Analysis of Adenosine Triphosphate (ATP) as a rapid, quantitative compliance test for ships’ ballast water

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Technologies for Monitoring Ballast Water Discharge Standard Compliance

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EXECUTIVE SUMMARY

The principle objective of this project was to devise a simplified ATP method for shipboard ballast water compliance testing. To meet the objective above, the ATP method needed to be rapid, sensitive and free from the requirement for laboratory resources and complicated extraction techniques such as sonication, high heat, grinding, bead-beating, centrifugation, etc. The ATP method for ballast water compliance testing also required cell-specific calibration so that the measured ATP concentration could be converted to equivalent live cell concentrations consistent with numerical ballast water discharge standards (BWDSs).

The final ATP method described here results in a simple ‘wet soak’ extraction technique that yields ATP values roughly 3-5x higher than those derived from traditional oceanographic boiling Tris buffer protocol. The samples can be read immediately after a 30 minute wet-soak, or frozen indefinitely for analysis of large sample batches at a later time. All phases of the operation can be executed without power requirements using a battery powered laptop computer. The ATP measurement integrates all live organisms including heterotrophs and autotrophs.

A model for calculating equivalent live cell concentrations from biochemical ATP assays was constructed for the unique 10-50 µm regulated ballast water organism size class. The calibrated cell-specific ATP content (pg ATP/cell) for 15 µm equivalent spherical diameter cells, corresponding to the mean cell size within the 10-50 µm group, was 2.62 pg ATP/cell. Thus, a ballast water sample with a size fractionated (10-50 µm) ATP concentration of 26.2 ng ATP/L is calculated to be equivalent to 10 live cells/mL, corresponding to the ballast water discharge standard (BWDS) limit for the 10-50 µm size class. The ATP concentration of unknown ballast water samples can be compared to the equivalent ATP-based BWDS limit of 26.2 ng ATP/L for the purpose of quantitative compliance monitoring.
INTRODUCTION: PROJECT OBJECTIVES

The discharge of ships’ ballast water is considered one of the primary vectors in the spread of aquatic invasive species (Baldwin 1977; William et al., 1988; Hallegraph and Bolch, 1991, 1992; Ruiz et al., 1997; Gollasch et al., 2000; MacIsaac et al., 2002). The maritime industry and international, federal and state agencies have made significant efforts to foster the development of commercial ballast treatment systems that will remove or inactivate planktonic organisms from ballast water for the purpose of abating the aquatic invasive species problem (IMO 2004). Unfortunately, existing regulations, such as IMO D-2 and USCG Final Rule, require exhaustive analytical procedures to verify biological inactivation efficiency of treatment systems relative to regulatory performance standards (IMO 2004; EPA 2010). The labor, time, and workforce required to fully substantiate successful adherence to ballast performance standards precludes such exhaustive tests for routine ship inspections.

Thus, at this time there is a need for a simple, rapid analysis of viable/living plankton for the purpose of ballast treatment verification through Port State Control, either onboard ship, at the dock, or in nearby facilities shortly after conclusion of sampling (Welschmeyer and Maurer, 2012). Moreover, such a method should be available to ships’ crew so that non-technical personnel can easily verify ballast treatment performance before routine shipboard de-ballasting operations.

In this report we summarize efforts, supported by Project BAA # HSCG32-13-R-R00016 US Coast Guard Research and Development, to develop and optimize the well-documented analysis of adenosine triphosphate (ATP) for specific application as a rapid ballast water compliance test (McElroy, 1947; Holm-Hansen and Booth, 1966; Karl 1980; Stanley 1989).

The results reported herein, correspond to the main objectives of the project, and fall broadly into three categories:

1. Development and optimization of an ATP method for natural communities of planktonic protists that uses a simple wet-soak extraction process. The method is free of tedious extraction techniques such as tissue grinding, bead beating and boiling extraction, thus lending itself to convenient shipboard testing without laboratory support.
2. Application of the new ATP protocol to full-scale ballast water treatment tests under a variety of treatment technologies, e.g., filtration, oxidation, UV irradiation, etc.
3. Development of a predictive model to be used in converting measured concentrations of ATP (ng/L) into equivalent estimates of numeric live organism concentrations for direct compliance checks against ballast water discharge standards (BWDSs), e.g., live organisms/mL in the size class 10-50 µm.

Below, we provide a short background on the history of ATP determinations and problems relative to ballast water testing conditions, and follow with three sections addressing the project objectives listed above.
BACKGROUND: ATP ANALYSIS AS A MARKER FOR LIVING BIOMASS

The measurement of ATP has been an active subject of research for decades because of the fundamental role ATP plays in the metabolism of all living organisms (McElroy 1947; Karl 1980; Lomakina et al., 2015). Three characteristics of ATP have lead to its continued use as a marker for living biomass in ecological studies of microorganisms in aquatic research:

1) All living cells are dependent on ATP as the primary energy currency of life and therefore must maintain a reserve of cellular ATP for basic metabolism.

2) ATP is depleted rapidly from stressed and/or dying cells (Hamilton and Holm-Hansen, 1966; Kennicutt, M.C., 1980; Eguchi et al., 1997)

3) The required cellular ATP content in healthy cells is reasonably constant and proportional to cellular biomass, for instance, as indicated by cellular particulate organic carbon, POC (Fig. 1) and thus, ATP can be expected to scale predictably with microorganism cell size.

The seminal work of Holm-Hansen (1970), shown in Fig 1, indicated a relatively constant ratio of cellular POC to cellular ATP (ca. 250 g C/g ATP) for cell biomass levels (POC) spanning...
almost seven orders of magnitude; this ratio is still in use today as a convenient means of converting relatively simple measurements of ATP into reasonable estimates of living organic carbon (Karl 1980; Hewes et al., 1990). That the cellular ATP content (pg ATP/cell) scales predictably as a function of cell carbon suggests it should also scale well with cell size, specifically cell volume. To our knowledge a systematic verification of ATP cellular content (pg ATP/cell) as a function of cell volume has not been completed, though it has been assumed often (Sinclair et al., 1979; Hunter and Laws, 1981; Verity and Langdon, 1984). The project described here includes a calibration effort to predict the expected ATP content for any cell size specified in ballast water discharge standards; this experimentally-determined relationship will be used as the primary model for making rational conversions of ATP, per se, into numeric live cell concentrations for the purpose of quantitative ballast compliance monitoring (detailed in Part 3 of this report).

Generally, the basic techniques and resources for measuring ATP are well developed and readily available (Stanley 1989; Lomakin a et al., 2015). In microbial ecology, ATP is a routine measurement (Karl 1980). The long-term data set collected at the Hawaii Ocean Time Series is now in its 28th year of data collection, using the routine boiling Tris ATP extraction method for natural communities of planktonic organisms in the central N. Pacific Ocean (Karl 1993). We have been using this same method (boiling Tris extraction) for over 12 years in our own ballast-related work. The technique is well developed and moderately simple, however, it requires reasonable laboratory resources. Our research shipboard workspace for ballast water testing includes a well-outfitted lab and we have the space and time to execute the ATP assay with little difficulty. However, this situation is not likely to be enjoyed during commercial ship inspections by non-scientific staff that will be responsible for ballast water compliance monitoring; this includes ships’ crew, USCG inspectors, and local Port State Control.

