How Might Mixing Bias Protozoan-experiments that Use the Common Micro-alga *Isochrysis galbana*?

Naomi DOWNES-TETTMAR and David J.S. MONTAGNES

School of Biological Sciences, University of Liverpool, Biosciences Building, Crown Street Liverpool, UK

**Summary.** Although there are strong recommendations to mix microalgal cultures, to improve productivity, there is only anecdotal evidence to encourage protozoologists to mix cultures, to maintain constant quality of microalgae. *Isochrysis galbana* is extensively used in laboratory experiments associated with applied and pure protozoan studies; thus, there is a need to assess how routine mixing may bias experiments. Although, mixing of cultures harms some microalgae, there is no indication of how mixing affects *I. galbana*. We address this problem by assessing mixing effects on key experimental variables: specific growth rate, cell volume, nutritional quality (as carbon and nitrogen content and ratio), and production (the product of growth rate and carbon or nitrogen content). Treatments, quantified as dissipation ($\varepsilon$) m$^{-3}$s$^{-1}$ were: mixed once per day ($\varepsilon=0$), rotated on a plankton wheel ($\varepsilon=5.3 \times 10^{-5}$), and shaken on agitating tables ($\varepsilon=8.2 \times 10^{-4}, 2.32 \times 10^{-3}$). Mixing levels regularly used in experimental work (no-mixing, plankton wheel, and low agitation) did not alter *I. galbana* specific growth rate and thus need not be carefully controlled for, but exceptionally high-mixing did significantly reduce growth rate. Cell volume decreased at exceptionally high-mixing, but otherwise there was no size-bias caused by mixing. Carbon and nitrogen levels were highest at low-mixing but were otherwise generally unaffected by mixing. The nutritional quality of *I. galbana* (i.e. carbon:nitrogen) remained unaltered by mixing. Production was doubled at low-mixing, compared to all other treatments (which did not differ). Overall, *I. galbana* is a relatively robust species that withstands mixing levels well within those experienced in the laboratory and still grows at exceptionally high-mixing levels. Thus, within reason, protozoologists using *I. galbana* do not need to be rigorous in their monitor of mixing. Furthermore, meta-analyses that examine this species need not be too concerned with variation in mixing methods as a confounding factor. However, the data suggest that gentle mixing, using a plankton wheel, is preferable to no-mixing.

**Key words:** Biochemical composition, carbon, experimental design, growth, nitrogen, phytoplankton, production, nutritional quality.

**INTRODUCTION**

Although there are strong recommendations to mix microalgal prey cultures to maintain their constant quality (Stein 1973), there is only anecdotal evidence to encourage protozoologists to mix their prey. In fact, key texts that outline routine maintenance methods (e.g. Fogg 1965, Stein 1973, Boney 1975, Becker 1994, Andersen 2005) fail to provide guidance regarding the application, extent, or consequences of mixing. There is, admittedly, a belief by many protozoologists that mixing of cultures is important, but to what extent? Here we address this question, focusing on an extensively used experimental species in marine protozoology, to provide guidance for future work.

Since its description ~60 years ago (Parke 1949), *Isochrysis galbana* has been extensively and routinely used in laboratory experiments associated with applied and pure marine sciences; every year 100s of studies...
examine this species. In these works, *I. galbana* is often employed as a model to investigate various algal processes (e.g. light responses, Thompson et al. 1990; temperature responses, Montagnes and Franklin 2001; clonal differences Sayegh et al. 2007). Furthermore, for the last 35 years *I. galbana* has routinely been used as a prey for a wide range of marine protozoa (e.g. Gold 1973, Steecker et al. 1988, Bernard and Rassoulzadegan 1990, Verity 1991, Christaki et al. 1996, Montagnes 1996, Dolan and Simek 1997, Jakobsen and Hansen 1997, Strom and Morello 1998, Klein Breteler et al. 1999, Johansson and Coats 2002, Kimmance 2006, Guermazi et al. 2008). The quality of *I. galbana* grown in experimental culture is thus a focus of concern, and studies have examined the impact of several key environmental variables (e.g. light, temperature, salinity, nutrients) on a range of attributes, including specific growth rate, composition (e.g. carbon and nitrogen content), predator-prey dynamics, and prey choice (e.g. Thompson et al. 1990, Montagnes and Franklin 2001, Montagnes et al. 2001).

