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NOTE

CULTURE CONDITIONS INFLUENCE CELLULAR RNA CONTENT IN ICHTHYOTOXIC FLAGELLATES OF THE GENUS *PSEUDOCHATTONELLA* (DICTYOCHOPHYCEAE)[1]

Simon M. Dittami[2] *and Bente Edvardsen*[2]

Marine Biology, Department of Biology, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway

Cell counts are the standard measure to quantify harmful algae and the basis for decisions on measures necessary to protect human health. Molecular detection methods have been developed for a range of algal species and genera, but these methods generally quantify DNA or RNA, and corresponding cell numbers are inferred based on the assumption that the cellular nucleic acid content is constant over time and in different conditions. Here, we tested this assumption for ichthyotoxic flagellates of the genus *Pseudochattonella* (Dictyochophyceae) under different light, temperature, salinity, and nutrient conditions. Our results show changes in cellular RNA contents of nearly one order of magnitude depending on the condition and also the time of exposure, rendering it difficult to anticipate per-cell RNA yields even if environmental conditions are known. However, cellular RNA content was positively correlated with cell size and growth rate across our experiments, and total RNA was comparable to cell number as a predictor for total biovolume. These results demonstrate the importance of considering the variability of RNA levels for comparisons with cell counts and provide a valuable aid for the interpretation of data from RNA-based detection methods.

Key index words: **cell size; growth conditions; harmful algae; molecular detection methods; ribosomal RNA contents**

RESEARCH NOTE

Continuous monitoring of marine microalgae is important to understand the ecology and distribution of different algal species and the dynamics of marine ecosystems. In the case of harmful algal species, monitoring programs are also fundamental as an early warning system to prevent damage to farmed animals and humans. Presently most monitoring programs rely on light microscopic cell counts to quantify algae (Humbert et al. 2010). These, along with toxin assays, are also currently the basis for decisions about harvesting closures in the presence of harmful algae. Cell counts are labor intensive and identification is frequently only possible to genus-level. This is problematic when toxic and closely related nontoxic species may co-occur.

One approach to these problems has been the development of molecular detection methods such as fluorescent in situ hybridizations (FISH, e.g., Not et al. 2002, Groben and Medlin 2005), sandwich hybridizations (e.g., Scholin et al. 1996, Diercks et al. 2008), quantitative PCR (e.g., Galluzzi et al. 2004, Handy et al. 2006), and microarrays (Metfies and Medlin 2005, Gescher et al. 2008). These methods rely on the recognition of species-specific RNA or DNA sequences and usually allow the quantification of this sequence, but, with the exception of FISH, not the algal cells themselves. In most cases target sequences are located in the ribosome regions (Humbert et al. 2010) and the corresponding probes can be used either to detect ribosomal RNA (rRNA) directly, rRNA-derived cDNA, or genomic DNA coding for rRNA (rDNA). The use of rRNA or cDNA has two distinct advantages over rDNA: First, RNA is rapidly degraded compared with DNA, which favors the detection mainly of live cells. Second, total RNA is constituted mainly of rRNA resulting in a high ratio of target to nontarget sequences. It can therefore be used directly in microarray assays without prior amplification of the target region (Peplies et al. 2006).

In some instances, rRNA content and cell number have been shown to correlate well, especially under standard laboratory conditions (Ayers et al. 2005). However, very little information is available about how environmental conditions and growth rate affect rRNA content in eukaryotes. In bacteria, the per-cell rRNA content has been shown to depend strongly on growth rate and nutrient availability, varying over 10-fold between starved cells in stationary phase and nutrient-replete cells in logarithmic growth phase (Fegatella et al. 1998). Furthermore, relative rRNA abundance has been successfully used as a proxy for bacterial activity (Lami et al. 2009, Campbell et al. 2011).

[1]Received 18 January 2012. Accepted 24 February 2012.
[2]Authors for correspondence: e-mails simon.dittami@bio.uio.no, bente.edvardsen@bio.uio.no.

