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The critical adenosine triphosphate (ATP) concentration in treated ballast water

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ABSTRACT

Measuring Adenosine triphosphate (ATP) provides a proxy to check compliance with IMO's ballast water D-2 standard: <10 cells mL^{-1} in the 10–50 μm size class. Measured with standard boiling techniques the ATP concentration in aquatic eukaryotic microorganisms is 0.6 mol m^{-3}. Model calculations with 10–50 μm spherical organisms show their ATP content is 0.2 to 20 pg $cell^{-1}$, in line with several cell lysis data. However, at 10 cells mL^{-1}, these ATP contents lead to a maximum of only 2 to 200 pg ATP mL^{-1}, at least 7.5× below a D-2 test kit 1500 pg ATP mL^{-1} upper limit. Different cell shape and ATP extraction scenarios to reach 1500 pg ATP mL^{-1} are discussed but remain improbable. Because cell lysis data are inconclusive, and a novel phosphoric acid-benzalkonium chloride method indicates up to 3× higher ATP concentrations, an independent test kit validation and a comparison of all three techniques are recommended.

1. Introduction

ATP is a reliable indicator of living microbial biomass in aquatic environments (Karl, 1993). For a long time it was assumed that ATP was a " universal energy currency" only, but recently it has been shown that ATP keeps hydrophobic proteins dissolved in the cell's cytoplasm, explaining its high cellular content (Patel et al., 2017). A recent publication on the ATP content of microorganism shows that the concentration of this biomass proxy is not only high but also quite constant in eukaryotic aquatic microorganisms such as diatoms and ciliates (Bochdansky et al., 2021). In theory, its' relatively constant and high cell content makes ATP a useful proxy for the concentration of viable cells in, for instance, ships' ballast water (van Slooten et al., 2015; Bradie et al., 2018a; Bradie et al., 2018b).

Ships' ballast water may contain invasive aquatic microorganisms and to prevent their world-wide spread the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments or BWM (IMO, 2004). Regulation D-2 of the BWM stipulates the standards that must be fulfilled in ballast water discharge: <10 viable organisms ≥50 μm in minimum dimension m^{-3}, and <10 viable organisms ≤10–50 μm in minimum dimension mL^{-1} (IMO, 2004). This minimum dimension is the diameter of spherical organisms or the width of elongated ones. There is no limit to the length of the organisms. Because microscopic methods to count these organisms are time-consuming and difficult to perform on board of ships, a range of rapid and relatively easy to use techniques and instruments, so-called "compliance monitoring devices" or CMDs have been developed.

CMDs are now widely used to rapidly check the performance of newly installed ballast water treatment systems. Most of these devices do not directly count organisms but use proxies such as natural chlorophyll fluorescence, fluorescence from an added vital stain such as Fluorescein DiAcetate (FDA), or ATP. In the case of chlorophyll fluorescence, the red fluorescence intensity can be used as a proxy for the phytoplankton biomass and by using a quenching light intensity the physiological status of the cells ("live" or "dead") may be assessed as well. FDA is added to a sample and is converted by esterase enzymes to a green-fluorescent dye whose intensity is the proxy for vital organism biomass. As will be introduced below, ATP is another proxy for living biomass. A general problem arises with biomass as a proxy because regulation D-2 is linear: its' unit is number of organisms per mL while biomass is related to the organism's three-dimensional volume.

Because proxies as chlorophyll, FDA fluorescence and ATP are related to volume, they require either a conversion to organisms per mL or a validation to establish a threshold analytical value such as maximum fluorescence, to be able to conclude if a sample is compliant with D-2. The conversion factors used are technique or instrument dependent, are established by the CMD manufacturer and are often unknown. The outcome of CMDs is therefore usually not presented as organism concentrations but as compliance vs non-compliance, meaning

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that it is assumed that the water tested does or does not comply with IMO's D-2 standard.

The measurement of ATP as biomass proxy has a long history, starting in the 1960s (Holm-Hansen and Booth, 1966). The basic process is filtration of the organisms, extraction of the ATP from the filter in boiling Tris buffer, and measuring the ATP quantity as photons emitted in the luciferin-ATP-luciferase reaction. The extraction procedure was recently improved by freezing the filter or whole water samples in liquid nitrogen before boiling in hot water (Bochdansky et al., 2021). In another recent change to exclude boiling liquids, the extraction procedure was modified by using P-BAC, a mixture of benzalkonium chloride with phosphoric acid in a Tricine buffer (Welschmeyer and Kuo, 2016). A recently developed CMD ATP-kit (Luminultra®'s B-QUA, https://www.luminultra.com/ballast-water-management/) extracts ATP using cell grinding by beads followed by lysis with a proprietary lysis solution (Lo Curto et al., 2018). This extraction procedure probably leads to higher ATP concentrations than a previous ATP-CMD method with cold water extraction that had a detection limit of 2.5 ± 0.5 *T. rotula* cells mL^{-1} and was not further developed for use in ballast water analyses (van Slooten et al., 2015).

