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Baseline

Validation of an adenosine triphosphate (ATP) model for 10–50 μm plankton

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ABSTRACT

A recent model demonstrated that the adenosine triphosphate (ATP) content of spherical aquatic organisms with a 10 to 50 μm diameter is between 0.16 and 19.9 pg cell^{-1}. Here, the model is validated by comparing microscopy-based counts with ATP concentrations from a commercial ATP kit.

The measured ATP content of both freshwater and marine organisms 10 to 50 μm size range falls in the 0.16 to 19.9 pg cell^{-1} model range. On average, freshwater organisms contain 0.33 pg ATP cell^{-1}, have a spherical equivalent diameter (SED) of 13 μm, while marine organisms have 0.89 pg ATP cell^{-1} and a SED of 18 μm. In addition, their 13 to 18 μm size is within the 10 to 50 μm ballast water size range and in agreement with the 15 μm mean SED of a coastal plankton size-distribution model.

This study concludes that the ATP-model is reliable, emphasizing the need for caution when converting three-dimensional biomass proxies into linear cell concentrations.

1. Introduction

Adenosine triphosphate (ATP) is a coenzyme known as the primary energy carrier molecule in all organisms, both prokaryotic and eukaryotic. A second role is to solubilize proteins in cells (Patel et al., 2017). As ATP is present in metabolically active cells, quantifying the amount of ATP can serve as an indicator of the number of living single-celled organisms. Furthermore, ATP has been established as a dependable marker for assessing living microbial biomass in aquatic environments. Recent studies have shown that the concentration of ATP varies among eukaryotic aquatic microorganisms depending on their cell volume. For instance, the ATP concentration may increase exponentially with an increase in cell volume of dinoflagellates, raphidophytes and chrysophytes compared to some diatoms species (Hyun et al., 2018) but remain relatively constant, as observed in diatoms and ciliates (Bochdansky et al., 2021). Because the analysis of ATP levels can be conducted within a short timeframe (<1 h) and is quite sensitive (able to detect 1–2 living cells), ATP bioluminescence-based methods have been suggested as a promising tool for conducting indicative analysis for ballast water compliance monitoring (van Slooten et al., 2015; Bradie et al., 2018a; Bradie et al., 2018b; Hyun et al., 2018).

Recently, a novel ATP model was introduced that computed the ATP content of aquatic organisms with a spherical equivalent diameter (SED) ranging from 10 to 50 μm to be 0.16 to 19.9 pg cell^{-1} (Peperzak, 2023). The selection of this size class aligns with the *International Convention for the Control and Management of Ships' Ballast Water and Sediments* or BWMC (IMO, 2004). Regulation D-2 of the BWMC specifies the required standards for ballast water discharge as follows: <10 viable organisms ≥50 μm in minimum dimension m^{-3}, <10 viable organisms ≤10–50 μm in minimum dimension mL^{-1}, and the maximum concentrations for three indicator microbes (IMO, 2004).

The ATP model consists of a number of basic calculations that were repeated twice as worst-case scenarios. The basic calculations consisted of the computation of 1) the volume of spherical organisms in 1 μm steps from 10 to 50 μm SED, 2) multiplication with the mean ATP concentration that was measured with hot-TRIS and hot-water extraction techniques in uni-algal cultures of *Thalassiosira weissflogii* (0.6 mM), *Skeletonema costatum* (0.6 mM), as well as in a combination of a) 13 other species of eukaryotic phytoplankton and b) 5 mixed assemblages (with a total of 65 species), that included both freshwater and marine species who were subjected to a range of light- and nutrient-limitations (0.6 mM) (Fig. 7 in (Bochdansky et al., 2021)) and 3) multiplication with the molar mass of ATP (507 g mol^{-1}). This first calculation resulted in an ATP content for 10 to 50 μm SED organisms between 0.16 and 19.9 pg

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cell^{-1} (Peperzak, 2023).

