphate (ATP) assay presented here solely focused on the 10–50 μm organism.

ATP is a coenzyme that is the main energy carrier molecule in the cells (prokaryotic and eukaryotic) of all organisms (Gollasch et al., 2012). Consequently, quantifying the amount of ATP present can indicate the number of living single-celled organisms, because ATP indicates the presence of metabolically active cells. In addition, ATP analyses can be completed in <1 h, and the equipment needed is simple to operate. Therefore, an ATP bioluminescence-based method has recently been recommended as a prospective tool for vessel onboard indicative analysis (First et al., 2016; Maurer, 2013; van Slooten et al., 2015).

In this study we evaluated a ATP bioluminescence-based method as a potential tool for detection of 10–50 μm organisms in indicative ballast water analysis for assessing compliance with the IMO and USCG discharge standards. However, because this method involves bulk measurements similar to that for detecting living algal cells using PAM fluorometry, it is also necessary to qualify the ATP values to enable them to be directly translated into organism numbers. Therefore, the ATP assay involved three steps. Firstly, we evaluated the ATP concentration for cell numbers <10 cells, using a laboratory dilution technique. Secondly, we measured the cell volume to calculate the relative ATP/cell volume ratio. Thirdly, we conducted the full-scale (flow rate: 250 $m^3\ h^{-1}$, Test water tank capacity: 400 m^3) BWMS tests using a natural plankton community to verify that the method was appropriate for determining live/dead phytoplankton. The ATP concentrations were assessed in parallel with epifluorescence microscopy. Finally, based on our findings we estimated ATP thresholds for measurement of compliance with the D-2 discharge standard.

2. Material and methods

2.1. Experiment design

2.1.1. ATP assay

In this study the ATP concentration was measured using a Glomax Microplate Luminometer in combination with the CellTiter-Glo 2.0 Assay (Promega, Madison, USA). The ATP analysis was conducted in three discrete steps: (1) sample concentration, (2) ATP extraction, and (3) detection using a luminometer. Cells in a water sample (50 mL) were concentrated by centrifugation (3000 rpm, 10 min; Hanil Scientific Inc., Gimpo, Korea). The supernatant was carefully removed using a 1-mL pipette. The pellet, containing any cells present in the sample, was suspended in 1 mL of phosphate-buffered saline (PBS), and subsamples (100 μL) were dispensed into the wells of a 96-well cell culture plate (white flat bottom; Thermo Fisher Scientific, Roskilde, Denmark). Finally, 100 μL of CellTiter-Glo Assay 2.0 was added to each subsample in the culture plate and mixed thoroughly for 2 min in the dark to induce cell lysis. Allow the plate to incubate at room temperature for 10 min to stabilize the luminescent signal. The resulting luminescence was immediately determined using a luminometer, and measurements were recorded as relative luminescence units (RLU). All RLU values were converted to ATP concentration using a calibration curve ($R^2 = 1.0$, $y_{ATP\ concentration\ (pg\ mL^{-1})} = 0.1571X_{RLU\ value} + 814.45$) obtained after dilution of the standard solution (rATP, 10 mM, Cat.#P1132, Promega, USA) to a certain gradient.

2.1.2. Laboratory experiment

To determine the ATP concentration as a function of species, and cell concentration and volume, we obtained nine phytoplankton species from the Library of Marine Science, Korea Institute of Ocean Science and Technology (KIOST), Republic of Korea. These included two diatoms (*Chaetoceros simplex*, *Skeletonema costatum*), five dinoflagellates (*Alexandrium tamarense*, *Heterocapsa circularisquama*, *H. triquetra*, *Prorocentrum minimum*, *Scrippsiella trochoidea*), one Raphidophyceae (*Chattonella marina*), and one Chrysophyceae (*Tetraselmis suecica*). Of these, *H. circularisquama* strain was kindly provided by the National Research Institute of Fisheries and Environment of Inland Sea, Japan. Cultures of the phytoplankton were maintained in 70-mL culture tubes (Corning Inc., New York, USA) containing f/2 medium (Guillard and Ryther, 1962) under a light:dark cycle of 14:10 h at 22.8 °C. All materials in contact with the phytoplankton were sterilized prior to use to minimize the possibility of contamination during testing. To

Table 1
Geometric shapes and equations for the calculation of biovolume.