In this study, we examined the effect of different growth conditions on the cellular total RNA yield as a proxy for cellular rRNA content in a group of eukaryotic microalgae: ichthyotoxic flagellates of the genus *Pseudochattonella* (Dictyochophyceae). Blooms of both species within this genus, *P. verruculosa* (Y. Hara and M. Chihara) Tanabe-Hosoi, Honda, Fukaya, Inagaki and Sako and *P. farcimen* (Eikrem, Edvardsen, and Throndsen) Eikrem are frequent in Japanese coastal (Yamaguchi et al. 1997) as well as Scandinavian waters (e.g., Edler 2006), where they have, on some occasions, been associated to fish kills (Aure et al. 2001). PCR, qPCR, dot blot hybridization, and microarray probes have been developed for both species (Dittami et al. in preparation), and the microarray probes will be included on a prototype array for the RNA-based detection of toxic algae, developed in the framework of the FP7 EU project MIDTAL (Microarray Detection of Toxic ALgae, http://www.midtal.com). Considering the increasing number of molecular detection systems being employed to monitor algae, our data will allow researchers to make well-informed decisions on the choice of methods based on facts, and, if RNA is chosen, to estimate the variance introduced for comparisons with cell counts.

Algal strains of *P. farcimen* (UIO109, UIO110, and UIO111; one culture each) and *P. verruculosa* (NIES670 and JG8, two cultures each) were grown in IMR½ medium (Eppley et al. 1967) supplemented with 10 nM selenite under control conditions, i.e. salinity 25, temperature 16°C, and irradiance 40 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} with a 12:12 h light:-dark regime. Each of these cultures was considered a biological replicate for the respective species and was used to test the effect of different salinities, temperatures, light intensities, and nutrient limitation in four independent experiments (Fig. S1, see supplementary material). At the start of each experiment 9 × 4 mL of each of the seven dense, growing cultures were transferred to 9 × 40 mL of fresh (stress) medium in 50 mL cell culture flasks. Three cultures were kept under control conditions, and the remaining six were exposed to two different stressors for each experiment: For salt stress, salinities of 15 and 35 were chosen, temperatures were adjusted to 4 and 20°C, and irradiance was set to 2 and 800 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. These treatments were previously shown to significantly reduce growth in *Pseuchattonella* spp. (Skjelbred et al. 2012). Finally, treatments with IMR½ medium based on 75% natural sea water and 25% dH_2O without added phosphate or nitrate were tested. For the temperature experiment cultures were grown in different temperature-controlled culture rooms, but growth conditions were identical otherwise.

Experiments were run for 3 d. Every 24 h one mL of culture was fixed with one drop of Lugol's solution for counting and cell size measurements, and 2 × 20 mL (technical replicates) of one flask for each condition and each of the seven cultures were harvested by gentle filtration (water aspirator) on 25 mm cellulose nitrate filters (3 μm pore size). Filters were placed in 2 mL cryotubes with 0.5 mL of TRI Reagent (Ambion, Applied Biosystems, Foster City, CA, USA) and ca. 150 μL of acid-washed glass beads (213–300 μm) and were frozen at −80°C. Within 2 weeks of freezing, samples were agitated for 2 × 15 s at 6,000 rpm using a Precellys 24 homogenizer (Bertin, Montigny le Bretonneux, France) and total RNA was extracted according to the TRI Reagent protocol. Finally, total RNA was quantified using a Nanodrop 1000. Technical duplicates were used to estimate the effect of possible differences in extraction efficiency and then averaged for further analysis. Although the extraction efficiency was rather constant, RNA yields obtained in our experiments are likely to underestimate the actual per-cell RNA content, as an extraction efficiency of 100% is rarely reached.

Cell concentrations were determined using a Fuchs-Rosenthal counting chamber and counting at least 2 × 400 cells. If cell estimations varied more than 10% between duplicated counts a third count was performed and all counts averaged. Cells of each sample were photographed at 200× magnification using a Nikon D5000 camera with a Zeiss Axio Scope A1. Length (a) and diameter (b) of 20 cells per condition, time point, and replicate were measured using ImageJ (Abramoff et al. 2004). Cell volumes (V) were estimated assuming an ellipsoid rotation body around the longer axis (a) using the formula $V = \frac{\pi}{6} ab^2$. The average volume for each replicate culture was then used for further analysis. Average growth rates (r) over 3 d were calculated from cell concentrations (C) as $r = \frac{\mathrm{In}(C(t_1)) - \mathrm{In}(C(t_2))}{(t_2 - t_1)}$.

For comparisons of our cell volumes with data obtained from live or non-Lugol's fixed samples it should be noted, that we may be systematically underestimating the actual cell volume, as Lugol's -fixation results in slight shrinkage of cells.

Statistical analyses were carried out in R 2.10.1 using a separate Repeated Measures Analyses of Variance (ANOVA) for each experiment and species, matching observations made for each culture/strain. Condition and time (d 1, 2, and 3) were used as predictors and cellular RNA yield and cell size as response variables. A Kolmogorov–Smirnov test was used to confirm that the data did not differ significantly from a normal distribution ($P > 0.1$ in all cases). The relationship between average cellular RNA yield, cell volume, and average growth rate was explored using Pearson correlations.

