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Preservation of marine planktonic ciliates: losses and cell shrinkage during fixation

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ABSTRACT: For enumeration of microzooplankton (20–200 µm size fraction), including planktonic ciliates, water samples are usually fixed and preserved, then concentrated by sedimentation in Utermöhl chambers and examined with an inverted microscope. However, losses of ciliates may occur during fixation and handling, and fixation may shrink cells. Estimates of abundance with several commonly used fixatives were compared in samples from the North Atlantic and subarctic Pacific Oceans and in samples from cultures. Buffered formaldehyde has the advantage that it allows epifluorescence microscopy to be used, but, on average, ciliate counts from the North Atlantic were 56% higher (95% CI 30 to 82%) in samples fixed with 10% acid Lugol's solution than in samples fixed with 2% formaldehyde. Ciliate counts from the subarctic Pacific were 23 to 49% higher in samples fixed with 10 or 20% acid Lugol's solution than in samples fixed with 5% acid Lugol's solution. Fixation with 10 or 20% acid Lugol's solution results in significantly higher cell counts than fixation with formaldehyde or dilute acid Lugol's solution, but shrinks cells severely and often distorts their morphology. Bouin's solution yields cell counts that are usually similar to those with 10% acid Lugol's solution, but with less shrinkage. No single fixation method is ideal for all purposes; fixative-specific and assemblage- or taxon-specific correction factors are necessary for accurate estimates of cell numbers and cell volumes/biomass.

KEY WORDS: Microzooplankton · Ciliates · Oligotrichs · Fixation · Shrinkage · Cell losses · Inverted microscope technique

INTRODUCTION

Ciliates are an important component of the protistan plankton in marine, estuarine and fresh waters (e.g. Beers & Stewart 1969, Rassoulzadegan & Gostan 1976, Pace & Orcutt 1981, Smetacek 1981, Sorokin 1981, Revelante & Gilmartin 1983). In open waters, the ciliate assemblage is usually dominated by non-loricate, oligotrichous taxa (Sorokin 1991). Planktonic ciliates are important as consumers of pico- and nanoplankton (Rassoulzadegan et al. 1988). Autotrophic and mixotrophic ciliates also contribute to primary production in the microplankton size fraction (Jonsson 1987, Stoecker et al. 1987a). Planktonic ciliates are, at times, important components of the diet of copepods and other macrozooplankton (Stoecker & Capuzzo 1990, Gifford 1993b).

Microplankton, including ≥ 20 µm ciliates, are usually collected as part of whole water samples, fixed and preserved, concentrated by sedimentation, and then enumerated using the inverted microscope method (Hasle 1978). Filtration and staining techniques suitable for nanoplankton (< 20 µm protists) (reviewed by Sherr & Sherr 1993) are generally not suitable for enumerating the larger microzooplankton that typically occur at lower densities than nanoplankton in open, coastal waters and oceanic waters. An exception is the Quantitative Protargol Technique (QPS) which has been used to enumerate and identify both nano- and microplanktonic ciliates and dinoflagellates from coastal waters (Montagnes & Lynn 1987, Bockstahler & Coats 1993).

A number of fixatives/preservatives have been used in conjunction with the inverted microscope technique to enumerate microplankton in seawater samples, including dilute formaldehyde (Beers 1978, Dale & Burkill 1982, Stoecker et al. 1987a), a modified Bouin's solution (Dolan & Coats 1990), and various concentra-

tions of acid Lugol's solution (Gifford 1988, 1993a, Sherr & Sherr 1993). Although dilute acid Lugol's solution is recommended for enumeration of flagellates (Throndsen 1978) and has been used to fix and preserve ciliates (Revelante & Gilmartin 1983, Putt & Stoecker 1989, Ohman & Snyder 1991, Jerome et al. 1993) use of higher concentrations may reduce ciliate losses (Gifford 1993a, b). Live counting of ciliates often results in significantly higher estimates of ciliate abundance than does counting of preserved samples (Sorokin 1981). For example, Dale & Burkill (1982) found that live counting of ciliates from coastal waters often resulted in up to 20% higher estimates of abundance than did counting of samples preserved with 0.4% (final conc.) neutral formaldehyde. This raises the question of the importance of cell losses during fixation and whether fixation method influences estimates of abundance in microzooplankton assemblages.