Species	Simulated shape	Volume (V)
Tetraselmis suecica	Prolate spheroid	$V = \frac{\pi}{6} \cdot b^2 \cdot a$
Alexandrium tamarense *Prorocentrum minimum*	Ellipsoid	$V = \frac{\pi}{6} \cdot a \cdot b \cdot c$
Skeletonema costatum	Cylinder + 2 half spheres	$V = \pi \cdot b^2 \cdot (\frac{a}{4} - \frac{b}{12})$
Heterocapsa triquetra	Double cone	$V = \frac{\pi}{12} \cdot a \cdot b^2$
Chattonella marina *Heterocapsa circularisquama* *Scrippsiella trochoidea*	Cone + half sphere	$V = \frac{\pi}{4} \cdot a \cdot b^2$
Chaetoceros simplex	Prism on elliptic base girdle view	$V = \frac{\pi}{4} \cdot a \cdot b \cdot c$

verify the relationship between ATP concentration and cell number for each species, a 10-mL culture sample was diluted with sterilized filtered seawater (0.2 μm) to approximately 100–150 cells mL^{-1} in a 15 mL conical tube (SPL Life Sciences, Pocheon, Korea). This was used to prepare a dilution series (2%, 4%, 6%, 12%, 25%, 50%, 100%; six replicates per dilution) using 0.2 μm filtered and sterilized seawater as the diluent. Three replicates of each dilution were used to determine the ATP concentration, while the other three were used for determination of cell number and volume. Enumeration of living cells was performed for each dilution using a Sedgwick Rafter chamber to count bright green fluorescent cells (Axioplan II Epifluorescence Microscope, Carl Zeiss, Germany) staining with fluorescein diacetate (FDA)/chloromethylfluorescein diacetate (CMFDA) (Steinberg et al., 2011). The volumes of 20 cells per species were also measured using a light microscope (Axioplan II, Carl Zeiss, Germany) equipped with an imaging analysis system. The cell length and width were measured individually according to their unique geometric shapes, and the cell volume was calculated based on the method described by Sun and Liu (2003). The characteristics of each species are shown in Table 1.

2.1.3. Full scale land-based test

In winter–spring 2016 two land-based tests, one involving brackish water (salinity approximately 19 psu) and other involving seawater (salinity approximately 30 psu), were conducted in a barge in Masan Bay, Republic of Korea. Seawater for the tests was drawn by pumping from the surface of Masan Bay into a 400 m^3 test water tank in the barge. For the brackish water tests, the bay water was diluted with tap water that had been aerated overnight to remove any residual Total Residual Oxidant (TRO). The BWMS (250 $m^3\ h^{-1}$) in the barge involved three treatment steps: (i) filtration (50 μm stainless steel element); (ii) UV + TiO_2 (200–400 nm two medium-pressure lamp); and (iii) electrolysis (maximum TRO < 15 ppm). At uptake the water, including various organisms, was filtered using the filtration system, processed with UV + TiO_2 and electrolysis, and the treated water was transferred into a 200 m^3 tank (Fig. 1). After 5 days the water was re-treated using UV + TiO_2, then discharged following neutralization if the TRO concentration was <0.2 mg L^{-1}. Control water was pumped into the control tank and discharged after 5 days without treatment. Abiotic water parameters (temperature, salinity, and dissolved oxygen) were measured directly in each sample using a data sonde (YSI 6600; Yellow Springs Instrument, Yellow Springs, OH, USA). To determine live and dead phytoplankton and their composition, a 1 L water sample was collected at three time points during the test (Begin: 75%, Middle: 50%, End: 25%). A treated water sample was taken continuously to reach >100 L sample volume and was concentrated using a 7–8 μm (diagonal size) mesh size plankton net during each test cycle. The community compositions of 10–50 μm organisms and the number of each species in both the control and treated samples were determined using an epifluorescence