Our results show the per-cell RNA content in *Pseudochattonella* spp. to vary by nearly one order of magnitude depending on the culture conditions: mean RNA yields ranged from 0.80 to 5.7 pg $\cdot$ cell^{-1} in *P. farcimen* and from 0.25 to 1.9 pg $\cdot$ cell^{-1} in *P. verruculosa* (Fig. 1). Corresponding cell volumes

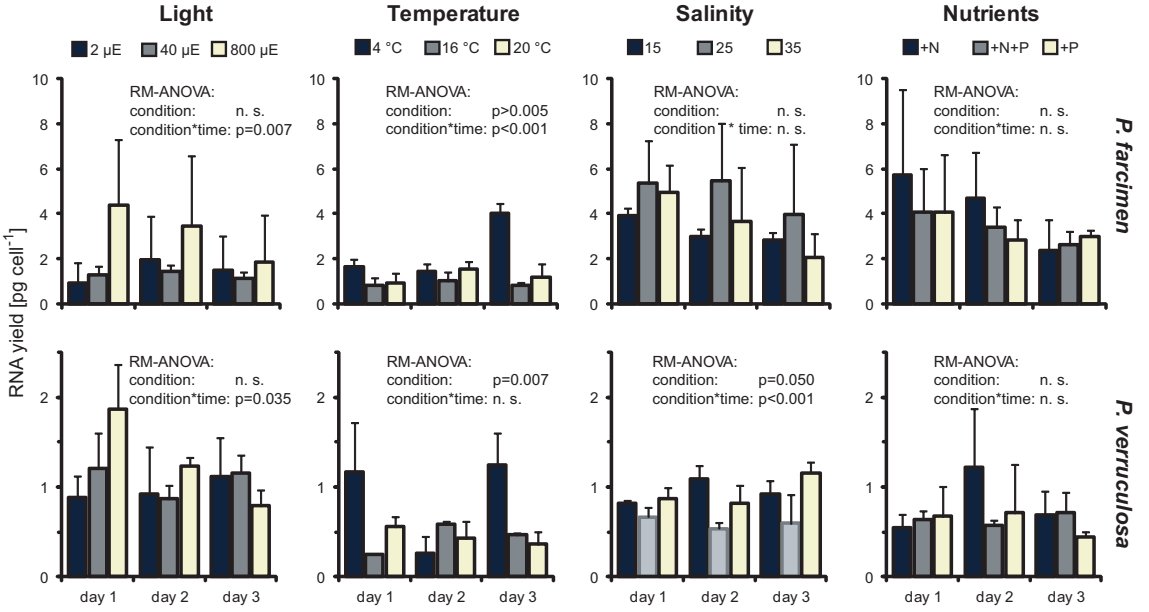

FIG. 1. Mean RNA yield for both *Pseudochattonella* species under various culture conditions ($n = 3$ for *P. farcimen*, $n = 4$ for *P. verruculosa*; error bars = SD). The statistical significance of the effects of the treatment (condition) as well as the interaction term (condition × time) as assessed by a Repeated Measures (RM)-ANOVA is indicated in the graphs (n. s. = not significant; $P > 0.05$).

ranged from 252 to 727 µm^3 and 147 to 437 µm^3, respectively (Table S1 supplementary material; Fig. 2). Culture conditions significantly influenced the RNA yield in *P. farcimen* in the light- and the temperature experiment (Fig. 1). In *P. verruculosa,* the RNA yield was significantly altered in the light, temperature, and also the salinity experiments (Fig. 1). In the nutrient experiment high variability was observed in both species, but there was no systematic difference among the treatments, possibly because the cells were not sufficiently limited only 3 d after the transfer. In addition, we observed variations in extraction efficiency, although these were small compared with the changes in RNA contents induced by the experiments: the average difference between two technical replicates was 28 ± 20% (mean ± SD), and the effect of extraction efficiency on our data further decreased by averaging two replicate extractions for each biological replicate.

Finally, we detected general and statistically significant trends across all experiments. Most importantly there was a positive correlation between RNA yield and cell volume (*P. farcimen* $P < 0.001$, *P. verruculosa* $P = 0.004$; Fig. 2a, c). In addition, RNA yield and growth rate were positively correlated in *P. farcimen* ($r = 0.43$, $P = 0.010$; Fig. 2b), but a similar trend in *P. verruculosa* ($r = 0.27$) was not significant (Fig. 2d).