The model calculations were repeated twice with higher values for the ATP concentration to account for worst-case scenarios (underestimation of the ATP concentration). Firstly with the maximum ATP concentration (2 mM) measured in eukaryotic phytoplankton (Bochdansky et al., 2021). Secondly, the model was fed with values 1.5 and 3× the mean (0.6 mM) and maximum (2 mM) ATP concentrations, i.e. 0.9 to 6 mM (Table 2[1] in Peperzak (2023)). These higher ATP concentrations were chosen because ATP-extraction techniques other than hot-TRIS and hot-water, such as by P-BAC and in the Luminultra® assay, may yield more ATP.

The range in ATP content was also specifically reported for 33 and 15 μm organisms in the 10–50 μm SED range. These are the average sizes of spheres in that range (33 μm) and of coastal phytoplankton (15 μm) (Welschmeyer and Kuo, 2016) (Table 2[1] in Peperzak (2023)). The model results were compared with the (non)-compliance limits of a commercially available extraction Compliance Monitoring Device (CMD, Luminultra® ATP) as used in ballast water compliance tests to extract ATP using cell-grinding by beads followed by lysis with proprietary lysis solutions (Lo Curto et al., 2018).

In recent years, several compliance monitoring devices (CMDs) have been made commercially available to verify the efficacy of BWMS and assess compliance with Regulation D-2. These devices utilize indicators such as natural chlorophyll fluorescence, fluorescence from vital markers like Fluorescein Diacetate (FDA), or ATP, instead of directly quantifying organisms. However, CMDs that rely on fluorescence signals or ATP concentrations as proxies for quantifying living organisms face limitations in establishing a linear relationship. This is primarily due to their design, which aims to indicate pass or fail outcomes rather than providing a precise count of organisms (Casas-Monroy et al., 2023). A fundamental cause of this mismatch is the use of biomass proxies as an indicator of organism concentration: whereas Regulation D-2 follows a linear approach, measuring the number of organisms per mL, biomass proxies are directly linked to the three-dimensional volume of the microorganisms (Peperzak, 2023).

To determine if a sample is compliant with Regulation D-2, proxies such as chlorophyll and ATP, require either a conversion to organisms per mL or validation to establish a threshold analytical value such as maximum fluorescence. Conversion factors are technique- or instrument-dependent, determined by the CMD manufacturer, with the underlying basis being frequently unknown. CMD results are typically not expressed in organism concentrations per unit volume, but as compliance vs non-compliance (and sometimes a third intermediate value) indicating whether the ballast water sample tested complies with Regulation D-2. Recently, the IMO approved a standard protocol for testing the accuracy and precision of CMDs (IMO, 2023). In addition, the International Standards Organization published methods for evaluating the performance of CMDs (ISO, 2023). However, until now no such evaluations have been published and the IMO protocol and ISO methods do not directly assess the principles or calculations inherent to each device.

The aim of the current study is to validate the recent ATP model (Peperzak, 2023) using empirical data obtained during independent ballast water tests with natural samples where cell concentrations, in the 10 to 50 μm size range, analysed by microscopy (cells mL^{-1}), and ATP concentrations (pg mL^{-1}) analysed with a commercially available kit, were simultaneously measured. The combination of cell and ATP concentrations should render ATP content per cell (pg cell^{-1}). Because the cell sizes are constrained between 10 and 50 μm, the model predicts that the ATP content per cell should be between 0.16 and 19.9 pg cell^{-1}. If the ATP model is correct, it could be used to evaluate the sensitivity of thresholds set for CMDs using ATP as a proxy measure.

[1] The values in Table 2 are the ATP contents (pg ATP/cell) for 1 organism, not for 10 organisms/mL as in the legend.

2. Methods

The ATP model developed by Peperzak (2023), described above, was verified using organism concentration (cells mL^{-1}) of the regulated size class (here 10 to 50 μm), and the mean intracellular ATP concentration of eukaryotic phytoplankton (here 0.6 mM) as reported by Bochdansky et al. (2021).

