Effects of Fixation on Cell Volume of Marine Planktonic Protozoa†

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The effects of fixation on the cell volume of marine heterotrophic nanoflagellates and planktonic ciliates were investigated. Decreases in cell volume depended on the combination of the protozoan taxa and the particular fixative. For a particular fixative and protozoan species, degree of shrinkage was independent of physiological state. The volume of fixed cells was found to be approximately 20 to 55% lower than the cell volume of live organisms. For the heterotrophic microflagellates, the fixatives ranked, in order of decreasing effect on cell volume, as glutaraldehyde, formaldehyde, acid Lugol's solution, and modified van der Veer solution. With oligotrichous ciliates and a tintinnid ciliate, formaldehyde caused less shrinkage than glutaraldehyde or acid Lugol's solution. With the aldehyde fixatives, the microflagellates were found to shrink more than the ciliates. Differential effects of fixation on cell volumes may result in an underestimation of the biomass of certain protozoan taxa in natural samples.

Protozoa are major consumers of bacterioplankton (14, 31, 36) and phytoplankton (11, 19; D. A. Caron, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984) in marine planktonic ecosystems. In addition to their role as grazers, heterotrophic protozoa, rather than bacteria, may also be the major nutrient remineralizers in open ocean (17, 45). The microbial food web is considered to be an important source of metabolic activity in open ocean (30, 35, 46); thus, data on the abundance and activity of heterotrophic protozoa are crucial to understanding the flow of energy and material in the ocean.

Heterotrophic microflagellates and ciliates usually dominate the protozoan biomass; however, because they overlap in size with phytoplankton and some micrometazoans, they cannot be separated from these groups by size fractionation. Estimates of the biomass of heterotrophic protozoa are usually based on cell counts and microscopic measurements of cell size. Carbon is widely used as a currency of biomass to compare different groups of organisms or to calculate the efficiency of energy transfer between trophic levels, although biovolume (4) and wet weight (39) are sometimes also reported. Cell volume to cell carbon conversion factors for protozoa have been reported for a few species of flagellates (7, 13, 23) and ciliates (15, 41), but conversion factors derived for phytoplankton have also been used to estimate the biomass of heterotrophic protozoa (11, 28, 42).

Fixation can cause cells to shrink (6, 16, 17) or increase in volume (22) and, thus, may affect cell volumes. Some fixatives also affect retention of ingested food particles (37), and this, in turn, affects cell volume estimates for protozoa. Volume estimates of heterotrophic protozoa, from which biomass was calculated, have been based on live (38), Lugol's solution-fixed (23), and glutaraldehyde-fixed (Caron, Ph.D. thesis) and formaldehyde-fixed (1–3, 5, 21, 33, 34) cells. Although volume to carbon conversion factors may vary with fixatives and taxa, values from the literature are often applied without consideration of sources of variation. Variability in the percentage of shrinkage or swelling could

result in the overestimation or underestimation of biomass from fixed samples. Therefore, data on the effects of fixation on cell volume may be useful in both accurate estimation of the biomass of protozoa from field samples and secondary production from field and laboratory data. The objectives of the present study were to (i) compare the effects of commonly used fixatives on cell volume of marine phagotrophic protozoa, including heterotrophic flagellates and ciliates; (ii) determine whether protozoan taxa vary in response to fixation; and (iii) determine whether physiological state (i.e., growth rate) affects cell volume change due to fixation.

MATERIALS AND METHODS

Culture of flagellates and ciliates. Two species of heterotrophic flagellates (a chrysomonad, *Paraphysomonas imperforata* strain HM-1, and an unidentified species [strain HM-2]) and three species of ciliates (a tintinnid, *Favella* sp. strain JunFan, and two oligotrichous ciliates, a strictly heterotrophic species, *Strobilidium spiralis* strain Stro, and a mixotrophic species, *Strombidium acutum* strain Gpgr [26]) were used. *Paraphysomonas imperforata* and HM-2 were fed *Isochrysis galbana* strain Iso. A *Favella* sp. was fed *Heterocapsa triquetra* strain A984; *Strombilidium spiralis* and *Strombidium acutum* were fed a mixture of *Heterocapsa pygmaea* strain Gymno, *Isochrysis galbana* strain Iso, and *Pyrenomonas salina* (formerly *Chroomonas salina*) strain 3C. The algae were grown in f/2 medium (18) without silicic acid. The heterotrophic flagellates were inoculated into 500 ml of the algal cultures in 1-liter flasks and kept in the dark at 20°C. A *Favella* sp., *Strombilidium spiralis*, and *Strombidium acutum* were grown at 15°C; the protocols have been described previously (26, 41).