The biomass of ciliate assemblages is typically estimated from numerical abundance and cell size using volume:carbon conversion factors. Shrinkage, caused by fixation and preservation may affect estimates of cell biomass based on measurements of cell volume (Choi & Stoecker 1989, Putt & Stoecker 1989, Ohman & Synder 1991, Jerome et al. 1993). Field and laboratory experiments were done to address the question of how fixation procedure affects estimates of ciliate abundance and biomass. Experiments were done at 2 oceanic stations in order to compare the effects of various fixatives on estimates of cell number in natural microplankton assemblages. Because it is difficult to compare fixation losses for individual species or to compare changes in cell volume in natural assemblages, experiments on fixation and preservation of laboratory cultures representative of the Oligotrichidia (*Strombidium*) and the Choreotrichida (*Strobilidium*, non-loricate and *Favella*, loricate) were also performed.

METHODS

Fixation experiments with natural assemblages. In the North Atlantic experiment, water samples were collected with Niskin bottles from 4 depths in the upper water column (z = 0 to 34 m) on 3 dates during late spring 1989. Sampling was done from the RV 'Atlantis II' in the vicinity of 47° N, 18° W as part of the JGOFS North Atlantic Bloom Experiment (Sieracki et al. 1993). Whole water samples (950 to 990 ml, depending on fixative) from each Niskin bottle were drained through silicon tubing into 3 jars containing different fixatives: 2% formaldehyde buffered with hexamethylamine (Throndsen 1978), 10% acid Lugol's solution (Throndsen 1978), and 5% Bouin's solution (stock solution from Sigma Diagnostics) (all final conc., v/v).

In the oceanic subarctic Pacific experiment, seawater was collected from the middle of the mixed layer (z = 35 m) at Stn P near 50° N, 145° W. A teflon-lined 30 l Go-Flo bottle was used to sample from the RV 'Thomas G. Thompson' as part of the SUPER (SUbarctic Pacific Ecocystem Research) program. Whole water samples (1600 to 1900 ml, depending on fixative concentration) were drained through silicon tubing into jars containing 3 concentrations of acid Lugol's solution: 5%, 10% and 20% (final v/v).

Several months later, 100 ml subsamples were concentrated by sedimentation using Utermöhl chambers and examined with an inverted microscope (Hasle 1978). All aloricate ciliates in the settling chambers were enumerated at 200× or 250× magnification. Loricate ciliates (tintinnids) were absent from the North Atlantic samples and were present, but rare, in the subarctic Pacific samples. Tintinnids are not included in the total counts. In the North Atlantic samples, only total counts of aloricate choreotrichs and oligotrichs were made. In the samples from the subarctic Pacific, the aloricate ciliates were categorized on the basis of geometry as spheres or cones; oral membranelles and tail structures were not included in the estimates of cell geometry. The large mixotroph *Laboea strobila* was enumerated separately from other cone-shaped ciliates. Ciliate densities were corrected for the volume of fixative in each sample and expressed as cells l^{-1}.

Fixation experiments with cultured ciliates. Fixation of ciliate cultures with 2%, 5% and 10% acid Lugol's solution, 2% buffered formaldehyde, and 5% Bouin's solution were compared in the laboratory to confirm and expand the results of the comparative fixation experiments done with natural assemblages. Three cultures representative of important ciliate taxa in the oceans were used: *Strombidium capitatum* (an oligotrich), *Strobilidium spiralis* (an aloricate choreotrich) and a tintinnid, *Favella* sp. (a loricate choreotrich). Culture methods are described in Stoecker et al. (1987b). The 3 cultures were mixed together and triplicate ~150 ml samples were preserved in each fixative. Samples were stored for several weeks before 100 ml subsamples were settled in Utermöhl chambers and each species enumerated using the inverted microscope technique.