These data show that variations in cellular RNA content are an important factor to consider for comparisons between cell-counts and RNA-based detection methods in *Pseudochattonella* spp. Since we chose different algal strains of each species as replicates, trends highlighted are valid for several different genotypes. Our initial consideration was that environmental metadata on light- and nutrient availability as well as temperature and salinity could assist the prediction of cellular RNA contents. However, in several cases the interaction term (time × condition) was significant, indicating that the effect of a particular treatment was not constant over time. In addition, even after considering the effect of our treatments, the variability in our data, and thus the variability between strains was still high (see Fig. 1, standard deviations). Both the high variability among strains and the influence of time on the interactions make it difficult if not impossible to reliably estimate per-cell RNA yields to be expected in a field sample containing *Pseudochattonella* spp. purely based on environmental metadata.

The general trends observed across all experiments may, however, aid the comparison of cell counts and RNA-based detection methods. Cell volume is easy to record and can explain 22% (*P. verruculosa*) to 46% (*P. farcimen*) of the variability observed in the per-cell RNA content, as indicated by the positive correlation between cell volume and per-cell RNA yield. RNA therefore is a better proxy for total biovolume (cell number × volume) than it is for cell number alone, as previously proposed e.g. for fungi (Koliander et al. 1984). Although a large proportion of the variability in the per-cell contents due to differences in the culture conditions still remains unaccounted for, a direct comparison of the predictive value of cell number and RNA content for total biovolume (Fig. 3), showed both measures to perform nearly equally well (similar R^2-values). Moreover, this

