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Adenosine triphosphate (ATP) as a metric of microbial biomass in aquatic systems: new simplified protocols, laboratory validation, and a reflection on data from the literature

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Abstract

The use of adenosine triphosphate (ATP) as a universal biomass indicator is built on the premise that ATP concentration tracks biomass rather than the physiological condition of cells. However, reportedly high variability in ATP in response to environmental conditions is the main reason the method has not found widespread application. To test possible sources of this variability, we used the diatom *Thalassiosira weissflogii* as a model and manipulated its growth rate through nutrient limitation and through exposure to three different temperatures (15°C, 20°C, and 25°C). We simplified the ATP protocol with hot-water or chemical extraction methods, modified a commercially available luciferin-luciferase assay, and employed single-photon counting in a scintillation counter, all of which increased sensitivity and throughput. Per-cell ATP levels remained relatively constant despite changes in growth rates by approximately 10-fold in the batch culture (i.e., nutrient limitation) experiments, and approximately 2-fold in response to temperature. The re-examination of related literature values revealed that average cellular ATP levels differed little among taxonomic groups of aquatic microbes, even at the domain level, and correlated well with bulk properties such as elemental carbon or nitrogen. Fulfilling multiple cellular functions in addition to being the universal energy currency requires ATP to be maintained in a millimolar concentration range. Consequently, ATP relates directly to live cytoplasm volume, while elemental carbon and nitrogen are constrained by an indeterminate pool of detrital material and intracellular storage compounds. The ATP-biomass indicator is sensitive, economical, and can be readily standardized among laboratories and across environments.

When firefly beetles were ground up with sand and water, strong bioluminescence ensued with the addition of adenosine triphosphate (ATP), resulting in an almost perfectly linear concentration response (McElroy 1947). Subsequently, and because of the high sensitivity of the reaction, ATP was proposed as an indicator of microbial biomass on Earth, and to detect extraterrestrial life (Levin et al. 1964). Holm-Hansen and Booth (1966) adopted the ATP-biomass method for use with aquatic microbes. In aquatic systems, the ATP method is useful as it excludes the vast reservoir of nonliving organic carbon found in the detrital fractions (Yanada and Maita 1995; Volkman and Tanoue 2002).

Over the decades, the method has been applied in a variety of aquatic habitats, such as oceans, lakes, and streams (Hobbie et al. 1972; Karl et al. 1977; Taylor et al. 1986; Hewes et al. 1990; Karl et al. 1991; Hammes et al. 2010; and routinely at the Hawaiian Ocean Time Series station). The method has proved especially effective in systems where biomass estimates with other methods were exceedingly difficult to employ, such as in soils (Dyckmans et al. 2003), on mineral particles (Taylor and Gulnick 1996), in benthic environments (Bernhard 1989), sediment traps (Taylor 1989), the cryosphere (Helmke and Weyland 1995), activated sludge (Chen 2000), ballast water on ships (Bradie et al. 2018), groundwater (Eydal and Pedersen 2007; Pedersen 2013), drinking water (Siebel et al. 2008), and the deep biosphere (Kimura et al. 2003).

In a major review of cellular nucleotides in marine systems, Karl (1980) concluded that, "although the accuracy of the ATP-biomass technique has been questioned and numerous

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difficulties in its application have been cited, it is still by far the most convenient and reliable method for estimating total microbial biomass in most environmental samples." Curiously, the same publication has been frequently cited by critics of the ATP-biomass method.

Our perception of the role of ATP has undergone a paradigm shift in recent years. ATP occurs in much higher concentrations in cytoplasm than is required for energy metabolism alone (Patel et al. 2017). In addition to being the universal energy currency, other functions have been attributed to ATP, such as serving as a biological hydrotrope to keep hydrophobic proteins dissolved in cytoplasm (Patel et al. 2017; Pu et al. 2019). This role requires ATP concentrations to be homeostatically maintained at millimolar concentrations, which aligns well with the application of ATP as a biomass indicator. With these more recent findings as a backdrop, we revisited the ATP-biomass method, simplified extraction and measurement procedures, tested the variability of cellular ATP concentrations in laboratory experiments with a diatom, and critically examined previously published values.

Use materials and procedures

Culture of diatoms

Xenic cultures of *Thalassiosira weissflogii* were grown in an f/2$^+$ medium (Guillard 1962) in 34 psu GF/F-filtered artificial seawater (Instant Ocean) at 66 μmol m^{-2} s^{-1} continuous illumination. For the first batch culture experiment (i.e., the nutrient limitation time course), diatoms were cultured in 6 × 2 L Erlenmeyer flasks, covered with aluminum foil, and filled with 1 L of f/2$^+$ medium. Then, 1 mL of an exponentially growing starter culture was transferred to each flask as inoculum, and the experiment was conducted at room temperature (approximately 20°C). For the second set of experiments, diatoms were grown in 2-liter flasks containing 1 liter of f/2$^+$ medium with loose plastic screw caps at the same illumination. Two flasks for each temperature treatment were immersed in water baths at 15°C, 20°C, and 25°C with temperature-controlled baths. Separate smaller batch cultures of the same alga grown in an f/2$^+$ medium at room temperature and the same light conditions were used to test extraction and analytical procedures.

Extraction of ATP

A variety of extraction procedures were tested; however, most resulted in a strong inhibition of the luciferin-luciferase reaction. Inhibitory effects were observed with detergents such as Triton X-100 and Dodecyltrimethylammonium bromide (DTAB), as well as with digestion with acid and bases that needed to be neutralized in a separate step, and thus, had a high salt content. These chemicals interfered with the bioluminescence assay in a similar way to the chemical extraction procedure discussed below. We found that a simple extraction procedure using boiling-hot ultrapure water was highly compatible with the ATP assay, corroborating the findings by Yang et al. (2002). We also explored a chemical extraction procedure using P-BAC, a mixture of benzalkonium chloride (1% w/w fin. conc.) with phosphoric acid (5% w/w fin. conc.) in a 25 mM Tricine buffer (Welschmeyer and Kuo 2016). Note that the density of 85% phosphoric acid is 1.6845 kg L^{-1}, and concentrations are expressed in weight percentages.

To compare the hot-water with the P-BAC extraction protocols directly, we designed an experiment in which the concentration of ATP is the same for the final step of the luminescence assay (Fig. 1). For these experiments, 5 mL each of six samples was filtered onto GF/F in the same filter rack (6-positions of 25 mm glass filtration manifold fitted with a stainless-steel grid [Millipore]) and using the same vacuum. The time required from transferring the filters from the filter rack to the first extraction step was also the same in the liquid-nitrogen hot-water and P-BAC procedures. For the hot-water extraction procedure, the filters were placed in 15 mL polypropylene centrifuge tubes (Falcon) and shock frozen in liquid nitrogen. The centrifuge tubes can be stored at −80°C or used after a few minutes. However, boiling-hot ultrapure water must be poured directly on the still frozen filters to inactivate ATPases and not be thawed slowly. The centrifuge tubes were then vortexed for 10 s each and immersed in a beaker with boiling-hot water for ~ 15 min (Fig. 1A). After cooling to room temperature, ultrapure water was added to the 5 mL mark in the centrifuge tubes and vortexed again for a few seconds to mix the sample homogenously. For P-BAC extraction, samples were soaked at room temperature for ~ 30 min in cryovials filled with 1 mL P-BAC (Welschmeyer and Kuo 2016; Fig. 1B). Samples can be stored frozen at this stage or analyzed after the extraction interval. For the final analysis using the firefly reagent, 50 μL from the hot water or 10 μL from the P-BAC extractant were used. This approach kept the ATP concentrations the same in the final analytical step for both extraction methods (i.e., 50 μL from 5 mL extractant = 10 μL from 1 mL extractant). To a subset of samples, a 50 μL of standard (ranging in concentrations from 3.24 to 162 nM) depending on the type of sample, was added, chased with 3 mL of ultrapure water for dilution (*see* the Discussion section for the requirement of a dilution step in the P-BAC procedure). Finally, 50 µL of firefly extract was added (CellTiter-Glo 2.0). For test samples, we used cultures of *T. weissflogii* grown in an f/2$^+$ medium, collected surface water from the Elizabeth River at the Old Dominion University Sailing Center (16 September 2020, Latitude: 36.885 N, Longitude: 76.308 W, salinity: 16.4 psu, temperature: 24.0°C), and mesopelagic water offshore at 300 m (06 October 2020, latitude: 36.734 N, longitude: 74.629 W, salinity: 34.4 psu, temperature: 12.7°C).

Growth rate experiments with *T. weissflogii*

In all experiments, sampling occurred daily, beginning at the day of inoculation of the f/2$^+$ medium. After agitating the flasks to resuspend and evenly mix settled cells, 25 mL were withdrawn from each flask using a sterile disposable pipette.