The recent publication that the ATP concentration in eukaryotic aquatic microorganisms is high and constant (Bochdansky et al., 2021), opens the possibility to calculate IMO's D-2 standard in 10–50 μm cell concentrations in terms of ATP concentration. The practical validation method for CMDs is to microscopically measure the organisms, presume viability by using a vitality stain and to count the organisms of interest. On the other hand, in CMD ATP-analyses size, here 10–50 μm, is confined using membrane filtration. This includes filtration through a 50 μm filter to remove the ≥50 μm size fraction, collection of the 10–50 μm organisms on a 10 μm filter to remove <10 μm organisms, and extraction of the 10–50 μm ATP (Bradie et al., 2018a; van Slooten et al., 2015; Lo Curto et al., 2018). The specific ballast water, size-related application of ATP analysis is, besides its novelty, the reason there are no validations between B-QUA, P-BAC and the traditional hot Tris boiling or the liquid nitrogen hot water boiling procedures.

The main goal of this study is to examine the ATP concentrations that CMDs should be able to measure to detect (non)-compliance with the D-2 standard, which are related to ATP extraction efficiency, the ATP content of cells of varying size (10–50 μm) and hence volume, and the ATP detection limit. Basically, of the four methods in use the first three, the hot Tris (Holm-Hansen and Booth, 1966), the P-BAC (Welschmeyer and Kuo, 2016) and liquid nitrogen-hot water methods have been compared (Bochdansky et al., 2021); they had ATP extraction efficiencies between 80 and 100 %. For eukaryotic phytoplankton, it was concluded that the ATP concentration in the cytoplasm of aquatic eukaryotic microorganisms was relatively constant at 0.6 mol m^{-3}, with a range of 0.1 to 2 mol m^{-3} (Bochdansky et al., 2021). On the other hand, an earlier study claimed a 3 to 5 time improvement by the P-BAC method compared to the hot Tris method (Welschmeyer and Kuo, 2016). The fourth, B-QUA method, was validated with microscopic counts of culture and field samples (Lo Curto et al., 2018), compared with other CMDs (Bradie et al., 2018a) and compared with the P-BAC method (Bradie et al., 2018b) but not with the hot liquid methods. Unfortunately, ATP-comparison studies are scarce, were not performed independently, i.e. without involvement of the improvers, and were not performed on all four methods simultaneously.

The approach of this study is theoretical. Two models are described to calculate ATP concentrations that are based on the volume of well-known small aquatic life-forms: spheres, disks, and cylinders, in samples with 1 or 10 cells mL^{-1}: the practical detection limit and the limit of non-compliance (D-2). Their minimum dimensions, diameter or width, are 10 and 50 μm (1 μm above D-2). To get a feeling of the three-dimensional nature of ATP content and biomass, the spherical equivalent diameter (sed) of the average volume of spherical organisms in the 10–50 μm range (ca. 20,000 μm^3, sed = 33 μm) will be used in a number of calculations. In addition, calculations with organisms with a sed of 15 μm are made. A sed of 15 μm is the mean coastal plankton size derived from a size-distribution model that also predicts that the likelihood that cells smaller than 15 μm are observed in natural samples is >80 % (Welschmeyer and Kuo, 2016). Their initial ATP content is that of aquatic microorganisms, based on hot-boiling methods: 0.6 mol m^{-3} (Bochdansky et al., 2021).

The model outcomes are discussed in relation to: (1) the B-QUA-analysed ATP content of *Tetraselmis suecica* (Lo Curto et al., 2018), (2) B-QUA-analysed ATP content of field samples from North and Baltic Sea (Lo Curto et al., 2018), (3) ATP measurements and comparisons with natural plankton cell counts in CMD validation studies by Martinez-Romero et al. (unpublished) and (Bradie et al., 2018a). Then, a number of worst-case scenarios is examined if non-compliance can be reached at higher ATP concentrations then 0.6 mol m^{-3}: (1) the highest value of the range: 2.0 mol m^{-3} (Bochdansky et al., 2021), (2) high Luminultra® precursor efficiencies of 1.5 to 3× the hot boiling Tris method: 1.8 to 6 mol m^{-3} which is comparable to the increased efficiencies of the P-BAC method (Welschmeyer and Kuo, 2016).