To compare cell volumes, 30 to 31 cells of each species from each replicate were measured with a calibrated ocular micrometer. Cell volume was estimated using an ellipsoid approximation of cell shape for all 3 species. Oral membranelles and tail structures were not included in the estimates of cell volume. Only the cell inside the lorica of *Favella* sp. was measured.

Statistical analyses. Two-way analysis of variance was done using SAS. All tests of significance were performed at the $p = 0.05$ level. A Bonferroni (LSD) cor-

rection to the significance level was used when multiple comparisons were made (Kleinbaum et al. 1988).

For the North Atlantic data it was assumed that ciliate counts for any 2 preservatives are related in a linear fashion. Replicate counts were not made, however it seems unlikely that the standard errors of the dependent variables are constant over the range of observed cell densities (156 to 9489 l^{-1}). Thus, ordinary least squares regression is inappropriate. Instead, a quasi-likelihood approach (McCullagh & Nelder 1991) was employed. Using this approach, we fit a linear relationship and also included the assumption that the variance of the dependent variable is proportional to its mean. Parameter, variance and proportionality constant estimates were computed using STATA (Hilbe 1993). Approximate 95% confidence intervals for the mean were based on large sample normal approximations. Negative estimates of the intercept were truncated to zero.

RESULTS

In the North Atlantic samples, the ciliate abundance estimates from water fixed with 10% acid Lugol's solution were significantly higher ($p = 0.006$) than those of water fixed with 2% buffered formaldehyde (Fig. 1A). On average, estimates for samples preserved with 10% acid Lugol's solution were 56% (95% CI of 30 to 82%) higher than the formaldehyde based estimate for the same samples (Fig. 1A). The estimates of ciliate abundance in samples preserved with acid Lugol's solution were, on average, about 7% higher than the corresponding estimates for samples preserved with Bouin's solution, but the differences between samples were not significant (Fig. 1B).

The fixation experiment from the subarctic Pacific indicates that the concentration of acid Lugol's solution employed has a significant effect on abundance estimates (Table 1). Abundance estimates from samples fixed with 10% acid Lugol's solution were significantly higher than estimates from corresponding samples fixed with 5% acid Lugol's solution. Although the average abundance estimates for conical ciliates were higher in the treatment with 20% rather than 10% acid Lugol's solution, the estimates of total ciliate abundance were not significantly different between these 2 treatments. The interaction between ciliate category (cone, sphere or *Laboea*) and percent acid Lugol's solution was not statistically significant (Table 1). However the average abundance of *Laboea* sp. and other ciliates with a conical morphology was greater with the higher concentrations of fixative. Differences between species

Table 1. Abundances of 3 morphological types of ciliates (cells l^{-1}) in samples from the sub-arctic Pacific preserved with 5%, 10%, and 20% acid Lugol's solution (L). Abundances were corrected for the volume of fixative. Means of 3 replicates (SD in parentheses). ns: non-significant ($p < 0.05$)

	5% L	10% L	20% L
Laboea strobila	204 (22)	263 (39)	304 (79)
Conical morphology[a]	442 (74)	556 (80)	654 (170)
Spherical morphology	400 (104)	544 (51)	492 (108)

2-way ANOVA

Source of variation	Mean square	df	*F* value	
% Acid Lugol's	45306	2	5.52	$p < 0.05$
Morphological type	210983	2	25.69	$p < 0.001$
Interaction	6093	4	0.74	ns
Error	8213	18		