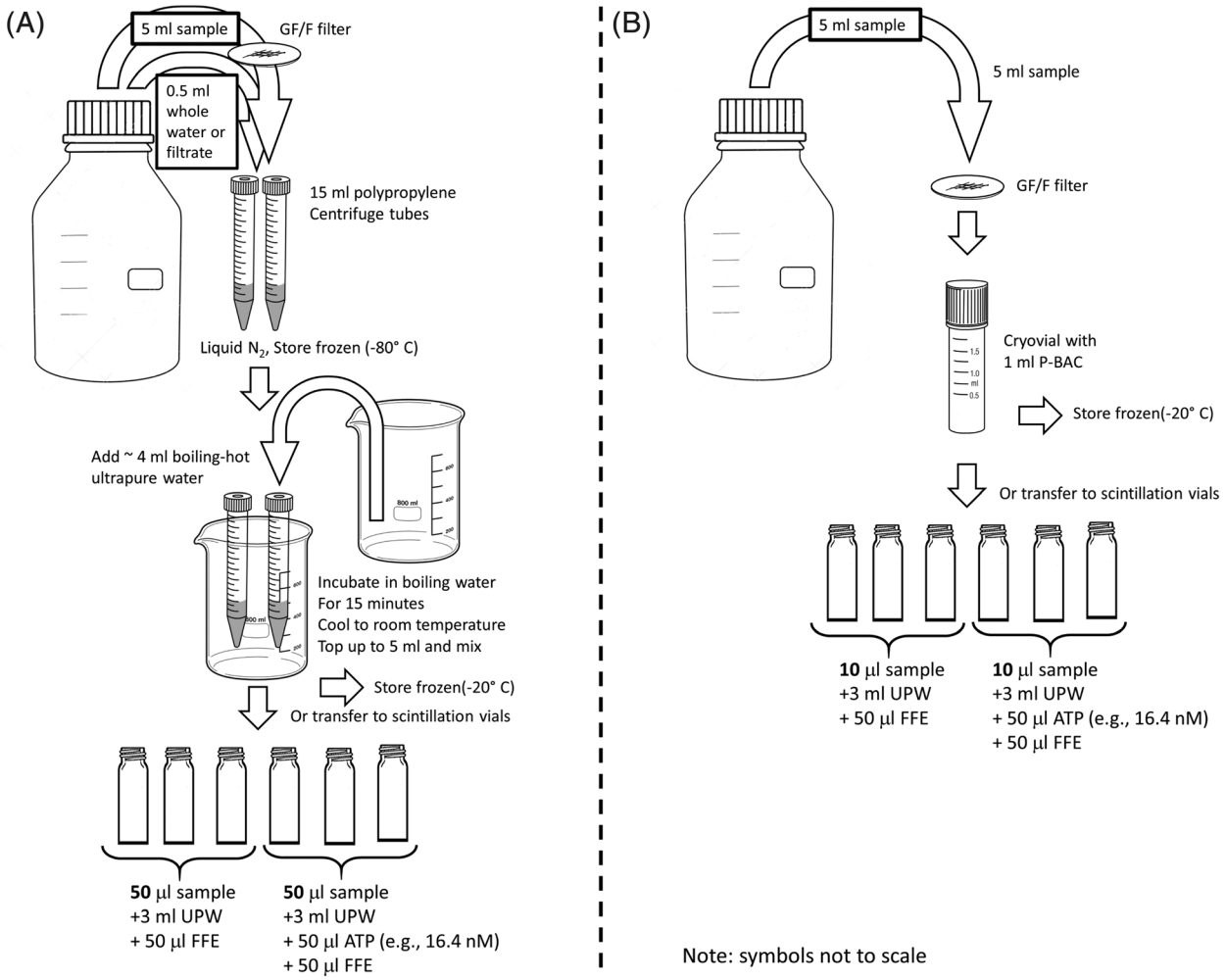

Fig 1. Workflow of ATP extraction and analysis. (**A**) GF/F filter, whole water, or the filtrate are extracted in hot ultrapure water (UPW). Liquid nitrogen pretreatment increases extraction efficiency to almost that of the P-BAC chemical extraction. (**B**) The P-BAC extraction method for particulate ATP is simple and useful in the field where no liquid nitrogen or hot water is available. The P-BAC method does not allow for assessment of the dissolved fraction. A known quantity of ATP as precisely determined by spectrophotometry is added as internal standard to a subset of the samples, followed by firefly extract (FFE, e.g., Cell-Titer Glo 2.0).

Then, 5 mL were immediately filtered through a 25 mm diameter, 0.2 μm polycarbonate filter (Isopore type GTTP) at a vacuum of 200 mbar in a filter station preloaded with six filters. The filtrate was captured in 15 mL polypropylene centrifuge tubes (Falcon) placed below the filter funnel inside the vacuum flasks. The filters were quickly transferred into 15 mL polypropylene centrifuge tubes, and $\sim$ 4.5 mL boiling-hot ultrapure water (18.2 MΩ) was added. The centrifuge tubes were vortexed for a few seconds and then immersed in a beaker containing hot water (> 90°C). The hot ultrapure water breaks up cells and inactivates ATPases (Yang et al. 2002). For unfiltered whole water, triplicate samples of 0.5 mL were added to 15 mL centrifuge tubes chased by $\sim$ 4 mL boiling-hot water and vortexed. Of the filtrate, 0.5 mL was transferred to the centrifuge tubes and treated in the same fashion as the whole water samples. All tubes were kept in hot-water baths for approximately 15 min. The samples were subsequently cooled to room temperature and analyzed the same day. Since ATPases are largely inactivated at this stage, samples can also be frozen at −20°C or −80°C for later analysis if desired. Using a muffled Pasteur pipette, ultrapure water was added dropwise to exactly the 5 mL mark (markings on the 15 mL Falcon centrifuge tubes are sufficiently accurate). For analysis, 50 μL of sample, 3 mL of ultrapure water, and 50 μL of the ATP working stock (for the internal standard only, see below), and 50 μL of firefly extract (CellTiter-Glo 2.0, Promega Corp.) were combined in 6 mL plastic scintillation vials (Pico Prias, Perkin Elmer) and briefly vortexed.

We programmed the scintillation counter to run samples in sequence repeatedly up to 10 times, and only used the data of the second cycle. The other cycles were used to observe the decaying luminescence signal (Supplementary Fig. 1).

Repeated runs are typically unnecessary, and if samples are run only once, we recommend providing a reaction time of approximately 30 min before the first vial is counted to count over the more linear time trajectory. We always alternated samples and corresponding internal standards.

Standards

Adenosine-5′-triphosphate disodium salt hydrate (Millipore Sigma) was dissolved in ultrapure water (Barnstaed Nanopure, 18.2 MΩ) and serially diluted to a stock concentration of ~ 10 μM using ultrapure water. The stock solution was then divided into individual 15-mL polypropylene centrifuge vials and stored frozen at −20°C. The exact concentration of the ~ 10 μM standard was determined using a spectrophotometer (Shimadzu UV-2401PC) at a wavelength of 259 nm, a 1 cm cuvette and a molar absorptivity coefficient of 15.4×10^3 M^{-1} cm^{-1} (Karl 1993) in the equation

$$C = \frac{A}{l \times e} \quad (1)$$

where C is the molar concentration of ATP (M), A the absorption, l the path length of the cuvette (cm), and e the molar absorptivity coefficient (M^{-1} cm^{-1}). Even after many repeated thawing and freezing cycles over the course of 1 yr, the concentration of the stock solution remained exactly the same (i.e., 16.4 μM). A working stock solution was prepared daily by diluting 250 μL of the stock solution to 250 mL with ultrapure water in a volumetric flask (1000× dilution to 10 nM). This working stock was added as the internal standard (see above).

Calculations

ATP concentrations in the samples were calculated as displayed in Eq. 2:

$$\mathrm{ATP(nM)} = \frac{[\mathrm{ATP}] \times \left(\mathrm{CPM_{sample}} - \mathrm{\bar{C}PM_{blank}}\right)}{\mathrm{\bar{C}PM_{standards}} - \mathrm{\bar{C}PM_{samples}}} \frac{V_{\mathrm{std}} \times R}{V_{\mathrm{extr}}} \quad (2)$$

where [ATP] is the concentration of the internal standard (i.e., 16.4 nM), $\mathrm{CPM_{sample}}$ the photon counts per minute for the sample, $\mathrm{\bar{C}PM_{blank}}$ the average value of 4 to 6 blanks (50 μL of firefly reagent added to 3 mL ultrapure water only), $\mathrm{\bar{C}PM_{standards}}$ the average counts per minute for the standard vials, $\mathrm{\bar{C}PM_{samples}}$ the average value of replicate samples, V_{std} the volume of the standard added to the scintillation vial in μL, R the ratio between volume of the extract (numerator) and volume of sample filtered (denominator), and V_{extr} the volume of the extract added to the scintillation vial (μL). If many samples are counted, as in our case, the most accurate procedure is to calculate the standard values by using a regression equation with time stamp of counting as explanatory variable and then applying this regression to every sample based on its time stamp in the counting protocol. This procedure, rather than using the values from the adjacent vials, reduces variance caused by standard-to-standard variability. However, if ATP concentrations are expected to be very different between samples, we recommend subtracting the values of the individual vials from those containing the internal standards instead. To arrive at the final ATP values in the results, the values were further multiplied by two to correct for the extraction efficiency difference between material collected on filters and whole water samples (Fig. 2). This factor was highly consistent between the two main experiments and in other experiments with *T. weissflogii*.

