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Estimating Carbon Biomass Using DNA: Phytoplankton as a Case Study

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Abstract: Background/Objectives: Estimating carbon content for cells is often necessary but difficult. In many biological, oceanographic, and marine biogeochemical studies, information on phytoplankton species composition and their biomass contribution to the community is essential. However, it is technically challenging to estimate the biomass of individual species in a natural assemblage. DNA analysis has the potential to profile species composition and estimate species-specific carbon biomass simultaneously. However, this requires an established relationship between carbon biomass and DNA content with species resolution using a measurable DNA index such as rDNA. **Methods:** In this study, DNA, rDNA, and carbon contents were measured for species from major phytoplankton phyla grown in different growth stages and under different nutrient and temperature conditions. Correlations between these parameters were examined. **Results:** Our data resulted in significant log-log regression equations: Log C = 0.8165 × Log DNA + 2.407 (R^2 = 0.9577, $p < 0.0001$), Log rDNA = 0.7472 × Log DNA − 0.0289 (R^2 = 0.9456, $p < 0.0001$), and Log C = 1.09 × Log rDNA + 2.41 (R^2 = 0.9199, $p < 0.0001$). Furthermore, similar strong regression functions were found when incorporating previously published data on a wide range of organisms including bacteria, plants, and animals. **Conclusions:** Carbon biomass is significantly correlated with DNA and rDNA abundances in phytoplankton and other organisms. The regression equations we developed offer a tool for estimating phytoplankton carbon biomass using DNA or rDNA and serve as a foundation for establishing similar models for other organisms.

Keywords: DNA content; carbon content; rDNA copy number; correlation; phytoplankton; biomass; estimation

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1. Introduction

Information of carbon (C) content from living organisms is critical for studying biogeochemical cycles of carbon but not easy to obtain. For instance, C biomass estimation for natural marine phytoplankton at community, class, or species levels is very important in biological oceanographic research; it is essential for balancing the C budget and for assessing the contribution of each species or class in the community to primary production,

biogeochemical processes, and carbon export in the ocean [1,2]. The information of C biomass may not be accurately represented by cell abundance as contributing species being compared are likely to have different cell sizes. However, while community C biomass is tedious to measure, it is practically impossible to measure C for individual species or groups of species in a natural community. Indirect methods exist, e.g., based on cell size versus carbon content correlations, such that the C content of a cell can be estimated from cell size; then, the carbon biomass of a population can be estimated from cell abundance multiplied by the inferred C content. Indeed, several such regression equations have been developed separately for different functional groups of plankton (protists) [1,3–6]. This method has proven very useful and remains the only available method, yet it is time-consuming and requires extensive technical expertise in taxonomy if the cell size-based carbon biomass information were to be attributed to species. Furthermore, the method is not applicable to species that are unable to be cultured and are only identified from the rapidly growing body of environmental DNA (eDNA) data and hence no cell size data are available (e.g., *TARA Oceans*). Often, researchers are forced to use conversion constants for functional or size groups of phytoplankton to convert cell abundance to carbon biomass [2].

As a polymer of a ribose bound to pyrimidine or purine bases, DNA is carbon-rich and, as the genetic material, is relatively stable for a given species. In the lifetime of a cell, DNA changes from the G1 phase (preparation for DNA duplication) of the cell cycle to the G2 phase (completion of DNA duplication) by a factor of two, during which cell size and carbon content also increase. If DNA can be used as an estimator of C biomass, it will remarkably simplify C biomass estimation and be highly useful, particularly because DNA data have become easily achievable thanks to technological innovation. The potential of DNA to be a carbon estimator was demonstrated decades ago [7,8], although these studies did not examine the potential effects of variations in DNA and C content associated with different growth stages and growth conditions [9,10]. Indirect evidence in support of the potential is also found. First, there is a positive correlation between DNA content and cell size for both uni- and multi-cellular organisms [11–18] either because a larger cell size requires a larger nucleus for structural stability, or a larger cell size requires more functional genes [11,19]. Second, C content is positively correlated with cell size [1,3,4,20–25]. Given the above, we hypothesize a quantitative DNA–carbon relationship could potentially be established for phytoplankton regardless of growth stage and growth conditions, such that C biomass can be estimated by measuring DNA quantity whether from the community, functional group, or species levels.

Measuring DNA quantity per cell for individual species or groups of species from the natural assemblages is also challenging, although flow cytometry can potentially help. Therefore, a more accessible DNA marker is required if the C-DNA relationship is to be useful. For this purpose, ribosomal RNA genes (for brevity, rDNA) can potentially be a choice. The rDNA copy number, which is relatively easy to measure, has consistently been found to correlate with DNA content in eukaryotes [16,26,27]. Moreover, based on recent analysis of certain published data, the rDNA copy number has been found to correlate with cell size over a wide taxonomic and organismal size range [16,28–33]. These along with the relationship between C and DNA contents hypothesized above, led us to further hypothesize that a correlation existed between rDNA and C content in phytoplankton, such that species- or group-specific rDNA abundance can be used to estimate carbon biomass.

Analyzing rDNA has become easier than C measurement, but more importantly, it offers species resolution that C or cell size measurement does not. The hypervariable V4 region and V9 region of the SSU rDNA have been widely used to study biodiversity and plankton community structure in the marine ecosystem [28,34,35]. However, SSU rDNA is a multi-copy gene, and its abundance (copy number) varies with species, creating bias

toward rDNA-rich species [36,37]. For instance, dinoflagellates have large genomes and numerous copies of rDNA, and hence tend to be overestimated in phytoplankton samples. One possible solution is to calibrate the sequence abundance data using a species-specific rDNA copy number per cell, but there is no rDNA copy number database covering an adequate number of species to make this approach useful. Developing an rDNA-to-C regression is potentially a better solution to the problem not only because it is expected to be more consistent but also because C biomass is a better measure for biogeochemical and oceanographic applications.