***A priori* comparisons (LSM)**

5% L vs 10% L	$p < 0.05$
10% L vs 20% L	ns

[a] Not including *Laboea*

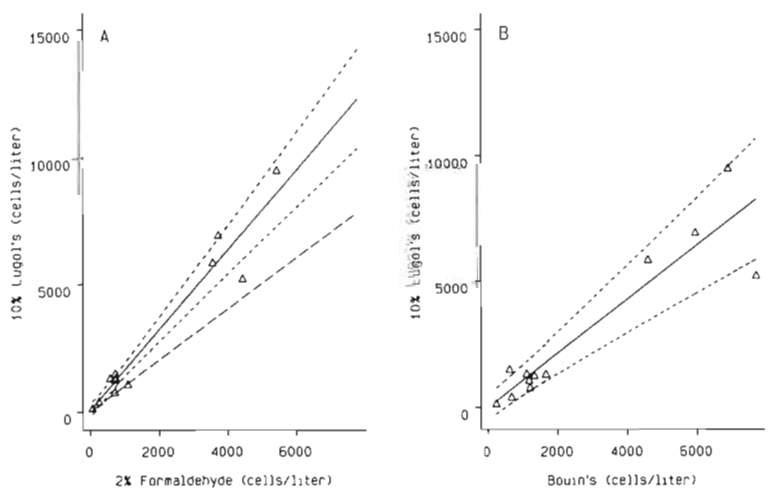

Fig. 1. Relationship of abundance estimates of planktonic ciliates (cells l^{-1}) for samples from surface waters of the North Atlantic fixed and preserved with (A) 10% acid Lugol's solution versus 2% buffered formaldehyde and (B) 5% Bouin's solution. The linear relationship was estimated using the model $Y = b_0 + b_1X$, where X is the estimate with 2% formaldehdye (A) or 5% Bouin's solution (B) and Y is the estimate with 10% acid Lugol's solution. Average abundance with 10% Lugol's predicted from the estimated slope (b_1) and intercept (b_0) (———) with 95% confidence interval (------). The predicted line which would arise if $b_0 = 0$ and $b_1 = 1$ (– – – –) is shown in (A). Parameter estimates (95% CI): Lugol's on formaldehyde (A) $b_0 = 57$ (0, 318); $b_1 = 1.56$ (1.30, 1.82), Lugol's on Bouin's $b_0 = -8$ (0, 499), $b_1 = 1.07$ (0.76, 1.37)

may have existed but may have been below the 'limit of detection' for this experiment.

In the laboratory experiments, interactions between species and fixation treatment were significant for both abundance estimates (Table 2) and cell size (Table 3). Although abundances of all 3 species varied with fixation treatment, between-treatment differences were only significant in *Strombidium capitatum* (Table 2). For this oligotrich, the estimates with 2% buffered formaldehyde were significantly lower than the estimates with 5% or 10% acid Lugol's solution (Table 2). For example, the estimate of *S. capitatum* cell density with 2% formaldehyde was only 64% of the estimate with 10% acid Lugol's fixation.

Fixation method influenced cell size in all 3 species (Table 3). Cell volumes in the 10% acid Lugol's treatment were significantly lower than cell volumes with the 2% acid

Table 2. Abundances (cells 100 ml^{-1}) of cultured ciliates preserved with 2%, 5%, 10% acid Lugol's solution (L), 2% buffered formaldehyde (F) and 5% Bouin's solution (B). Means of 3 replicates (SD in parentheses). ns: non-significant ($p < 0.05$)

	2% L	5% L	10% L	2% F	5% B
Strobilidium spiralis	151 (5)	140 (4)	167 (9)	138 (14)	165 (9)
Favella sp.	288 (12)	264 (11)	277 (20)	246 (11)	234 (24)
Strombidium capitatum	227 (15)	272 (32)	300 (39)	193 (7)	251 (23)

2-way ANOVA

Source of variation	Mean square	df	*F* value	
Preservative	3549	4	6.87	
Species	54983	2	106.48	
Interaction	1597	8	3.09	$p < 0.05$
Error	516	30		

***A priori* comparisons (LSM)**

	S. spiralis	*Favella* sp.	*S. capitatum*
2% L vs 10% L	ns	ns	ns
2% L vs 5% L	ns	ns	ns
5% L vs 10% L	ns	ns	ns
2% F vs 2% L	ns	ns	ns
2% F vs 5% L	ns	ns	$p < 0.05$
2% F vs 10% L	ns	ns	$p < 0.05$
5% B vs 10% L	ns	ns	ns