Organisms concentrations (cells mL^{-1}) were determined using vital markers (e.g., FDA or FDA-CMFDA) and counted using epifluorescence microscopy. Simultaneously, ATP concentrations (pg ATP mL^{-1}) measured using the commercially available B-QUA kit (LuminUltra®, Canada) were obtained from two independent studies. Marine and freshwater data in Casas-Monroy et al. (2023) were collected in 2018 during ballast discharge after treatment under normal operational conditions of BWMS (primarily from ships arriving to Vancouver on the Pacific coast). Samples in 2019 were collected as paired uptake (before treatment) and discharge (after treatment) events from ships operating within the Great Lakes-St. Lawrence River. Samples in 2020 and 2021 were collected as natural water (not from ships) from Hamilton Harbour, Lake Ontario, and St. Andrews, New Brunswick, Canada. The data in Romero-Martinez et al. (2023, unpublished) were collected during a laboratory study using untreated and UV-treated natural marine Wadden Sea water.

Paired data covering a range of cell concentrations in both freshwater and marine water environments were analysed separately. The detection limits were 1 cell mL^{-1} for epifluorescence microscopy and 0.5 pg ATP mL^{-1} for the ATP estimation. Due to the wide concentration range (>3-log) of organisms, a log transformation was applied to the data: log(x + 1) for organism concentrations and log(y + 0.5) for ATP estimations. The transformed data were then analysed using a linear regression model fitted with the lm() function using R programming language (version 4.3, 2023). Extracted variables included mean, standard errors, t-values, and *P*-values for each dependent variable (see Annex 1). Next, the null-hypothesis was tested that there is no significant difference in the slopes (relation between the concentrations of organism and ATP) and intercepts (the ATP concentrations at zero organisms) of the freshwater and marine water samples, respectively. This test was done by multiplying the SE's with the t-value for the correct degrees of freedom ($df_{FW} = 23$, $df_{MW} = 27$) at a two-tailed probability of 0.05 to obtain the 95 % confidence intervals. If the intervals of two variables do not overlap the null-hypothesis is rejected and the variables are significantly different at $P < 0.05$.

The mean ATP content of the freshwater and marine organisms was calculated for 10 organisms mL^{-1} using the respective regression slopes. Because these slopes are the log-log ATP-organism concentrations, the antilog of the y-value (pg ATP mL^{-1}) was calculated and divided by 10 (organisms mL^{-1}). Next the SED's belonging to the calculated ATP contents for the freshwater and marine organisms were obtained from the ATP model (Peperzak, 2023).

3. Results and discussion

Linear regression analysis indicates a linear positive relationship between log-transformed organism and ATP concentrations (Fig. 1).

In freshwater samples, the slope (0.53 ± 0.25) was much lower, although not significantly, than in marine samples (0.93 ± 0.22). The probable reason is that in freshwater, contrary to marine environments, species such as diatoms are smaller (Litchman et al., 2009).

In a mixture of taxa and even in a singular species, the reported ATP contents will be variable. Part of the variability is related to the analysis, i.e. differences and variability in extraction procedures and variability in the luminescence assay (Bochdansky et al., 2021; Karl, 1980). ATP is present in the cell's cytoplasm and, therefore, the presence of vacuoles will decrease the ATP content of a cell. For instance, in diatoms the vacuole volumes differ intraspecifically (Strathmann, 1967), meaning that the cytoplasm volume and hence ATP content are variable in the