In this study, we used 11 phytoplankton species to represent all major phyla of microalgae and measured the cellular DNA content, C content, and rDNA copy number under different growth stages, phosphorus-nutrient, and temperature conditions. Based on this measured dataset, we inferred three robust correlations between the three parameters: C content vs. DNA content, rDNA content vs. DNA content, and C content vs. rDNA content. We also extended the analysis by including previously reported data and found highly similar correlations to the ones we developed from phytoplankton. These correlations are expected to be useful for oceanographic and marine biogeochemical research as well as in other fields, where estimating the carbon biomass contribution of individual taxa to a mixed species community is required.

2. Materials and Methods

2.1. Cultures, Treatments, and Sample Collection

Eleven species from major phyla of phytoplankton were selected to cover phylogenetic diversity, ecotypic diversity, and cytological diversity (Table 1). The strains were grown under different conditions in batch cultures (~0.2 L, in Corning Cell Culture flasks). The tested conditions included nutrient availability, temperature, and growth stage. Two temperatures were used, one at or near the optimal (mostly 15 °C or 20 °C depending on the species, but 25 °C for tropical *Symbiodiniaceae* species) and the other being 5 °C higher. For nutrient variation, we used the L1 complete medium (36 μM phosphate) as the control and L1-based with reduced phosphate (3 μM phosphate) for the phosphorus-limited condition; it has been shown that phosphate limitation elicits strong impacts on growth and cell size [10,38]. Each treatment and control were set up in triplicate. For the growth stage experiment, cultures were maintained for 14 days, and samples were collected on days 2, 5, 8, 11, and 14 to capture different growth stages. For the nutrient and temperature experiments, cultures were grown for 5 days, and samples were collected only on days 2 and 5 without covering different growth stages. All samples were collected during the afternoon, and cell counts were taken for each culture at the same time. At each sampling event, three types of samples were collected: 1 mL for cell count fixed in Lugol's, ~one million cells for measuring DNA content and the rDNA copy number, and ~one million cells for measuring C content. Except for cell count, cells for other measurements were harvested using filtration, with membranes of appropriate pore size (0.22 μm for cyanobacteria, 3 μm for eukaryotes) and material type (for measurement of C, using GF/F pre-combusted at 450 °C for five h). Cells on the membranes were washed with autoclaved 0.22 μm-filtered seawater and frozen at −80 °C until analyses.

Table 1. Species from diverse lineages of algae investigated in this study.

Class	Species	Source	Type
Dinophyceae	*Amphidinium carterae*	CCMP1314 [a]	Photosynthetic, athecate, HAB
	Prorocentrum shikokuense	CCMAXU-364 [b]	Photosynthetic, thecate, HAB
	Fugacium kawagutii	CCMP2468 [a]	Photosynthetic, symbiotic, athecate
Bacillariophyceae	*Ditylum brightwellii*	CCMP2227 [a]	Centric, large-sized, common
	Thalassiosira pseudonana	CCMP1335 [a]	Centric, small-sized, model
	Odontella sinensis	CCMP1815 [a]	Centric, large-sized
Haptophyceae	*Emiliania huxleyi*	PMLB92/11 [b]	Cocolithophorid, model
	Isochrysis galbana	CCMA-079 [b]	Small-sized, model
Raphidophyceae	*Heterosigma akashiwo*	CCMA369 [a]	HAB
Chlorophyceae	*Dunaliella tertiolecta*	CCMP1320 [a]	Model
Cyanophyceae	*Synechococcus bacillaris*	CCMP1333 [a]	Coastal ocean, North Atlantic

[a] Center for Collections of Marine Algae at Xiamen University. [b] National Center for Marine Algae and Microbiota. HAB: Species involved in Harmful Algal Blooms

2.2. Measurement of DNA Content

DNA content can be measured using two methods: DNA extraction followed by spectrophotometric measurement or a fluorometric DNA stain followed by flow cytometric analysis. Both methods have their advantages and disadvantages. Both have sources of uncertainties: flow cytometric analysis relies on proper standards and has been found to generally overestimate [39], while DNA extraction suffers from DNA loss during the extraction procedure [40]. In our study, the DNA extraction method was preferred as DNA was needed for qPCR to quantify rDNA, and because it is possible to calibrate for DNA loss occurring during the extraction process [27,40]. DNA extraction followed the recently developed protocol, which features long incubation and bead beating of the samples followed by a microscopic check to ensure complete cell lysis [27,40]. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) [41].

2.3. Measurement of Carbon Content

Cells collected on 25 mm GF/F filters described above were processed in a Fisons NA 1500 series II elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA) following the EPA method 440 [42]. The procedure was as recently reported [43]. Based on the cell concentration measured as described above and the volume of culture sampled, the C content per sample was divided by (cell concentration x sample volume) to yield C content per cell.

2.4. Quantification of rDNA Using Quantitative PCR (qPCR)

qPCR was carried out for all cultures to estimate the rDNA copy number per cell. Species-specific primers were used throughout this study for each species, for both eukaryotes (18S) and prokaryotes (16S), as shown in Table S1. Standards were prepared by amplifying a ~1.5 kbp rDNA region from the target species, quantifying it, and making 10-fold serial dilutions [44]. Efficiencies and R^2 were used to evaluate the quality of qPCR and accuracy, with PCR efficiency > 80% and R^2 > 0.998 as minimal criteria. Melt curves were generated to check the specificity of the amplification. Technical details were as reported in Yuan et al. [40] and Saad et al. [27]. The rDNA copy number per cell was estimated by multiplying the copy number per nanogram of genomic DNA measured from

qPCR by genome size. The documented rDNA copy numbers from the sequenced genomes were used, where applicable, to check accuracy.