Here we test the hypothesis that mixing will affect the outcome of experimental studies that use *I. galbana*, as mixing microalgal cultures, in general, can alter their quality by reducing nutritional gradients, improving gas transfer, preventing sedimentation, and ensuring equal light exposure (Jiménez et al. 1996), but excessive mixing can be detrimental to some microalgae, disrupting nutrient uptake, causing cell wall damage, inducing filament loss, and increasing mortality (Thomas and Gibson 1990, Borowitzka 1997). If *I. galbana* responds like other microalgae, then this is a concern for experimental protozoologists, and if it is not affected, then this observation will provide extremely useful time-saving guidance for researchers.

Surprisingly, virtually nothing is known regarding mixing effects on *I. galbana*, except that it is not affected by low-level turbulence (i.e. a dissipation rate of $10^{-4}$ m$^2$ s$^{-3}$; Havskum 2003). A review of the literature also reveals that a wide range of mixing levels are used in experimental work, and these are rarely quantified: some studies mix cultures once a day, others continuously mix them, and in many studies mixing it is not mentioned (e.g. Davidson et al. 1992, Kleppel and Burkart 1998, Martel 2006). Thus, we test if variation in mixing could alter the outcome of experiments that use *I. galbana*, and determine if future experiments should carefully consider mixing. To this end, we have assessed the impact of mixing *I. galbana* cultures over a range of levels that cover, and extend beyond, the spectrum potentially used in laboratory incubations. We have focused on examining mixing effects on key experimental variables examined by others and applicable to protozoan-related experiments: growth rate, cell volume, carbon and nitrogen content of cells (as proxies of nutritional quality), and production (the product of growth rate and carbon or nitrogen content).

**METHODS**

*Isochrysis galbana* Parke 1949, CCAP 927/1 was maintained in artificial seawater, enriched with f/2 (Guillard 1972) at 16 ± 1°C under a constant irradiance of 85 µmol photons m$^{-2}$s$^{-1}$. Cultures (sample volume 175ml) were grown in 250 ml plastic, rectangular tissue culture flasks. Four replicated treatments were applied (n = 3): non-mixing, where flasks were only mixed (by gentle rotation) once daily when sampled; low-mixing, where flasks were rotated on a vertically oriented ‘plankton wheel’; medium and heavy mixing, where flasks were placed on horizontal agitating tables that moved back and forth, rather than rotating (Table 1).

For the agitated samples, mixing was characterised as dissipation ($\varepsilon$, m$^2$ s$^{-3}$); $\varepsilon = U^2/L$, where $U$, the velocity (m s$^{-1}$), was determined from the speed of agitation (Table 1), and $L$, length (m), was determined as the cube-root of the sample volume (0.056 m). For the rotated samples (low-mixing), turbulence was also assessed as dissipation, but this was a more complex calculation. In this case $U$ was determined from the kinetic energy of rotation of the fluid and the bottle together, in the first instance assuming it was a ridged body; then assuming that the fluid rotates with the bottle, kinetic energy was converted to velocity by

$$U = (0.5 I \omega^2 / M_f)^{1/3}$$

where $M_f$ is the mass of the water, $\omega$ is the angular rotation (2π/rotation time), and $I$ is the moment of inertia, which is $(M_f + M_b) r^2$ (where $r$ is the radius of rotation and $M_b$ is the mass of the flask), but assuming $M_b \gg M_f$ this approximates to $M_f r^2$; $U$ then simplifies to $(0.5 \pi \omega^2)^{1/3}$. Furthermore, mixed samples are likely to experience shearing as well as a dissipation effect (e.g. Havskum 2003); thus we have made estimates.

**Table 1.** Characteristics of the four mixing regimes that *Isochrysis galbana* was exposed to. Calculations are described in the text.