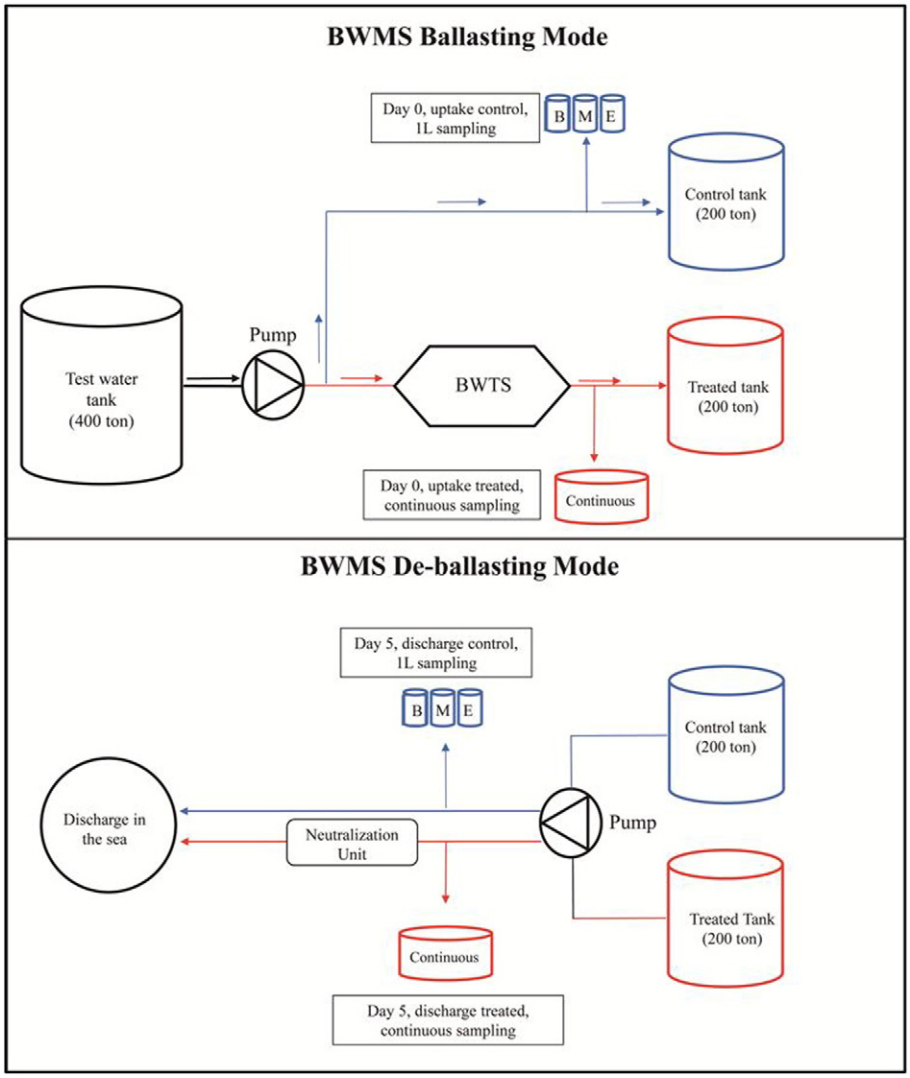

Fig. 1. Ballast water management system (BWMS) schematic diagram, showing ballasting and de-ballasting modes.

microscope (Axioplan II; Zeiss, Jena, Germany) at an appropriate magnification (Rines and Hargraves, 1988; Shim, 1994; Tomas, 1997), following staining with FDA/CMFDA, as described by Steinberg et al. (2011). Analyses for living organisms and the ATP concentration were carried out to determine the relationship between these parameters. The number of living organisms in each sample was determined in triplicate, and the ATP concentration was measured in triplicate or duplicate.

2.2. Data analysis

The results are presented as the means and standard deviations of the raw data. All datasets had normal distributions and homogeneous variances. The confidence level for statistical tests and CIs was 95% ($\alpha = 0.05$). The significance of differences in the ATP concentration between non-treated and treated samples in the land-based test was assessed using the *t*-test. The relationship between living cell numbers and ATP concentration were fitted using simple linear regressions.

3. Results and discussion

3.1. Linearity between cultured phytoplankton species and the ATP value

To prepare for the more stringent USCG Phase II standard, the low detection limits of instruments used for compliance monitoring of ballast water is crucial. Many previous studies have suggested that further validation experiments will be needed to confirm the accuracy of bulk measurement instruments under lower cell concentration conditions (e.g., Gollasch et al., 2015). In this study the ATP assay was conducted using samples having low cell concentrations, and ATP concentrations were detected at concentrations <5 cells 0.1 mL^{-1} for all species studied (Fig. 2).

For each species the ATP concentration was plotted against the cell concentration to investigate the relationship between these parameters (Fig. 2). Significant linear relationships were found for all species ($R^2 > 0.90$; $P < 0.05$), which is a finding consistent with those of van Slooten et al. (2015), who reported a correlation ($R^2 = 0.73$) between the concentration cells of the diatom *Thalassiosira rotula* and the RLU signal. However, in accordance with the IMO G2 guidelines, the indicative analysis may be undertaken to establish whether a ship is potentially in compliance with the IMO D-2 discharge standard. Therefore, bulk measurement analyses including ATP and PAM fluorometry must be reported in terms of the concentration of living cells in a defined size range. In the present study the ATP concentration for each species varied widely, ranging from 846 pg mL^{-1} for *C. simplex* to 12,454 pg mL^{-1} for *C. marina*. The diatom species (*C. simplex* and *S. costatum*) showed the lowest ATP concentration relative to other phytoplankton species (Fig. 2a,b). The finding that different species exhibit different ATP concentration is consistent with the results of Maurer (2013), who found that the ATP concentration of 16 phytoplankton species varied and was dependent on cell size, which ranged from 134 μm^3 for *Haematococcus* sp. to 792,253 μm^3 for *Coscinodiscus* sp.