2. Methods

2.1. Model calculations

2.1.1. Spherical organisms

First, it was assumed that the ballast water organisms are spherical, which means that their diameter (D) is also the minimum dimension allowed (10–50 μm). Second, in these spheres the ATP content in picogram was calculated from their volume ($V = 4/3 * \pi * (D/2)^3$), the mean ATP concentration in *Skeletonema costatum*, *Thalassiosira weissflogii* and eukaryotic phytoplankton of 0.6 mol m^{-3} (Bochdansky et al., 2021) and a molar mass of ATP = 507 g mol^{-1} (see Annex 1 for an example). To exemplify the three-dimensional characteristic of ATP content, the range of diameters was 10 to 100 μm, i.e. double that of the D-2 upper limit (size class 10–50 μm). Furthermore, in a number of cases, the ATP content was calculated for organism of 33 μm, which is the average size of spheres in the 10–50 μm range, and for 15 μm sized organisms which is the average size in the 10–50 μm range for coastal phytoplankton (Welschmeyer and Kuo, 2016). Third, although <10 viable organisms mL^{-1} of ≤10–50 μm in minimum dimension are allowed by IMO, the ATP contents were calculated for the lowest, detection limit (1 per mL) and the first non-compliant concentration (10 per mL). Fourth, in addition and partly as extreme cases, the calculation for 10 cells were repeated with the maximum ATP concentration in eukaryotic phytoplankton of 2.0 mol m^{-3} (Bochdansky et al., 2021) and with values 1.5 and 3× the Bochdansky et al. (2021) values in case the Luminultra® ATP extraction method would have these increased efficiencies compared to hot-boiling.

In all calculations the possible false contribution of <10 μm organisms such as bacteria to the measured ATP concentration in the 10–50 μm fraction were not considered. The B-QUA targets bacteria (as part of <10 μm organisms) as well as ≥50 μm organisms (mainly zooplankton) and uses membrane filters to produce the distinct size fractions. The 10–50 μm size fraction therefore presumably has little interference from bacteria (≪10 μm). Epi-bacteria may be present on larger particles such as detritus, but it is expected that much of this detritus will end up in the <10 μm fraction. In addition, if a sample is treated by a ballast water system many of the bacteria are expected to have been killed. However, in the end, there may be bacterial ATP present in the 10–50 μm fraction so a non-compliance threshold should be higher than as it would be based on axenically cultured 10–50 μm organisms.

2.1.2. Cylindrical organisms

In practice organisms in treated ballast water are often observed as small (<20 μm) circular or spherical shapes (L. Peperzak, unpublished). Nevertheless, many species such as diatoms are disk or cylinder shaped

(Kraberg et al., 2010). Cylindrical diatoms as *Rhizosolenia* spp. have the potential to extend to great length (≫50 μm), increase their biomass and ATP content, but according to IMO regulations most still need to be classified as 10–50 μm organisms. Therefore, a second set of calculations was performed with disks and cylinders.

First, a disk was defined as a circular shape with diameter D and thickness D/2, such as common coastal *Thalassiosira* spp. (Table 1). Second, a cylinder is also a circular shape with diameter D but with a length D*10 such as *Rhizosolenia* spp. (Table 1). Third, the disk diameters that were used in the calculations, as defined by the minimum and maximum IMO dimensions, were 10 to 50 μm, assuming that in microscopy the cells lay flat. Fourth, the two cylinder diameters used in the calculations were the minimum (10 μm) and the maximum plus 1 μm (50 μm) IMO dimensions, with lengths up to 1000 μm (>10× the diameter: length ratio) (Table 1). Fifth, the ATP concentrations were calculated from shape volumes and at 1 and 10 per mL to include the detection limit and the first non-compliant concentration, using the methods as for spherical organisms.

Comparison: The modelled ATP values, based on the hot-boiling method, were compared with ATP data in peer-reviewed publications for the 10–50 μm size class and the detection limits and the IMO D-2 compliance guidelines of LuminUltra® for the B-QUA PLUS Ballast Water Monitoring Kit. These Luminultra® guidelines are given in pg ATP/mL: "most likely compliant" (<500 pg ATP mL^{-1}), "signal close to limit" (500 to 1500 pg ATP mL^{-1}) and "most likely not compliant" (>1500 pg ATP mL^{-1}).

Definitions: A false negative compliance test is a test result declared negative (D-2 compliant or pass, <10 cells mL^{-1}), where it is in fact positive.