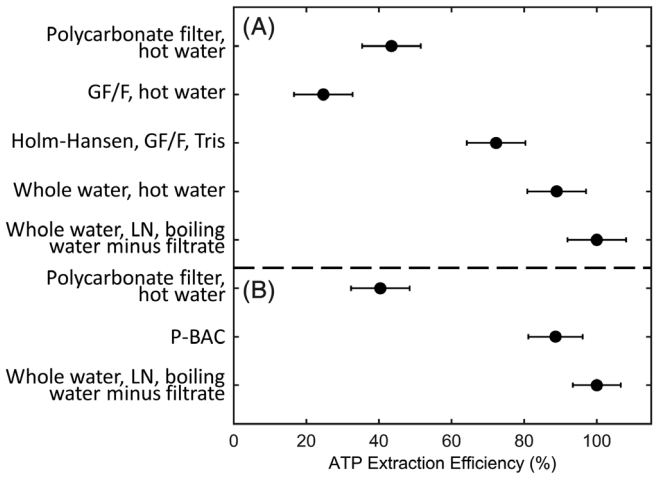

Fig 2. Efficacy of the extraction protocols tested on *Thalassiosira weissflogii* using filtered (5 mL each) and unfiltered (whole water, 0.5 mL each), the latter of which was corrected for ATP in the filtrate (dissolved ATP). Data of the two experiments (**A** and **B**) were normalized to 100% extraction efficiency as defined by the whole water minus filtrate treatment. Data points represent means, and error bars indicate comparison intervals of a Tukey–Kramer multiple comparison test. Where intervals overlap, the difference between treatments is not significant at the $\alpha = 0.05$ level. The GF/F-Tris treatment is the classical Holm-Hansen extraction procedure (Karl 1993). P-BAC = chemical extraction according to Welschmeyer and Kuo (2016).

Cell counts

T. weissflogii was enumerated in a Z2 Coulter Counter Multisizer with a 100 μm orifice tube. Seven measurements each were made for particle sizes larger than 10 μm, the first values discarded, and the next six measurements recorded (the first measurement may still contain air bubbles).

Growth rate was calculated applying first-order kinetics,

$$g = \frac{1}{T} \ln\left(\frac{N_t}{N_{t-1}}\right) \quad (3)$$

where g is the instantaneous growth rate (day^{-1}), T is the time interval between samples (days), and N_t and N_{t-1} are the abundance of cells mL^{-1} at time t and $t - 1$.

Then, 5 mL of each sample was preserved in 2% (fin. conc.) 0.2 μm syringe-filtered formaldehyde stabilized with 10% methanol for bacterial counts. The following day, 1 mL of the formaldehyde-preserved sample was filtered onto 25 mm diameter 0.2 μm pore-size black polycarbonate filters (Isopore type GTBP). The filters were transferred onto microscope slides. One or two drops of a mounting medium containing DAPI (Vectashield, Vector Laboratories) was added, covered with a cover slip, and stored at −20°C until counting. Bacteria were enumerated under an epifluorescence microscope (100 × oil immersion objective lens, 2 × loupe, 10 × ocular magnification, equipped with a DAPI filter cube) by counting 60 fields (0.025 mm^2 each) per slide (30 vertical, 30 horizontal across the filter). Field sizes were determined using a stage micrometer with 0.01 mm increments.

T. weissflogii carbon was calculated using the equation provided by Strathmann (1967) as 110 pg $cell^{-1}$ based on a diameter of 15 μm. This per-cell carbon value matched well the raw data on *T. weissflogii* (formerly *Thalassiosira fluviatilis*) in Strathmann (1967) and was very similar to the values calculated using equations reported in Menden-Deuer and Lessard (2000; average between regressions of small and large diatoms). For prokaryotes, 20 fg carbon $cell^{-1}$ was used. This number is suitable for bacteria grown in culture but likely lower for prokaryotes found in the wild (Fukuda et al. 1998).

Literature comparisons

To convert ATP per dry weight from various literature sources (e.g., tables in Karl 1980) into molar concentrations (moles of ATP per-cell volume), we needed to make assumptions regarding the dry mass density per-cell volume. We used a universal conversion of 0.2 g cm^{-3} (pg μm^{-3}) dry-matter content with the acknowledgment that the dry-weight to volume ratio may change from 0.1 to 0.57 g cm^{-3} (Bratbak and Dundas 1984; Norland et al. 1987; Simon and Azam 1989). A factor of 0.2 g cm^{-3} is also a suitable approximation for bacteria given a similar wet-weight to dry-weight relationship (Bakken and Olsen 1983). To test the conversion factor on algae, we calculated the dry-weight to volume ratio of a wide range of phytoplankton from small to large, from *Thalassiosira pseudonana* to *Lingulodinium* (previously *Gonyaulax*) *polyedra* using the allometric volume to carbon conversions in Menden-Deuer and Lessard (2000). Using a 50% carbon to dry-weight conversion, we arrived at 0.26 g cm^{-3} for *T. pseudonana* (the smallest alga in Menden-Deuer and Lessard 2000) and 0.13 g cm^{-3} for *L. polyedra* (the largest alga in Menden-Deuer and Lessard 2000), which brackets the conversion factor we used. For *Skeletonema costatum*, we used the mean of ATP (0.041 pg $cell^{-1}$; Sakshaug 1977) and the range of cell volumes for *S. costatum* as reported in Strathmann (1967) to calculate molar concentrations of ATP. In the absence of other information for ciliates, we used the lorica volume of tintinnid ciliates in Verity and Lagdon (1984) to calculate molar concentrations of ATP from pg per cell in that taxonomic group.

Statistical analysis

Residuals were tested for normality using the Komolgorov–Smirnov test (Lilliefors 1967). Since, in several cases, residuals did not conform to the normality assumption of parametric tests, *p*-values were based on 10,000 randomizations of the data and custom *F*-distributions (Manly 2007).

Assessment

Test of the extraction protocols

In extraction experiments with *T. weissflogii*, we achieved the highest ATP yield with (1) whole samples of the cultures shock frozen in liquid nitrogen with subsequent hot-water extraction; (2) GF/F-filtered cultures boiled in Tris buffer for 5 min according to the protocol by Holm-Hansen and Booth (1966); (3) the P-BAC extraction protocol; and (4) the liquid nitrogen hot-water (LNHW) extraction on a GF/F filter (Fig. 2; Table 1). ATP retained using 0.2 μm pore-size polycarbonate filters was twofold lower than the P-BAC protocol and

Table 1. Comparison of hot-water extraction with liquid nitrogen pretreatment (LNHW) and phosphoric acid-benzalkonium chloride in Tricine buffer (P-BAC) extractions on a diatom culture (T.w. = *Thalassiosira weissflogii*), a sample from the brackish Elizabeth River (E.R.), and an offshore sample from the mesopelagic (300 m depth, Mesopel.). *n* = number of samples. All samples were filtered onto GF/F filters prior to extraction.

		LNHW extraction		P-BAC extraction		Ratio	Wilcoxon rank-sum
Sample	*n*	Mean ATP concentration (nM)	SD ATP concentration	Mean ATP concentration (nM)	SD ATP concentration	P-BAC/LNHW	z-value, *p*
T.w.	12	88.16	12.17	104.98	28.96	1.19	−4.19, $p<0.0001$
T.w.	12	70.33	6.27	84.30	14.21	1.20	−4.19, $p<0.0001$
E.R.	12	4.20	0.40	4.93	0.36	1.17	−4.19, $p<0.0001$
Mesopel.	12	0.22	0.023	0.26	0.015	1.18	4.19, $p<0.0001$

the boiling-hot water extraction methods (Fig. 2). The lowest ATP values were observed using GF/F filters in combination with hot-water extraction but without the boiling-water bath. The coefficient of variation (i.e., the percent standard deviation of the mean) was lowest for the hot-water extraction of the GF/F filters (CV = 9%), followed by P-BAC (CV = 10%), the whole water extraction after liquid-nitrogen fixation (CV = 11%), and the hot-water extraction of the polycarbonate filters (CV = 12%). The traditional method of boiling in Tris buffer displayed the highest coefficient of variation in replicate samples (CV = 27%). The difference between ATP obtained by whole water extraction and ATP retained on polycarbonate and GF/F filters cannot be explained by ATP in the filtrate as they only made 5.7% and 0.9% of the ATP in this stationary phase diatom culture, respectively.