The relationship between the ATP concentration and cell volume for 10 living cells was investigated by measuring the ATP concentration/cell volume ratio for all species studied; the cell volumes varied from 142 μm^3 for *S. costatum* to 20,525 μm^3 for *A. tamarense*. There was an increase in the ATP concentration with increasing cell volume ($R^2 = 0.99$), but the trend was exponential rather than linear (Fig. 3a). For example, the small volume (142–7,636 μm^3) species *C. simplex*, *S. costatum*,

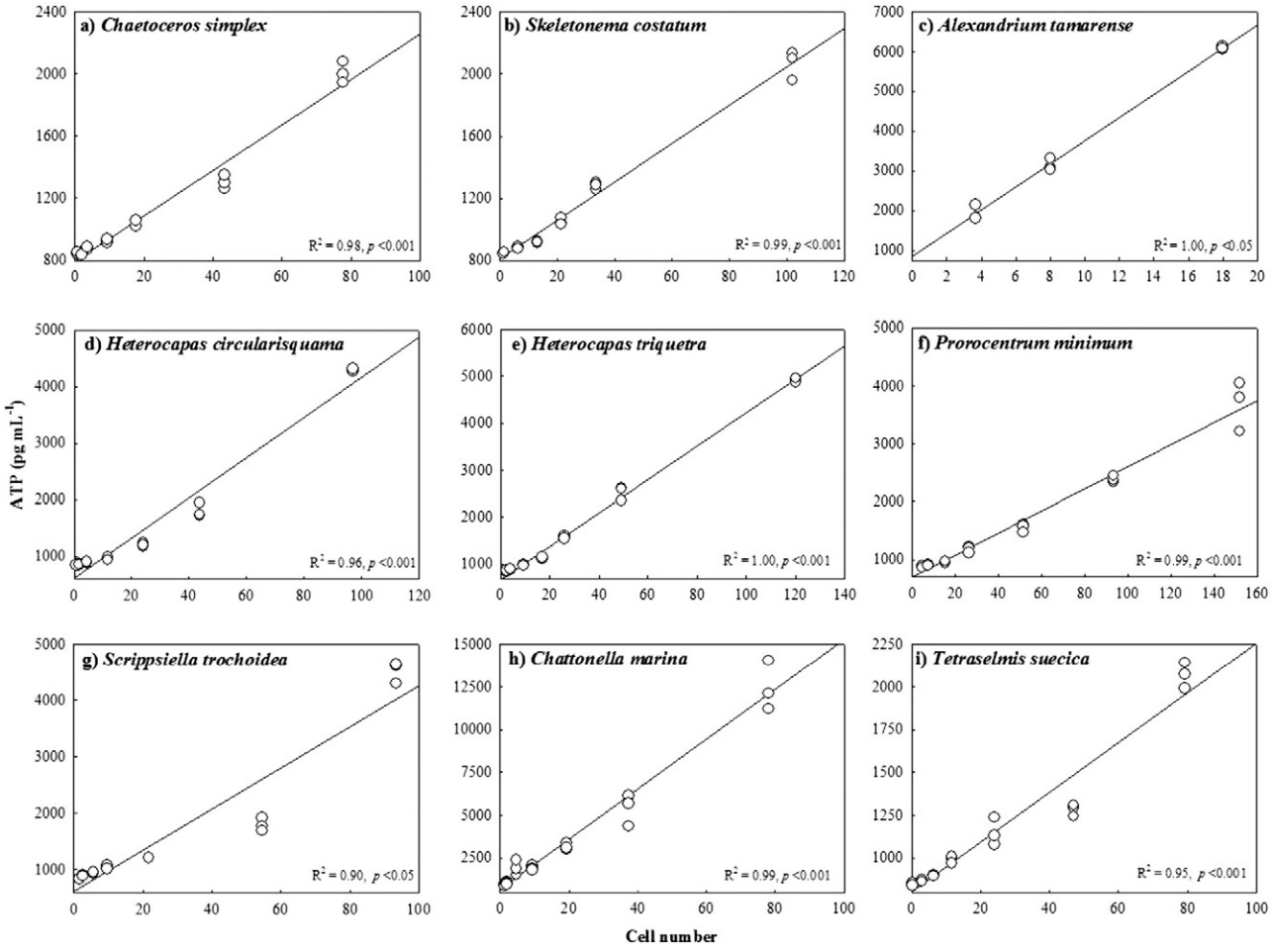

Fig. 2. Relationship between the ATP concentration and the cell concentration for each cultured species.