3. Results and discussion

3.1. Spherical organism model

The mean ATP content in aquatic microbes is quite high due to its multiple functionality with a mean 0.6 mol m^{-3} in phytoplankton (Bochdansky et al., 2021). Yet, the cells are so small that the model calculation shows that in individual 10 to 50 μm spherical organisms the ATP content is just 0.2 to 20 pg. Theoretically, the B-QUA detection limit is sufficiently low at 0.2–0.8 pg ATP mL^{-1} (Annex 2), which is $^1/_3$ to $^1/_{10}$ of the ATP concentration from 10 organisms mL^{-1} of 10 μm diameter. Contrary to practical findings (Trindade de Castro and Veldhuis, 2019), it is assumed here that the filter that retains the ≥10 μm organisms, has a 100 % efficiency, meaning that there is no loss of 10–50 μm organisms into the filtrate and also that <10 μm organisms or ≥ 50 μm organism that passed the 50 μm filter are not retained on the 10 μm filter. However, the theoretical non-compliance limits, for 10 cells mL^{-1}, are 2 to 200 pg mL^{-1}, which is 750 to 7.5× below the B-QUA limit of 1500 pg ATP mL^{-1} (Fig. 1). This implies that the B-QUA limit is too high.

First, to check the model the only B-QUA culture validation data available is used: the ATP content of *Tetraselmis suecica* of 0.08 pg $cell^{-1}$, derived from the regression equation in Fig. 11 of Lo Curto et al. (2018). For the spherical model calculation the volume of *T. suecica* is needed. Its volume increases with nutrient concentrations and is variable from 252 to 905 μm^{-3} (Fabregas et al., 1985), which means a spherical equivalent diameter (sed) of 8–12 μm. Using the spherical model an ATP content of 0.08 to 0.28 pg ATP $cell^{-1}$ is calculated which agrees with Lo Curto (Lo Curto et al., 2018). This agreement does suggest that the *T. suecica* cells were at their smallest, or that the B-QUA method somehow underestimated the ATP content of in the sample. If the standard analysis procedure was followed, including the 10 μm filtration step, a fraction of these small (8–12 μm sed) cells may have passed the filter. In any case, this B-QUA data appears to confirm the validity of the ATP model.

Second, the model ATP data can be compared with B-QUA validation and field measurements. First, (Lo Curto et al., 2018) give two regression equations based on validations with Baltic Sea and North Sea plankton in the 10–50 μm range. The Baltic Sea regression (assuming the vertical axis in Fig. 10 is concentration ATP per mL (Lo Curto et al., 2018)) yields 0.3 pg ATP $cell^{-1}$. According to the model this ATP content equates to 12 μm diameter spherical organisms, which is close to the mean 15 μm sed according to the coastal plankton size-distribution model (Welschmeyer and Kuo, 2016). However, the North Sea regression (assuming the vertical axis in Fig. 5 is indeed in ng ATP/mL (Lo Curto et al., 2018)) yields 92 pg ATP $cell^{-1}$. According to the model this ATP content equates to 83 μm diameter spherical organisms, which is clearly unrealistic. If the unit in the North Sea regression would be pg ATP mL^{-1}, the ATP content would be 0.09 ATP $cell^{-1}$, close to the lower *T. suecica* measured and calculated lower ATP values, but the diameter then is <10 μm which is also unrealistic. Requests for clarification to Lo Curto and co-authors have not been answered.

Third, in a CMD validation study using NIOZ (Royal Institute for Sea Research) harbour water, the mean ATP content of treated and untreated 10 to 50 μm organisms studied was 1.9 pg $cell^{-1}$ (Martinez-Romero et al. unpublished), which according to the ATP model is equivalent to spherical organisms with a 23 μm diameter. In a comparison of CMDs, including a B-QUA precursor (SGS-ATP) and other counting techniques, the concentrations ranged from 20 to 200 pg ATP mL^{-1} in samples with 10 to 40 cells mL^{-1} (Bradie et al., 2018a). Using the model, 40 to 10 spherical organisms with 0.6 mol ATP m^{-3} with respectively a 23 μm or 32 μm diameter would yield that range of ATP concentrations, i.e. also within the 10 to 50 μm range. Values of 23 and 32 μm are also in the range of 15 μm (average size-distribution model) to 33 μm (average 10–50 μm sed). It is concluded that the simple spherical model appears to deliver relevant ATP contents.

Organisms that have undergone ballast water treatment, i.e. pumped

Table 1
Coastal phytoplankton species of different shapes and sizes used to define model disks (*Thalassiosira* spp.) and cylinders (*Rhizosolenia* spp.). Data from Kraberg et al. (2010). D = diameter (μm), H = thickness or length (μm).

