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Evaluation of double formalin—Lugol's fixation in assessing number and biomass of ciliates: an example of estimations at mesoscale in NE Atlantic

Hera Karayanni[a,b], Urania Christaki[b,c,*], France Van Wambeke[a], Andrew P. Dalby[b]

[a] *Microbiologie Marine, CNRS, UMR 6117, Campus de Luminy, case 907, 13 288 Marseille cedex 9, France*
[b] *Hellenic Centre for Marine Research, P.O. Box 712, 19013, Anavissos, Greece*
[c] *Université du Littoral Côte d'Opale, MREN, 32 Avenue Foch, 62930 Wimereux, France*

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Abstract

Ciliated protozoa are potential grazers of primary and bacterial production and act as intermediaries between picoplankton and copepods and other large suspension feeders. Accurate determination of ciliate abundance and feeding mode is crucial in oceanic carbon budget estimations. However, the impact of different fixatives on the abundance and cell volume of ciliates has been investigated in only a few studies using either laboratory cultures or natural populations. Lugol's solution and formalin are the most commonly used fixatives for the preservation of ciliates samples. In the present study, the aim was to compare 0.4% Lugol's solution and 2% borated-formalin fixation and evaluate the need of counting duplicate samples each using a different fixative. For this, a large number of samples ($n = 110$) from the NE Atlantic was analyzed in the frame of POMME program (Multidisciplinary Mesoscale Ocean Program). We established a statistically significant relationship ($p < 0.0001$) between Lugol's and formalin fixed samples for both abundance ($r^2 = 0.50$) and biomass ($r^2 = 0.76$) of aloricate ciliates which showed that counts were higher in Lugol's solution by a factor of 2 and a non-taxon specific cell-loss in formalin. However, loricate ciliate abundance in our samples which were represented primarily by *Tintinnus* spp. did not show any difference between the two treatments. Abundance and biomass of mixotrophic ciliates (chloroplast-bearing cells) were for various reasons underestimated in both treatments. Our results show that unique fixation by formalin may severely underestimate ciliates abundance and biomass although their population may not alter. For this reason, Lugol's solution is best for the estimation of their abundance and biomass. However, for counts of mixotrophs and the evaluation of the ecological role of ciliates in carbon flux, double fixation is essential. Compromises regarding the fixatives have lead to severe underestimations of mixotrophs in studies conducted by now.

Keywords: Double formalin; Lugol's fixation; Ciliates

1. Introduction

Ciliated protozoa, aloricate oligotrichous taxa and loricate choreotrichs (tintinnids), are the most abundant microzooplanktonic organisms in the 20–200-

* Corresponding author. Université du Littoral Côte d'Opale, MREN, 32 Avenue Foch, 62930 Wimereux, France.
E-mail address: Christaki@mren2.univ-littoral.fr (U. Christaki).

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μm size range (Dussart, 1965). They are ubiquitous in marine waters where they feed on picoplankton (Sherr et al., 1986; Bernard and Rassoulzadegan, 1993; Christaki et al., 1999), nanoplankton (Pilling et al., 1992), or on other constituents of the microplankton (Bernard and Rassoulzadegan, 1990). The only pathway through which small primary producers can enter into the food web and reach higher trophic levels is from grazing by protozoa (flagellates and ciliates), which are potential food-web intermediaries that repackage picoplankton production in a form available to copepods and other large suspension feeders (Levinsen and Nielsen, 2002). There are several mixotrophic taxa among ciliates which can sequester photosynthetically functional chloroplasts from a variety of chromophytic and chlorophytic algae (Jonsson, 1987; Stoecker et al., 1987). These can contribute up to the half the biomass of oligotrichs (Stoecker et al., 1987; Pitta and Giannakourou, 2000) and up to 20% of microplanktonic primary production in some coastal waters (Stoecker, 1991).