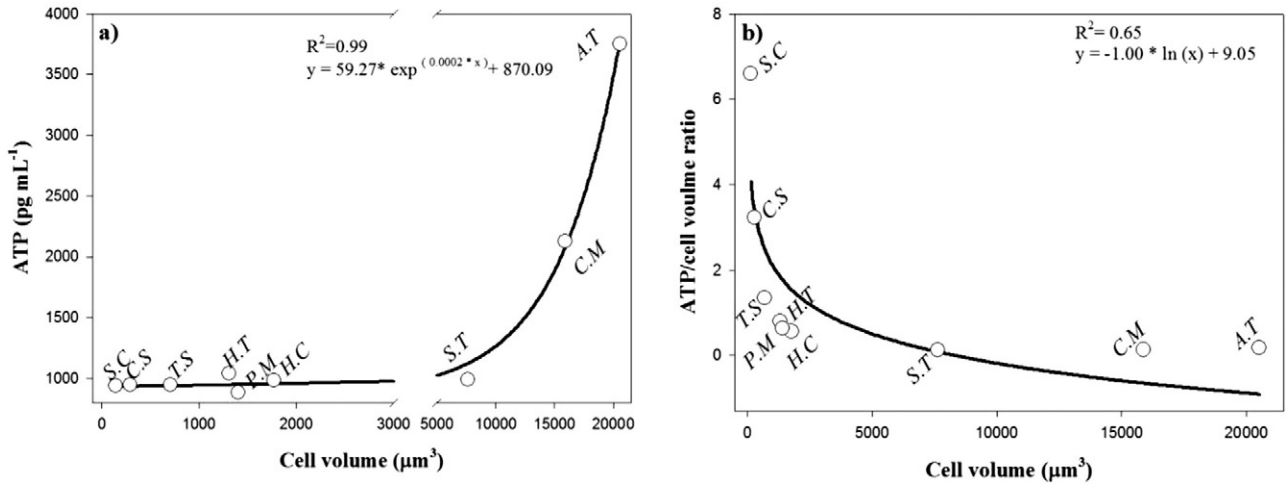

Fig. 3. Relationships between (a) the ATP concentration and cell volume, and (b) the ATP concentration/cell volume ratio and cell volume for each species when 10 living cells exist.

H. circularisquama, *H. triquetra*, *P. minimum*, *S. trochoidea*, and *T. suecica* yielded low ATP concentration (883–1,041 pg mL^{-1}), but the largest cells (*A. tamarense*: 20,525 μm^3; *C. marina*: 15,887 μm^3) exhibited >2-fold higher ATP concentration (*A. tamarense*: 3,752 pg mL^{-1}; *C. marina*: 2,126 pg mL^{-1}) (Fig. 3a). No distinctive difference of ATP concentration between the small-sized species with increasing cell volume might be influenced by other parameters, since the difference of cell volume between the small phytoplankton species is not relatively large. According to the previous studies, cellular ATP concentration is also affected by various parameters e.g., nutrient, photo period, growth cycle, species-related metabolic states (e.g., Holm-Hansen, 1970; van Slooten et al., 2015). Among various parameters, therefore, species-specific metabolic rate might be influenced the cellular ATP concentration as well as organism size. In contrast, the ATP concentration/volume ratio for individual species decreased logarithmically with increasing cell volume (Fig. 3b; $R^2 = 0.65$). This trend could in part be because large phytoplankton species have more vacuolar space than do small cells (Becker, 2007; Maurer, 2013). This vacuolar space contributes to cell volume but not to cellular carbon (Strathmann, 1966), and is likely to contain less ATP than other areas of the cell (Verity and Robertson, 1992). It appears that larger cells have more open intracellular space and less ATP per unit cell volume, resulting in the trend of decreasing ATP concentration/ cell volume with increasing cell volume.