Species	Minimum diameter	Maximum diameter	Minimum thickness or length	Maximum thickness or length	D/H (minimum)	D/H (maximum)
Thalassiosira spp.						
T. rotula	8	60	5	20	2	3
T. punctigera	40	185	20	120	2	2
T. minima	10	50	5	33	2	2
T. hendeyi	40	110	30	90	1	1
				Mean:	**2**	**2**
Rhizosolenia spp.						
R. imbricata	5	40	–	650	–	0.1
R. styliformis	20	100	–	1500	–	0.1
R. hebetata	15	40	–	750	–	0.1
R. setigera	5	50	–	600	–	0.1
				Mean:		**0.1**

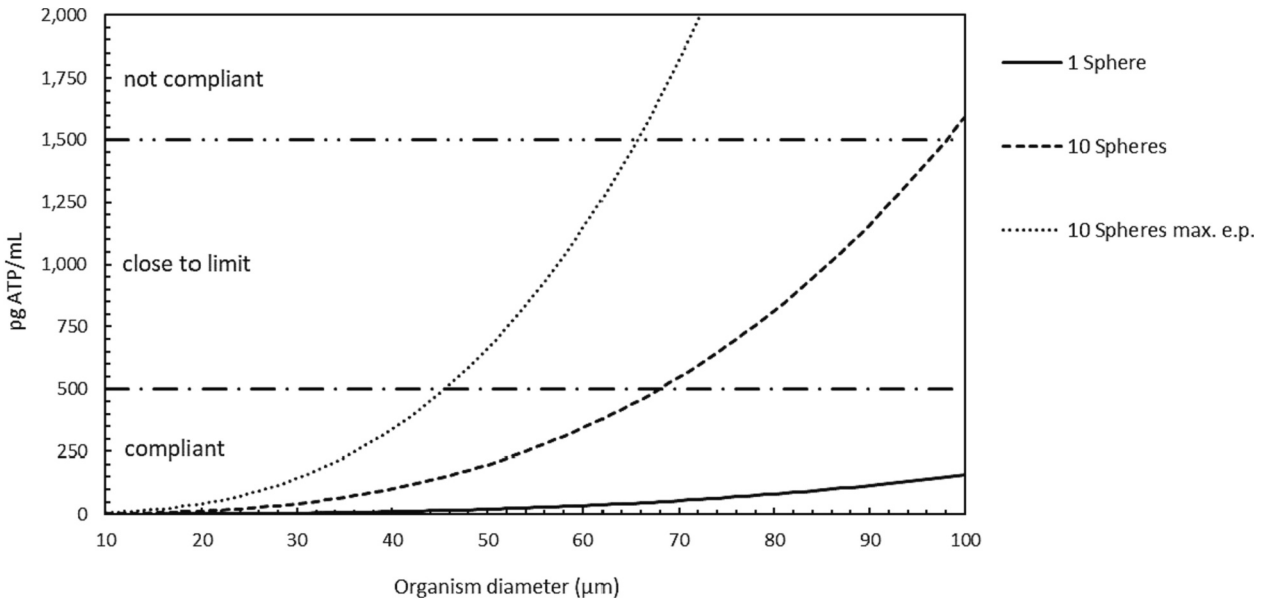

Fig. 1. ATP concentration of spherical organisms from 10 to 100 µm in diameter, modelled for 1 sphere (mean ATP = 0.6 mol m^{-3}), 10 spheres (mean ATP = 0.6 mol m^{-3}) and a worst case of 10 spheres with a maximum ATP = 2.0 mol m^{-3} as reported for eukaryotic phytoplankton (max. e.p.). The horizontal lines depict the Luminultra® guidelines for IMO D-2.

at high turbulence, filtered, and treated by chemicals or UV are stressed and it could be expected that they have reduced energy levels and hence ATP concentrations. In mammalian cells lowered ATP content is an indication of programmed cell death (Eguchi et al., 1997). ATP concentrations also decrease when sludge microorganisms, mostly bacteria, are treated with toxic chemicals (Dalzell and Christofi, 2002). On the other hand, the high millimolar ATP content is related to cytoplasm volume and is relatively constant despite differences in growth rate (Bochdansky et al., 2021). This could mean that stress, when it does not decrease cytoplasm volume or destroy organisms, may not considerably reduce ATP concentrations. Because there are arguments for both a decrease and a relatively constant ATP concentration, the use of 0.6 mol ATP m^{-3} in the model calculations remains a fair approximation.

The model shows that even when 10 of the largest (50 µm) spherical organisms are present in 1 mL ballast water, which would exceed the D-2 compliance standard, their ATP yields only 200 pg mL^{-1} (Fig. 1). This low ATP concentration means that according to the LuminUltra® guidelines, the ballast water would be falsely classified as compliant. In fact, 10 organisms per mL of nearly 100 µm, are needed to reach the non-compliance threshold of 1500 pg mL^{-1} (Fig. 1). This not only twice the maximum size allowed by IMO but it is also unlikely that such large spherical organisms are present because ballast water is usually pre-treated by <50 µm filters before chemical or UV-disinfection (Peperzak et al., 2022). Therefore, a number of worst-case scenarios are tested in which the ATP concentration is higher than 0.6 mol m^{-3}.