Table 3. Mean cell volume ($\mu m^3 \times 10^6$) of cultured ciliates preserved with 2%, 5%, 10% acid Lugol's solution (L), 2% buffered formaldehyde (F) and 5% Bouin's solution (B). Means of 3 replicates (SD in parentheses), except for 2% L means of 2 replicates (range in parentheses)

	2% L	5% L	10% L	2% F	5% B
Strobilidium spiralis	45.0 (40.1–49.9)	40.2 (3.6)	35.8 (1.9)	60.4 (2.4)	38.2 (1.8)
Favella sp.	73.5 (70.1–76.9)	74.4 (2.1)	61.3 (3.6)	108.9 (0.6)	76.2 (8.5)
Strombidium capitatum	47.3 (42.6–52.0)	43.2 (1.6)	31.4 (3.5)	77.1 (6.3)	45.8 (5.4)

2-way ANOVA

Source of variation	Mean square	df	*F* value	
Preservative	1948×10^6	4	102.25	
Species	4860×10^6	2	255.15	
Interaction	71×10^6	8	3.71	$p < 0.05$
Error	19×10^6	27		

***A priori* comparisons (LSM)**

	S. spiralis	*Favella* sp.	*S. capitatum*
2% L vs 10% L	ns	$p < 0.05$	$p < 0.05$
2% L vs 5% L	ns	ns	ns
5% L vs 10% L	ns	$p < 0.05$	$p < 0.05$
2% F vs 2% L	$p < 0.05$	$p < 0.05$	$p < 0.05$
2% F vs 5% L	$p < 0.05$	$p < 0.05$	$p < 0.05$
2% F vs 10% L	$p < 0.05$	$p < 0.05$	$p < 0.05$
5% B vs 10% L	ns	$p < 0.05$	$p < 0.05$

Lugol's treatment for all 3 species. For *Strombidium capitatum* and *Favella* sp., cell volumes with 10% acid Lugol's solution were significantly lower than with 5% acid Lugol's solution. For all 3 species, cell volumes with 2%, 5% and 10% acid Lugol's solution were significantly lower than with 2% buffered formaldehyde. For example, the cell volume of *S. capitatum* preserved in 2% buffered formaldehyde was 2.45× the volume of similar cells preserved in 10% acid Lugol's solution. For *Favella* sp. and *S. capitatum*, but not *S. spiralis*, volumes of cells preserved with 5% Bouin's solution were significantly higher than those preserved with 10% acid Lugol's solution. In all cases, volumes of cells preserved with 2% formaldehyde were significantly higher than those preserved with 5% Bouin's solution.

DISCUSSION

Method of fixation can influence estimates of ciliate numerical abundance significantly (Pace & Orcutt 1981, Revelante & Gilmartin 1983; R. Leakey, P. Burkhill & M. Sleigh summarized in Rassoulzadegan 1991). The combined results of our field and laboratory experiments indicate that higher cell counts are obtained in either strong (10 or 20%) acid Lugol's solution or Bouin's solution than in 2% acid Lugol's solution or 2% formaldehyde. Higher non-loricate ciliate densities in samples fixed with 1% acid Lugol's (final conc.) than in samples fixed with formaldehyde have been reported previously by Pace & Orcutt (1981) and Revelante & Gilmartin (1983). However, our data indicate that counts of oligotrichs are significantly higher in strong (≥10%) than in the dilute (≤2%) acid Lugol's solution. Low concentrations of acid Lugol's solution (usually 0.5 to 2%) have traditionally been used to preserve microplankton samples (Sherr & Sherr 1993) although Gifford (1993a) recommends 10% for ciliates. The data in Table 1 suggest that for certain ciliates (*Laboea* and other ciliates with a conical morphology) slightly higher counts may be obtained with 20% than with 10% acid Lugol's solution. However, with 20% acid Lugol's solution, ciliates may be difficult to identify and size because of severe shrinkage, distortion of morphology and dark coloration. The iodine color can be bleached with sodium thiosulfate (E. Sherr & B. Sherr summarized in Rassoulzadegan 1991, Sherr & Sherr 1993), laundry bleach (Gifford pers. obs.) or light (Gifford pers. obs.).