2.5. Use of Previously Reported Data

Previously reported data of cellular DNA content, C content, and rDNA copy number were collected [1,15,16,26,31,36,45–59]. The data covered microbes, plants, and animals, including many model organisms. For instance, microbes included *Escherichia coli*, *Bacillus subtilis*, *Caulobacter crescentus*, and *Mycobacterium tuberculosis*; algae included the chlorophyte *Chlamydomonas reinhardii*; plants included *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, and *Oryza sativa*; animals included *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Bombyx mori*, and *Artemia salina*. All three parameters, genome size, rDNA copy number, and carbon content, were available except for some organisms that missed one of the three parameters. For some unicellular organisms, equations between cell volume and rDNA copy number: LogV = 1.22 LogrDNA − 0.61 [29], and between cell volume and C: LogC = 0.866 LogV − 0.46 [20] were used to calculate the missing C content and rDNA copy number. The units of the data were converted when needed. These collected data were overlayed to our established regression lines to examine their fitness to the regression.

2.6. Statistical Analysis

Correlation analyses were conducted to relate measured variables to each other: total C content vs. DNA content, rDNA copy number vs. DNA content, and C content vs. rDNA copy number. Based on the literature data mentioned earlier, we predicted a linear correlation (Pearson) relationship of log-transformed measured variables (Equation (1) shown below). Still, we tried other functions and compared their degrees of fit (R^2 and p values) as well.

$$Y = aX + b \quad (1)$$

where Y = log (rDNA or C), X = log (rDNA or DNA), a and b are constants.

To evaluate the applicability of the DNA (rDNA)-to-C regression models, differences between species were assessed. Statistical analyses such as ANOVA were performed to assess the influence of environmental conditions on the regression, e.g., the effects of growth conditions and growth stages on the deviations from the regression line. All statistical analyses were conducted using GraphPad Prism 10.0.0. Brown–Forsythe and Bartlett's tests were used to test the assumption of homogeneity of variances, confirming the appropriateness of parametric statistical analysis.

3. Results

3.1. Variability of DNA Content, C Content, and rDNA Copy Numbers

We determined the DNA content by DNA extraction from a known number of cells and the spectrophotometric measurement of the isolated DNA solution, the C content by the EPA standard method, and the rDNA copy number by the qPCR method. No significant differences were found among the replicate cultures grown under the same condition, i.e., within-treatment (one-way ANOVA, $p > 0.05$), verifying the consistency of our measurements. The DNA content of the 11 phytoplankton species included in this study under all conditions ranged from 0.0024 ± 0.0007 pg/cell (*Synechococcus bacillaris*, $n = 33$) to 212.90 ± 64.34 pg/cell (*Odontella sinensis*, $n = 33$) (Figure 1A, Table 2).

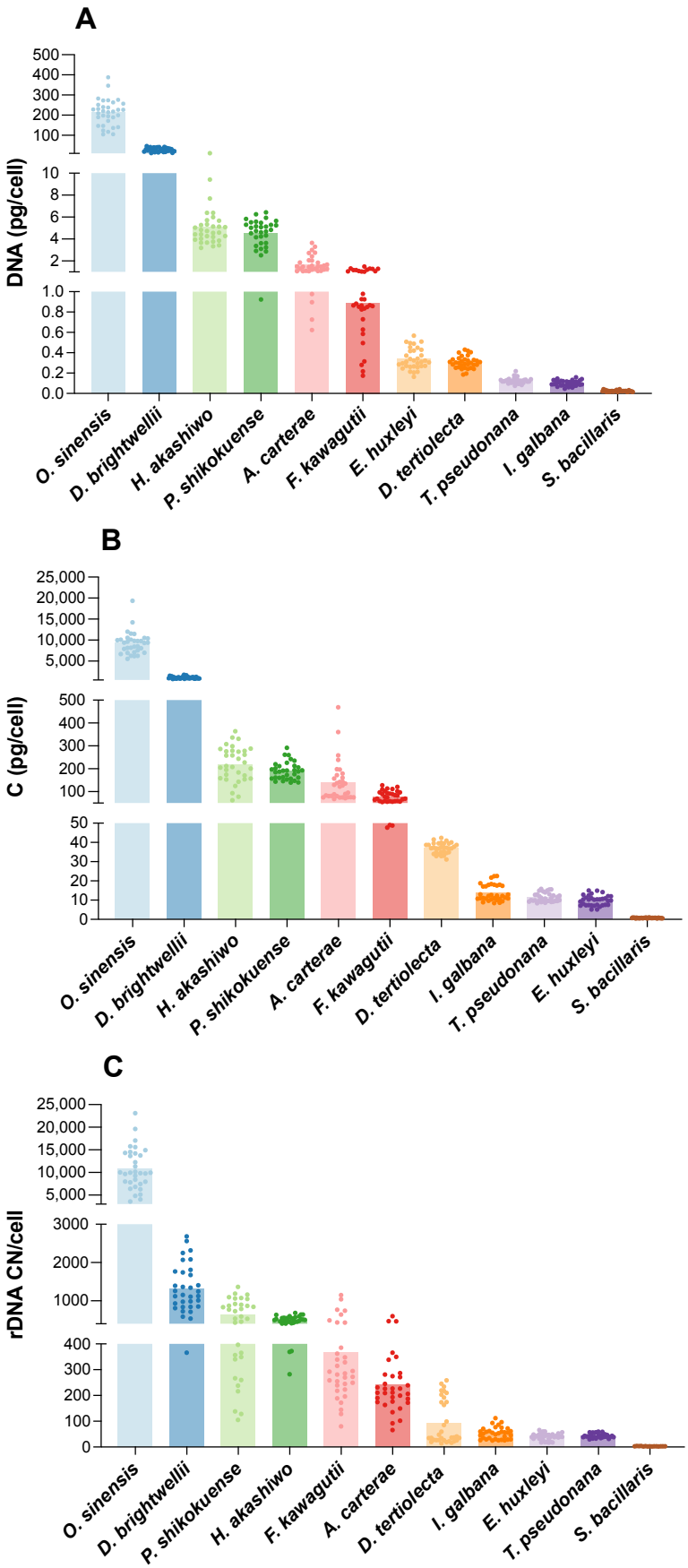


Figure 1. Measured DNA content (**A**), C content (**B**), and rDNA copy number (**C**) per cell of the 11 phytoplankton species.