3.2. Spherical organisms and non-compliance

Several worst-case scenarios can be envisaged. The maximum value in the range of hot-boiling ATP concentrations in eukaryotic phytoplankton is 2 mol m^{-3} (Bochdansky et al., 2021). In addition, the other extraction methods may yield higher values than 0.6 mol m^{-3}. This would be truly remarkable because the hot-boiling (Tris) method and its data have been in use for more than half a century (Holm-Hansen and Booth, 1966). On the other hand, Patel et al. (2017) mention a general ATP cell concentration of 5 mol m^{-3} which is 2.5 to 8× higher than the range for aquatic microorganisms (Bochdansky et al., 2021). Welschmeyer and Kuo (2016) compared the hot-boiling (Tris) method with that of a Luminultra® B-QUA precursor: QGO-M, and with a new, P-BAC extraction. The comparison, performed with natural water and four phytoplankton cultures and compared to the traditional hot boiling procedure, yielded an average 1.5 to 3× higher ATP yields with the lysis method, and 2 to 4× higher yields with the P-BAC method. Higher P-BAC ATP yields compared to a B-QUA precursor (SGS-ATP) were also reported in a ship-board experiment for ≥50 µm and <10 µm organisms, but the 10–50 µm organisms were not compared (Bradie et al., 2018b). If the highest P-BAC yield is correct, i.e. 4× relative to the hot-boiling 0.6 to 2.0 mol m^{-3}, the range of aquatic microorganism ATP concentrations could be 2 to 8 mol m^{-3}, with a middle value of 5 mol m^{-3}, compliant with Patel et al. (2017).

First, in a worst case where 10 organisms of 45 µm diameter per mL have the maximum hot-boiling ATP content of 2.0 mol m^{-3} (Bochdansky et al., 2021), the compliance threshold of 500 pg mL^{-1} would be succeeded. However, the LuminUltra® guidelines allow for some flexibility because surpassing the compliance threshold leads to a warning that the signal is "close to the limit." The limit for non-compliance is 1500 pg mL^{-1} (Fig. 1). The LuminUltra® non-compliance threshold would only be reached with 10 organisms per mL of >65 µm diameter with 2.0 mol ATP m^{-3} (Fig. 1). Such large spherical organisms are >50 µm and therefore outside the D-2 standard.

Second, the high Luminultra® precursor QGO-M efficiencies of 1.5 to 3× the hot boiling Tris method (Welschmeyer and Kuo, 2016) can be applied to the spherical ATP model, although the validation and field data as discussed above contradict this. Nevertheless, in that case the average and maximum ATP per organism of 0.6 to 2.0 mol m^{-3} (Bochdansky et al., 2021) would change to 0.9–1.8 and 3–6 mol m^{-3}. Note that this range also includes the general ATP concentration of 5 mol m^{-3} (Patel et al., 2017). The calculations were done at 10 organism mL^{-1}, as before for 10, 15, 33 and 50 µm spherical organisms. Table 2 shows that even at 1.5 to 3× increased efficiencies of the lysis method none of the 10, 15 and 33 µm organisms at the non-compliant concentration of 10 organisms mL^{-1} would reach the 1500 pg ATP mL^{-1} Luminultra® non-compliance limit. In fact, only 10 of the largest organisms (50 µm) at the highest ATP value (Bochdansky et al., 2021) and the highest (3×) efficiency (Welschmeyer and Kuo, 2016) reach >1500 pg ATP mL^{-1}. Such large organisms are not a highly likely scenario in ballast water treatment.

Table 2
ATP content of 10 spherical organisms (mL^{-1}), the non-compliant IMO concentration, at the average and maximum ATP concentration from the literature (hot-boiling method) used in the spherical model in mol m^{-3}, multiplied by 1.5 and 3, the possible increased efficiency when using lysis ATP extraction. The Luminultra® non-compliance limit is 1500 pg ATP mL^{-1}. The IMO size range is 10–50 μm. 15 μm is the average from a (10–50 μm) size-distribution model and 33 μm is the average 10–50 μm sed.

pg ATP/cell	Spherical model		Increased efficiency			
	0.6 mM	2 mM	0.6 mM × 1.5	2 mM × 1.5	0.6 mM × 3	2 mM × 3
10 μm	0.2	0.5	0.2	0.8	0.5	1.6
15 μm	0.5	1.8	0.8	2.7	1.6	5.4
33 μm	6	19	9	29	17	57
50 μm	20	66	30	100	60	199

3.2.1. Disk-shaped organism model

According to the spherical model, the highest ATP concentration of a spherical organism of 50 μm diameter is 20 pg mL^{-1} (Fig. 2), and this value is used as a reference for the calculations of other shaped organisms. Of these largest spherical organisms, 75 mL^{-1} are needed to reach non-compliance in the ATP assay (1500 pg ATP mL^{-1}). Ten disks that have a thickness that is half their diameter have an ATP content that is 75 % of spheres of the same diameter (Fig. 2). Such disks contain well known species such as *Thalassiosira* spp. (Kraberg et al., 2010), that have also been used in ATP laboratory studies (Bochdansky et al., 2021; van Slooten et al., 2015) and have been detected in treated ballast water (Stehouwer et al., 2013; Stehouwer et al., 2015). But even above the D-2 compliance limit, at 10 cells mL^{-1}, and at the maximum IMO size allowed (50 μm), such disk-like species would still lead to a relatively low ATP concentration (150 pg mL^{-1}), and therefore a compliant test result and a false negative B-QUA test.