The first objective of the work reported here was to optimize a new extraction method for ATP that requires a simple ‘wet soak’ for plankton samples collected on common filters, similar to the common solvent extraction method for chlorophyll a. Such a method would be adequately simple for execution by non-technical staff during relatively short (<1 h) inspections on board vessels, and would require almost no laboratory resources.

PART I. THE P-BAC ATP EXTRACTION TECHNIQUE

Table 1 below is drawn from the exhaustive review by Karl (1980) which shows the wide assortment of buffers, acids, bases and solvents that have been used as extractants in quantitative ATP determinations. Strehler and McElroy (1957) described one of the earliest routine analytical protocols for ATP, which utilized simple boiling water as the extractant. The seminal work in oceanographic applications of ATP analysis (Holm-Hansen and Booth, 1966) argued that boiling Tris buffer provided equivalent extraction efficiency with higher precision. The Tris boiling method remains in routine use today for analysis of suspended prokaryotic and eukaryotic plankton (Karl 1993). However, more complex samples, such as marine sediments,
have been shown to need modifications including strong acid extraction such as 5% sulfuric acid Karl and Lomak (1975) or 5% phosphoric acid (Karl and Craven 1980). Numerous reviews of ATP analysis make it clear that the sample matrix influences the performance of various extraction agents and some reflection and experimentation is needed on the part of the user to

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optimize ATP extraction for best results on specific sample types, such as sediments (Bancroft et al., 1976), soils (Eiland 1979; Martens 2001; Lehtokari 1983), waste sludge (Patterson et al., 1970), yeasts (Stanley 1989) and some algae (Larsson and Olsson, 1979). More recently there has been a move to use detergents/surfactants for cell disruption in ATP work and we were motivated to use the popular surfactant benzalkonium chloride (BAC, the active ingredient in Lysol disinfectant) as suggested by Siro et al., (1982) and Hattori et al., (2003). Both of those studies used a simple, short ‘wet soak’ of biological tissue in BAC solution yielding acceptable ATP extraction efficiencies for their respective test organisms, fungi and bacteria. The results in Siro et al. were rapid enough to be incorporated in a continuous-flow ATP extraction routine.

In this project, we generally exercised a tact of ‘trial and evaluation’ when making decisions for an optimized extraction scheme; detailed methods can be found in Kuo (2015). Key points and results will be identified in the report herein. One theme was certainly consistent with much of the literature on ATP methods – the best extraction mixtures often carried an undesired inhibition of the ATP luminescence reaction. That is, the presence of the extraction reagents in the luciferase luminescence mixture can cause measurable reduction in the luminescence output, and therefore, decreased sensitivity. Our objective was to devise an extraction scheme that yielded the highest efficiency of ATP extraction (the ‘ideal’ goal for any organic extraction technique) but that also yielded adequate sensitivity for detecting low levels of living microorganism in the 10-50 µm size class, commensurate with levels well below ballast water discharge standards, e.g., <10 live cells/mL.

All ATP data reported herein were determined with special effort to a) reduce the luminescence inhibition through appropriate dilution of sample extraction fluid and proper buffering and to b) execute ATP calibration with ATP standards that were ‘matrix matched’ to mimic the chemical composition of the extraction fluids. The steps for these procedures are detailed in the Standard Operating Procedure that accompanies this report as Appendix 1.

A recent commercial product-line of ATP measuring tools from LuminUltra Technologies Ltd. (Canada) was used as a modern reference technique to judge the performance of our ATP protocol as it was being developed. LuminUltra provides a lysing agent, UltraLyse, that is designed for rapid extraction of ATP from microorganisms in industrial water, without the need for physical extraction assistance from heat, sonication, bead-beating, etc. (similar to our objectives). UltraLyse is a proprietary reagent, though the material safety data sheet (MSDS) notes the presence of BAC at unspecified concentrations. We were helpfully advised by product manager, Dave Tracey (LuminUltra), that mixed plankton samples may need an extra ‘softening’ reagent to condition the cell walls for more efficient release of ATP. That product, LumiClean, was used in conjunction with UltraLyse to maximize ATP extraction; both reagents are included in their ATP kit model QGO-M. As shown in Fig. 2 the outcome of ATP extractions with natural water from Elkhorn Slough, CA were ca. 4x higher with LuminUltra technique relative to the standard boiling Tris method. Interestingly, the ATP content of the cell softening rinse, LumiClean, was ca 40% of the total extraction signal. The routine LuminUltra procedure is to
discard the LumiClean rinse, since it is regarded as cell softening reagent, not an extractant; clearly that was not the case. Inspection of the LumiClean MSDS identified the active ingredient to be dimethyl sulfoxide (DMSO), a well known ATP extraction solvent (see Table 1; Palumbi et al., 1987). Thus, in order to correctly calculate the total ATP concentration using the LuminUltra kit for planktonic organisms, one must measure ATP in two solutions, one organic (DMSO), the other aqueous (buffer), requiring a unique ATP standardization for each reagent, due to luminescence inhibition by each reagent.

The extra steps in operating the LuminUltra ATP method as in Fig. 2 were not in line with our primary objective for a simple rapid ATP method, even though it was clear that greater ATP extraction would result over the standard boiling Tris method. We commenced a systematic selection of appropriate reagents and mixture ratios to yield a rapid method that met or exceeded the performance of the modern LuminUltra protocol (termed by the manufacturer as ‘2nd generation ATP technology’) while requiring the simplest protocols possible. We noted that BAC alone could not extract as efficiently as the Ultralyse-LumiClean combination, but the addition of a strong acid, such as phosphoric acid or sulfuric acid (Karl and Larock, 1975; Karl and Craven, 1996; Christensen and Devol 1980) in combination with BAC provided the optimized mix.

A final extract combination of 5% phosphoric acid (w/w) and 1% BAC (w/w) in 25mM Tricine buffer yielded the best results; we termed the reagent ‘P-BAC’. As shown in Fig. 3 the extraction is rapid; a simple room temperature filter-soak in a 1 mL volume (snap cap vial) of P-BAC for 20-30 minutes brings the ATP extraction to a uniform completion. If desired, the

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Figure 2. Relative ATP concentrations from Moss Landing Harbor water (concentrations normalized to 100). Standard LuminUltra extraction consisted of a single-pass (5 mL) of the cell softening reagent, LumiClean, followed by a 1 mL pass of the extraction reagent UltraLyse through a syringe-based filter apparatus.

![Figure 2](image)

Figure 3. Room temperature extraction times of 20 minutes or greater yields uniform ATP extraction that remains constant over the 100-minute time frame for this experiment. Subsequent trials showed constant results for several hours at room temperature with no ATP degradation.

![Figure 3](image)
extraction tubes can be frozen for later analysis (up to at least 6 months, with hold-time tests still under observation). The extraction procedure is thus quite similar to the technique for common chlorophyll a extraction in 90% acetone (Welschmeyer 1994; Chavez et al., 1995).

Figures 4 and 5 provide comparisons of the three extraction procedures, P-BAC, boiling Tris and LuminUltra (QGO-M), for natural samples and cultured phytoplankton, respectively. In all cases, P-BAC meets or exceeds the extraction efficiency of LuminUltra reagents while the P-BAC protocol also uses 10x less reagent volume, requires only a single dilution for proper reduction of the luminescence inhibition issue, and is easily stored frozen for later analysis if desired (see Appendix 1 for complete details of operating procedures).