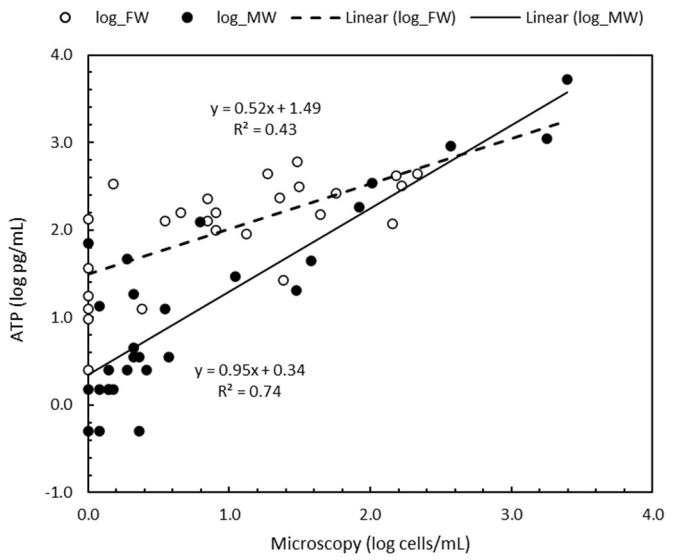

Fig. 1. ATP concentration as a function of the microscopically-counted vital organisms. Lines are linear regressions for freshwater (FW, o) and marine (MW, •) data separately.

same species. Cellular carbon and ATP are both measures of cell biomass. Because carbon: volume ratios differ between small and large diatoms and between phylogenetic groups (Menden-Deuer and Lessard, 2000) extra variance in average cellular ATP content is expected in mixed assemblages as likely to be encountered in ballast water testing.

In addition, the intercept in freshwater samples (1.47 ± 0.32) is significantly ($P < 0.05$) higher than in marine samples (0.35 ± 0.28). In linear terms, this is 1.1 (MW) to 30.6 (FW) pg ATP mL^{-1}, at a concentration of 0 organism mL^{-1}. The probable reason is that the freshwater samples contained cyanobacteria, and perhaps other <10 μm colonial (filamentous) species, which contributed to the measured ATP concentration because they were retained on the 10 μm filter during sample preparation. In microscope analysis organisms <10 μm (in minimum dimension) are not counted: they are not in the discharge standard of IMO. Colonies with individual cells of <10 μm will also not be counted. In addition, a 10 μm filter will retain some <10 μm species (Trindade de Castro and Veldhuis, 2019). Variation in community composition between freshwater and marine water systems is to be expected, with freshwater environments often having a higher abundance of cyanobacteria, which are typically smaller than 10 μm in size.

Based on the regression slopes, the mean ATP content of freshwater organisms is 0.33 pg ATP $cell^{-1}$ while the marine organisms have 0.89 pg ATP $cell^{-1}$. These values are in line with the ATP content calculated with the ATP model: 0.16 (10 μm) to 19.9 pg $cell^{-1}$ (50 μm). If there were to be 10 organisms in 1 mL of water, and each organism contributes either 0.33 or 0.89 pg of ATP, the total ATP concentration in the water would be 3.3 (FW) or 8.9 (MW) pg mL^{-1}.

The value of 0.89 pg ATP $cell^{-1}$ for marine species can be compared to a small number of available literature data. Using Baltic Sea data from Lo Curto et al. (2018), the ATP content was calculated to be 0.3 pg ATP $cell^{-1}$, equivalent to a SED of 12 μm (Peperzak, 2023). Maurer (2013) using the boiling TRIS method, reports a relation between ATP $cell^{-1}$ and cell volume in marine cultures. For a SED of 18 um (which we found for marine samples), this regression yields 0.46 pg ATP $cell^{-1}$, which is also lower than our 0.89 pg ATP $cell^{-1}$. Higher ATP and hence larger SEDs were found in two studies. Using the CellTiter-Glo 2.0 assay in marine samples, Hyun et al. (2018) found the correlation between ATP and cell concentrations to be 2 pg ATP $cell^{-1}$, corresponding 23 um SED organisms if the ATP concentration is 0.6 mM. Likewise, with data from a P-BAC ATP-extraction assay performed on marine samples (Bradie et al., 2018a), the calculated ATP content is 5–6 pg $cell^{-1}$ which is equivalent to cells with 34 um SED (using 0.6 mM ATP) or to ca. 1 pg ATP $cell^{-1}$ and 20 μm SED, using 3 mM ATP if P-BAC extraction leads to a 5× higher yield. Our value of 0.89 pg ATP $cell^{-1}$ for marine species appears valid because it is the median value of five studies. The calculated range of ATP contents emphasises the need for a simultaneous comparison and validation of the different ATP extraction and detection techniques (Peperzak, 2023).

