The Effects of Formalin and Lugol’s Iodine Solution on Protozoal Cell Volume

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With 2 Figures and 1 Table

Key words: Preservatives, protozoa, cell biovolume, time course

Abstract

The effects of formalin, Lugol’s iodine solution and a mixture of these preservatives on the cell volumes of two protozoan species (the flagellate Chilomonas sp. and the ciliate Cyclidium sp.) were studied using phase contrast microscopy. While the percent of live volume was affected by the preservative used and protozoan type, storage time was of overriding importance. Within 10 min., cells exposed to formalin and formalin + Lugol’s solution increased in biovolume, but by 24 h cell shrinkage was in the range of 6 - 19% of live biovolume, formalin fixed samples having the least amount of shrinkage. After one month of storage, cell biovolume was reduced to between 60 and 65% of live biovolume in all three preservatives.

Introduction

Several studies have documented that heterotrophic protozoa can play an important role in the energetics and trophodynamics of planktonic food webs (PACE & ORCUTT 1981; AZAM et al. 1983; PORTER et al. 1985; SHEEHY et al. 1986; CAPRIOLO 1990; REID et al. 1990; MÜLLER & GELLER 1993). Such analyses require an accurate determination of the standing stock of protozoa in order to estimate food demand and carbon availability to grazers. For this, the biovolume of heterotrophic protozoa is calculated from measurements of the cell size of preserved specimens examined microscopically. Biomass is then determined using appropriate conversion factors for density, and the proportions of water and carbon per cell. An accurate determination of cell volume is crucial for obtaining a correct estimate of biomass (PUTT & STOECKER 1989).

Estimates of biovolume should take into consideration any deviation from live volume resulting from the use of preservatives. While the number of studies is small, available data indicate that protozoal cell size can both increase and decrease upon preservation and that changes can be related to the type and concentration of preservative used and to the taxa of protozoa under study (BORSHEIM & BRATBAK 1987; CHOI & STOECKER 1989; PUTT & STOECKER 1989; JEROME et al. 1993). However, there still is disparity concerning the degree of change in biovolume brought about by different preservatives and surprisingly little consideration has been given to other factors that could affect the estimate of biovolume. For example, the biovolume of preserved samples could change over time but time course experiments are rare, the work of WIACKOWSKI et al. (1994) serving as a notable exception. Additionally, most work has been done with a few taxa of cultured protozoa and there is little information to indicate whether organisms in natural samples respond similarly to preservatives. We report here results of time course experiments on the effects of two widely used preservatives (formalin and Lugol’s iodine solution) on the cell size of two protozoan taxa, the ciliate Cyclidium sp. and the flagellate Chilomonas sp. from laboratory culture and natural pond water, respectively.

Materials and Methods

A surface water sample (~13 l) was collected from a farm pond at the Stroud Water Research Center, Chester Co., (39°53'N, 75°45'W) Pennsylvania, USA in early November, and stored at room temperature in the laboratory. Sub-samples (150–200 ml) were taken and filtered through a 50 μm mesh sieve to remove larger grazers. The filtrate was incubated for 5 days at room temperature in the dark which allowed the growth of flagellates and ciliates. The predominant taxa were the flagellate Chilomonas sp. and the ciliate Cyclidium sp. Cyclidium was subcultured in Cerophyl (Agri-Tech, Inc., Kansas City, Missouri) previously bacterized with White Clay Creek bacteria and...
subsamples of this culture, maintained in the dark in a water bath shaker at 25 °C, were used as source material for experiments. Continued incubation of the pond-water sub-samples resulted in profuse growth of *Chilomonas* sp. within another week, which served as source material for experiments with this organism.