Sampling and fixation. Protozoa were preserved with the following fixatives: acid Lugol's solution (43), 2% glutaraldehyde (10), 1% formaldehyde (43), and a modified van der Veer fixative (final concentrations, 2% glutaraldehyde and 2% tannic acid) (44; D. A. Caron, personal communication). Since various concentrations of glutaraldehyde have been used to preserve heterotrophic flagellates, the effects of 1, 2, and 3% glutaraldehyde on *Paraphysomonas imperforata* were also compared. Cell volumes of fixed protozoa were compared with cell volumes of live organisms. Nickel sulfate (24) and Polyox (40) were used to reduce motility of protozoa to measure live cells microscopically. Working concentra-

tions of slowing agents were determined for each species of protozoa. Size measurements of live cells, in the presence and absence of slowing agents, were compared to detect the effects, if any, of these slowing agents on cell volume of live protozoa. Comparisons between live cells with and without nickel sulfate were made with a Coulter counter, but all other comparisons were based on microscopic measurements.

Because the growth stage of a protozoan can influence the cell volume in both heterotrophic flagellates (12) and ciliates (20), samples were collected from batch cultures during various growth phases characterized by cells of different sizes. At each sampling, three 5-ml replicate samples were prepared for each treatment (fixed or live samples of heterotrophic flagellates). A final volume of 10 to 25 ml was prepared for ciliates, depending on cell density in the cultures, which varied from <5 to 50 cells per ml. For the ciliates, a batch culture was sacrificed at each sampling to obtain sufficient cells. Samples from batch cultures representative of different growth stages were then compared.

Size measurements and shrinkage calculation. Sizes were measured by using light microscopy, with magnification of ×1,000 for flagellates and ×400 for ciliates. Dimensions of 20 to 30 cells from each replicate sample were measured with a calibrated ocular micrometer for ciliates and flagellates. Diameters of flagellates and oligotrichous ciliates were measured. The volume of the protoplast of a *Favella* sp. was calculated assuming that the shape of protoplasm is either a cone or a paraboloid, depending on cell shape. Shrinkage (percent) was calculated as (fixative-treated diameter/live diameter)^3 × 100 except for the *Favella* sp., for which it was calculated as (fixative-treated volume/live volume) × 100.

RESULTS AND DISCUSSION

Neither Polyox nor NiSO$_4$ influenced the cell volume of *Paraphysomonas imperforata* (tested by one-way analysis of variance [ANOVA]; $P > 0.5$); thus, these two agents appear suitable for microscopic determination of live cell dimensions. Although the Coulter counter was used to compare the volumes of untreated and NiSO$_4$-treated cells of *Paraphysomonas imperforata*, it was not possible to make similar comparisons with the other protozoan species because of low culture density. With NiSO$_4$, the cells could be concentrated before microscopic examination, and, thus, it was possible to measure cells from samples with low protozoan densities. The working concentrations of NiSO$_4$ were 0.025, 0.0025, 0.07, and 0.002% (wt/vol) for HM-2, *Strobilidium spiralis*, *Strombidium acutum*, and the *Favella* sp., respectively.

Samples were taken at five growth intervals for heterotrophic microflagellate cultures. The average volumes of live cells varied with growth stage (Fig. 1 and 2) and were significantly different between samplings (tested by one-way ANOVA; $P < 0.01$). Under the same fixation conditions, shrinkage, in terms of percent live volume, was not statistically different among cells from different growth phases (i.e., sizes) (tested by one-way ANOVAs; $P > 0.05$). However, there were significant differences among fixatives on shrinkage of both species of heterotrophic microflagellates (tested by one-way ANOVA; $P < 0.01$).