![Comparison of P-BAC_60min, UltraLyse_60min, and boiling Tris ATP extraction efficiency](image)

**Figure 4.** Relative ATP extraction efficiencies on natural samples collected on GF/F glass fiber filters. Note that in all cases P-BAC yielded highest ATP concentrations.
Figure 5. ATP extraction efficiencies (normalized to 100) for three ATP extraction methods on cultured phytoplankton. Red bar sections refer to ATP measured in the LuminUltra cell softening rinse (see text). In all cases shown here, P_BAC extraction methodology yielded the highest ATP concentrations.
Important Considerations for Routine ATP measurements.

1. **Luminescence Inhibition.** The current optimized P-BAC extraction solution consists of 5% (w/w) phosphoric acid and 1% (w/w) benzalkonium chloride (BAC) combined in a solution of 25 mM Tricine (stock Tricine set to pH 7.8). This solution provides simple ‘wet soak’ extraction capabilities with no need for physical extraction assistance. However, like many mixed reagents used in ATP extraction fluids (including LuminUltra’s UltraLyse and LumiClean), the P-BAC mix (at full strength) will inhibit the output luminescence of the luciferin-luciferase reaction significantly. Fig. 6 shows the required Tricine dilution of the P-BAC solution to yield a constant, ATP-normalized, Response Factor (RLU/(ng ATP/L)); we recommend a 100x dilution to ensure minimization of the luminescence inhibition problem while maintaining a luminescence response that is well within the sensitivity range for analysis of treated ballast water. The standard dilution for sample extracts that we recommend is 10 µL P-BAC extract + 990 µL Tricine pH 7.8; for ATP calibration standards (1x10⁻⁷ M) the recommended dilution is 10 µL ATP std. + 10 µL P-BAC + 980 µL Tricine pH 7.8, thus creating ‘matrix-matched’ reagents that are equivalents with respect to P-BAC for both sample mixtures and ATP standard mixtures.

2. **Optics and sample volumes in ATP luminometry.** The reaction between luciferin-luciferase and ATP generates a luminescent signal, which is directly proportional to the mass of ATP contained in the sample matrix within the luminometer cuvette. However, it is important to recognize that the response is also 1) volume-dependent, 2) optically-dependent and 3) reagent-dependent on the final composition of analyte in the cuvette solution. All three of the above factors contribute effects to the small 150 uL droplet of fluid that is ultimately measured for luminescence as described in the recommended protocol (Appendix 1). Thus, the volumetric addition of enzyme and sample must be constant for all sample assays, including the ATP standard, which is used for generating the calibration response factor (RF). Specifically, the reagent chemistry and volume of the ATP standard must be mixed in identical proportions to the sample matrix for analysis in...
the luminometer. Linearity and proper calibration in ATP luminescent measurements can only be maintained with standardized 1) volumes, 2) cuvette shape and, 3) reagent chemistry; in particular, the latter must maintain uniform concentrations of P-BAC extraction fluid to normalize the luminescence inhibition properties of the P-BAC solution in both samples and standards (see Appendix 1).

PART 2. ATP MEASUREMENTS FROM SURFACE OCEAN SAMPLES AND BALLAST TREATMENT TESTS.

In this section we provide examples of ATP determination made on surface ocean samples collected during shipboard tests on the training vessel T/V Golden Bear, providing side-by-side comparisons of the new P-BAC extraction method and the popular ‘boiling Tris’ method. Examples are also given for full-scale ballast treatment tests using a variety of treatment technologies to demonstrate the quantitative reduction in ATP for treated ballast water relative to uptake water.

Fig. 7a gives the station locations for sea surface samples that were collected from T/V Golden Bear in summer 2014 while steaming from San Francisco to Busan, S. Korea. The corresponding data in Fig. 7b shows the relation between the P-BAC method and boiling Tris method for ATP determination; the derived regression relation shows that on average the P-BAC extractions yielded 6.2-fold more ATP than the traditional boiling Tris method for sample concentrations that range over 40-fold (from ca. 40 – 1600 ng ATP/L).

Figure 7. A) Station locations for sea surface samples collected during a trans Pacific crossing in April 2014. Samples were collected by surface bucket and were processed using GF/F filters for both P-BAC and boiling Tris ATP extraction methods. Extracts were frozen and analyzed 2 months later at Moss Landing Marine Laboratories. B) Regression of both extraction methods show 6.2-fold higher ATP values using P-BAC extraction.

Samples in warmer southern water were collected on the same cruise two weeks later during a transect from Manila to Saipan (Fig 8a). In this case P-BAC ATP levels were ca. 3.4-fold greater than those determined with boiling Tris.
Overall, our experiences thus far in evaluating ATP from P-BAC extractions and traditional Tris extractions have always shown P-BAC to be higher, by ca. 3x to 5x. The data given in Figs. 7 and 8 were collected on fine pore GF/F filters which capture most organisms down to a nominal size of 0.7 µm; the ATP concentrations from these open ocean sites thus reflect levels of ATP that might be deposited in local harbors after open-ocean ballast exchange (e.g., 100-300 ng ATP/L). The application of the new P-BAC ATP method to ballast water testing of regulated size classes of organisms is relatively straightforward. For simplicity, we have usually directed efforts to evaluating the 10-50 µm protist size class to maintain consistency with BWDSs for the same size class (see Part 3). This relatively narrow size range can be easily sampled by filter size-fractionation. A nylon mesh, 10 um pore-size filter (Millipore Corp. NY00010, 25mm dia.) provides fast flow rates and allows easy particle capture by simple gravity filtration. A nominal 600 mL filtration volume is expected to yield adequate biomass for determining living ATP-based biomass at levels well below BWDSs. The use of finer pore size filters would of course capture more particles and living biomass and could be viewed as a more stringent test of ballast treatment effectiveness; below, we will also provide examples of such ballast water data collected on glass fiber GF/F filters (nominal 0.7 µm pore) by vacuum filtration.

Ballast water treatment tests from ports visited during a Golden Bear training cruise to harbor locations in Busan, Kobe, Guam and San Francisco in 2013 are given in Fig. 9. The treatment system was oxidant-based, utilizing shipboard electrochlorination technology (Balpure system, Severn-Trent). During these early cruises we were still employing routine boiling Tris ATP extraction, since the P-BAC development was in its early stages. We discuss the early ATP Tris data in ballast treatment tests here specifically to highlight the obvious reductions in ATP that resulted from full-scale ballast water treatment systems (in this case, oxidant-based electrochlorination). In some cases in Fig. 9, the uptake ATP concentrations were reduced by over two orders of magnitude relative to discharge water by the treatment protocol, which included a 5-day ballast tank hold. Control samples in Fig. 9 refer to ballast tank water that
underwent no treatment, but which was held for 5 days in ‘control’ ballast tanks. In all cases shown in Fig. 9, IMO-approved fluorescein diacetate (FDA) cell counts passed BWDS with no detectable live cells. The Balpure system received IMO type approval in 2014.