Table 2. Current data compared with published data.

Species	DNA Content (pg/cell)		C Content (pg/cell)		rDNA Copy Number/Cell	
	This Study	Published Data	This Study	Published Data	This Study	Published Data
Amphidinium carterae	1.63 ± 0.73	0.48 [60]	141.15 ± 87.8	95.46 [4]	240.38 ± 109.77	840 [57]; 84 [58]
Prorocentrum shikokuense	4.64 ± 1.02	6 [8]	190.09 ± 37.72	190.5 [43]	649.41 ± 346.19	628 [61]
Dunaliella tertiolecta	0.30 ± 0.06	0.30–0.37 [62]	37.05 ± 2.69	37.98 [8]	93.41 ± 84.68	
Isochrysis galbana	0.10 ± 0.03	0.09 [a]	13.8 ± 4.18	6.97 [1]	53.33 ± 22.58	
Emiliania huxleyi	0.34 ± 0.1	0.17 [63]	9.77 ± 2.64	4.57 [1]	39.66 ± 13.09	47.6 [52]
Thalassiosira pseudonana	0.13 ± 0.03	0.032 [64]	11.41 ± 2.28	11.79 [8]	43.96 ± 8.27	
Ditylum brightwellii	28.92 ± 9.45	12.9 [14]	1,071.37 ± 248.76	911 [3]	1323.04 ± 590.58	36,896 [57]; 11,000 [58]
Odontella sinensis	212.90 ± 64.34		9275.52 ± 2609.27		10,772.71 ± 4474.86	
Heterosigma akashiwo	5.08 ± 1.68	3.65 [40]	219.43 ± 75.45		502.88 ± 89.02	
Fugacium kawagutii	0.89 ± 0.32	0.94 [65]	78.63 ± 22.85		365.77 ± 240.95	470 [27]
Synechococcus bacillaris	0.0024 ± 0.0007	0.0024 [66]	0.71 ± 0.14	0.6 [21]	2.47 ± 0.69	3 [50]

[a] GenBank: Accession No. GCA_018136815.1.

The C content ranged from 0.71 ± 0.14 pg/cell (*S. bacillaris*, $n = 33$) to 9275.52 ± 2609.27 pg/cell (*O. sinensis*, $n = 33$) (Figure 1B, Table 2). Similarly to the case of DNA, the estimates for the within-treatment triplicate cultures for each species showed no significant variation (one-way ANOVA, $p > 0.05$).

The rDNA copy number ranged from 2.47 ± 0.69 copy/cell (*S. bacillaris*, $n = 33$) to 10772.71 ± 4474.86 copy/cell (*O. sinensis*, $n = 33$) (Figure 1C, Table 2). The estimates for the within-treatment triplicate cultures for each species showed no significant variation (one-way ANOVA, $p > 0.05$).

Besides our measured data, we also collected published data to extend the coverage of species as much as possible. For cyanobacteria, we only measured C, DNA, and rDNA for one species, *S. bacillaris*, but from previous reports, we obtained DNA content and rDNA copy numbers for 80 cyanobacteria species [49,50]. With the addition of these published data, the range of DNA content and rDNA copy number per cyanobacterial cell were 0.0036 ± 0.0020 pg/cell and 2.0924 ± 0.8679 copy/cell. Unfortunately, cellular carbon data were not available for these cyanobacteria.

3.2. Variable Effects of Growth Stage, P-nutrient Condition, and Temperature on DNA Content, C Content, and rDNA Copy Number

All 11 phytoplankton species were grown under three different conditions, optimal condition (optimal temperature and complete L1 medium), P-limited condition, and elevated temperature. In optimal conditions, the samples were collected at exponential and stationary phases. In the P-limited conditions and elevated temperature conditions, the samples were collected at the exponential phase. The results showed that the growth stages, phosphorus limitation, and the elevated temperature had significant influence on some phytoplankton species.

Growth stages had significant influence on the DNA content of six species (*Amphidinium carterae*, *Heterosigma akashiwo*, *Isochrysis galbana*, *Emiliania huxleyi*, *Thalassiosira*

pseudonana, and *O. sinensis*), the C content of six species (*A. carterae*, *E. huxleyi*, *Prorocentrum shikokuense*, *I. galbana*, *Fugacium kawagutii*, and *T. pseudonana*), and the rDNA copy number of four species (*A. carterae*, *E. huxleyi*, *I. galbana*, and *O. sinensis*) (one-way ANOVA, $p < 0.05$) (Figure S1).

Elevated temperatures had significant influence on the DNA content in six species (*A. carterae*, *P. shikokuense*, *Dunaliella tertiolecta*, *E. huxleyi*, *Ditylum brightwellii*, and *S. bacillaris*), on the C content in five species (*P. shikokuense*, *E. huxleyi*, *H. akashiwo*, *D. brightwellii*, and *S. bacillaris*), and on the rDNA copy number in one species (*F. kawagutii*) (one-way ANOVA, $p < 0.05$) (Figure S2). All the DNA content from the six species (*A. carterae*, *P. shikokuense*, *D. tertiolecta*, *E. huxleyi*, *D. brightwellii*, and *S. bacillaris*) increased at higher temperatures. The C content increased in *P. shikokuense*, *E. huxleyi*, *D. brightwellii,* and *S. bacillaris* and decreased in *H. akashiwo*. The rDNA copy number from *F. kawagutii* decreased.