Ciliates are therefore very important organisms for our understanding of food-web dynamics and as such, require accurate quantification of abundance and feeding mode in order to generate useful carbon budget estimations, among other data. Preferentially, living material in combination with specific equipment would be required for accurate determination of cell volume (Putt and Stoecker, 1989), quantification (Sime-Ngando et al., 1990; Lynn and Montagnes, 1991) and identification of various species of ciliates (Maeda and Carey, 1985; Sime-Ngando et al., 1990; Montagnes and Lynn, 1991). Unfortunately, this is not always feasible as analysis is time-consuming requiring long-term sample storage. A compromise is to use a fixative to preserve the samples in situ. However, there are challenges associated with this.

The two fixatives traditionally used for this purpose are Lugol's solution and formalin (Burkill et al., 1993; Stoecker et al., 1994b). Their effect on the determination of ciliate abundance and cell volume has been investigated in only a few studies using either laboratory cultures (Putt and Stoecker, 1989; Ohman and Snyder, 1991) or natural populations (Sime-Ngando et al., 1990; Leaky et al., 1994). Current observations of the effects of both fixatives used on ciliates are summarized below.

For abundance estimates, Lugol's seems to be the most effective fixative (Revelante and Gilmartin, 1983; Stoecker et al., 1989; Leaky et al., 1994) and cell loss may be minimal compared to live counting (Sime-Ngando et al., 1990). Low concentrations of Lugol's solution have traditionally been used to preserve microplankton samples (Sherr and Sherr, 1993), however, the effectiveness of Lugol's for long-term storage of samples is unclear. Cell loss may increase with storage time (Sime-Ngando and Groliere, 1991), however, this does not appear in cell loss experiments conducted by Ohman and Snyder (1991). Comparison of cell volumes of live and fixed cells has shown that relative to Lugol's, formalin is more likely to minimize shrinkage of ciliates (Putt and Stoecker, 1989; Choi and Stoecker, 1989; Ohman and Snyder, 1991). Another disadvantage of Lugol's solution is that it masks chlorophyll fluorescence of mixotrophic forms and fluorescence of natural or labeled ingested prey, used as feeding indicators in grazing experiments. Hence, to obtain estimates of the trophic status of ciliates (autotrophs, mixotrophs and heterotrophs), samples are fixed with borated-formalin solution (final concentration 2%). However, various studies have shown that although formalin allows observing chlorophyll fluorescence, it can cause severe loss of cells (Revelante and Gilmartin, 1983; Stoecker et al., 1989, 1994a). It is thus evident that no single fixation method is ideal for all purposes. Notwithstanding, since ciliate counting is quite time consuming and sample storage problematic, it is difficult to analyze duplicates with both formalin and Lugol's fixation and different compromises regarding the fixatives are necessary (e.g. Dolan and Marrasé, 1995; Pitta et al., 2001).

In order to compare Lugol's and formalin fixation, duplicate samples from the same water sample were examined each using a different fixative. We needed to establish a quantitative relationship between formalin and Lugol's counts, therefore, a large number of samples (110 in total) from different stations, depths and seasons in an oceanic area (NE Atlantic) were analyzed. We evaluated in particular the accuracy of mixotrophic ciliate numbers from both fixation techniques (duplicate counting). Additionally, we compared the two fixatives in terms of full (containing cell) versus empty tintinnid loricas.