Electrochlorination shipboard ballast tests:
ATP >10 um

Results from tests of a UV treatment system measured with all three ATP extraction protocols are given in Fig 10. Not too surprisingly, all three extraction methods showed the same general trend – a significant decrease in ATP of almost 2 orders of magnitude relative to the original uptake water. The experiment was made under full-scale processing of 200 m³ of uptake water aboard T/V Golden Bear, followed by a 5-day hold period in the ballast tank and a second UV irradiation during the discharge event. In this experiment the final discharge ATP concentrations were similar for all extraction methodologies (5-11 mg ATP/L) although P-BAC was again the superior extractant, as evidenced by the relative ATP concentrations measured (all samples were processed from the same continuously-integrated sample collections made over the full uptake and discharge events).
The large reduction in ATP after UV treatment application (fig. 10) is, of course, consistent with working concept of ATP-based tracking of live/viable biomass. However, the full-scale shipboard experiments reported here are not consistent with the laboratory, bench-scale UV experiments of First and Drake (2011) who also measured ATP (using LuminUltra products) and saw relatively little, or no change in ATP concentration after UV treatment utilizing small-scale germicidal lamps in a benchtop UV cross-linker device. We suspect that the hold-time of the samples after treatment in full scale testing could affect the results significantly. Treated water was held for 5 days in the ballast tank for all data given in Fig. 10. The result is typical of most ‘successful’ experiments we have executed, where 2- to 5-day hold times were practiced (‘success’ here is defined by numeric 10-50 µm counts that passed BWDSs made with cytometric/microscopic FDA-based live counts or MPN grow-out techniques). On the few occasions that we have measured ATP in land-based tests requiring ‘t-zero’ samples, where only one UV exposure was given and no hold-time was practiced (<4 hours between collection and extraction), the results were considerably more modest, with ATP only reduced by approximately half; we conclude that a short 4 h period between the last UV dosage and the immersion into extraction fluid is too short a period to allow full decomposition of cellular ATP.
Generally, our experiences has shown that all tests passing IMO and/or USCG BWDSs for the 10-50 µm size class have yielded treatment ATP levels that are quite low (<15 mg ATP/L) and clearly reduced relative to the uptake concentrations. Our objective in Part 3 of this report will be to provide the quantitative model that rationalizes a logical means of converting ATP concentration per se, as ng ATP/L, into equivalent numerical live cell concentrations (cells/L) reflective of regulated BWDSs.

We have tested four unique and independent treatment technologies that all show similar treatment results when evaluated by the P-BAC ATP protocol developed here (Fig. 11). Those treatment technologies include A) hypochlorite (from seawater electrochlorination), B) UV irradiation, C) chlorine dioxide and 4) high pH from NaOH treatment. All the experiments shown in Fig. 11 also yielded FDA/CMFDA live counts that passed BWDSs (e.g., <10 live cells in the 10 – 50 µm class).

**A. Hypochlorite treatment**

![Graph A]

**B. UV treatment**

![Graph B]

**C. Chlorine dioxide treatment**

![Graph C]

**D. NaOH treatment (freshwater)**

![Graph D]

Figure 11. Examples of successful ATP responses to 4 independent treatment technologies, A) sodium hypochlorite oxidation, B) UV irradiation, C), chlorine dioxide, and D) NaOH high pH. All samples were collected on 10 µm filters and processed for ATP extraction with P-BAC as described in this report.
PART 3. AN EMPIRICAL MODEL FOR CONVERTING ATP CONCENTRATIONS TO EQUIVALENT NUMERIC LIVE CONCENTRATIONS FOR BALLAST WATER COMPLIANCE TESTING.

The final objective in our project was to link the biochemical estimate of relative living biomass, based on ATP (ng/L), to the numerically-defined concentrations of regulated BWDSs, e.g., live cells/mL. Essentially, we ask - how can we convert a bulk measurement of ATP (ng ATP/L) to an equivalent ‘live numerical concentration’ (live cells/mL) for natural organisms of varying sizes? We start by simplifying the selection of an appropriate target for analysis on the basis of size as summarized in the color panel to the left (Fig. 12) In this model we take the position that the primary desire for compliance monitoring is that it be simple, convenient, and rapid. Thus, we eliminate efforts to measure zooplankton (>50 µm) because the required large sample volumes (several m³) preclude this organism size-class from ever being considered ‘convenient’ (Miller et al., 2011). That is, the sampling volumes, capture techniques (zooplankton nets or caged filters) and overly-specified requirements for isokinetic flow control and large piping specifications ensure that compliance monitoring of the >50 µm size class could never be termed, simple, convenient, and rapid. We eliminate the smallest size class (<10 µm) because existing BWDSs do not specify total counts, nor total biomass, of all living organisms within the <10 µm size class, but rather, the BWDSs identify three unique bacterial indicator species, E. coli, Enterococcus and Vibrio cholerae, some of which are rarely detected even in untreated challenge water. Instead, we have chosen to focus specifically on the 10-50 µm class because it represents the logical ‘target’ for simple, rapid tests. A critical (and simple) feature of the 10-50 µm group that we exploit here is its well-defined upper and lower size limits; it represents a relatively small ‘size bin’ within the almost infinite continuum of organism sizes found in oceans and lakes (from bacteria to whales). If we can define a mean size for the 10 – 50 µm group and if we can define the expected ATP cellular content for organisms of any specified size, especially for the ‘mean size’ within 10 – 50 µm, we can easily convert an integrated 10-50 µm bulk ATP concentration (captured by size fractionation) to its equivalent numeric live organism concentration.

Figure 12. Strategy for selecting a logical rapid compliance target among all regulated ballast water size categories.
The size distribution of organisms in aquatic environments has been studied extensively for decades (Sheldon and Sutcliffe, 1972; Platt and Denman, 1978; Gasol et al., 1997; Huete-Ortega et al., 2010). It is well known that the smallest organisms dominate numerically while larger organisms are scarce; the gradient from massively abundant to vanishingly rare is surprisingly predictable (Vidondo et al., 1997). However, the error limits of the existing size distribution models may be quite large when considering the full range of sizes in nature (from say, bacteria to whales), but within smaller limits (say for instance, our 10-50 µm ballast water category) we can constrain the uncertainty reasonably well. However, even within the relatively narrow 10-50 µm size class, the range of expected cellular traits, such as ATP per cell (pg ATP/cell), can vary widely because those traits are expected to scale with the biomass, or volume, of each cell (as predicted by simple geometry; vol = 4/3πr³). Thus, the calculated ATP content for 10 µm vs. 50 µm cells of equivalent spherical diameter (ESD) cells could potentially differ by 125x, a sobering disappointment if this represents the true expected error range that randomly sized cells might convey when we measure ATP collectively for all cells in the 10-50 µm size class. The microscopist may only see a 5-fold difference in the visible diameter of a 10 µm vs. a 50 µm organism, however, the measured biomass-related parameters of the cells, such as internal ATP content (related directly to cell volume), can differ by approximately two orders of magnitude between the extremes of 10 µm to 50 µm. One might ask, what if all the cells in a ballast water sample are 10 µm ESD; what if all the cells are 50 µm? Fortunately, we can quickly put this worry to rest, theoretically and empirically (see below).