The P-limited condition had the least influence on the content of DNA, C, and rDNA in the 11 phytoplankton species we examined. Compared to the control, the cellular DNA content only significantly decreased in *F. kawagutii*; the cellular C content only significantly increased in *P. shikokuense*; and the cellular rDNA copy number only decreased significantly in *F. kawagutii* (one-way ANOVA, $p < 0.05$) (Figure S2).

3.3. Strong Correlation of Carbon Content with DNA and rDNA Content at Species Level

The potential correlation among C content, DNA content, and rDNA copy number were first explored in individual species at different growth stages. In most cases, the trends of the three parameters followed each other well, which showed strong positive correlations. Figure S4A shows an example of the correlations in *I. galbana* at different growth stages (Pearson's correlation, $r > 0.9$, $p < 0.0001$). Moreover, the correlations were explored in individual species under different growth conditions. Positive correlations were found in most species as well. Figure S4B shows the example of the positive correlations in *F. kawagutii* across different growth conditions (Pearson's correlation, $r > 0.9$, $p < 0.0001$). Generally, it reveals that the C content, DNA content, and rDNA copy number were correlated at the species level, suggesting that these parameters varied in a corresponding manner in response to different growth stages, P-nutrient conditions, and growth temperatures. This verifies our hypothesis that a correlation exists between DNA content, C content, and rDNA content at the species level.

3.4. Strong Correlations of Carbon Content with DNA and rDNA Contents at Community Level

To examine whether the three parameters form unified correlations across species, multi-model regression analyses were performed separately among three parameters using all the data from the 11 phytoplankton species and the 80 genome-sequenced cyanobacteria. The linear regression of log-transformed data was the best-fit regression model (Figure 2). With qPCR-measured rDNA data alone, the equation between rDNA and DNA is $Y = 0.7037 X + 0.1308$ ($R^2 = 0.9190$, $p < 0.0001$, $n = 362$). When data from the 80 genome-sequenced cyanobacteria were added, the correlation between rDNA and DNA was strengthened (Figure 2B).

For the C content vs. DNA content relationship, the regression equation is

$$\mathbf{Y = 0.8165\ X + 2.407}\ (R^2 = 0.9577,\ p < 0.0001,\ n = 362) \quad (2)$$

For the rDNA content vs. DNA copy number relationship, the regression equation is

$$\mathbf{Y = 0.7472\ X - 0.0289}\ (R^2 = 0.9456,\ p < 0.0001,\ n = 442) \quad (3)$$