Glutaraldehyde caused the most shrinkage of the microflagellates, yielding 38 to 47 and 45% of live volume for *Paraphysomonas imperforata* and HM-2, respectively (Table 1). Higher glutaraldehyde concentrations caused more shrinkage of *Paraphysomonas imperforata* than did
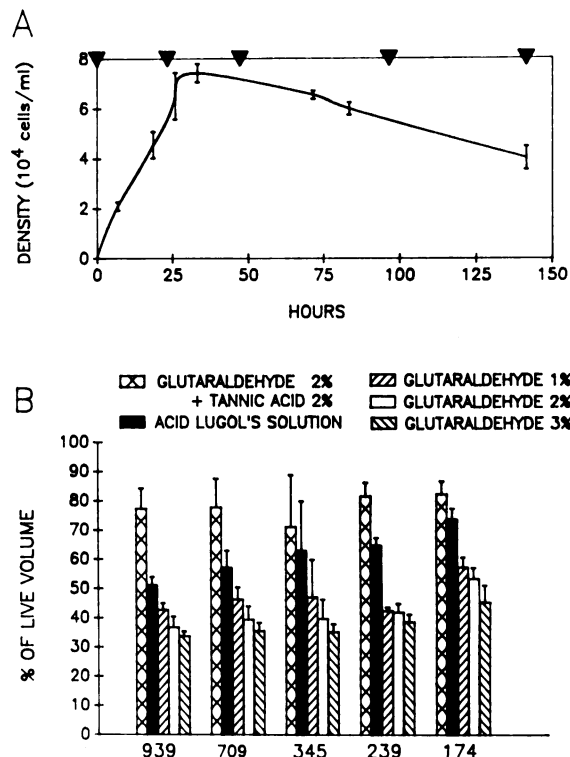

FIG. 1. Cell density change of *Paraphysomonas imperforata* after inoculation (A) and percent live cell volume (average ± standard error) of *P. imperforata* with different fixatives at the times indicated in panel A (B). (A) Filled triangles indicate times at which samplings were taken for size measurements. (B) Average live volume (cubic micrometers) for each sampling time is given below each set of bar graphs.

lower concentrations (Table 1). Of the fixatives tested, a mixture of glutaraldehyde and tannic acid (modified van der Veer fixative) caused the least change in cell volume; the average volume of fixed cells was 78% of the live cell volume for *Paraphysomonas imperforata* and 82% for HM-2 (Table 1). *Paraphysomonas imperforata* and HM-2 fixed with acid Lugol's solution were 62% of live volume. HM-2 fixed with formaldehyde yielded 64% of live volume (Table 1).

One size of *Strombidium acutum*, two sizes of the *Favella* sp., and three sizes of *Strobilidium spiralis* were obtained from the batch cultures in different growth phases (Fig. 3) (tested by one-way ANOVA; $P < 0.01$). As for the heterotrophic ciliates, the percentage of shrinkage was independent of cell size (tested by one-way ANOVAs; $P > 0.05$). However, there were significant differences among the effects of different fixatives on the percent shrinkage of each ciliate species (Fig. 3) (tested by one-way ANOVA; $P < 0.01$). Formaldehyde had the least effect on cell volume (Table 1): *Strobilidium spiralis*, 80%; *Strombidium acutum*, 83%; and the *Favella* sp., 87% of live volume. Glutaraldehyde caused the most shrinkage in the two naked ciliates: *Strobilidium spiralis*, 54%; and *Strombidium acutum*, 64%. With the loricate ciliate, the *Favella* sp., acid Lugol's solution caused the most shrinkage (70%; Table 1).