The scientific literature (reviewed nicely by Blanco et al., 1994; Vidondo et al. 1997) clearly shows that the likelihood of finding 10 µm vs. 50 µm cells is not equivalent within the 10-50 µm size bin. In Figure 13 we give a representative size distribution model for coastal microorganisms based on over 40 years of empirical and theoretical analysis, summarized from over 30 careful studies listed in Fig. 13.

The early size distribution work of Sheldon et al (1972, 1977) led to the provocative hypothesis that the biomass of all organisms in the ocean (from bacteria to whales) was nearly equivalent across the expected size range of ocean biota (now known as the ‘Sheldon-type’ distribution). In other words, within the ballast water 10-50 µm size class, 10 µm cells are expected to be 125-fold more numerous than 50 µm cells, given a Sheldon-like biomass size spectrum. All recent size distribution analyses (by microscope, particle counter, flow cytometry and net sampling) have found that the Sheldon-type distribution actually underestimates the observed biomass levels of small particle size classes (Platt and Denman 1978, 1985; Cavender-Bares et al. 2001); see Fig. 13.

The estimated biomass concentration for any given cell size along a size distribution x-axis is commonly modeled as,

\[ \text{Eq. 1. biomass/Liter} = aW^b \] (see Fig. 13)
where a is a constant (an appropriate assumption for single-celled, poikilothermic protists within the size range 10-50 µm), W is body weight (or cell volume or biomass) and b is the exponent that defines the slope of the typical log-log plot of environmental biomass (y-axis) vs. cell volume (x-axis) (Blanco et al, 1994; Vidondo et al, 1997). The log-log Sheldon-like distribution has a flat slope of zero (b=0) when log biomass (y) is plotted against log cell volume (x). However, nearly all modern measurements have suggested that the observed planktonic size distribution is typically more negative; b ≈ -0.1 for coastal environments and b ≈ -0.2 for open
ocean (reviewed in Cavender-Bares et al. 2001). Thus, the true numeric organism concentrations are heavily skewed toward the smallest size classes (Platt 1985); e.g., the smaller the organism - the more abundant it will be (Fig. 13). Mathematical integration of the ballast-related, 10-50 µm biomass size distribution spectra described by Eq.1 and shown in Fig. 13 (in terms of numeric densities) yields a mean characteristic diameter (equivalent spherical diameter, ESD) of 16.52, 15.71, and 15.01 µm for b set to 0, -0.1 and -0.2, respectively. Given the uncertainties of the model above, we have rounded down, and assumed a convenient value of 15 µm (ESD) to be an appropriate mean size for the 10-50 µm size class (until otherwise proven incorrect). Corresponding probability calculations show that numeric counts of cells smaller than the mean diameters cited above should be observed with >80% likelihood; that is, the 10-50 µm ballast regulation class is expected to be dominated in biomass, and certainly in numbers, by small cells.

Now that we have defined and computed the theoretical mean diameter (ESD) of cells in the 10-50 µm size class, we can ask - does any of this hold true for ballast water samples? To test this, we analyzed 50 water samples from locations typical of marine harbors (Seattle WA, Port Angeles WA, San Francisco CA, Vallejo CA, Moss Landing CA, and Hundested, Denmark). The size distributions of live protists (FDA-tagged) were measured flow-cytometrically using a broad range of size calibration beads as in Cavander-Bares et al. (2001). The result of measuring over 174,000 individually-sized organisms is summarized in Fig. 14. We predicted a mean size for the 10-50 µm size class of roughly 15 µm and we measured (flow-cytometrically) a mean of 17.45 µm (Fig. 14); thus, within the error limits of the theoretical models, and the uncertainties of our measurements, our assumptions seem to be reasonable. We will continue to use 15 µm as a well-rounded (easy to memorize) estimate of the mean organism size (ESD) within the 10-50 µm regulated ballast water size class for the remaining discussion below.

It is clear that the mean cell size within the 10-50 µm class is likely to be significantly closer to 10 µm than to 50 µm for natural communities. Since the mean expected cell diameter of the 10-

![Figure 14. Flow-cytometric size distribution analysis of coastal harbor environments given in terms of numeric densities. Organisms were identified as live phytoplankton on the basis of red chlorophyll fluorescence and green fluorescein fluorescence from FDA viability tagging. (see text)](image-url)
50 µm class is calculated to range from roughly 15-17 µm this greatly reduces the >100-fold range of possible biomass-based responses, pessimistically estimated earlier for the extremes of 10 µm vs. 50 µm; the anticipated error range is now reduced to less than 2-fold, for mean sizes of 15-17 µm.

Our next task was to construct a model for predicting the ATP content (pg ATP/cell) for any cell size within the 10-50 µm size class, but especially the mean cell size. This task was accomplished empirically for the new P-BAC ATP method by measuring the ATP content and cell volume of several cultured marine and freshwater phytoplankton grown in the lab (Fig. 15). The data were produced by making simultaneous measurements of mean cell size (Coulter Z2 particle analyzer; cell volume displacement principle), fluorescence-based numeric cell counts (Applied BioSystems Attune flow cytometer) and cell-specific ATP content (pg ATP/cell) using the P-BAC extraction method. The resulting data in Fig. 15 show the expected linear cell-specific ATP content as a function of cell volume, with a regression slope (log-log) approaching

![Graph showing the relationship between log cellular ATP content (fg ATP/cell) and log cell volume.](image)

**Figure 15.** Log Cellular ATP content (fg ATP/cell) as a function of log cell volume. Cell volume was measured with a Coulter Z2 particle analyzer; cell numbers were enumerated flow cytometrically (Applied Biosystems Attune cytometer, red fluorescence). ATP was measured with the P-BAC extraction method. All phytoplankton cultures were grown under identical day/night incubation conditions and harvested in early exponential growth. Taxa included: (marine): *Amphidinium*, *Dunaliella*, *Isochrysis*, *Porphyridium*, *Prorocentrum*, *Tetraselmis*, *Thalassiosira pseudonanna*, *Thalassiosira weissflogii* (freshwater): *Cosmarium*, *Haematococcus*, *Scenedesmus*. Vertical dotted lines mark 10 µm and 50 µm size limits (equivalent spherical diameter, ESD). Red arrows show cell-specific calibration responses calculated for 15 µm ESD from regression equation. Y-axis gives cellular ATP as log (fg ATP/cell); the resulting regression prediction is converted to pg ATP/cell (specifically, 2.62 pg ATP/cell) to provide simple conversion of environmental ATP ballast water concentration measured as ng ATP/L into numeric concentration, as live cells/mL (see text).
1.0 (e.g., 1.0191 from regression eq. in Fig. 15). The predicted ATP content for the extreme size limits of 10 µm and 50 µm, calculated from the derived equation \( \log \text{ATP (fg/cell)} = 1.0191 \times \log \text{Vol (µm}^3) + 0.1096 \), and converted to pgATP/cell, are 0.760 and 104.4 pg ATP/cell respectively, giving a cell-specific ATP content that ranges 137-fold over the 10-50 µm size range; remarkably close to the range of 125-fold that would be calculated on the basis of geometry alone \( (\text{vol} = 4/3 \pi r^3) \).