2. Materials and methods

Sampling was conducted in the NE Atlantic Ocean (16–22° W, 38–45°N) within the framework of POMME (Multidisciplinary Mesoscale Ocean Program). The study covered an area of 500 km (east–west) by 750 km (north–south) centred on 41.5°N, 19°W (16W–22°W/38N–45°N) (Fig. 1). For ciliate enumeration, 500 ml samples were taken from the upper 100 m at different increments (5, 10, 20, 30, 40, 50, 60, 80, 100 m) during the winter (February–March), spring (April–May) and fall (late August–October). Data for fixative comparison were gathered from 110 samples corresponding to 48 study sites (10, 17 and 21 sites for winter, spring and fall sampling, respectively). The 500-ml samples were each gently mixed and then 250-ml sub-samples (duplicates) were decanted into two opaque glass bottles. One duplicate was fixed with borax-buffered 37% formaldehyde (borax purity: 99.8%, formaldehyde purity: 37–38%, stabilized with 10% methanol) solution filtered on 0.2-μm cellulose filters and the other with acid Lugol's solution in order to obtain final concentrations of 2% and 0.4%, respectively. Fixative solutions were diluted with Millipore Milli-Q purified water (deionized; decarbonized TOC < 15 ppb). One litre of acid Lugol's solution contained: 50 g iodine (I); 100 g potassium iodide (KI); 100 ml acetic acid (CH_3COOH); and Milli-Q purified water. The samples were always added to the fixative so that the preserved ciliates experienced at least the minimum target fixative concentration at all times (Gifford and Caron, 2000). The treatment bottles were gently inverted once in order to ensure proper mixing of the fixative. The samples were then stored at 4 °C in the dark until analysis (1–6 months).

In the laboratory, samples were left to settle for 3–4 days at 4 °C. Before examination, the top 150 ml of the sample was gently siphoned off using a low vacuum pump. The bottom 100 ml of the sample was transferred into Hydro-Bios Kiel combined plate settling chambers (model no. 435 025), allowing it to settle for a minimum of 16 h and then examined with an Olympus IX-70 inverted microscope at 400 ×. The

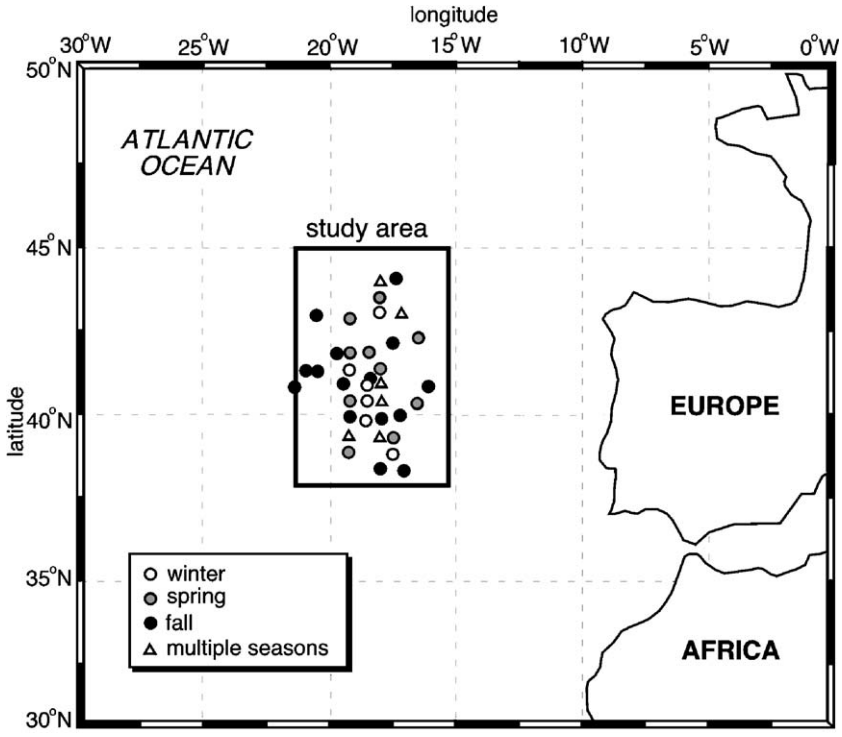

Fig. 1. Sampling sites for fixatives comparison during POMME cruises, winter to fall 2001.

microscope was equipped for transmitted light, phase contrast and epifluorescence microscopy. Blue light excitation (DM 500-nm dichroic mirror, BP 450 to 480-nm exciter filter, BA 515-nm barrier filter and a 100-W mercury burner) was used to detect chlorophyll autofluorescence and to distinguish plastidic from non-plastidic ciliates. Lugol's fixed samples were examined with phase contrast while borated-formalin fixed samples were enumerated using epifluorescence microscopy. In order to compare Lugol's with formalin counts for mixotrophic ciliates, mixotrophic oligotrich species with very distinctive gross morphologies (Laval-Peuto and Rassoulzadegan, 1988), *Laboea* sp. and *Tontonia* spp. were counted in Lugol's fixed samples as well. On several occasions, the settling efficiency at each stage of the settling process (opaque bottles and settling chambers) was examined by resettling the supernatant solution and then examining it microscopically for the presence of ciliates, however, examination of the supernatant solution did not show any cell loss.