Comparing the LNHW and the P-BAC extraction methods directly using GF/F filters, in both cases the P-BAC extraction was highly efficient and yielded ATP values $\sim 20\%$ higher than the LNHW procedure (Table 1). The tradeoffs of efficiency and practicality are discussed in detail in the Discussion section. P-BAC exerted a much greater matrix effect than samples treated with pure water. In situations with complex matrix effects, it is advisable to use either the Internal Standard Method (ISM), or the Standard Addition Method (SAM) instead of external standards (Harris and Lucy 2020). The SAM, however, requires multiple standard additions for each sample and is, thus, impractical with large sample numbers. We were able to validate the ISM with the SAM method (Supplementary Fig. 2) and, thus, recommend using internal standards for both hot-water and P-BAC extraction protocols.

Batch culture experiments with *T. weissflogii*

In six replicate cultures, *T. weissflogii* grew exponentially until day 6 and then continued with a linear growth pattern (Supplementary Fig. 3). The relationship between ATP and cell numbers remained linear as the slope after the log–log transformation was close to 1 (i.e., 1.079; Fig. 3A). The instantaneous growth rate dropped from an average maximum value of 1 to approximately 0.1 d^{-1} during the experiment (Fig. 3B). There was no apparent decrease in ATP levels despite the abrupt 10-fold decrease in growth rates (Fig. 3C). There, however, was a significant, gradual, and small increase in cellular ATP levels over the course of the experiment, although the regression was driven primarily by a few low values on day 2 (Fig. 3C). In all incubations, the contribution of bacteria to total carbon biomass based on 110 pg $cell^{-1}$ in *T. weissflogii* and 20 fg $cell^{-1}$ for bacteria ranged between undetectable and 0.6% of total carbon and was, thus, negligible (Supplementary Fig. 4).

Effect of temperature-induced growth rate variability on ATP levels

In experiments at three different temperatures (15°C, 20°C, and 25°C), *T. weissflogii* initially followed an exponential

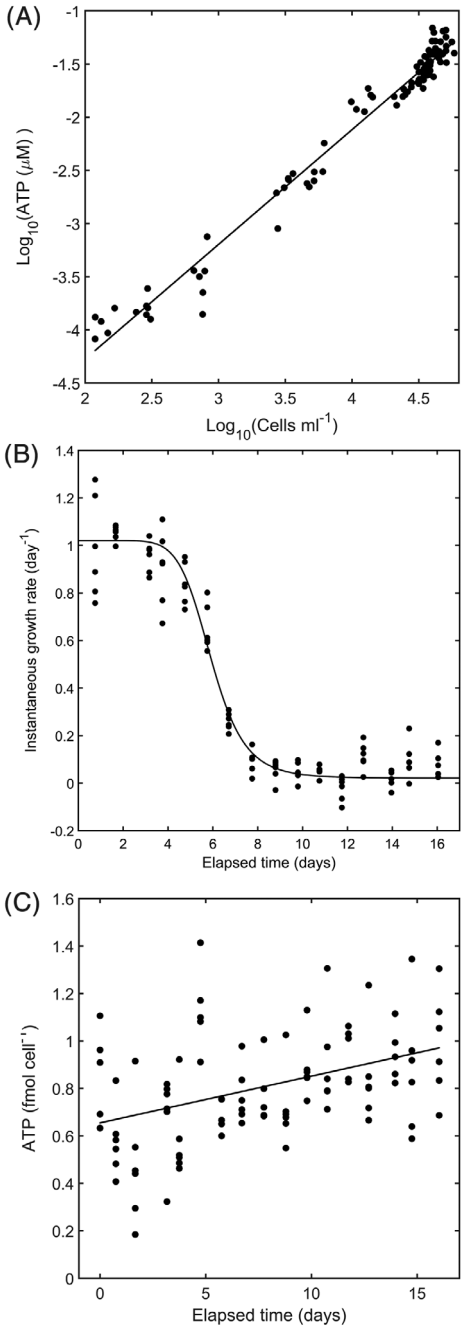

Fig. 3. (**A**) Relationship between ATP and cell numbers in six replicate batch cultures of *Thalassiosira weissflogii*. Solid line represents linear regression on the log10-transformed ATP and cell count data is log10 (ATP) = −6.43 + 1.079 × log10(cells mL^{-1}). (**B**) Instantaneous growth rates (IGR, unit: day^{-1}) in six batch culture replicates of the diatom *T. weissflogii* over time (days). Sigmoid model fit in (**B**): IGR = 0.02096 + (1 − (time$^{7.921}$/[5.926$^{7.921}$ + time$^{7.921}$])), and (**C**) ATP per cell over the course of the experiment in the same cultures. Regression: ATP fmol $cell^{-1}$) = 0.649 + 0.0197 × elapsed days. The slope was significantly different from zero ($n = 99$, $F = 18.8$, $r^2 = 0.163$, $p < 0.0001$), however, the average per-cell ATP values changed very little over the dramatic decrease in growth rate.

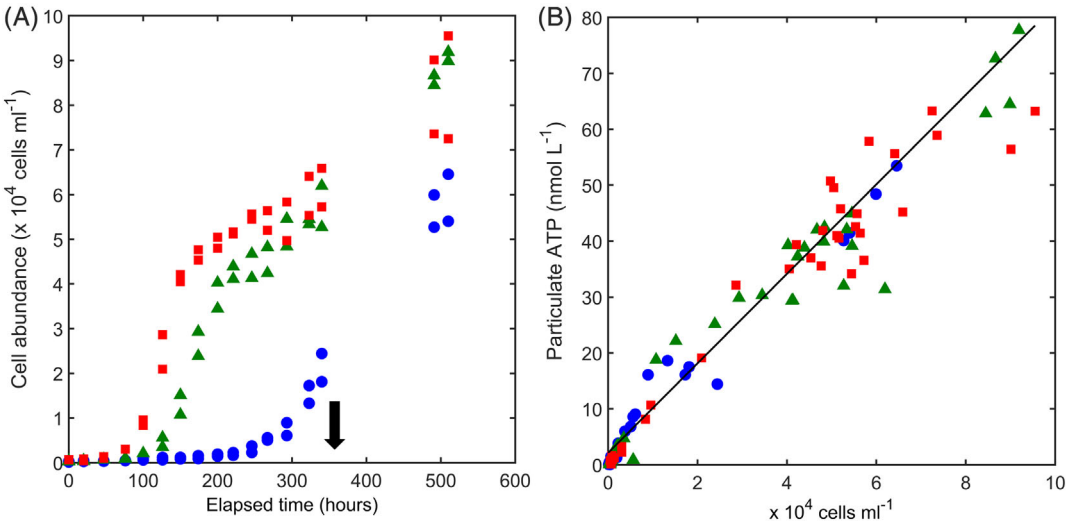

Fig 4. (**A**) Growth of six batch cultures of *T. weissflogii* at three temperatures: 15°C (blue circles), 20°C (green triangles), and 25°C (red squares). Black arrow indicates where temperature baths were switched off and growth resumed at room temperature in all six cultures. (**B**) Relationship between particulate ATP and cell abundance of the cultures at three temperatures, representing three different growth rates. Regression line: ATP = 2.1439 + 8 × 10^{-4} cells mL^{-1}.

Table 2. Parameters of the exponential growth by *Thalassiosira weissflogii* at three temperatures (Fig. 4). The equation is $y = a \times e^{bx}$, where y is cells mL^{-1} and x is the elapsed number of days since the beginning of the experiment. "*n*" indicates the number of data points included in fitting the exponential model. The model was only fit over the exponential growth phase, which had different lengths depending on the temperature, and before nutrient limitation set in. The Q_{10} was 2.18 from minimum to maximum growth rate between 15°C and 25°C.

Flask #	Temperature (°C)	*n*	From day	To day	*a*	*b* (d^{-1})	r^2
1	15	14	1	14	30	0.474	0.996
2	15	14	1	14	21	0.477	0.992
3	20	7	1	7	52	0.739	0.996
4	20	7	1	7	116	0.764	0.995
5	25	5	1	5	128	1.031	0.999
6	25	5	1	5	221	0.767	0.998

growth trajectory and then tapered to a linear growth (Fig. 4A). The time of the change from exponential to linear growth was consistent with delayed nutrient limitation at delayed biomass accumulation at lower temperatures (Fig. 4A). Diatom growth rate varied consistently with temperature (Table 2; Fig. 4A); however, there was no significant difference in the slopes of the relationship between ATP and cell concentrations at the three temperature groups (Fig. 4B; ANCOVA, homogeneity of slope test, $n = 102$, $F = 0.37$, $p = 0.690$).