The data in Fig. 15 are important; they form the quantitative foundation of the calibrated, rapid compliance tests described here. In Fig. 15 we see that the cell-specific ATP content for any cell size can be predicted from the regression equation given. In reverse logic, if one measures the ambient environmental ATP concentration (ng ATP/L) of an appropriately size-fractionated ballast water sample (e.g., 10-50 µm), one could calculate the equivalent number of cells required to generate that measured ATP concentration, for any specified cell size (cell volume). We choose to predict and use the ATP cellular content for the mean cell size within the 10-50 µm class (e.g., 15 µm); it serves as the best estimate of average conditions.

Conversion of ballast water ATP concentration to equivalent live cell concentrations. The final application of the ATP method for compliance checks against ballast water discharge standards (BWDSs) is simple. The calibration constant is given in Fig. 15 for the average 15 µm cell (ESD); it is 2.62 pg ATP/cell. The appropriate dimension for typical environmental ATP concentrations in natural surface water is ng ATP/L. Thus, for example, if one measures a concentration of 26.2 ng ATP/L within the 10-50 µm particle range for any ballast water sample (by 10-50 µm filter size fractionation) we would calculate the equivalent numerical live concentration to be:

\[
\text{Eq. 2} \quad \text{Live organisms/mL} = \frac{\text{Sample ATP (ng ATP/L)}}{(2.62 \text{ pg ATP/cell})}, \quad \text{or…}
\]

\[
26.2 \text{ ng ATP/L}/(2.62 \text{ pg ATP/cell}) = 10 \text{ live organisms/mL}
\]

For example, going back to the size-fractionated ballast water ATP measurements given in Fig. 11, we see that treated ballast water yielded 10-50 µm ATP estimates of 8.00, 0.07, 7.77, and 1.73 ng ATP/L; we would calculate (from Eq.2) corresponding estimates of live cell concentrations (referenced to 15 µm ESD) to be 3.05, 0.26, 2.97 and 0.66 live cells/mL, respectively. Thus, all tests in Fig. 11 would be judged to have passed the BWDS limit of 10 live cells/mL (all tests in Fig. 11, in fact, passed by flow-cytometric FDA numeric counting and/or MPN grow out technique).

Thus, within a narrow size range, such as the convenient IMO/USCG 10-50 µm range, we can argue that reasonable estimates of live numeric counts can be made by measuring the total live biomass, as ATP (ng/L), in a suitably size-fractionated sample followed by computation of the numerical equivalent concentration using the 15 µm size-based cellular calibration response of 2.62 pg ATP/cell, taken from the regression eqns. of Fig. 15 and substituted into Eq. 2.

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CONCLUSIONS

The principle objective of this project was to devise a simplified ATP method for shipboard ballast water compliance testing. To meet the objective above, the ATP method needed to be rapid, sensitive and free from the requirement for laboratory resources and complicated extraction techniques such as sonication, high heat, grinding, bead-beating, centrifugation, etc. The ATP method for ballast water compliance testing also required cell-specific calibration so that the measured ATP concentration could be converted to equivalent live cell concentrations consistent with numerical ballast water discharge standards (BWDSs).

The final ATP method described here results in a simple ‘wet soak’ extraction technique that yields ATP values roughly 3-5x higher than those derived from traditional oceanographic boiling Tris buffer protocol. The samples can be read immediately after a 30 minute wet-soak, or frozen indefinitely for analysis of large sample batches at a later time. All phases of the operation can be executed without power requirements using a battery powered laptop computer. The ATP measurement integrates all live organisms including heterotrophs and autotrophs.

A model for calculating equivalent live cell concentrations from biochemical ATP assays was constructed for the unique 10-50 µm regulated ballast water organism size class. The calibrated cell-specific ATP content (pg ATP/cell) for 15 µm equivalent spherical diameter cells, corresponding to the mean cell size within the 10-50 µm group, was 2.62 pg ATP/cell. Thus, a ballast water sample with a size fractionated (10-50 µm) ATP concentration of 26.2 ng ATP/L is calculated to be equivalent to 10 live cells/mL, corresponding to the ballast water discharge standard (BWDS) limit for the 10-50 µm size class. The ATP concentration of unknown ballast water samples can be compared to the equivalent ATP-based BWDS limit of 26.2 ng ATP/L for the purpose of quantitative compliance monitoring.
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**APPENDIX I**

ATP Standard Operating Procedure
Analysis of Adenosine Triphosphate (ATP) as a rapid, quantitative compliance test for ships’ ballast water
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Abbreviations:

ATP Adenosine Triphosphate
AMP Adenosine Monophosphate
BWDS Ballast Water Discharge Standard
ESD Equivalent Spherical Diameter
RF Response Factor (RLU/(ATP concentration))
RLU Relative Luminescence Unit (measure of luminescence)
SOP Standard Operating Procedure
USB Universal Serial Bus
PURPOSE

The determination of Adenosine Triphosphate (ATP) from natural, whole-cell protists serves as a rapid method to assess ships' ballast water for compliance relative to ballast water discharge standards (BWDSs). The Standard Operating Procedure (SOP) here provides details on the application of a newly developed ATP extraction assay for ballast water compliance testing. The method is specifically configured to yield easily measured ATP concentrations that scale proportionally with the total living protist biomass within the 10 - 50 µm size class. The method has been designed for ease of use on board ships or in non-laboratory settings. A calibrated conversion factor allows simple calculation of the equivalent numerical live concentration of cells in the 10-50 µm size class, thus providing compliance evaluation against BWDSs. The method responds to both heterotrophic and autotrophic living organisms within the 10-50 µm size class thus satisfying the requirements of US Coast Guard regulations for BWDSs.

SCOPE AND APPLICATION

The protocol described here utilizes a portable, but highly sensitive, hand held luminometer (LuminUltra Photon Master) to measure ATP response based on the well-established ATP luminescence method. The technique is based on enzymatic (luciferase) conversion of ATP to AMP with resulting luminescence generated in direct proportion to ATP concentration. This technique has been used for decades in marine science plankton work to assess total living biomass and has been reviewed extensively (Holm-Hansen 1971; Karl 1980). The key ingredients in the new method described here, include 1) the application of a simple ‘wet-soak’ extraction method that yields approximately 5x more ATP than previous extraction methods and 2) the use of modern synthetic luciferase enzyme reagents with a) stability for long-term frozen storage and b) extended room temperature stability during real-time assay measurements. The extraction is processed in miniaturized 1 mL volumes with no need for physical extraction mechanisms such as tissue grinding, sonication, bead-beating or high heat, thus providing easy execution on board ships where lab facilities are assumed to be absent.

The ATP concentration is determined from the response factor (RLU/(ng ATP/L)) derived from a stable ATP standard measured at the time of sample analyses. The conversion of ATP concentrations into numerical equivalent concentrations is based on the size-dependent relationship of cellular ATP content to organism cellular volume (biomass). In this case, the cellular ATP content was determined for a wide variety of marine and freshwater phytoplankton (protists) over a 1000-fold range in cell volume. The resultant size-dependent response curve provides a calibrated means of converting the measured concentration of ATP within the 10-50 µm size class to the equivalent numerical concentration of cells expected, on average, for the 10-50 µm size class (Fig. 1). Thus the biomass-based ATP concentration can be referenced to numerically-based BWDSs for the purpose of compliance evaluation.
The lower level of detection for this assay scales proportionally with the volume of sample filtered. At the nominal recommended filtration of 600 mL for ‘treated’ ballast water the technique is capable of resolving <1 ng ATP/L captured on a 10 µm filter, which corresponds to <0.4 live cells/mL (15 µm equivalent spherical diameter, ESD).