All ciliates in each sample were enumerated and assigned to one of the following groups: oligotrichs and tintinnids. The former is comprised of aloricate (naked) taxa of Oligotrichida and Choreotrichida and the latter loricate species (order Choreotrichida, suborder Tintinnina, Montagnes and Lynn, 1991). Oligotrich ciliates were grouped into four taxa (Table 1) on the basis of shape, size, visible ciliature and morphology and identified wherever possible to the species level based on the works of Maeda and Carey (1985) and Maeda (1986). Furthermore, *Strombidium* spp. was separated into three morphological types (morphotypes): conical, spherical and prolate-spheroid. In addition, oligotrich ciliates were separated into four cell-volume categories from $<10^3$ to $>10^5$ μm^3 (Montagnes et al., 1988; Leaky et al., 1992). Tintinnids were identified based on the lorica shape and dimensions by reference to Jörgensen (1924) and Kofoid and Campbell (1929). Empty loricas were counted separately.

Biovolumes of all oligotrich taxa and morphotypes identified in this study (Table 1) were calculated using the linear dimensions of all cells present in 22 randomly selected samples for each treatment. Linear dimensions (length and diameter) were measured at 400 × using a calibrated ocular micrometer on the microscope. Equations for a sphere, prolate spheroid or cone were applied depending on cell shape. Oral membranelles and tail structures were not included in the estimation of cell geometry (Stoecker et al., 1994a). Biovolumes were converted to biomass using volume-to-carbon conversion factors 0.19 and 0.14 pg C μm^{-3} for Lugol's and formalin preserved samples, respectively (Putt and Stoecker, 1989).

Table 1
Z adjusted–Values (Mann–Whitney rank-sum test) resulted from the comparison of cell-volumes of different morphotypes and size classes identified in formalin and Lugol's fixed samples

	Size class (μm^3)			
	$<10^3$	10^3–10^4	10^4–10^5	10^5–10^6
Laboea strobila	–	–	1.05 (6, 11)	–
Lohmaniella spp.	–	1.94 (13, 11)*	1.55 (8, 10)*	–
Tontonia spp.	–	1.18 (2, 12)	0.47 (7, 9)	–
Strombidium spp. 1 (conical morphology)	0.58 (9, 47)	−0.41 (14, 25)	2.26* (24, 35)	–
Strombidium spp. 2 (spherical morphology)	◇	1.55 (9, 47)	0.74 (10, 23)	–
Strombidium spp. 3 (prolate spheroid)	−0.66 (4, 16)	4.16*** (101, 137)	−2.87** (127, 85)	−0.30 (7, 14)

In parentheses: (valid *N* in formalin, valid *N* in Lugol's).
◇: No data for formalin.
*$p<0.05$.
**$p<0.01$.
***$p<0.001$.

To test for significant differences between abundance or biomass in Lugol's and borated-formalin samples, the paired *t*-test was used. To determine whether the mean cell volumes of different taxa differ between the two treatments, the Mann–Whitney test was used as the data sets for most taxa studied were small. In both cases, the hypothesis tested was that there was no difference between the means or the medians of the two populations. Statistical analysis was carried out with Statgraphics plus 4.0. and Statistica.

3. Results

Aloricate ciliates of the family Strombidiidae (*Strombidium* spp., *Laboea* sp. and *Tontonia* spp.)