Changes in protozoan volume in response to fixation varied with species and fixatives, but responses of each species to a fixative were constant irrespective of its physiological state. In this study, the effects of fixatives on the heterotrophic flagellates were, in order of increasing shrink-
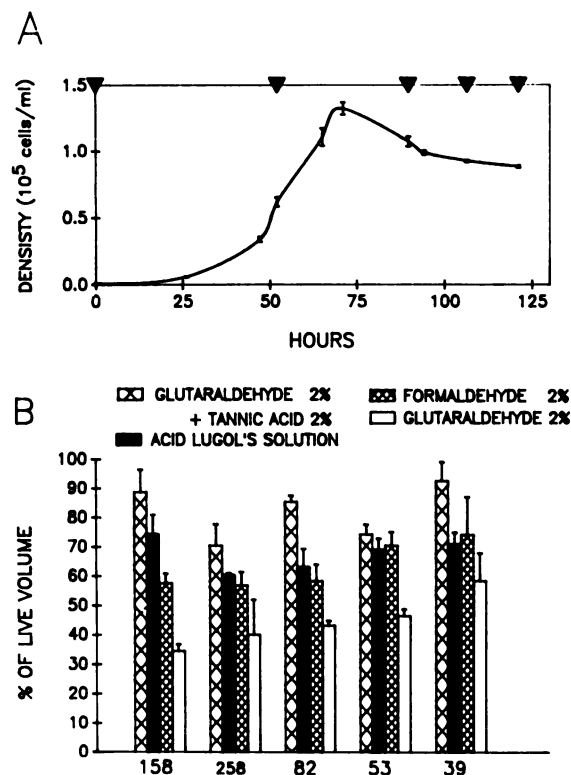
FIG. 2. Cell density change of HM-2 after inoculation (A) and percent live cell volume (average ± standard error) of HM-2 with different fixatives at the times indicated in panel A (B). (A) Filled triangles indicate times at which samplings were taken for size measurements. (B) Average live volume (cubic micrometers) for each sampling time is given below each set of bar graphs.

FIG. 3. Percent live cell volume (average ± standard error) of different species and sizes of ciliates after fixation: (A) *Favella* sp.; (B) *Strombidium acutum*; (C) *Strobilidium spiralis*. Average live volume or diameters are given below each set of bar graphs. Symbols: ■, Lugol's solution; ▨, 2% formaldehyde; □, 2% glutaraldehyde.

age, van der Veer fixative < Lugol's solution < formaldehyde < glutaraldehyde; for the oligotrichous ciliates, formaldehyde < Lugol's solution < glutaraldehyde; and for the tintinnid, formaldehyde < glutaraldehyde < Lugol's solution (Table 1). Under the same fixation procedures, the flagellate volumes decreased in volume more than ciliates and among the ciliates the oligotrichous ciliate decreased in volume more than the tintinnid.

Sieracki et al. (37) reported that particle retention by a phagotrophic chrysomonad flagellate was affected by the method of fixation. Van der Veer's solution (2% acrolein, 2% glutaraldehyde, 1% tannic acid) resulted in better particle retention by the flagellate than glutaraldehyde and formalde-
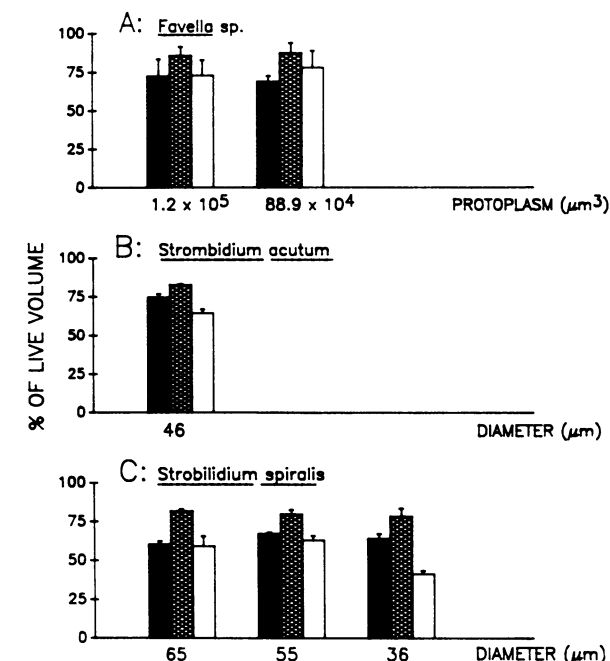
hyde. Shrinkage of flagellates by fixatives showed the same trends as particle retention. Modified van der Veer's solution (2% glutaraldehyde, 2% tannic acid) caused the least shrinkage. We hypothesize that particle egestion may be a major cause of shrinkage in phagotrophic flagellates as a result of fixation. Autotrophic flagellates, which do not generally ingest particles, show less shrinkage than phagotrophic flagellates upon fixation. For example, Chlorophyte, Prymnesiophyte, and Prasinophyte flagellates are reported to shrink to 69, 60, and 85%, respectively, after fixation with 2.5% glutaraldehyde (6), whereas in this study heterotrophic forms fixed with 2 or 3% glutaraldehyde shrank to 37 to 42% of their live volume.