Reanalysis of data in Sakshaug (1977)

We reanalyzed data from a frequently cited paper on the relationship between ATP and growth rate in phytoplankton (Sakshaug 1977). To verify the faithfulness of the data reconstruction, we replotted the original data in Supplementary Fig. 5. Using these data, we reconstructed the per-cell carbon, nitrogen, and ATP values. While in these examples ATP was loosely correlated with carbon in *Asterionella japonica* (Fig. 5A), ATP and carbon were uncorrelated in *S. costatum* (Fig. 5C). In contrast, ATP tracked cell nitrogen very well in both cultures with explained variances (r^2) of 0.997 and 0.927 (Fig. 5B,D).

Reanalysis of data in Stuart (1982)

Data were taken from table 1 and fig. 1 in Stuart (1982) and then combined and are displayed in Fig. 6. Supplementary Fig. 6 demonstrates that data from the figure from Stuart (1982) were faithfully reproduced. There is a significant regression fit between ATP and carbon of the microbial community with and without the lowest value (Fig. 6). The two resulting linear regressions straddle the slope based on the carbon to ATP weight ratio of 250 originally proposed by Holm-Hansen (1970).

Discussion

Methodological considerations

Problems of the bioluminescence assay and improvements of the ATP method were already discussed in detail in Schram and van Witzenburg (1989) and Karl (1993). The luminescence peak of the original luminescence assay can be quite complex, but peak integration can be entirely avoided with our method and by using the commercial bioluminescence assay, which is very similar to that employed by Hammes et al. (2010). The basis of this assay is a patented thermostable recombinant luciferase that provides a protracted and stable luminescence signal with a half-life of more than 3 h

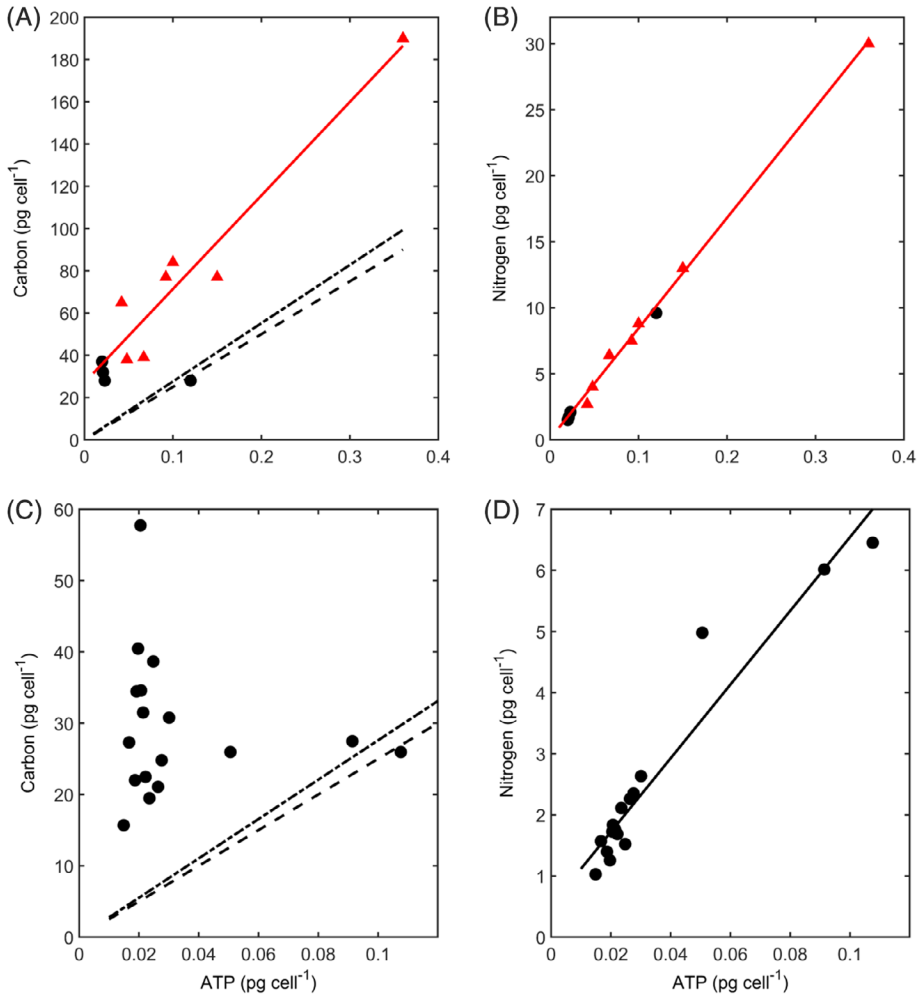

Fig 5. Relationship between carbon and nitrogen, and ATP in experiments by Sakshaug (1977). (**A** and **B**) Data from table III in Sakshaug (1977): *Skeletonema costatum* (black circles) and *Asterionella japonica* (red triangles). Linear regressions for Asterionella only: carbon = 27 + 443 ATP, $n = 7$, $r^2 = 0.916$, $F = 54.64$, $p = 0.0111$†; nitrogen = 0.07 + 83.7 ATP, $r^2 = 0.997$, $n = 7$, $F = 1521$, $p = 0.0003$†. (**C** and **D**) Data from figs. 4 and 5 in Sakshaug (1977). Linear regression for Skeletonema: nitrogen = 0.51 + 60.3 ATP, $n = 16$, $r^2 = 0.927$, $F = 176$, $p < 0.0001$†; carbon was not significantly correlated with ATP in Skeletonema, $n = 17$, $F = 0.243$, $p = 0.595$†. For comparison, the carbon : ATP ratios of 250 : 1 (dashed line, Holm-Hansen 1970), and 276 : 1 (dash-dotted line, Paerl and Williams 1976) are added. †All *p*-values based on 10,000 randomizations (Manly 2007).

(Promega technical manual, Celltiter-Glo 2.0 Assay). The final dilution of the reagent (i.e., Celltiter-Glo) in our modification is much higher than suggested by the manufacturer. Instead of adding the reagent to the sample at a 1 : 1 ratio, we added either 10 or 50 μL of sample and diluted that with 3 mL of ultrapure water. Fifty microliters of the firefly reagent was then added to this mixture. In our experience, this approach led to lower sample-to-sample variance and a stronger luminescence signal because of the dilution of salts present in seawater that would otherwise suppress the luciferin–luciferase reaction. Adding hot water or P-BAC was also more efficient in releasing ATP and inactivating ATPases than using the reagent on a 1 : 1 basis with the sample. Internal standards rather than a separate calibration curve were used to counter any matrix effects such as those caused by ions, humic acids, turbidity, and residual enzymes (Strehler 1968; Karl 1993). While scintillation counters have been used previously for ATP assays (Addanki et al. 1966; Skórko 1984), the addition of the firefly extract had to be timed, and the trajectory of light formation and dissipation recorded precisely in the original method. In contrast, the application of a scintillation counter together with the protracted luminescence in the Celltiter-Glo assay is ideal, especially when vials with samples and internal standards are alternated. While luminescence detectors are broadly available, many aquatic laboratories have scintillation counters to measure primary production using the ^{14}C-incorporation method (Stemmann Nielsen 1952). Scintillation counters are superior light detectors with the capability of single-photon counts. No cooling of the photomultiplier tube should be employed to maintain samples at room temperature during counting.

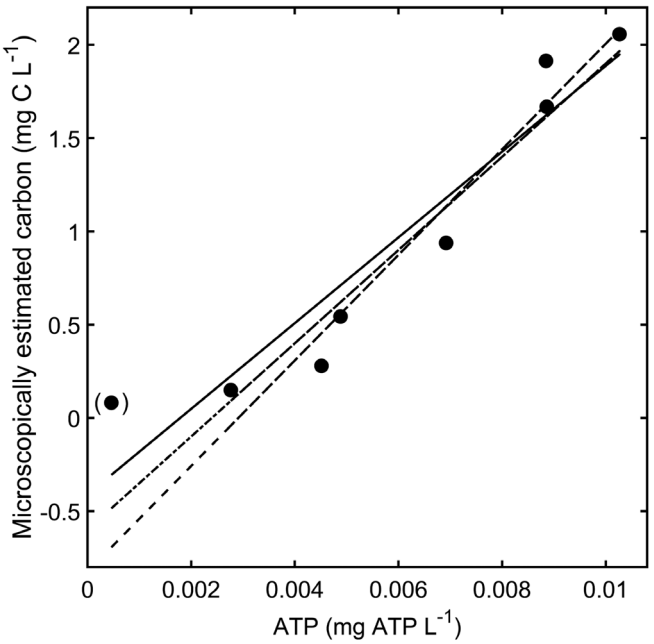

Fig 6. Reanalysis of data of microbes on decomposing kelp detritus in Stuart (1982). This paper was highly critical of the ATP – biomass method despite a very close agreement between ATP and carbon. The linear regressions fall close to that proposed by Holm-Hansen (1970). Linear regression: carbon = −0.411 + 230 ATP, $n = 8$, $r^2 = 0.902$, $F = 54.93$, $p = 0.0003$. Linear regression without lowest value (in parenthesis): carbon = −0.824 + 283 ATP, $n = 7$, $r^2 = 0.958$, $F = 113.3$, $p = 0.0009$ (dashed line). For comparison, the ATP : carbon ratio of 250 proposed by Holm-Hansen (1970) is shown (dot-dashed line).