**Storage experiments.** For these experiments, source material was sampled and the dimensions of 28–30 live specimens were obtained (length and width were measured using a calibrated eyepiece micrometer in a Zeiss Universal phase contrast microscope at a magnification of 250×). The dimensions of live organisms were measured when the organisms stopped at the edges of cover slips, air bubbles, or particles (debris) in the culture medium, thereby avoiding the use of any chemical means of slowing them down. In all experiments, different slide preparations were made every five to seven minutes to avoid deformation of cells (that would consequently lead to wrong cell volume) and drying of the specimen from the heat of the microscope. Replicate samples of source material were fixed with formalin (F, 2% final concentration), Lugol’s iodine solution containing 1.0 g I₂ and 1.5 g KI per 25 ml water according to Kudo (1966), or a combination of the two (L+F) (Sherr & Sherr 1993). Fixed samples were stored in the dark at 4 °C and at scheduled times (see Table 1) the sizes of 28–30 specimens were measured. We selectively measured the sizes of *Chilomonas* sp., ignoring other protozoa that were encountered in the pond water sample. Biovolumes were calculated assuming that the protozoa were oblate spheroids (v = 4/3πab², where a and b are the major and minor semi-axes).

An arc-sin-square root transformation was applied to proportional data. ANOVA and multiple range tests were performed using SAS software (Version 6.08, SAS Institute, Cary, NC) on a Digital MicroVAX 3100 computer. Other statistical tests were performed using Stat-View (Version 4.02, Abacus Concepts, Berkeley, CA) software on an Apple Macintosh computer.

**Short term experiments.** In order to examine the short-term effects of the preservatives on protozoan size, duplicate samples were taken 10 minutes after the preservatives were added to samples fixed for storage experiments, and also 2 h later. Sizes of organisms were obtained as described, although fewer organisms were measured so that measurements were made close to the desired time.

### Results

**Storage experiments**

The mean live biovolumes of the large flagellate *Chilomonas* sp. and the small ciliate *Cyclidium* sp. were 1850 ± 566 (X ± SD, n = 5 experiments) and 1107 ± 331 μm³ (X ± SD, n = 7 experiments), respectively. Biovolumes for preserved specimens at each sampling time, expressed as a percentage of live biovolume, are presented in Table 1. These data were tested for significance using a 3-way ANOVA with preservative, storage time and protozoan species as main effects. The effects of preservative (p < 0.01) and storage time (p < 0.001), but not protozoan species (p > 0.05), were statistically significant in a model that explained 74.6% of the variance in the data. Although the effects of the most widely used preservatives for protozoa, Lugol’s solution and formalin, were not significantly different from each other, the effect of the two preservatives combined (L+F) was significantly different from the formalin fixed samples [Tukey’s Studentized Range (HSD) test, p ≤ 0.05]. We suspected that this significance between preservatives was due to the differences measured during the first 48h, and when those times were removed from the analysis the effect of treatment was no longer significant (3-way ANOVA, p > 0.05) in a model that explained 56.8% of the variance.

Time was the most significant variable in each of the 3-way ANOVAs, accounting for 10 times and 3 times more variance than the next most important parameter in the respective analysis. Except for differences between successive samples (e.g., 24h and 48h, 48h and 1 week, etc.), the differences in biovolume between times were statistically significant [Tukey’s Studentized Range (HSD) test, p ≤ 0.05]. The biovolume of the 1 month samples was significantly lower than in all other samples [Tukey’s Studentized Range (HSD) test, p ≤ 0.05].

<table>
<thead>
<tr>
<th>Preservative</th>
<th>Organism</th>
<th>Percent of live volume after storage for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td><strong>Formalin</strong></td>
<td><em>Chilomonas</em> sp.</td>
<td>93.8 ± 4.0 (4)</td>
</tr>
<tr>
<td></td>
<td><em>Cyclidium</em> sp.</td>
<td>94.0 ± 5.9 (4)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>93.9 ± 4.7 (8)</td>
</tr>
<tr>
<td><strong>Lugol’s solution</strong></td>
<td><em>Chilomonas</em> sp.</td>
<td>85.8 ± 6.3 (4)</td>
</tr>
<tr>
<td></td>
<td><em>Cyclidium</em> sp.</td>
<td>82.0 ± 0.0 (2)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>84.6 ± 5.3 (7)</td>
</tr>
<tr>
<td><strong>Formalin+ Lugol’s solution</strong></td>
<td><em>Chilomonas</em> sp.</td>
<td>80.5 ± 9.0 (4)</td>
</tr>
<tr>
<td></td>
<td><em>Cyclidium</em> sp.</td>
<td>82.0 ± 5.3 (3)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>81.2 ± 7.1 (7)</td>
</tr>
</tbody>
</table>