3.2.2. Cylindrical organism model

As discussed for the spherical model, the ATP concentrations measured to date relate to cells in the 20 to 30 μm size range. However, in a comparison study (Bradie et al., 2018a) at <10 cells mL^{-1} (≈5 mL^{-1}) three relatively high ATP values and apparent outliers of 350–550 pg ATP mL^{-1} were measured in the 10–50 μm fraction. At these low and compliant cell concentrations, using the Fig. 2 model, the length of 5 cylindrical cells that yield such high ATP concentrations (≈500 pg ATP mL^{-1}) would need to be 400 μm (10 μm diameter). Theoretically, this could be achieved by certain *Rhizosolenia* species (Table 1), but they would have to pass the filter perpendicularly. Bradie et al. (2018a, 2018b) mention that one outlier measurement was dropped because a large organism was seen. Perhaps the filter was damaged and some other, non-observed large organisms, passed too. However, non-compliance was still not reached in the Bradie et al. (2018a, 2018b) study.

Non-compliance in the ATP test can be reached theoretically for cylindrical cells depending on their concentrations, cell diameter (maximum 50 μm) and length. Cylindrical cells with a diameter of 10 μm at a concentration of 10/mL^{-1} need to reach >600 μm in length to achieve 1500 pg ATP mL^{-1} (Fig. 2). A species as *R. setigera* would qualify (Table 1). Cylindrical cells with a diameter of 50 μm and a length of 500 μm (the definition of the shortest cylinder with this diameter) would yield >1500 pg ATP mL^{-1} and non-compliance already at a concentration of 1/mL^{-1} (Fig. 2). In fact, a 50 μm cylindrical cell of 250 μm length would already contain 1500 pg ATP. A formidable species as *R. styliformis* would theoretically qualify (Table 1). If the ATP content of cylindrical cells would be a factor of 5 higher, say 5 mol m^{-3} (Patel et al., 2017), the calculated lengths of the organisms would be 5× shorter for non-compliance, but still in the order of 50 to 100 μm for *R. setigera* and *R. styliformis* cells. As will be discussed below, the rarity of large cells in the sea compared to small ones, ballast water treatment, and filtration to achieve the 10–50 μm size fraction make the presence of such large cells in CMD ATP analysis unlikely.

The discrepancy between the large number of small spherical cells that are needed for non-compliance and the small number of very large cells for the same, is related to the fact that ATP is related to biomass and hence to volume, a three dimensional variable and not a linear variable as cell counts. The chance however that such large cells are taken up in ballast water is extremely small given that the abundance of species in the sea decreases with their size (Raven, 1996; Trindade de Castro and Veldhuis, 2019; Welschmeyer and Kuo, 2016). There is even an 80 % chance that coastal plankton organisms are smaller than 15 μm (Welschmeyer and Kuo, 2016), so the chance for long or wide organisms to be observed in ballast water appears to be very small.

Large cells are not only rare, but the question is also how long cylindrical *Rhizosolenia*-type cells would end up in treated ballast water. This water has been treated by filters that have mesh sizes smaller than 50 μm, before chemical or UV-disinfection, storage in ballast tanks and subsequent discharge under turbulent conditions (Peperzak et al., 2022). It is not surprising that the species that survive ballast water treatment are generally small, *Thalassiosira* spp., *Chaetoceros* spp. and *Nitzschia* spp. (Stehouwer et al., 2013; Stehouwer et al., 2015). Furthermore, before ATP analysis Luminultra® prescribes filtration over a 50 μm filter to remove interfering organisms such as >50 μm