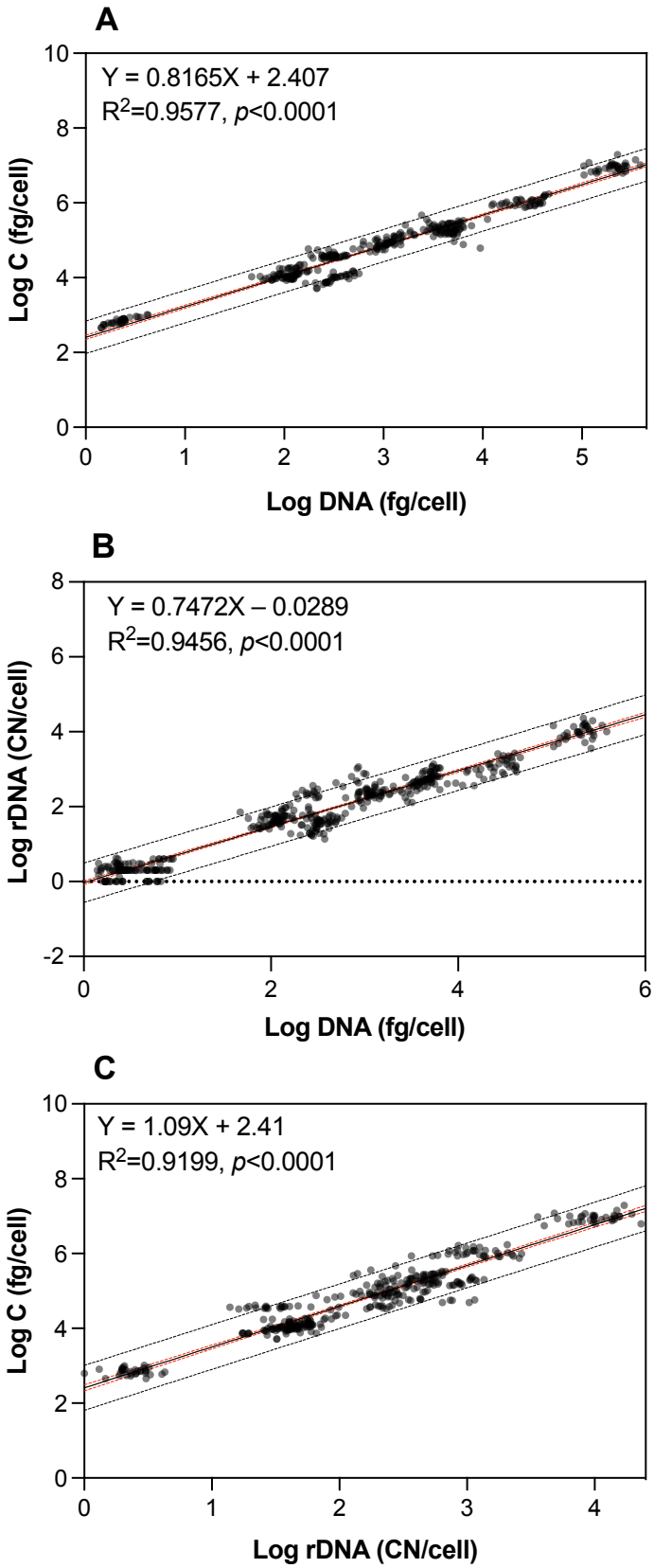


Figure 2. Log-log correlations of C content vs. DNA content (**A**), rDNA copy number vs. DNA content (**B**), and C content vs. rDNA copy number (**C**) for 11 phytoplankton species across different growth stages, P levels, and temperatures. Data of 80 cyanobacteria species from a previous study were included in (**B**) [49,50]. The solid line is the regression line, the red dashed line depicts the 95% confidence interval, the black dashed line denotes the 95% prediction interval, and the black dotted line is the zero baseline in (**B**).

For the C content vs. rDNA copy number relationship, the regression equation is

$$\mathbf{Y = 1.09\ X + 2.41}\ (R^2 = 0.9199,\ p < 0.0001,\ n = 362) \quad (4)$$

4. Discussion

4.1. Measurement Validation

To validate our measurement accuracy, we compared our results with existing data from the literature and databases for various species. The mean values of the DNA contents in each species are very close to previous reports (Table 2). For instance, DNA content in *I. galbana* was estimated to be 0.1 pg/cell in the present study and 0.09 pg/cell in a previous study [67]. For *F. kawagutii*, the estimate was 0.89 pg/cell in the present study and 0.94 pg/cell in a previous study [65].

The accuracy of C content measurements was also verified by the high similarity between our data and published data (Table 2). For example, *D. tertiolecta* was estimated to have a C content of 37.05 pg/cell in our study and 37.98 pg/cell in the literature [8]. *T. pseudonana* had a C content of 11.41 pg/cell in our study and 11.79 pg/cell in the literature [8].

The rDNA copy numbers were also compared with the published data. Most of our measurements were very close to the available reported data (Table 2). For instance, *E. huxleyi* (39.66 copy/cell) was similar to a previous estimate (47.6 copy/cell) [52]. *S. bacillaris* (2.47 copy/cell) was close to the published data (3 copy/cell) [50].

Overall, many of our measured parameters corresponded with published data (Table 2). This is especially true for the C measurement, of which the method was consistently and reliably used for years in different research [68]. Of the seven species (*A. carterae*, *D. tertiolecta*, *I. galbana*, *E. huxleyi*, *T. pseudonana*, *S. kawagutii*, and *S. bacilarris*) in our study that had genome sequencing data with estimated genome sizes, five species had DNA contents very close to the sequenced results, which verified the reliability of our DNA measurement. The only exceptions were *A. carterae* and *T. pseudonana*, for which DNA content appeared to be overestimated by ~3.5-fold. Even higher overestimation (~20-fold and ~11-fold) occurred in another study [8]. So, these two species might have potentially unknown characteristics that significantly influence DNA measurements. Regarding the rDNA copy number, we compared our data with the limited published results (Table 2); *A. carterae* and *D. brightwellii* showed variations with our data. For *A. carterae*, our measurements fell within the range of previous studies, indicating acceptable consistency. However, for *D. brightwellii*, we observed a significant discrepancy, with our values differing by over tenfold from existing data. This substantial variation may arise from differences in methodologies, primers, or operational procedures across studies. The consistency within our study enhances the reliability and value of our results compared to data from disparate sources. Overall, while some variations between studies exist, they are generally within reasonable limits. The alignment of our data with the literature values supports the validity of our measurements.

4.2. Effects of Growth Stage, P-nutrient Condition, and Temperature

Growth stages had significant influence on the C content, DNA content, and rDNA copy number of some species. Some variations were observed, most frequently on the second day and the fourteenth day, corresponding to the early exponential and late stationary stages when the highest and the lowest cell division rates were expected (Figure S1). During the exponential stage, the active cell division happens with the active synthesis of DNA and C. The variations were within a factor of two, in general, except for *A. carterae* which might have some unknown influencing characteristics as discussed above, and in

some cases were very small, reflecting the range of dynamics of growth rate variation in different species. Only *T. pseudonana* showed significantly lower C content, and *O. sisnensis* showed a significantly lower rDNA copy number on the second day, reflecting the lag phase at the beginning of the cultures. On the contrary, during the late stationary stage, the cell division rates decreased, and so did the content of DNA and C per cell on average. However, *H. akashiwo* and *T. pseaudonana* showed significantly higher DNA content on the fourteenth day, which cannot be explained with current data.

Elevated temperatures had significant influence on the C content, DNA content, and rDNA copy number in some species. Cell mitosis is very sensitive to temperature, and elevated temperature will activate cell cycle progression [69], causing more cells to cycle out of the G1 phase, which has the lowest amount of DNA (single copy of the genome) and conceivable rDNA as well. Conversely, cells in the later stages would have higher cellular contents of C and DNA. We observed that cell size and C content changed in parallel with the cell cycle progression. For instance, G2-phase cells contain 2N DNA and are larger than G1-phase cells. This aligns with our observations that samples from the stationary growth phase (where most cells are in G1 phase) and exponential phase (where some cells are in the S and G2 stages) both fit the regression model. In regard to the decrease of rDNA copy number in *F. kawagutii*, the negative impact of the elevated temperature might be due to the slowdown of its growth and the arrest of the cell cycle at the G1 phase, reducing the rDNA copy number.