(N.D. = Not Determined; N.A. = Not Applicable)
Although the differences between protozoan taxa were not statistically significant in either 3-way ANOVA, the amount of shrinkage for the protozoan species also was examined without the influence of treatment or time using a Tukey's Studentized Range (HSD) Test. The range tests (conducted with and without the inclusion of the data ≤ 48 h) each indicated that shrinkage of the flagellate Chilomonas sp. (grown in pond water) was significantly greater than Cyclidium sp. (grown in bacterized Cerophyl) (p ≤ 0.05).

**Short-term experiments**

In the two experiments that were conducted to examine short-term effects, the volume of Cyclidium sp. fixed with L+F increased by 29% in 10 minutes and by 10% in 2 h (Fig. 1). Formalin fixed samples showed an increase of 17% and 9% in 10 minutes and 2 h, respectively. In contrast, samples fixed with Lugol’s solution shrank by 8 and 3% in 10 minutes and 2 h, respectively (Fig 1). Differences in these data were not statistically significant (Kruskal-Wallis tests, p > 0.05) but there was a consistent trend to slightly larger cell volumes when formalin was used. Formalin fixation of Chilomonas sp., Cyclidium sp. and another unidentified ciliate sp. led to an increase of biovolume by 16–26% within the first 10 minutes of exposure, while Chilomonas sp. fixed with Lugol’s solution showed a decrease of about 10% (Fig. 2). Only the statistically significant difference involved fixation with Lugol’s solution (Mann-Whitney test, p = 0.01).

**Discussion**

During the first 48 h of storage, samples fixed with Lugol’s solution or Lugol’s solution plus formalin experienced greater shrinkage than samples fixed with formalin. PUTT & STOECKER (1989) also found that the biovolume of three ciliate species stored in Lugol’s solution was 76% of the biovolume in formalin, although they did not report the length of storage. Even so, any advantage to the use of formalin for short storage times (with respect to changes in biovolume) disappeared over a longer storage time. In addition, other disadvantages that may be pertinent to the purposes of a given study have been reported for formalin [e.g. egestion of ingested bacteria (Sieracki et al. 1987) or loss of cells (PACE & ORCUTT 1981; Bloem et al. 1986)]. We have not found other studies with data for two days of preservation with which to compare our results.

It is noteworthy that we found that storage time explained the greatest amount of variance in the data. This suggests that length of exposure to a preservative may be an even more important parameter affecting shrinkage than the choice of preservative, at least among those used here. We can compare our data for 2 weeks of storage to those reported by BORSHEIM & BRATBAK (1987), who showed that cells of the microflagellate Monas sp. preserved for 2 weeks had biovolumes in the range of 57.3–76.2% of live volume when stored in formalin and 50.0–65.3% when stored in acid Lugol’s solution. In our work the mean percentage of live biovolume for the species we studied dropped to about 72% by 2 weeks in all the three treatments and to 61.5–65.2% of live biovolume in 1 month, a maximum shrinkage of ~ 40%. WIAKOWSKI et al. (1994) reported that after 1 month cells of two species stored in acid Lugol’s solution showed shrinkage of approximately 25 and 35 %, while cells stored in formalin showed an increase of approximately 15% for one species.
and a decrease of about 10% for the other. In their studies, changes between 1 and 2 months of storage were minimal. Choi & Stoecker (1989) reported that the volumes of fixed protozoa were 20 to 55% smaller than live cell biovolumes. Sherr & Sherr (1993) summarized much available data and concluded that shrinkage could be as great as 45 to 75% of the live cell volume. Those values are greater than measured here, but the length of storage was not reported in many of those studies.