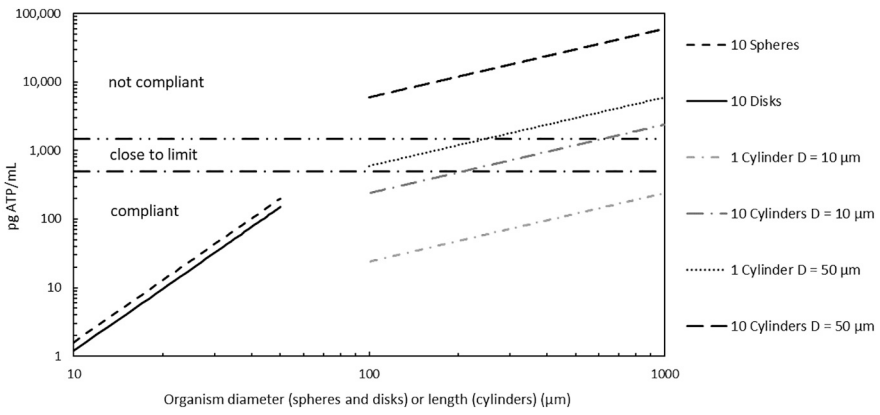

Fig. 2. ATP concentration of different shapes and concentrations with a mean ATP = 0.6 mol m^{-3}: 10 spheres with a 50 μm diameter (D), 10 disks with D = 50 μm and a thickness of D/2, 1 and 10 cylinders with D = 10 μm or D = 50 μm diameter and a minimum length of 100 μm. For comparison reasons the length for the 50 μm cylinders was originally set at 10*D (500 μm) but was extrapolated to 100 μm. The horizontal lines depict the Luminultra® guidelines for IMO D-2 compliance in pg ATP/mL. Note that the vertical axis now has a logarithmic scale.

zooplankton. Unless the long cylindrical cells move perpendicular to and through the filter, any large treatment-surviving organisms are likely to be removed from the 10–50 μm size fraction during sample preparation. The combination of ballast water treatment and the ATP sample preparation make it very unlikely that long cylindrical *Rhizosolenia*-type cells would lead to a non-compliant (>1500 pg ATP mL^{-1}) result.

4. Conclusions and recommendations

At present, ballast water CMDs are not yet validated independently, i.e. other than by their manufacturers or users. IMO is preparing a validation protocol for CMDs aimed to assess non-compliance with the D-2 standard. This protocol will probably be published in 2023 and independent validation results are not to be expected earlier. It is recommended that such validations are performed at relevant concentrations, with differently shaped microorganisms, excluding the 10 μm border-sized *T. suecica*, and that attention is given to possible contamination by smaller organisms such as bacteria.

In addition to the validation of ballast water CMDs, a validation of the different ATP extraction techniques seems worthwhile. Although a review has recently been published (Bochdansky et al., 2021), a simultaneous comparison of hot-boiling methods, cell lysis and P-BAC seems warranted given the disparate results achieved till today.

The model calculations presented here, with 10–50 μm organisms, which are at their maximum dimension (spheres, disks), show that non-compliant ballast water ATP results at the present Luminultra® guidelines are extremely improbable. Treated ballast water is also unlikely to contain long cylindrical cells, with a 10–50 μm diameter, which are needed to reach non-compliance. An independent validation by the IMO protocol can show the correctness of these conclusions.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

This research in this submission was performed by me as a NIOZ senior research scientist. I am also employed by Peterson Control Union as a ballast water expert.

Data availability

Data will be made available on request.

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Annex 1. ATP per cell calculation example

Example of the calculation of the ATP content:
For a spherical organism of 10 μm diameter:
Diameter = 10 μm → Radius = r = 5 μm = 5×10^{-6} m.
Volume = $4/3 * \pi * r^3 = 4/3 \times \pi \times (5 \times 10^{-6}\ m)^3 = 523.6 \times 10^{-18}\ m^3$.
[ATP] = 0.6 mM = 0.6 mmol L^{-1} = 0.6 mol m^{-3} and ATP = 507.18 g mol^{-1}.
ATP in 10 μm spherical organism = $523.6 \times 10^{-18}\ m^3 \times 0.6$ mol $m^{-3} = 314.2 \times 10^{-18}$ mol ATP
314.2×10^{-18} mol ATP × 507.18 g $mol^{-1} = 159.3 \times 10^{-15}$ g ATP = 159 fg ATP ≈ 0.2 pg ATP.

Annex 2. Calculation of B-QUA detection limit

Variables (from B-QUA PLUS detailed protocol; RLU = Relative Light Unit):

RLUcATP10-50	RLU 10-50 um sample
RLUBN	RLU Blank Negative (blank control, should be <20 RLU)
RLUUC1	RLU from UltraCheck 1 (standard, should be >5000 RLU)
V10 [mL]	Volume filtrated: ≥200–1000 mL

Assuming the minimum measure RLUcATP10–50 = 10, a perfect blank = 0, a standard RLU = 20,000 and a sample volume = 200 and 1000 mL:

RLUcATP10-50	RLUBN	RLUUC1	V10 [mL]	cATP10-50 (pg/mL)
10	0	20,000	1000	0.15
10	0	20,000	200	0.77

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