Shock-freezing samples in liquid nitrogen break up cells and releases ATP, and because of the low temperature stops ATPase activity effectively. This is a very efficient way to preserve ATP quickly and, therefore, is useful for field deployments where liquid nitrogen is available. Previously, decreased ATP values were observed when samples were frozen in liquid nitrogen; however, that decrease was due to the addition of a cryoprotectant (Bajerski et al. 2018), which created a matrix effect. Adding boiling-hot water directly to the frozen samples and immersing them in a boiling-water bath brings the temperature of the samples to 100°C very quickly so that ATPases do not have much time to act on the released ATP before being inactivated. In our laboratory setting, it was possible to analyze all samples for ATP and Coulter Counter cell counts on the same day. However, preservation, slide preparation and cell counting under the microscope took many days.

Hot-water and P-BAC extraction procedures in comparison

Both the hot-water and P-BAC extraction procedures have advantages and disadvantages. The single most important advantage of the P-BAC method is the more complete extraction of ATP from filters, which exceeded even our best extraction protocol (i.e., the pretreatment of sample with liquid nitrogen before hot-water extraction) by approximately 20% (Table 1). In contrast, Welschmeyer and Kuo (2016) observed three to five times higher ATP values between P-BAC and the traditional ATP extraction in the Tris buffer. We are unsure what caused this high discrepancy, but it could be connected to their use of external standards in the presence of a complex sample matrix. The quenching of the firefly reaction by P-BAC is strong, and reliance on external standards with the addition of pure P-BAC may result in overcorrection.

The interference of P-BAC with the firefly assay means that sufficient dilution is important. For instance, adding 10 μL to 3 mL of ultrapure water provided good results, while adding 50 μL to 3 mL ultrapure water almost completely extinguished the bioluminescence reaction, likely by denaturing either the luciferin substrate or the luciferase enzyme, or both. Another practical issue is that pipetting small volumes, such as 10 μL, requires well-calibrated and highly consistent pipettes to obtain accurate results (i.e., pipettes selected on the basis of small standard deviations as determined by repeated pipetting of distilled water on a microbalance). The GF/F filter also needs to be completely immersed in the 1 mL P-BAC solution, and air pockets have to be removed as much as possible. This step may require forceps or pipette tips to be immersed in the P-BAC extractant, which can lead to contamination and carryover.

The advantage of the hot-water extraction is that the filtrate can be processed in a similar fashion (i.e., ATPases can be inactivated by the boiling-water bath). In some cases, it is useful to determine the dissolved ATP fraction, especially when it needs to be subtracted from the unfiltered whole water extract. A hot-water dispenser dedicated for ultrapure water is useful regardless of the extraction technique as it provides a supply of rinsing water for the filtration towers between samples. Thus, this simple extractant is already at hand in copious amounts.

Adenylate energy charge

There is a great deal of literature on the adenylate energy charge proposed by Atkinson (1967) to measure the energy status of a cell (see recent analysis by De la Fuente et al. 2014). A review of this literature is outside the scope of this manuscript; however, it is noteworthy that the adenylate energy charge is very limited in scope across all organisms ranging only from 0.7 to 0.95 (De la Fuente et al. 2014). For a review on adenylated energy charges in aquatic systems, *see* Karl (1980) and Jewson and Dokulil (1982). Since the adenylate energy charge is a ratio based on the relative distribution of ATP, ADP, and AMP, the absolute values are independent of those ratios. More relevant for the current analysis is whether the absolute concentrations of ATP in cells change appreciably, and whether they reveal something about the "energetic state" of a cell. Recently, however, there has been a shift away from the notion that ATP's only function is that of energy currency. If ATP's sole function

was energy currency, there would be no need for ATP concentrations in the millimolar range exceeding all metabolic needs by 1000-fold (Patel et al. 2017); nor does constraining the function of ATP to energy currency alone explain why the ATP concentration in cytoplasm is even higher than that in the mitochondria (Imamura et al. 2009).

ATP concentration in the cytoplasm of aquatic microbes

Detailed analysis of nucleotide pools of aquatic organisms is not available to the same extent as literature on laboratory cultures of bacteria including human pathogens. ATP values in cytoplasm have been mapped in giant amoeba (Ueda 1987), and with new single-cell techniques they can even be determined for much smaller prokaryotic cells (Yaginuma et al. 2015). While there is some cell-to-cell variability between individual bacteria, ATP concentrations display a clear mode around 1 mM (Yaginuma et al. 2015). Near millimolar ATP concentrations are indeed found in many microbes, including those from aquatic environments. We converted data from our experiments with *T. weissflogii* presented here, from a variety of prokaryotic and eukaryotic microorganisms, and from mammal erythrocytes into molar concentrations based on estimated cell volumes (Fig. 7). Using cell volume in the denominator is not entirely accurate because of the variable contributions of cell walls and vacuoles to the total cell volume, as well as the possible shrinkage of cytoplasm away from the interior of the cell walls (see below). Therefore, reported molar concentrations are likely minimum estimates of ATP concentrations in cell plasma (Fig. 7). What is initially striking is the wide range of values found within each group (Fig. 7). Variability is not necessarily tied to the organisms themselves but is likely exacerbated by different extraction protocols of the various investigators, matrix effects, and whether or not internal standards were chosen. Despite the variability, the average values fall narrowly between 0.62 and 1.37 mM ATP. For comparison, literature values of mammalian erythrocytes are also displayed with an average remarkably close to those of microbes. For microorganisms, the averages rank in sequence from lowest to highest from phytoplankton, bacteria, and tintinnids, to fungi (Fig. 7). The means determined by Sakshaug (1977) for *S. costatum*, the average of a variety of eukaryotic phytoplankton (Karl 1980), and the values of *T. weissflogii* in our experiments were almost exactly the same (Fig. 7). Statistical analysis is of limited value on the dataset in Fig. 7, because it is heavily compounded by different methods and authors. How to pool the data and whether to use summary data or unavailable raw data affect the statistical significance levels as well. However, the high molar concentrations of ATP in the millimolar range and the consistency of average values across a wide range of taxa are entirely consistent with the hydrotrope theory discussed above.

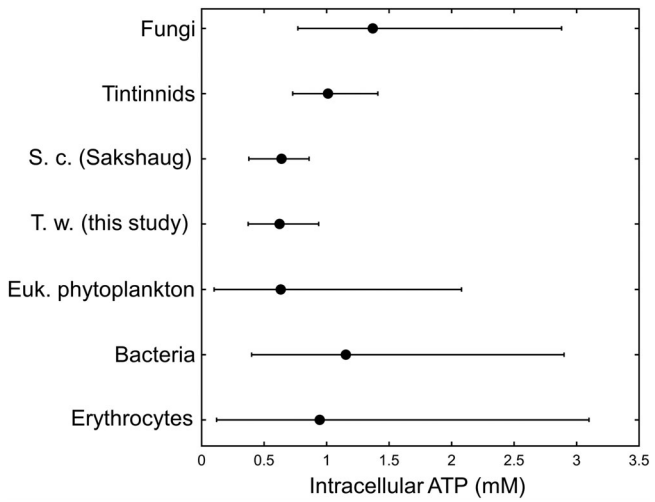

Fig 7. Mean and ranges of cellular ATP concentrations in a variety of taxonomic groups. Mammal erythrocytes (Miseta et al. 1993), bacteria (Karl 1980), eukaryotic phytoplankton (Karl 1980), T.w. (*Thalassiosira weissflogii*, this study), S.c. (*Skeletonema costatum*, Sakshaug 1977), loricate tintinnids (Verity and Lagdon 1984), and fungi (Karl 1980).