Our findings for samples preserved with formalin or Lugol’s solution differ from results reported by some other workers (Borsheim & Bratbak 1987; Putt & Stoecker 1989; Choi & Stoecker 1989), perhaps because different protozoan species and/or different concentrations (and sometimes formulations) of preservatives were used. The greater shrinkage caused by the L+F preservative on long-term storage may have been because the effects of the two preservatives were additive.

Borsheim & Bratbak (1987) reported that on the average 85% of the changes in volume of preserved samples took place during the first 2 h of storage. We studied changes during that time period in our short-term experiments and found that the immediate effect of formalin on protozoan biovolume differed from its effect after longer storage. Formalin, when used separately or in combination with Lugol’s solution (L+F), tended to distend the cell for at least 2 h. This is consistent with other reports indicating that cells may initially swell when preserved and then, after some hours, reach a stable volume that is smaller than live biovolume (Ohman & Snyder 1991). On the other hand, samples preserved with Lugol’s solution seem to shrink first and then regain some of their volume (Figs. 1 and 2). Given the limited data we have here, we can only say that although cells shrink when stored in fixatives, the onset of shrinking differs with preservative and the process should receive further study when cell size is a parameter important to research goals.

We have worked with fixatives from the classes of compounds most commonly used to preserve protozoa, i.e., the aldehyde group (formalin or gluteraldehyde) and Lugol’s iodine solution, usually with acetic acid or acetate amendment. While formalin can cause regurgitation of ingested cells as noted above, it has sometimes been used to insure preservation following treatment with Lugol’s solution (Sherr & Sherr 1993). Preservation in gluteraldehyde produced the most variable results in the study of Wiackowski et al. (1994). Samples fixed with Lugol’s iodine solution require decolorization with thiosulfate when epifluorescence microscopic staining is desired, because the iodine quenches fluorescence.

Most studies of the effects of preservatives on protozoan biovolume have been done with taxa isolated from marine environments, e.g., Monas sp. (Borsheim & Bratbak 1987), Strombidium spp. (Choi & Stoecker 1989; Putt & Stoecker 1989), Strombidium spiralis (Putt & Stoecker 1989; Jerome et al. 1993), Laboea strobila (Putt & Stoecker 1989), Euplotes sp. and Eutintinnus sp. (Jerome et al. 1993). There is much less work with freshwater taxa, although Wiackowski et al. (1994) studied the effects of four fixatives on Colpidium kleini, Halteria grandinella and Urotrichia armeta and Muller & Geller (1993) worked with four ciliate taxa (Urotricha furcata, Pelagostrombidium fallax, Strombidium lacustris and Pseudobalanion planctonicum). The organisms investigated here have not been used in other published studies concerning shrinkage and thus our work broadens the range of taxa for which data concerning fixatives is now available.

The significant difference in the changes in biovolume between the two species studied here could be affected by the study design in that the number of experiments for the two organisms differed. However, the degree of shrinkage for Chilomonas was almost always higher than it was for Cylidium, suggesting that the preservatives affected the two protozoan species studied differently. This is consistent with the studies of Choi & Stoecker (1989) and Putt & Stoecker (1989) who reported that changes in cell volume depended on protozoan taxa, and that microflagellates shrink more than ciliates in aldehyde fixatives. However, since all work to date but ours with Chilomonas has used cells from enriched cultures, it remains to be seen whether the same species from culture and in field-collected samples would respond differently to preservatives.

In conclusion, our study, albeit limited, has shown that the volumes of preserved protozoa were affected not only by the types of preservatives used and the taxa studied, but also, most of all, by the storage time. We recommend that storage time should be considered as a factor when microscopic measurements of preserved samples are made for biovolume estimation and subsequent cell size to carbon conversion.

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References


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