The IMO convention sets discharge limits on densities of live organisms by size class, and specifies that international vessels in foreign ports can discharge <10 individual 10–50 μm organisms (IMO, 2008). The relationship between the ATP concentration and the cell volume enables prediction of the concentration of living 10–50 μm organisms. Maurer (2013) and van Slooten et al. (2015) also used cell volume ATP concentrations as a measure of cell concentrations. To estimate the living cell concentration we assumed that the ATP concentration changed only with organism size. Using a non-linear regression model [$y_{ATP} = 59.27 \times \exp.(0.0002X_{cell\ volume}) + 870.09$] (Fig. 3a), the ATP concentration were converted to living cell number. The ATP concentration calculated for 10 living cells were 933 for *C. simplex*, 931 for *S. costatum*, 4,464 for *A. tamarense*, 954 for *H. circularisquama*, 947 for *H. triquetra*, 948 for *P. minimum*, 1,143 for *S. trochoidea*, 2,292 for *C. marina*, and 938 for *T. suecica*. The lower and higher ATP values for 10 living cells are 931 for *S. costatum* and 4,464 for *A. tamarense*, respectively. To estimate the pass and/or fail ATP concentration in this study in relation to D-2 standard, we selected the *T. suecica*. The reasons for using this species are that they are widely used for test species during the land-based test of BWMS around the world and their cell length (a: 11.97 ± 1.19 μm) and width (b: 10.56 ± 0.05 μm) is closely to 10 μm in this study. The ratio between the long and short axis of *T. suecica* is 1.13, so long axis diameter in *T. suecica* was 11.33 μm for cells having a short axis diameter of 10 μm, and 56.67 μm for cells having a short axis diameter of 50 μm. Therefore, cell volume in *T. suecica* ($\pi/6 \times b^2 \times a$) was 593 μm^3 and 74,143 μm^3 for cells having a short axis diameter of 10 and 50 μm, respectively (Table 1). Moreover, the ATP concentration/cell volume ratio for 10 living cells was 1.33 for *T. suecica*. Using this ratio and cell volume, the lower and upper ATP concentrations for 10 living cells would be (for example, 593 (74,143) × 1.33) 788 and 98,610 for cells having short length diameter of 10 μm and 50 μm, respectively. Based on this range, estimated the pass/fail ATP concentration in this study in relation to D-2 compliance would be: <788 (most compliant); 788–98,610 (close to the limit, with a need for further investigation); >98,610 (most non-compliant) (Table 5).

3.2. Application of ATP assay for full scale land-based test

To evaluate use of the ATP technique as an onboard compliance monitoring tool, full scale land-based tests were carried out using natural plankton assemblages in seawater and brackish water. Table 2 shows that the water temperature and salinity in each treatment remained relatively constant during the test period. A large diversity of organisms, including up to 9 taxa, were observed in the control uptake samples, which contained an average density of 518 ± 132 cells mL^{-1} for brackish water, and 850 ± 129 cells mL^{-1} for seawater (Tables 3 and 4).

Table 2
Changes in temperature and salinity in each treatment during land-based testing.

Land-based test									
Source water			Temperature (°C)	Salinity (psu)	Source water			Temperature (°C)	Salinity (psu)
BW[a]	Uptake	Control	4.93 ± 0.14	18.8 ± 1.25	SW*	Uptake	Control	6.20 ± 0.14	30.5 ± 2.27
		Treated	5.21 ± 0.63	18.79 ± 0.40			Treated	6.38 ± 0.34	30.6 ± 1.19
	Discharge	Control	5.13 ± 0.12	21.75 ± 1.99		Discharge	Control	6.68 ± 0.30	29.7 ± 3.08
		Treated	5.19 ± 0.16	19.64 ± 0.28			Treated	6.73 ± 0.27	27.9 ± 1.26

[a] BW: Brackish water; SW: Seawater. Values represent means ± standard deviations.

Table 3
List of living 10–50 μm organisms in the uptake control.

10–50 μm organism	Brackish water	Seawater
Bacillariophyta		
Asterionella glacialis	+	+
Chaetoceros spp.	++	++
Cylindrotheca closterium	+	+
Ditylum brightwellii	+	
Eucampia zodiacus		+
Navicular spp.		
Odontella longicruris	+	+
Pleurosigma spp.		
Pseudo-nitzschia spp.		+
Skeletonema spp.	+	+
Thalassiosira spp.	++	++
Dinoflagellata		
Alexandrium spp.		
Amphidinium spp.		
Gymnodinium spp.		
Heterocapsa spp.		+
Prorocentrum spp.		
Protoperidinium spp.		
Scrippsiella trochoidea		
Unidentified dinoflagellate	+	++
Cryptophyta		
Cryptomonas spp.	+	++
Ciliophora		
Ciliate		+
Unidentified phytoplankton (10–50 μm)		+

Notes: +: 1–100 cells mL^{-1}; ++,:101–500 cells mL^{-1}.

Following water treatment the living cell density for all groups was significantly reduced (to <1 cells mL^{-1}) compared with that in the control uptake water (Table 4). In the treated discharge samples, living cells of *Thalassiosira* spp. were found in the seawater, while both *Chaetoceros* spp. and *Thalassiosira* spp. were found in brackish water.