Considering the baseline level of ATP that was found (Fig. 1), which can range from ca. 1 to 31 pg mL^{-1} (y-intercept), the total concentrations of ATP would be 10 (MW) or 34 (FW) pg mL^{-1}. These results suggest that ATP concentrations as low as 50 pg mL^{-1} can be an indicator of non-compliance with Regulation D-2. These findings may also explain the high number of false negative results reported recently by Casas-Monroy et al. (2023) for samples assessed with the B-QUA device using a compliance threshold of 500 pg ATP mL^{-1}.

Furthermore, it may be useful to set compliance thresholds independently for freshwater vs. marine ballast water samples to consider the variation in community composition and the sizes of organisms in different water systems.

4. Conclusions and recommendations

The accuracy of the ATP model, using spherical organisms and a 0.6 mM ATP concentration, is confirmed. The measured ATP content of both freshwater (0.33 pg ATP $cell^{-1}$) and marine (0.89 pg ATP $cell^{-1}$) organisms in the 10 to 50 μm size range fall in the 0.16 to 19.9 pg $cell^{-1}$ model range. This demonstrates the applicability to set limits to ballast water (non)-compliance for any CMD based on ATP.

The distinctions observed between freshwater and marine samples are linked to the diverse community composition in these ecosystems. The ATP model can now be used to set threshold limits for ballast water compliance. This threshold should account for both sensitivity and specificity, ensuring precise evaluations while accommodating potential variations arising from different environmental factors and sample types. A precise and ecologically relevant threshold can thus be established to ensure accurate assessments of ballast water compliance, reducing the likelihood of false negatives, and providing a more effective environmental protection.

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Code availability

Not applicable.

CRediT authorship contribution statement

Louis Peperzak: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Oscar Casas-Monroy:** Writing – review & editing, Investigation. **Sarah A. Bailey:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: L. Peperzak reports a relationship with Peterson Control Union that includes: employment.

Oscar Casas-Monroy reports there is no activity that may be interpreted as a conflict of interest.

Sarah Bailey reports financial support was provided by Transport Canada. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have

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Data availability

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Appendix A

For freshwater samples:

```
a<-lm(BQUAlog_FW_MW ~ Logmic, data=fw)
```

```
Call:
lm(formula = BQUAlog_FW_MW ~ Logmic, data = fw)

Residuals:
     Min       1Q   Median       3Q      Max
-1.06861 -0.26861 -0.01752  0.25693  0.92596

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   1.4686     0.1563   9.398 2.43e-09 ***
Logmic        0.5272     0.1227   4.295  0.00027 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.4772 on 23 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.4451,            Adjusted R-squared:  0.421
F-statistic: 18.45 on 1 and 23 DF,  p-value: 0.0002699
```

For marine water samples:

```
b<-lm(BQUAlog_FW_MW ~ Logmic, data=mw)
```

```
Call:
lm(formula = BQUAlog_FW_MW ~ Logmic, data = mw)

Residuals:
    Min      1Q  Median      3Q     Max
-1.0284 -0.3284 -0.1545  0.2150  1.4455

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   0.3544     0.1351   2.624   0.0141 *
Logmic        0.9348     0.1085   8.612 3.16e-09 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.5655 on 27 degrees of freedom
Multiple R-squared:  0.7331,            Adjusted R-squared:  0.7232
F-statistic: 74.17 on 1 and 27 DF,  p-value: 3.158e-09
```

Annex 1. Results from the analysis with R.

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