The P-limited condition had the least influence on the content of C, DNA, and rDNA in the 11 phytoplankton species we examined. Phosphorus is required for nucleic acids (DNA, RNA), so phosphorus deficiency is expected to decrease the synthesis of DNA and rDNA. Under P-limited conditions, cells would continue growing and accumulate fixed carbon in the absence of division [10]; therefore, the increase in C content observed was as expected. However, not all cultures showed the effect of P limitation because different species have different capacities to store P from previous nutrient-replete cultures, and some species were not yet in P-limited physiologies at the time of sampling according to the comparison of growth curves (Figure S3). In addition, when phytoplankton grow on full media, except for the time when a large fraction of the cell population are in the S or G2 or M phase (typically in the afternoon or at dusk), the majority of cells are in the G1 phase; when P-limited media are used, phytoplankton cells are arrested in the G1 phase [10]. This is consistent with our observation in the current study that most of the species did not show significant decreases in DNA or rDNA content per cell.

4.3. Correlations of Carbon Content with DNA and rDNA Content in Phytoplankton

Strong correlations of C content with DNA and rDNA content were established. Notably, the correlation between C content and the rDNA copy number is not as strong (R^2 = 0.9199) as that between C content and DNA content (R^2 = 0.9577). In other words, according to the regression equations, rDNA content is a slightly weaker predictor (by ~3.8%), compared to DNA content, of C content. This was likely caused by the variations in rDNA estimation. Although the quality of qPCR was evaluated by the efficiencies (>80%) and R^2 values (>0.998), compared to the measurement techniques of DNA and C contents, the sensitivity of qPCR could render the technique susceptible to a bigger bias which was inevitable. Fortunately, the reliable data from the 80 sequenced cyanobacteria strengthened the relationship between DNA content and the rDNA copy number and diminished the influence of the qPCR bias. Our analysis indicates that C content, DNA content, and rDNA content are correlated in the group of phytoplankton, verifying our hypotheses at the community level for phytoplankton.

4.4. Extension of the Correlations to Other Organisms

Besides our own phytoplankton data, we also further explored the possible correlations among the three parameters in other unicellular microbes, including some other phytoplankton, archaea, ciliates, and bacteria. Not all C content, DNA content, and rDNA copy number data were available for each species we searched. For the missing DNA content data, we searched the literature and the NCBI database (National Center for Biotechnology Information). For the missing C content and rDNA copy number, the cellular volume was used as the bridge: based on the published regression equations correlating cellular volume with both C content and the rDNA copy number, the missing C content and rDNA copy number data were calculated from reported cell volumes. To ascertain whether the other unicellular microbes exhibit similar correlations, we incorporated the new data into the correlation figures and found they had strikingly similar trends (Figure 3). However, some newly added organisms, including species from ciliates, foraminifera, fungi, and dinoflagellates, showed notable rDNA copy number variability, creating outliers in the data (Figure 3B and C). Among ciliates, *Tetrahymena thermophila* and *Paramecium tetraurelia* exhibit notable differences in rDNA copy numbers among strains, likely influenced by growth stages, nutrient conditions, and mutation accumulation, which may enhance adaptation and lead to potential overestimation in community analyses [31,36]. In foraminifera, *Globigerinoides ruber* and *Orbulina universa* display considerable intraspecific variation in rDNA copy numbers, possibly reflecting life cycle-driven genomic changes [37,70]. Similarly, in fungi, *Candida albicans* and *Saccharomyces cerevisiae* have been reported to possess varying rDNA copy numbers within species, potentially affected by phylogeny, lifestyle, and intragenomic polymorphisms in the ITS region ([71,72]. In dinoflagellates, *Alexandrium catenella* and *Alexandrium taylori* exhibit significant variability in rDNA copy numbers [54,56]. Factors like culture conditions and age contribute to rDNA variability [54,56]. These results suggest that while general correlations among carbon content, DNA content, and rDNA copy number hold for many unicellular microbes, certain outliers may arise due to unique biological traits.

Beyond microbes, we further collected data from multi-cellular organisms, including plants and animals. Prokopowich et al. [26] reported DNA content and rDNA copy numbers for 94 species of animals and 68 species of plants, and found a log-log linear correlation (LogDNA = 0.6352 LogrDNA + 1.576, R = 0.3536, $p < 0.0001$). Additionally, the C content, DNA content, and rDNA copy number for humans were also collected [45–48,59]. Notably, variations in the rDNA copy number were observed within humans, with potential causes including cancer-induced rDNA loss due to DNA damage, replication stress, and alterations associated with DNA methylation. These changes are linked to factors such as aging, body mass, schizophrenia, and lifespan, paralleling the influences of growth stages or culture age in phytoplankton. Given rDNA's critical role in maintaining life, adaptation, and evolution, further research is essential [59,73–79]. When we incorporated these new data into our correlation figures, they revealed similar trends. In particular, the human data exhibited a remarkable fit with all the correlations (Figure 3). This result suggests the correlations among the three parameters potentially hold for a broader range of organisms.

With these data from microbes, plants, and animals, we examined whether general correlations exist for carbon content with cellular DNA content and rDNA copy numbers across all three domains of life. By integrating these datasets with ours, we again derived robust regression equations.