**METHODS**

The ATP concentration is determined in a 5-step process:

1) Collect ballast water organisms on a 25mm diameter, 10 µm pore-size filter by volumetric gravity filtration of 600 mL (this is the suggested volume for ‘treated’ ballast water).
2) Extract filters in 1 mL Reagent E (extraction fluid) for 30 min at room temperature.
3) Dilute extraction fluid 100x in Reagent B (dilution buffer).
4) Combine diluted sample (100 µL) with Reagent L (50 µL luciferase enzyme) in a disposable luminometer cuvette, followed by immediate 10 second reading on the LuminUltra Photon Master luminometer.
5) The resulting relative luminescence unit (RLU) derived from the luminometer is directly proportional to ATP concentration and is converted to the environmental ATP concentration (ng/L) by means of a calibrated response factor (RF) and simple analytical calculation.

It is suggested that all samples be collected, filtered and extracted as described in the completion of steps #1 and #2 above. At that point, the extraction tubes can be frozen indefinitely and processed in large batches at a later time, proceeding through steps #3, #4, and #5 above; or, unfrozen sample extracts may be processed in one batch at the end of the work-day.

**Setup**

Four reagents are used for the determination of ATP in sample ballast water:

1) Reagent E – Extraction Solution
2) Reagent B – Dilution Buffer
3) Reagent L – Luciferase Enzyme
4) ATP standard (Stock concentration: 1x10^{-7} Moles/L); the stock standard is diluted 100x to a final concentration of 507.18 ng ATP/L for routine calibration. (Molecular Weight of ATP = 507.18 g/mole)

Instrumentation includes:

1) Laptop computer with LumiCalc software installed; one USB port must be available to power the luminometer
2) LuminUltra Photon Master luminometer with disposable plastic round cuvettes. This instrument is powered through the computer USB connection, it contains no moving parts and requires no factory calibration services.
In preparation for sample measurements, all reagents should be thawed prior to use and equilibrated to room temperature. One vial of Reagent L (1.5 mL) will provide enough for ca. 30 assays. Once Reagent L is thawed, check for brown-colored precipitates that may appear in the bottom of the vial; they can be removed by actively rolling the Reagent L vial between the palms of your hands to warm the fluid and bring precipitates back into solution.

After booting up the laptop with Lumicalc software installed, the Photonmaster Luminometer can be plugged into the USB port; the yellow ‘Power’ lamp on the front panel of the luminometer should light. Once the Lumicalc software is started, the luminometer will light the green ‘Ready’ lamp on its front panel indicating communication has been established between luminometer and computer. The software will prompt for ‘Offline Mode’; hit ‘Continue’. Go to ‘Input/edit’ on the top ribbon bar; select ‘ATP Data’. Click on ‘Manual operation’ along the ATP Data ribbon; the instrument will be now ready for readings. Check the operation by clicking 'Read' and a luminescence value will be reported after a 10 second sample observation period; the reading with no cuvette should be <10 RLU. The Photon Master luminometer has no power button, nor instrument switches of its own; it is controlled entirely by the computer.

As noted above, the simplest processing scheme is to complete the extraction step for all samples, including a 30 minute room temperature extraction soak, followed by frozen storage for measurements in larger batches (frozen extracted samples can be stored for at least 1 year). Alternatively, once the extraction has continued for 30 min at room temperature, the 1 mL extraction tubes can be processed immediately for luminescence measurement or saved for ‘end-of-day’ processing in one batch; room temperature extracts should be read within 6 h of start.

**Operation**

1.1.1 *Sample collection*

Collect water samples in clean plastic containers with screw caps. Square polycarbonate 500 mL bottles are recommended for sampling 'treated' ballast tanks where the live organism concentration is anticipated to be low. The 500 mL bottle, when filled to the brim, provides nominally 600 mL fluid volume. This volume will provide adequate sample biomass to confidently detect <1 live organism/mL (15 µm ESD) from ‘treated’ ballast tanks. Collect sample water by passing the desired volume through a 50 µm nylon sieve, thus removing organisms >50 µm. If samples are to be stored for prolonged periods (1-2 days) before extraction, they should be kept in the dark at refrigerator temperature (ca. 4 degrees C) or, alternatively during sample transit, samples should be held in coolers with ice packs. Note: the coarse 50 µm filter will allow free gravity flow of sample ballast water into subsampling bottles; a small sheet of this filter material can be rinsed and reused. After use, the 50 µm sieve should be rinsed thoroughly with clean freshwater and dried before storage. The water may be processed immediately, as in 3.2.2. below, or stored at chilled temperature (ice cooler or standard refrigerator) for up to 48 h before processing.
1.1.2 **Sample extraction.**

Collect sample specimens by passing the desired sample volume through a 10 μm nylon filter as follows. Load the 10 μm nylon mesh filter into the plastic fritted filter funnel making sure that the filter is centered properly. Pour sample water through filter (nominal 600 mL filtration for ‘treated’ water); sample water should flow freely by gravity (see Section 8, Interferences). Remove filter from fritted base using stainless forceps, fold filter twice and insert filter into 1 mL Reagent E vial (extraction reagent); **tap vial on bench surface to ensure filter is fully immersed in extraction fluid** (all surfaces of filter must be in direct contact with Reagent E fluid to achieve complete cellular extraction). Extraction is complete in 30 min at room temperature. Samples may now be frozen for later analysis, or processed immediately for ATP determination.

1.1.3 **Sample dilution.**

Important: the extraction fluid yields highly efficient ATP extraction from all microbes, however, the sample extract must be diluted 100x with Reagent B (buffer) to reduce quenching of the luminescent assay reaction. Mix extraction vial either by vigorous shaking or vortexing, remove 10 μL of extract using precision pipett and add directly to prefilled dilution vials containing 990 μL Reagent B (dilution buffer); cap and shake well (final 100x dilution). This same dilution must be applied to the ATP standard during the calibration step (see below).

1.1.4 **Sample measurement.**

Carefully pipet 50 μL of Reagent L (luciferase enzyme) into a new, disposable luminometer cuvette; make sure to direct the fluid to the bottom of the cuvette, do not apply to side wall. Pipet 100 μL of the 100x-diluted sample (from 3.2.3., above) directly to the bottom of cuvette, mix gently for 3 seconds and insert into the luminometer optical chamber. Close the optics lid, click ‘Read’ on the Photon Master software. After 10 seconds, Luminescence will be recorded in the Photon Master data file. Dispose cuvette, and use new cuvettes for all subsequent samples. **Important:** use only one pipet for dispensing Reagent L; the luciferase pipet tip must not contact sample extraction fluids; otherwise, the luciferase reagent will become contaminated with a loss in luminescent activity.