<table>
<thead>
<tr>
<th>Regime</th>
<th>Method</th>
<th>Displacement (m)</th>
<th>Cycles</th>
<th>Velocity ($U$, m s$^{-1}$)</th>
<th>Dissipation ($\varepsilon$, m$^2$ s$^{-3}$)</th>
<th>Shear ($S$, s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Mixed daily</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>~0</td>
<td>~0</td>
</tr>
<tr>
<td>Low</td>
<td>Rotated on wheel</td>
<td>Wheel radius ~0.115</td>
<td>Rotation time 202 s</td>
<td>0.014</td>
<td>5.3 × 10$^{-3}$</td>
<td>0.25</td>
</tr>
<tr>
<td>Medium</td>
<td>Shaker table</td>
<td>Shaking distance ~0.06</td>
<td>77 cycles min$^{-1}$</td>
<td>0.077</td>
<td>8.2 × 10$^{-3}$</td>
<td>1.38</td>
</tr>
<tr>
<td>High</td>
<td>Shaker table</td>
<td>Shaking distance ~0.06</td>
<td>109 cycles min$^{-1}$</td>
<td>0.109</td>
<td>2.32 × 10$^{-2}$</td>
<td>1.95</td>
</tr>
</tbody>
</table>
of shear rate ($S, s^{-1}$), using the above parameters (i.e. $S=U/L$, see Table 1). However, for the purpose of this study we have focused on dissipation ($\varepsilon$) as a parameter to quantify mixing. See Tennekes and Lumley (1972) for a more detailed description of these estimates.

Specific growth rates ($\mu, d^{-1}$) were determined by regressing ln cell numbers vs. time, over the exponential growth phase. Cell volumes were determined from linear dimensions of >30 live cells, harvested from exponentially growing cultures, using an image analysis system (Scion image for Windows, Scion Corp., MD, USA); cells were assumed to be prolate spheroids. Carbon and nitrogen content (pg cell$^{-1}$) was determined from duplicate samples, from each replicate, by elemental analysis (NC 2500, CE Instruments). The C:N ratio, C and N per unit volume, and C and N production (i.e. the product of specific growth rate and biomass) were then determined.

Treatment effects on the above measurements were compared using one way ANOVA, followed by pairwise comparisons (Holm-sidak method, $\alpha=0.05$, $n=3$). All datasets were normally distributed and homoscedastic; Kolmogorov-Smirnov test).

**RESULTS**

There was a significant decrease in specific growth rate of *I. galbana* between the lowest and highest mixing levels (Fig. 1a), but non-mixing was not significantly different from low-mixing or medium-mixing. Cell volume was significantly higher in low-mixing compared with high-mixing, and there was an apparent decrease in volume with mixing, although there was no significant difference between all the other treatments (Fig. 1b).

The carbon and nitrogen analysis demonstrated that at low-mixing C and N cell quota was significantly higher than all other treatments, and there were no significant differences across the other mixing treatments (Fig. 1c). There was no significant effect of mixing on C:N ratio (Fig. 1d). The estimates of C and N per unit volume were significantly higher at high-mixing, and there were no significant differences between the other mixing treatments (Fig. 1e). Production (in terms of both C and N) was significantly higher at low-mixing compared with the other mixing treatments, and there were no differences between the other mixing treatments with the exception of N production where there was a small but significant difference between medium-mixing and high-mixing (Fig. 1f).

**DISCUSSION**

Given the extensive use of *Isochrysis galbana* in experimental protozoological works (see Introduction), there is a need to assess if routine maintenance will alter culture parameters and potentially bias experiments.

We have addressed this issue by assessing growth rate, volume, nutritional quality, and production at a range of mixing levels that cover and extend above those typically used in experimental laboratory practices (Table 1, Richmond 1987); each of these is, in turn, addressed below.

Growth rate is undoubtedly the most measured parameter associated with examining microalgae; for *I. galbana* it is used as an index of physiological response (e.g. to temperature Montagnes and Franklin 2001), as a component of population dynamic studies and ecosystem models (e.g. Kimmance et al. 2006), and as an integral part of other measurements (e.g. determination of grazing on microalgae, Kimmance et al. 2006).