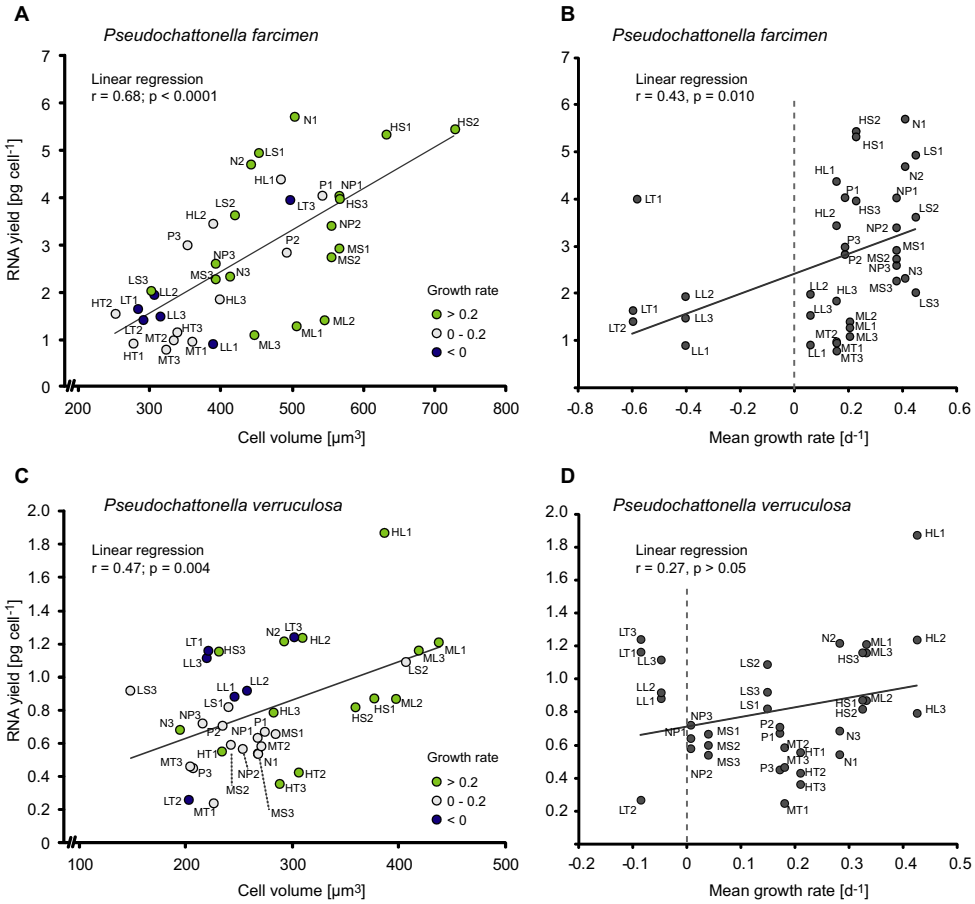

FIG. 2. Panels A, C: Correlation between RNA yield and cell volume over different experiments and culture conditions for *P. farcimen* (A; mean of three replicates) and *P. verruculosa* (C; mean of four replicates). The average growth rate (d^{-1}) in each condition is indicated by color coding. Panels B, D: Correlation between RNA yield and mean growth rate (d^{-1}) over different experiments and culture conditions for *P. farcimen* (B; mean of three replicates) and *P. verruculosa* (D; mean of four replicates). The black line indicates a linear regression comprising all data; r = Pearson correlation coefficient; P = significance of the correlation. Each data point is identified by a label: for the light (L)-, temperature (T)-, and salinity (S) experiments the first letter indicates high (H), medium (M) or low (L) levels of the factor indicated by the second letter. For the nutrients N and P indicate that nitrate or phosphate were added to the medium. ''1'' to ''3'' denotes on what day of the experiment the replicate cultures were harvested.

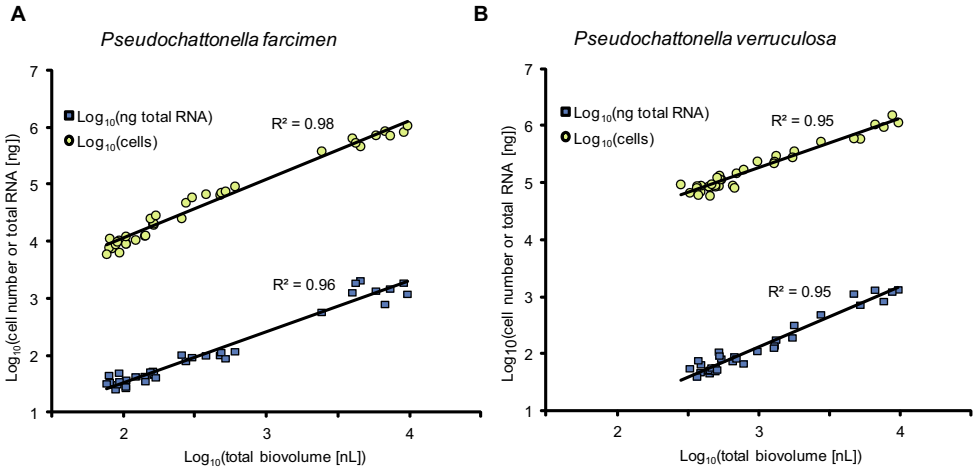

FIG. 3. Comparison of the predictive value of RNA and cell number for total biovolume (cell number × cell volume) in *P. farcimen* (A) and *P. verruculosa* (B). The graph shows $\log_{10}$-transformed data.

result was obtained despite the inherent bias of such a comparison in favor of cell numbers: Because cell numbers were used for the calculation of biovolume, any error introduced by inaccurate cell counts is not considered in the comparison of cell counts and biovolume.

In addition, the correlation between RNA yield and growth rate, although only significant for one of the tested species, needs to be considered. This type of correlation is well known in bacteria, were relative RNA contents may even be used as an indicator for growth rate (Rautio et al. 2003). For RNA-based detection methods this implies higher signals for well-growing algae and lower signals for stationary or dying algae, even though growth rate only explains a small part of the observed variation (18% for *P. farcimen*, $P = 0.01$; and 7% for *P. verruculosa*, not significant).

In summary, due to the substantial variation observed in the cellular RNA content of *Pseudochattonella* spp., RNA-based applications such as microarrays can only be considered semi-quantitative with respect to cell number. However, the observed correlation between cellular RNA level and cell volume as well as, to a certain extent growth rate, may be desirable in some applications, e.g. the monitoring of harmful algae. In *Pseudochattonella* RNA content and cell number performed equally well as proxy for total biovolume (biomass). Furthermore, ribosomal RNA is the main constituent of the ribosome and a motor of protein synthesis, and as such of high physiological relevance for the algae. Although these data indicate that RNA may be a valuable marker, additional studies will be required to ascertain this for different applications. Within the MIDTAL project, which focuses on the detection of toxic algae, for example, the predictive value of total RNA contents for toxicity will be evaluated using field data and comparing both RNA-derived microarray signals and cell counts for a range of toxic algae with quantitative toxin analyses.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Overview of the experimental setup. Seven independent cultures (five strains, there are currently only two *P. verruculosa* strains available) were each used in four experiments examining the effect of three different environmental conditions over 3 days. In each culture, we measured per-cell RNA yield, cell-volume, and growth rate (average over 3 days).

Table S1. Complete dataset used for analyses including cell numbers, growth rates, cell sizes, and RNA contents of all cultures in all conditions.