Consistent with the microscopy data, the greatest reduction in the ATP concentration was also observed between the control and treated samples (>97%; $P > 0.05$) (Fig. 4a and b). This result is consistent with previous studies involving full scale treatment systems applying peracetic acid, peroxide, electro-chlorination, and UV; these showed that the ATP concentration declined markedly (90–99%) following ballast water disinfection (De Lafontaine et al., 2009; Welschmeyer and Davidson, 2011; van Slooten et al., 2015). Moreover, the correlation plot revealed that the ATP concentration in control samples correlated well with the density of 10–50 μm organisms ($R^2 = 0.84$, $P < 0.001$), but this was not the case for treated samples (Fig. 5a and b). Maurer (2013) reported that the ATP concentration in culture, coastal, and bay plankton samples correlated well with the cell concentration and volume. Based on full scale land-based tests, van Slooten et al. (2015) also reported that the ATP concentration was more closely correlated with cell density than with FDA. Therefore, the absence of a correlation between the ATP concentration and the cell concentration in treated samples can be considered to indicate <1 living organism mL^{-1}.

Based on the linear regression model, the ATP concentration for 10 living cells in the control sample was estimated. Table 3 shows the diversity of organisms detected, and that large species including

Table 4
Abundance of living 10–50 μm organisms in each treatment during the test period.

Full scale land-based test							
Source water	Cell abundance (cells mL^{-1})			Source water	Cell abundance (cells mL^{-1})		
Brackish water	Uptake	Control	518 ± 132[a]	Seawater	Uptake	Control	850 ± 129
		Treated	0.24 ± 0.16			Treated	0.04 ± 0.07
	Discharge	Control	172 ± 70		Discharge	Control	1097 ± 298
		Treated	0.11 ± 0.11			Treated	0.31 ± 0.54

[a] Mean ± standard deviation.

Table 5
Estimation of pass/fail ATP concentration of 10–50 μm organism fraction at discharge ballast water in this study.

	Most likely compliant (pg mL^{-1})	Signal is close to the limit (pg mL^{-1})	Most likely non-compliant (pg mL^{-1})
10–50 μm organism	<788	788 to 98,610	>98,610

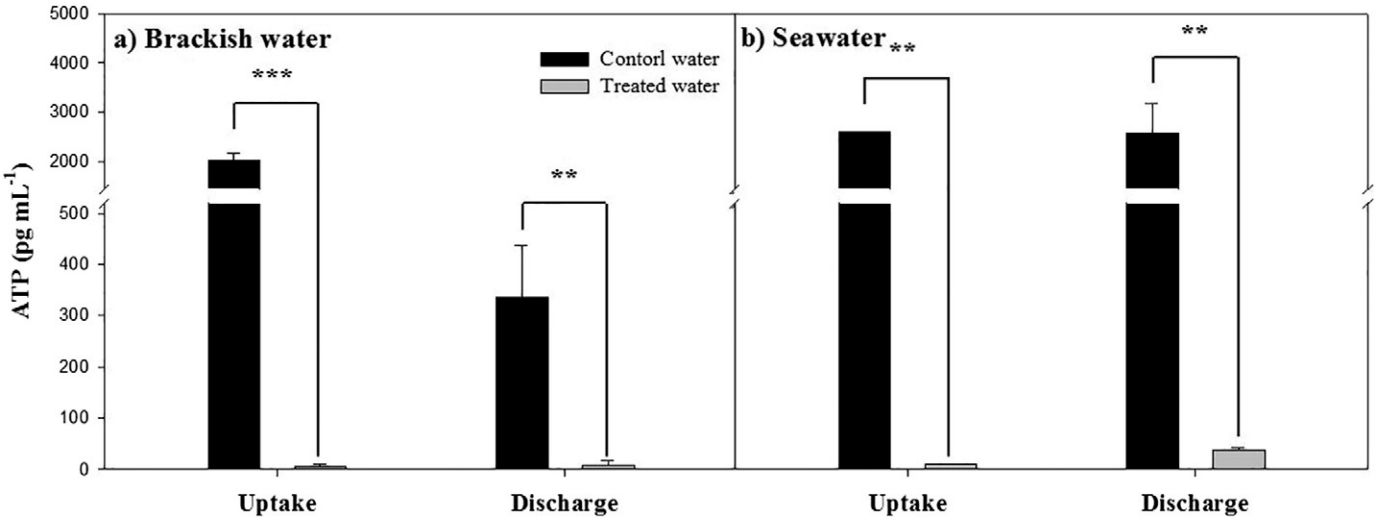

Fig. 4. Difference in the ATP concentration between the control and treated water during the full scale land-based tests using brackish water and seawater. Values and error bars represent the mean and standard deviation (n > 3), respectively, for all tests performed. *t*-test significance: **: $P < 0.05$; ***: $P < 0.001$.