We found that *I. galbana* growth rate decreased due to heavy mixing (a level far beyond that typically applied to laboratory cultures) but remained unaffected by the
other mixing treatments. This decrease in growth rate at an excessively high level could result from increased cell lyses and damage at the air surface interface (Havskum 2003) or hydrodynamic stress acting on the cells causing a physiological disruption (Thomas and Gibson 1990). This might be relevant if studies were interested in the impact of breaking waves on shallow-water, coastal populations, but otherwise mixing would have little ecological or laboratory significance. Clearly, *I. galbana* is a robust species, compared to other more sensitive microalgae (Thomas and Gibson 1990, Borowitzka 1997).

Small changes in prey cell size will also alter experiments with protozoa; *e.g.* prey choice and grazing experiments may be influenced by prey size (*e.g.* Jonsen 1986) and changes in volume may alter the cell nutritional quality (see below). However, *I. galbana* cell volume remains unaffected by lower mixing levels and only decreases with the influence of heavy mixing. Possibly heavy mixing inhibits nutrient uptake at the cell surface thus selecting for smaller cells, or possibly the small cell size is an adaptive mechanism to reduce cell lyses (see above). Regardless, of the cause, there does appear to be a potential size-bias caused by mixing. Protozoologists must also recognise the potential impact of mixing, on the nutritional quality of *I. galbana*; this is often determined by examining total carbon (C) and nitrogen (N) cell content (*e.g.* Mitra and Flynn 2005). We found that C and N quotas per cell reflect the overall trend observed in cell volume, with large cells at low-mixing having the highest C and N levels and small cells at high-mixing having the lowest C and N quotas. However, in terms of C and N per unit volume the relative densities of C and N were the same between non- and medium mixed treatments, but at high-mixing the smaller cells were more densely packed with C and N. We also found that there was no significant stoichiometric change in C:N ratio of cells; this ratio can be used as an index of prey quality, and our data thus suggests that, in this respect, mixing will have little influence on predator responses to *I. galbana*. Consequently, while mixing may affect growth rate and cell volume at high-mixing, the overall nutritional quality of *I. galbana* remains unaltered.

Finally, many experimental measurements (*e.g.* grazing rates, population dynamics) require estimates of production, rather than simply growth rate, as external stimuli may influence both growth rate and nutritional quality of cells (*e.g.* Montagnes and Franklin 2001). Furthermore, there are many cases where production needs to be maximised, such as when rearing batch cultures of algae as food in grazing and prey choice experiments (Montagnes *et al.* 2001). We found a clear trend across mixing treatments: as mixing is increased there is a decrease in production, although unmixed cultures had a significantly lower production than gently mixed ones. Note that production, being the derived product of growth rate and carbon or nitrogen content of cells accentuates the trends exhibited by these independently. Our results illustrate that low-mixing approximately doubles the productivity of cultures, supporting the case to use plankton wheels to optimise production.

In conclusion, mixing effects on *I. galbana* quality are contrary to those expected from past studies, which indicate that microalgae can be highly sensitive to mixing (see Introduction). In contrast, *I. galbana* is a robust species that withstands low to medium-mixing and is not prevented from growth even at unrealistically high-mixing levels. This suggests that within reason, protozoologists, and other researchers, using *I. galbana*, do not require rigorous monitoring of mixing and that meta-analyses that compare the many studies on this species (*e.g.* Sayegh *et al.* 2007) need not be concerned with variation in mixing methods.

Even though we suggest mixing variations are not important, we still found effects of extreme mixing levels and, therefore, can provide some guidance. For instance, *I. galbana* was deleteriously affected by our high-mixing treatment; fortunately, this level is rarely, if ever, used in the laboratory and would even be beyond levels experienced when samples are shipped in the post. Furthermore, we found that low-mixing, which reflects most laboratory conditions, is the most favourable treatment in terms of maximising cell size, production, and C and N content. Finally, there was a clear indication that continuous low-mixing, provided more productive cultures with cells containing more carbon and nitrogen than non-mixed (shaken once per day) cultures. Many researchers do not continuously mix their cultures, and this may result in some bias. Thus, we recommend that although *I. galbana* is relatively insensitive to mixing, experimental work should probably be conducted on gently mixed cultures, using a plankton wheel (*e.g.* Crocker and Gotschalk 1997), which provides homogeneous mixing at extremely low levels; *i.e.* lower than those achievable on a horizontal mixing plate.

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