It has been reported that naked ciliate species in closely related taxa can display quite different cell volume changes upon fixation; for example, *Lohmanniella spiralis* shrinks and *Strombidium reticulatum* swells (22). These differences may be due to difference in the concentration of fixatives,

TABLE 1. Shrinkage of protozoa after fixation

Fixative[a]	% Live cell vol, avg (SE)				
	Paraphysomonas imperforata (n = 15)[b]	HM-2 (n = 15)	*Favella* sp. (n = 10)	*Strombidium acutum* (n = 3)	*Strobilidium spiralis* (n = 3)
GT, 2%	77.93 (10.79)	82.28 (10.33)			
LU	61.96 (11.26)	67.68 (6.93)	70.31 (7.69)	74.48 (2.18)	63.85 (3.56)
FO, 1%		63.52 (10.17)	86.61 (6.22)	82.58 (0.49)	79.94 (3.71)
GL, 1%	47.16 (8.29)				
GL, 2%	42.22 (7.34)	44.58 (10.68)	75.87 (10.85)	64.32 (2.42)	54.48 (10.82)
GL, 3%	37.82 (5.47)				

[a] GT, 2%, 2% glutaraldehyde plus 2% tannic acid; LU, acid Lugol's solution; FO, 1% formaldehyde; GL, 1, 2, and 3%, 1, 2, and 3% glutaraldehyde.
[b] n, Number of means.

differences among species or strains, or perhaps difference in salinity or prey size. Under low salinity (osmotic pressure), dead cells may either shrink less than under higher salinity or, perhaps, increase in size due to the influx of water. Another possible explanation is that the size of the prey particles influences shrinkage. Particle egestion by phagotrophic microflagellates is inversely correlated with size of prey particle (J. W. Choi, manuscript in preparation); heterotrophic microflagellates shrink less when smaller prey are used as food source. The prey species used in this study were larger than those used by Jonsson (22) to feed ciliates.

The biovolume of phagotrophic protozoa is routinely estimated from size measurements of fixed cells and is then usually converted into a biomass currency, such as carbon or wet weight. Conversion factors for live phytoplankton (28, 42) have often been used for formaldehyde-fixed (21, 22, 29, 34), Lugol's solution-fixed (25), and live (39) protozoa. Conversion factors for live protozoa (23) also have been used for formaldehyde-fixed protozoa (33). Conversion factors based on the cell composition of live protozoa have been used for formaldehyde-fixed protozoa (2, 3, 5, 34). These estimates have generally underestimated the real biomass of heterotrophic protozoa because fixatives usually cause shrinkage of heterotrophic protozoa. Conversion factors from live protozoa (7, 15, 23) and from cell composition of live protozoa (5, 34), 0.04 to 0.10 g of C per ml, are in the same range as conversion factors for live phytoflagellates, but conversion factors for fixed protozoa are higher, 0.18 to 0.22 g of C per ml (7, 13, 41). The size of ingested prey is another factor that appears to influence shrinkage; conversion factors derived from the study of bactivorous flagellates may result in an underestimation of the biomass of natural populations which probably ingest both bacteria and larger prey. Herbivorous and carnivorous protozoa should tend to shrink more than bactivorous species under the same fixation procedures because of particle egestion. The biomass of heterotrophic protozoa in natural assemblages has probably been underestimated, by 20 to 55%, in investigations in which cell volumes were based on fixed samples but biomass conversions were based on live cells or on nonphagotrophic taxa.

ACKNOWLEDGMENTS

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