ATP levels and growth rates

While some authors have argued that there is a positive correlation between ATP and growth rates in microorganisms (Neidhardt and Fraenkel 1961; Bagnara and Finch 1973), the majority of studies support the notion that ATP was independent from growth rates (Franzen and Binkley 1961; Smith and Maaloe 1964; Forrest 1965; Hobson and Summers 1972). Most of the data are from bacterial cultures, and only a few are available on phytoplankton (see below). Our batch culture experiments with *T. weissflogii* were well replicated, and the use of the Coulter Counter produced very precise cell numbers during growth. The cell size of this diatom ($\sim 15\,\mu$m), its geometry, that it remains relatively monodispersed (i.e., does not clump easily; Logan et al. 1989), and that it is not chain-forming in our culture are all facts highly conducive to counting cells accurately with the Coulter Counter on which our growth rate estimates were based. While we did observe changes in cellular ATP levels with time in the batch culture experiments, the response was small (Fig. 3C). In contrast, growth rates dropped precipitously during the experiment when nutrient limitation set in (Fig. 3B). Also, an inverse relationship between ATP levels and growth would be counter to the notion that cellular ATP is higher at higher growth rates (*see* analysis of Sakshaug 1977 below).

Is ATP dependent on biomass or growth rate? Reanalysis of data by Sakshaug (1977)

Some of the most detailed analyses of phytoplankton ATP levels regarding growth are the experiments by Sakshaug (1977). At first, the data are compelling: a smooth curvilinear relationship between per-cell ATP levels and growth rate (fig. 5 in Sakshaug 1977; Supplementary Fig. 5). However, there are several aspects of this study that need to be revisited for context. First, the actual growth rates at the time of ATP measurements were not used in the comparison with ATP but the

growth rates expressed as the ratio of growth rates to maximum potential growth rates based on the extrapolation of data several days earlier were used. In this way, a term measured asynchronously enters into the equation, which is problematic because ATP turns over very quickly, and values several days before ATP measurements have no relevance. In our reanalysis of the digitized data, and using data points just prior to and after ATP sampling to calculate corresponding actual growth rates, we found an abrupt threefold step function in ATP from a growth rate of 0.43 to 0.49 instead (Supplementary Fig. 7). It is also important to recognize that it was the carbon to ATP ratio that changed with estimated growth rates (fig. 4 in Sakshaug 1977), and not necessarily cellular ATP concentration. In contrast to carbon, the nitrogen to ATP ratio remained constant over a wide spectrum of growth rates (fig. 4 in Sakshaug 1977; Supplementary Fig. 5A). In our reanalysis, we plotted data from figs. 4 and 5 in Sakshaug (1977) and combined them using trial number and growth rate to match them as closely as possible (note that some matching data points were missing in the original manuscript). In this fashion, and instead of ratios, the amount of ATP can be directly regressed against carbon and nitrogen (Fig. 5). While cellular ATP and carbon are only loosely related in one instance, ATP, and nitrogen correlated very well for both algae (Fig. 5). That ATP tracks well with any bulk property, such as the abundance of an element, strongly suggest that it correlates with biomass rather than a process (i.e., growth). In this context, it is important to recall that while there can be a significant contribution of nonprotein nitrogen to total cell nitrogen, most nitrogen in microalgae is bound in proteins (Lourenço et al. 2004).

The observed variability in ATP with growth rate can, thus, be accounted for by changes in the stoichiometry and cell size. It is well known that elemental ratios can change dramatically, especially in single-cell organisms with a variety of ecological factors forming the foundation of ecological stoichiometry (Geider and La Roche 2002; Sterner and Elser 2002). Beyond elemental ratios, there are also well-documented changes in cell volume and morphology with nutrient limitation (Harrison et al. 1977; Lomas et al. 2019), light and temperature (Jorgensen 1968), and growth rate in general (Prakash et al. 1973). Cytoplasm volume decreases even more drastically than cell volume during nutrient limitation, so much so that cytoplasm pulls back from the cell walls (Davidson et al. 2002). *S. costatum*, used in the nutrient limitation experiments by Sakshaug (1977), is one species that exhibits this behavior (Davidson et al. 2002), and decreases in ATP levels per cell can easily be a result of shrinking cytoplasm volume without at all affecting the molar plasma concentrations of ATP. If our goal is to determine "how alive" a system is, the abundance of live cytoplasm is arguably more relevant than the size of the hollow space inside a cell wall.

A similar discussion about the relationship between growth rate and ATP levels occurred in literature related to bacteria, in which there has been a long-held belief that ATP values change with growth rates in the prokaryote *Escherichia coli* (Gaal 1997). Schneider and Gourse (2004), however, demonstrated that some of the conclusions by Gaal (1997) et al were based on discrepancies in extraction procedures and not on an inherent relationship between ATP and growth rate. Based on further improvement in extraction protocols, in which individual nucleotides could be separated, Buckstein et al. (2008) demonstrated that hyperphosphorylated guanosine (ppGpp) in *E. coli* was inversely correlated with growth rate, while ATP and GTP levels remained constant over a fourfold growth rate range.

Translating ATP into carbon in complex microbial communities

For practical purposes and in models, it is desirable to express biomass in terms of carbon in complex communities (Holm-Hansen 1970; Hobbie et al. 1972; Karl 1980; Hewes et al. 1990). A frequently employed carbon to ATP mass conversion factor of 250 is based on a variety of unicellular algae, bacteria, and micro- and macrozooplankton from the field and the laboratory at various states of nutrient limitation (Holm-Hansen 1970, Holm-Hansen 1973). We calculated the ratio of carbon to ATP for *T. weissflogii* to be 280 in our experiments based on the following data: (1) ATP extraction using P-BAC with calibrated pipettes; (2) determination of the ATP standard using a spectrophotometer; (3) cell counts using the Coulter Counter; (4) cell volume measurements using a microscope; and (5) the cell volume to carbon conversion for diatoms reported in Menden-Deuer and Lessard (2000) (*see* Supplementary Table 1 for calculation details). Despite these many potential sources of error, 280 is remarkably similar to the 250 ratio in Holm-Hansen (1970), and even closer to the slope between carbon and ATP in live freshwater phytoplankton of 276 (Paerl and Williams 1976, see below).

Dealing with complex aquatic communities, however, it was pointed out early that problems arise when ATP is compared to parameters that are "inadequate" (e.g., carbon), and when there is no absolute biomass indicator available (Brezonik et al. 1975). One problem in determining ATP to carbon conversions in complex microbial communities is how to ascertain carbon values of live organisms when most of the particulate carbon in the ocean is detrital in nature. Estimates of nonliving particulate carbon range widely, from 50% to 90% (Eppley et al. 1977; Andersson and Rudehäll 1993; Yanada and Maita 1995; Volkman and Tanoue 2002). The proportion of non-living carbon is even larger when the portion of the gel phase retained by GF/F, polycarbonate or other filters in the submicrometer pore-size range (i.e., 0.2, 0.45, and 0.7 μm) is included (Verdugo et al. 2004).

To avoid the detrital pool and to determine carbon tied to living cells, estimates in most studies were based on microscopic cell counts and cell volume estimates, which are highly subjective, time consuming, variable, incomplete, and often

inaccurate. Despite these shortcomings, carbon values based on cell numbers and volumes have been demonstrated to compare surprisingly well to ATP in many cases (Holm-Hansen 1970; Holm-Hansen 1970; Brezonik et al. 1975; Paerl and Williams 1976; Stuart 1982, see reanalysis, below; Pridmore et al. 1984). Conversions from carbon to ATP were, overall, very close to the mass ratio of 250 suggested by Holm-Hansen (1970, 1973). Pridmore et al. (1984), studying New Zealand lakes, found carbon to ATP ratios of 287, with an interquartile range of 248 to 291. There were a few exceptions of extreme values larger than 400, which the authors attributed either to nutrient limitation or methodological difficulties due to the complexity of cell enumeration (Pridmore et al. 1984). For comparison, the slope of phytoplankton carbon and ATP in lakes of wide-ranging eutrophic status was 276 (Paerl and Williams 1976). In that study, the analysis was based on the identification of live phytoplankton via microautoradiography of ^{14}C, and the carbon to ATP ratio across many lakes did not vary by more than 17% (Paerl & Williams 1976).

Fairbanks et al. (1984) concluded that ATP is not a reliable indicator of biomass because carbon-to-ATP ratios in bacteria change with drought, glucose availability, and $(NH_4)_2SO_4$ levels. Glucose addition had the strongest effect, with a 5.9-fold effect on ATP levels per cell. The other two variables affected the carbon to ATP ratio by less than threefold. During the experiments, however, ATP and microscopic protocols were changed, and even the slightest differences in cell size estimates can lead to large changes in volume and, thus, in carbon estimates. More important, however, conditions of desiccation and the uptake of imbalanced, carbon-rich food are exactly the conditions during which cell size and stoichiometric balances of cells vary (Sterner and Elser 2002; Mooshammer et al., 2014).