**Calibration**

The ATP standard must be diluted 100x in a matrix-matched fashion, identical to the sample extract dilution. Add 980 μL Reagent B (dilution buffer) to a clean 1.5 mL vial, add 10 μL ATP standard solution and 10 μL Reagent E (extraction solution); mix thoroughly. The resultant 100x dilution of the stock ATP standard is now ‘matrix-matched’ to the sample extract due to the presence of 10 μL Reagent E in the diluted ATP mixture. Process the diluted ATP standard for luminescence determination in the same manner as sample extracts, e.g., 50 μL Reagent L + 100 μL diluted ATP standard added to a new, clean luminometer cuvette. The resultant luminescence response (relative luminescence unit, RLU) is used to generate the
response factor (RF) for calculation of environmental concentration of ATP in ballast water, where RF = RLU/(ng ATP/L). Ballast ATP is calculated as follows in Eq. 1;

\[
\text{(Eq. 1) Ballast Water ATP Concentration (ng ATP/L) =}
\]

\[
(\text{Sample RLU}/\text{RF}) \times \text{Extract Volume (L)} \times \text{Dilution (100)/Filtration Volume (L)}.
\]

Calibration should be performed for each batch of samples; the RF is expected to remain constant for at least 6 h at room temperature. If calibration is not possible, due to logistics such as time constraints, the expected RF for fresh Reagent L is approximately 100,000 RLU/(ng ATP/L) ± 20%. The final ballast ATP concentration could thus be calculated with 20% uncertainty if fresh Reagent L was used in the RLU determination for sample extracts.

**Maintenance**

The Photon Master luminometer has no maintenance requirements; it is a solid-state, computer-controlled device with no internal power supply. The instrument is expected to remain fully functional indefinitely, unless physically damaged. The luminometer performance is monitored through the empirical response factor, RF, generated during each calibration procedure. The expected nominal RF is approximately 100,000 RLU/(ng ATP/L) for fresh reagent standards prepared and measured as in Section 3.3. If the response should drop to 25% of that value, even with fresh reagents, contact LuminUltra Ltd. Canada for instrument service.

All reagents should be frozen when not in use (stable for at least 1 year when frozen).

**CALCULATION OF EQUIVALENT NUMERIC CELL CONCENTRATION**

The description below provides the means to estimate the equivalent numeric concentration of live cells corresponding to the measured ballast water ATP concentration. The exponentially-weighted mean cell size of the 10-50 µm plankton size class was calculated to be 15 µm (equivalent spherical diameter, ESD) based on plankton size distribution models for coastal systems (Welschmeyer 2016), which are characterized by a Log biomass vs. Log cell volume slope of -0.1 (Vidondo et al. 1997). A cell-specific ATP concentration of 2.62 pg ATP/cell for the 15 µm ESD cell size was determined empirically from cultured phytoplankton using the newly developed ATP extraction solution (Reagent E) and methodology described in this SOP (Fig. 1).

Thus, the total ATP concentration (ng/L) within the size-fractionated 10-50 µm size class, when divided by the mean cellular ATP quota of 2.62 pg ATP/cell, provides an estimate of the numerical concentration of 10-50 µm cells, assuming that 15 µm
is the mean cell size with the 10-50 µm fraction. For example, a measured concentration of 26.2 ng ATP/L, when divided by 2.62 pg ATP/cell is the equivalent of 10 live cells/mL, e.g., the allowable ballast water discharge standard for 10-50 µm organisms. ATP concentration significantly higher than 26.2 ng ATP/L for the 10-50 µm size class would constitute a likely non-compliant condition.

**METHOD DETECTION LIMITS**

<1 ng ATP/L, equivalent to ca. 0.5 live cells/mL for 15 µm ESD cells. (Assumes 600 mL filtered for extraction onto 10 µm filters). The detection limit is dependent on the volume filtered; e.g., the detection limit would be increased by a factor of two, by filtering half the volume of water.

**HEALTH AND SAFETY**

Reagent E (extraction solution) contains strong surfactants at an acidic pH; avoid skin contact.

**CAUTIONS**

Avoid skin contact with reagents.

**INTERFERENCES**

Reagent E is a strong extraction solution that provides efficient extraction of ATP from planktonic organisms. However, Reagent E also inhibits the luminescence of the luciferin-luciferase reaction. Reagent E must be diluted 100x with reagent B (buffer) **before** the addition of Reagent L (Lucifer in-luciferase) when luminometer readings are taken; otherwise the luminescence response will be lost.

The ATP assay is extremely sensitive and thus, easily contaminated with environmental ATP from biofilms from unclean surfaces; wear gloves, strive for a reasonably clean work area.

Reagent L is consumed in the presence of ATP. Therefore it is important to introduce only clean pipet tips into the Reagent L vial at the time of sample measurement; do not cross-contaminate pipet tips from sample extracts (containing ATP) into the Reagent L vial. If contamination is suspected, cap the Reagent L vial and invert to mix; recheck the calibration response factor with ATP standard as described Section 3.4 to check for loss of luminescence activity. Use the new response factor for all samples read **after** the contamination event.

Generally, the suggested 600 mL volume of treated ballast water will easily flow by gravity through the 10 um nylon mesh filters. However, silt laden ballast water may clog the filter before the full filtration volume is completed. In this case, choose a volume at least 3x smaller and repeat the filtration on a new filter. The ATP
sensitivity will still be adequate to detect equivalent numeric concentrations that are below the BWDS.

PERSONNEL QUALIFICATIONS AND RESPONSIBILITIES

Non-technical personnel can execute all aspects of the method; no special training or practice is required, though familiarization with pipet dispensing techniques should be reviewed prior to sample analysis.

SPECIAL EQUIPMENT AND MATERIALS

LuminUltra Photon Master Luminometer with data acquisition software.
Disposable plastic luminometer cuvettes.
Pipets capable of dispensing 10 µL, 50 µL and 100 µL.
Millipore NY00010, 25 mm, 10 µm pore size, nylon mesh filters,.
50 µm nylon sieve filter (50 µm on diagonal); this coarse filter can be reused after rinsing.

EQUIPMENT AND SUPPLIES

Instrument carry case
Squeeze bottle for rinsing labware (distilled water)
Plastic waste receptacle for fluids.
Test tube rack to hold samples
Sample extraction tubes, 1.5 mL, screw capped

QUALITY CONTROL

The luminometer response is not expected to drift. However, the luminescent reaction catalyzed luciferase will degrade over time as the luciferase ages; calibration of RF must be made during every measurement series.

REFERENCES


Fig. 1  Size-based measurements of cellular ATP content (fg ATP/cell). Cell volume was measured with a Coulter Z2 particle analyzer; cell numbers were enumerated flow cytometrically (Applied Biosystems Attune cytometer, red fluorescence). All phytoplankton cultures were grown under identical day/night incubation conditions. Taxa included: (marine): Amphidinium, Dunaliella, Isochrysis, Porphyridium, Prorocentrum, Tetraselmis, Thalassiosira pseudonanna, Thalassiosira weissflogii (freshwater): Cosmarium, Haematococcus, Scenedesmus. Vertical dotted lines mark 10 µm and 50 µm size limits (equivalent spherical diameter, ESD). Red arrows show cell-specific calibration responses calculated for 15 µm ESD from regression equations. The y-axis gives cellular ATP as log (fg ATP/cell); the resulting regression prediction is converted to pg ATP/cell (specifically, 2.62 pg ATP/cell) to provide simple conversion of environmental ATP ballast water concentration measured as ng ATP/L into numeric concentration, as live cells/mL. For example, ballast ATP concentration of 26.2 ng ATP/L corresponds to 10 live cells/mL [(26.2 ng/L)/(2.62 pg ATP/cell) = 10 cells/mL].