For cells preserved with 2% buffered formaldehyde, abundance estimates of oligotrichs were on average 63 to 64% of those obtained for cells preserved with 10% acid Lugol's solution (Fig. 1A, Table 2). Although cell losses are significant with this fixative, formaldehyde has the advantage that it can be used in conjunction with epifluorescence microscopy, and thus plastidic and non-plastidic cells can be distinguished (Stoecker et al. 1987a).

Because estimates of ciliate abundance from fixed samples were not compared to live counts, we do not know the accuracy of our estimates. However, Dale & Burkill's (1982) reported loss factors of up to 20% with dilute buffered formaldehyde (compared to live counts) and our estimated loss factor of 30 to 40% (compared to 10% acid Lugol's) with buffered formaldehyde suggest that the estimates obtained with 10 or 20% acid Lugol's solution or 5% Bouin's are close to maximum. However, our experiment with cultures of ciliates demonstrated that cell losses can be taxon-specific (Table 2). It is thus possible that some species are not fixed and preserved quantitatively with either strong acid Lugol's or Bouin's solutions.

Type of fixative as well as fixative concentration and ciliate taxa also influence biomass estimates through their effect on post-fixation cell volume (Table 3). Similar effects of fixation on ciliate cell volume have been reported by Choi & Stoecker (1989), Putt & Stoecker (1989), Ohman & Snyder (1991) and Jerome et al. (1993). Volume to carbon conversion factors for preserved planktonic ciliates, such as those of Putt & Stoecker (1989) and Ohman & Snyder (1991) are fixative- and probably taxon-specific. For example, if the volume to carbon conversion factor of 0.14 pg C μm^{-3} for oligotrichs preserved in 2% buffered formaldehyde (Putt & Stoecker 1989) is applied to oligotrichs preserved with 2% acid Lugol's solution or 5% Bouin's solution, biomass is probably underestimated by about 40%. If the same ciliates are fixed with 10% acid Lugol's solution, biomass is probably underestimated by about 59% with the formaldehyde conversion factor.

Fixation procedures can have a large effect on the estimation of planktonic ciliate abundance and biomass (Sime-Ngando et al. 1990, Ohman & Snyder 1991, Sime-Ngando & Groliere 1991, Jerome et al. 1993). Failure to account for losses during fixation can result in an underestimation of ciliate numbers and biomass (Fig. 1A, Tables 1 & 2). Failure to adjust volume:carbon conversion factors for cell shrinkage (Table 3) may underestimate ciliate biomass by >40%. Because some taxa appear to be more sensitive to these effects than others (Sime-Ngando & Grolière 1991, Jerome et al. 1993) the taxonomic composition of the ciliate assemblage probably affects the accuracy of estimates of ciliate abundance and biomass.

Correcting for cell losses in samples preserved in dilute acid Lugol's solution or 2% formaldehyde based on our data may not be appropriate for all samples, in part because of the taxon-specific effects of the different fixatives. Other factors may influence cell losses

and cell shrinkage as well. Losses may occur prior to fixation due to the mechanics of collection or manipulation of the sample (Gifford 1985). Osmotic affects (Jerome et al. 1993), due to the salinity, as well as the feeding history of cells (Choi & Stoecker 1989) may influence preservation and shrinkage. Cell losses in liquid samples may increase with storage time (Sherr & Sherr 1993), although this does not appear to be the case for marine ciliates preserved in acid Lugol's solution (Ohman & Snyder 1991, Gifford unpubl. data). For all of the above reasons, carbon:volume factors and factors correcting for cell loss and shrinkage should not be used indiscriminately. Individual investigators must check or derive correction factors for the particular microplankton assemblages and environmental conditions which apply in their own research. Further comparisons of numerical and biomass estimates obtained with different fixation and preservation methods and live techniques are needed before correction factors can be recommended for routine use.

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