It would make sense that ATP levels decrease with phosphorus limitation, as ATP is the biochemical most enriched in phosphorus, but ATP forms only a very small fraction of the total cellular phosphorus pool (Hunter and Laws 1981; Sterner and Elser 2002). In *T. weissflogii* (formerly *T. fluviatilis*), specifically ATP-to-carbon ratios varied sixfold during phosphorus limitation but remained relatively stable under light limitation (Hunter and Laws 1981). Both carbon and ATP contents covaried with light limitation and with the accumulation of biomass during the light phase (Hunter and Laws 1981). Cell ATP quota also dropped during iron limitation (Sakshaug and Holm-Hansen 1977). Carbon to ATP ratios were also reported to be as high as 5000 in Lake Kinneret during phosphorus limitation during a *Peridinium* bloom (Cavari 1976). From these studies, however, it is not possible to ascertain how much viable cell numbers, cell size, and, most important, live cytoplasm volume may have changed. If there is a minimum concentration of ATP that needs to be maintained in cytoplasm to keep proteins dissolved, a drop much below ~ millimolar concentration would be lethal.

Under which circumstances the carbon to ATP ratio deviates significantly from 250 : 1 in environmental samples still needs to be systematically addressed in future work. A broad recommendation is to "stay in ATP space" as much as possible. More research is needed regarding a systematic deviation of cells from the carbon to ATP ratios if carbon must be used as currency. Whether ATP is translated into biovolume, carbon, nitrogen, dry weight, or other metrics, the conversion should be made with caution. It is, therefore, useful to report always the original ATP values, which can accurately and consistently be determined. ATP values can then be compared among environments and across laboratories without introducing the subjectivity of microscope counts, biovolume determinations, and other analytical procedures. The high plasticity in terms of elemental ratios in microbial cells calls into question what biomass means, and ATP being representative of live cytoplasm is certainly a more suitable metric than particulate organic carbon.

Reanalysis of data by Stuart (1982) on benthic microbial communities

In the few studies available on benthic microbial communities, opinions diverge. Bancroft et al. (1976) determined a relatively good agreement with the Holm-Hansen's (1970, 1973) factor of 250, despite citing numerous problems with the enumeration of viable vs. nonviable cells, and the estimation of cell volumes. Cunningham and Wetzel (1978) found that organic acids (e.g., fulvic acids) that are abundant in sediments interfered with the accurate determination of ATP, but some of these matrix effects can be counteracted by the use of internal standards as in this study (also recommended by Karl 1993). Stuart (1982) strongly rejected a single factor conversion from ATP to carbon on the basis of experiments with microbial communities found on kelp detritus. We can demonstrate that the conclusions in the latter study were based on erroneous statistics, and that, in contrast, the data presented in Stuart (1982) strongly supported the notion of ATP as a biomass indicator and a mass conversion factor of 250 by accounting for an intercept. Our reanalysis of the digitized data indicated that the relationship between carbon and ATP is very tight (r^2 = 0.915; Fig. 6). Even more important, the slope between microscopically determined carbon and that based on ATP is close to 230 or 283 depending on whether the lowest data point (typically the least reliable in the analysis) is included or not (Fig. 6). The average between the slopes of the two regressions amount to 256, which is very close to the slope of 250 proposed by Holm-Hansen (1970; 1973). This consistency is even more remarkable given that Holm-Hansen (1970; 1973) used this ratio primarily with pelagic microbes, whereas Stuart (1982) examined a benthic microbial community on decomposing kelp. Contrary to the initial conclusion, Stuart's (1982) data are strong evidence that an ATP to carbon conversion factor of

approximately 250 can be extended to at least some benthic communities.

Conclusions

The determination of ATP as a biomass indicator has many advantages: (1) ATP is easily and consistently measured using standard luminescence detectors or scintillation counters; (2) ATP can be standardized among diverse environments and laboratories using simple spectrophotometric measurements, internal standards, and known molar absorptivity coefficients; (3) the ATP assay is highly sensitive and requires typically only a drop of water ($\sim 50\,\mu$L) in surface environments, which allows for unprecedented spatial and temporal resolution; (4) ATP represents live biomass and is not influenced by detritus, dead colloidal material, and other organic carbon sources; and (5) ATP can be used in environments such as sediments, biofilms, and marine snow, where accurate optical assessments of biomass via cell enumeration are impossible. We improved the ATP assay by means of either a simple hot-water or chemical extraction, by adding internal standards to the samples, by modifying the protocol of a commercially available firefly luminescence kit, and by using a scintillation counter as a highly sensitive light detector. Problems primarily arise when ATP values are translated into units of carbon, an element that may not directly translate into live cytoplasm volume. Observed independence of ATP from growth conditions, invariance across taxonomic groups, and even across pelagic and benthic communities, and that ATP must be maintained at high and constant concentration in the cytoplasm to serve its function as a hydrotrope, makes us cautiously optimistic that ATP is a reliable metric for biomass. More work is needed to explore the utility of ATP in the environmental context, and problems related to the dissolved pool, extraction procedures, and matrix effects must be addressed in each circumstance. However, the sensitivity, simplicity, and economy of the ATP assay are compelling. After all, ATP may be the closest metric we have for determining how alive a parcel of water truly is.

Comments and recommendations

There follows some practical considerations.

1. As the ATP assay is much more sensitive than other biomass indicator methods (e.g., carbon, chlorophyll), it is especially important to work very cleanly; even an imperceptibly small drop of water or dust particle with attached bacteria can increase the luminescence signal.
2. Muffling (450°C, 5 h) is suitable to remove residual ATP and ATPases from glassware.
3. Always calibrate automatic pipettes. As the ATP levels directly depend on the accuracy of the pipette used to prepare samples and standards, frequent calibration of the pipettes is recommended. Typically, we chose the same $50\,\mu$L fixed-volume pipette that provided consistent results for all pipetting during an experiment (i.e., low standard deviations between pipetting ultrapure water droplets using a microbalance).
4. By shock freezing in liquid nitrogen and storing samples at −80°C, freezer samples can be stored for months prior to analysis. Shock freezing also has the advantage of breaking up cells, which increases extraction efficiency. Do not use any cryoprotectants as this process is counterproductive for breaking up cells and they impede the luminescence reaction. Since ATPases are still very active, boiling-hot water must be added directly to frozen samples without delay, and then the samples must be immediately incubated in boiling water. If the samples thaw slowly, much ATP will be destroyed.
5. It is important to cool the samples to room temperature before analysis so as not to denature the luciferin protein. The sample and internal standard must also be kept at exactly the same temperature during analysis as the time course of the luminescence signal is highly temperature dependent. The main purpose of adding 3 mL of ultrapure water to the scintillation vials is the dilution of salts and detergents that would interfere with the reaction, but it also serves to equilibrate the temperature before adding the firefly extract.
6. Measure the stock solution in the spectrophotometer rather than relying on weighing in the standard. Using spectrophotometry, ATP can be determined in a pure standard at much higher accuracy than any analytical balance can provide. While ATP standards in ultrapure water last for years when frozen at −20°C, it is good practice to test the levels in the standard in the spectrophotometer occasionally before using it in the analysis.
7. Choose internal ATP standards at relatively high values to provide a strong contrast between internal standards and samples. The assay is linear over many orders of magnitude, and a good contrast between internal standard and sample readings improves accuracy.
8. Measure the luminescence of the sample, and sample with the internal standard in short succession, ideally by alternating the sample and internal standard vials. If the matrix effects are expected to be similar in a batch, regress the internal standards against time during scintillation counting, and then use this slope to determine the sample values at each time point. This point is especially important when hundreds of vials are counted. Using standard values based on the regression model reduces random variance between samples and internal standards and leads to better estimates of true sample-to-sample variability.
9. Like any other type of analysis, ATP measurements greatly benefit from replication with the simple rule to use "as many replications as practical." However, this may not be possible when ATP is measured in very small volumes, such as individual droplets.
10. In some aquatic systems, extracellular ATP is a high percentage of the total (Azam and Hodson 1977; Riemann 1979), so it may be necessary to account for it in

samples. Filtrate can be easily captured in centrifuge tubes placed underneath the filtration funnel inside the vacuum flask. This filtrate can be shock frozen and stored with the filters or whole water samples.

11. It is important to note that metazoans were not included in this comparison as cell sizes are more difficult to analyze in complex tissues (blood cells as a tissue composed of individual cells being the exception). However, ATP values based on dry weight were reported to be higher for zooplankton than for microbial cells (Skjoldal and Båmstedt 1977; Karl 1980). It is, therefore, advisable to exclude metazoans from the analysis, as suggested by Karl (1993), and focus on the microbial biomass only.
12. Measure ATP in the whole water, not just in the filtered component, and capture the filtrate for analysis. The maximum contribution of the dissolved fraction may only be a small fraction, and some of it may be, in part, caused by breakage or leakage of cells during filtration. As an added advantage, taking whole water samples is quick and can be automated very easily, which allows for higher replication and, thus, increased precision.
13. For the wet extraction with P-BAC, it is important to keep the dilution at 10 μL extract with 3 mL ultrapure water. A test with 50 μL extract to 3 mL ultrapure water quenched the luminescence signal completely.

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Conflict of Interest

None declared.

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