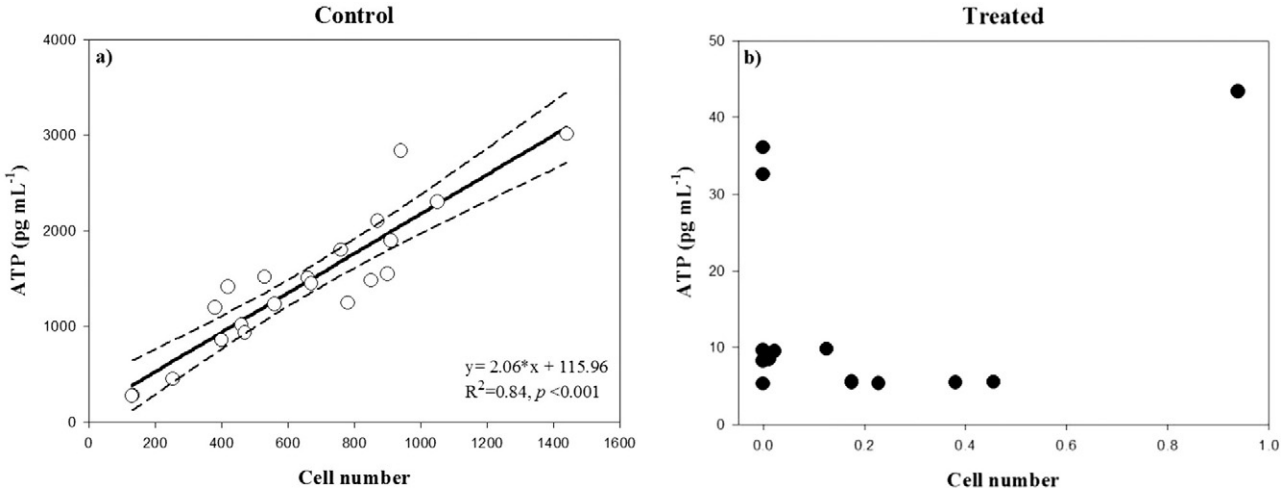

Fig. 5. Relationship between cell concentration and ATP concentration in the full scale land-based test for a) the control (n = 20) and b) treated water (n = 13).

Thalassiosira spp. dominated in the control uptake waters. However, Fig. 4 shows that the ATP concentration from 10 living cells from natural water was much lower (137 pg mL^{-1}) than the concentration from cultured cells (931–4,464 pg mL^{-1}). Maurer (2013) reported that fluorescein production in phytoplankton increased markedly when light is provided, and its concentration was closely correlated with the ATP concentration. Burkhardt et al. (1999) also reported that the growth rate for three marine phytoplankton species (*Phaeodactylum tricornutum*, *Thalassiosira weissflogii*, and *Scrippsiella trochoidea*) was largely insensitive to day length. Therefore, the ATP concentration in phytoplankton may be low in situations (e.g. the control tank) of lowered light intensity.

3.3. Establishing viability thresholds

Laboratory experiments based on the ATP guideline can indicate whether ballast water is D-2 compliant or likely to be non-compliant, and then full scale land-based tests can be applied using natural plankton communities. We found evidence that the ATP guideline is too high, because of the decline in ATP concentration when cells are exposed for too long to dark conditions. In these conditions the ATP concentration will be likely to be below the ATP concentration for cells cultured under ideal conditions. Moreover, the ATP concentration in phytoplankton is significantly affected by water temperature (van Slooten et al., 2015), total suspended solid (TSS) (First and Drake, 2013), species-specific metabolic state (Maurer, 2013), and nutrient levels (Holm-Hansen, 1970). Therefore, to validate the efficacy of the ATP assay for onboard monitoring, laboratory experiments will need to be performed under similar environmental conditions as that in the full scale land-based test.

4. Conclusion

The results of our study show that the ATP assay is a suitable method for indicative analysis. From laboratory and full scale land-based tests we found that the ATP assay had a lower detection limit (able to detect 1–2 living cells), and there was a significant positive correlation between both cultured and living natural plankton cells. Especially, as the cell volume increased, ATP concentration increased exponentially, while ratio between ATP concentration and cell volume decreased logarithmically. The relationships established in the study show that bulk measurements of living biomass based on the ATP assay can be converted into an estimate of the concentration of living cells. However, ATP concentration in living cells obtained from mono-culture experiment was much higher than living cells in the field test, and that therefore, any conclusions based on ideal growth condition can be hard to extrapolate directly to the field test results. Additional laboratory and full scale field tests under more realistic conditions are needed before it can be widely adopted as a compliance monitoring tool.

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