For the C content vs. DNA content relationship, the regression equation is

$$\mathbf{Y = 0.8329\ X + 2.337}\ (R^2 = 0.8591,\ p < 0.0001) \quad (5)$$

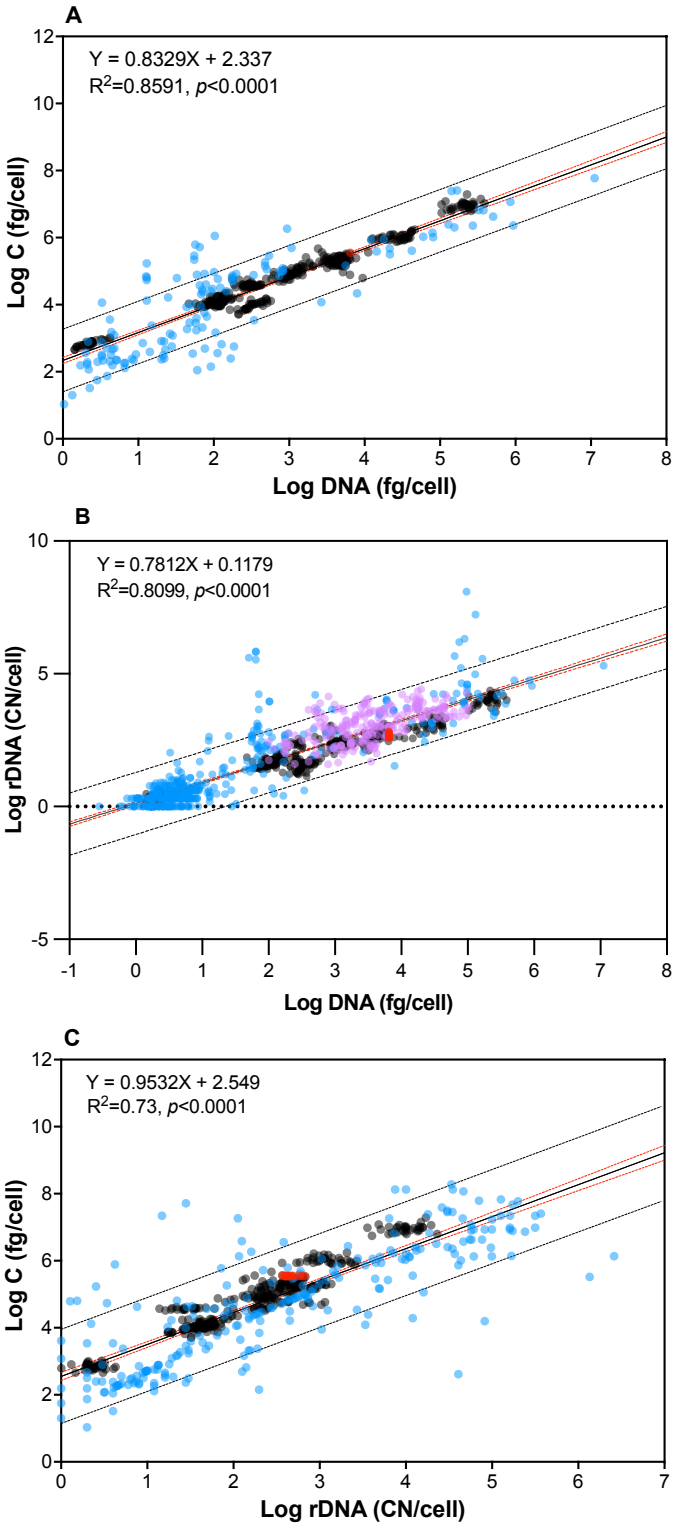


Figure 3. Log-log correlations of C content vs. DNA content (**A**), rDNA copy number vs. DNA content (**B**), and C content vs. rDNA copy number (**C**) for phytoplankton and other organisms. Black dots represent the 11 lab-cultured phytoplankton and 80 cyanobacteria. Blue dots represent published microbial data. Purple dots (only in (**B**)) represent the 94 species of animals and 68 species of plants' data. The red dots represent human data. The solid line is the regression line, the red dashed line represents the 95% confidence interval, the black dashed line denotes the 95% prediction interval, and the black dotted line is the zero baseline in (**B**).

For the rDNA content vs. DNA copy number relationship, the regression equation is

$$\mathbf{Y = 0.7812\ X + 0.1179}\ (R^2 = 0.8099,\ p < 0.0001) \quad (6)$$

For the C content vs. rDNA copy number relationship, the regression equation is

$$\mathbf{Y = 0.9532\ X + 2.549}\ (R^2 = 0.73,\ p < 0.0001) \quad (7)$$

These strong correlations demonstrate there is a high potential that universal correlations among cellular C content, DNA content, and rDNA copy numbers exist in a wide range of organisms, which will be helpful for studying ecology and biogeochemical processes as well as evolutionary biology. However, it is also important to recognize and further investigate potential outliers that may not conform to these patterns, as they could provide valuable insights into unique biological mechanisms.

5. Conclusions

In this study, the C content, DNA content, and rDNA copy number of 11 phytoplankton were measured. Growth stages, P-limited conditions, and elevated temperature conditions were used to reflect some of the environmental variabilities experienced by algae naturally and examine whether such variations would impact the correlations between our measured parameters. Based on the measurements, strong correlations between C content vs. DNA content, rDNA content vs. DNA content, and C content vs. rDNA content were established. Through the correlations, the total carbon biomass of a natural sample will be able to be determined directly according to the total DNA content, and the species-specific carbon biomass will be able to be estimated based on accurate corresponding rDNA copy numbers. Previous data of phytoplankton DNA content or reliable rDNA copy numbers in some related research can now be used to determine the corresponding carbon biomass as well. Additionally, our analysis suggests the existence of universal correlations among C biomass, cellular DNA content, and rDNA copy number across domains of life, highlighting a promising avenue for further exploration. The observed outliers may represent unique cytological or physiological features that also merit additional research.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/dna5010013/s1. Figure S1: Influence from growth stages on DNA content, C content, and rDNA copy number; Figure S2: Comparison of the DNA content, C content, and rDNA copy number from the optimal condition, P-limited condition, and elevated temperatures condition; Figure S3: Comparison of growth curves from the optimal condition, P-limited condition, and elevated temperatures condition; Figure S4: DNA content, carbon content, and rDNA copy number of *Isochrysis galbana* over 14-day experimental period, and of *Fugacium kawagutii* under optimal growth condition, P-limited condition, elevated temperature condition; Table S1: Primers for each phytoplankton.

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