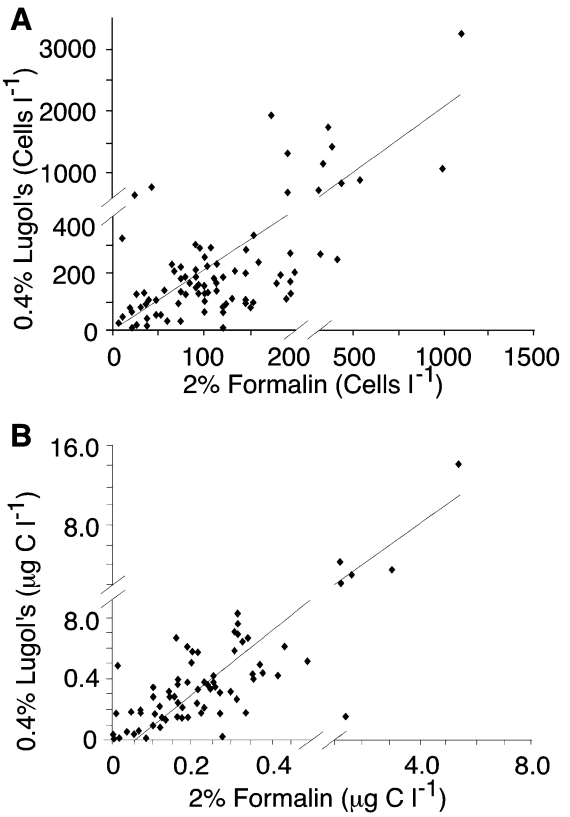

Fig. 2. Oligotrich ciliates abundance (A) and biomass (B) in Lugol's fixed samples plotted against abundance and biomass in formalin, respectively. Fitted regression lines: (A) $y=8.16439+2.0624x$, $r^2=0.50$, $p<0.0001$ ($n=110$) and (B) $y=-0.11771+2.06584x$, $r^2=0.76$, $p<0.0001$ ($n=110$).

dominated the ciliate community in numbers and biomass year round. Additionally, one taxon of Strobilidiidae, *Lohmaniella* spp., was identified in several samples, but it never became dominant. Loricate ciliates of *Tintinnus* spp. were dominant on two occasions (41.5°N, 18°W and 43.2°N, 18.5°W) during the spring. In 80 out of 110 duplicate samples counted, abundance and biomass of oligotrichs were higher in the Lugol's fixed samples (72% of samples). Abundance of oligotrichs in the 2% borated-formalin fixed samples ranged between 6 and 1091 cells l^{-1}, while in 0.4% acid Lugol's fixed samples, the range was 8–3252 cells l^{-1}. Their, biomass ranged from 0.002 to 5.44 µg C l^{-1} and from 0.01 to 14.09 µg C l^{-1} for formalin and Lugol's samples, respectively.

The paired *t*-test comparison showed that both abundance and biomass of oligotrichs were significantly different in Lugol's and formalin fixed samples ($p<0.0001$, $n=110$). A statistically significant linear relationship was calculated between Lugol's and formalin fixed samples for both cell abundance and biomass ($r^2=0.5$ and 0.76, respectively, $p<0.0001$, Fig. 2A,B) showing that abundance and biomass in Lugol's fixed samples were about 100% higher than in formalin (slope of the regression line = 2, Fig. 2A,B).

Biovolumes were calculated using linear dimensions of all cells identified in Lugol's and formalin-treated samples. Furthermore, cells of each taxon and morphotype were separated in 4 cell-volume categories: $<10^3$, 10^3-10^4, 10^4-10^5 and 10^5-10^6 µm^3. Mean cell volumes in Lugol's samples ranged between 75% and 119% of formalin fixed samples. In 4 out of 14 cases, no difference was observed between formalin and Lugol's fixed samples, while in 2 cases, cell-volume in Lugol's was greater than in formalin. Statistically significant differences between cell-volumes were observed only for one to two size classes of *Lohmaniella* spp., *Strombidium* spp. 1 and *Strombidium* spp. 3 (Table 1).

Mixotrophic oligotrichs belonged to the following taxa: *Strombidium* spp., *Laboea strobila* and *Tontonia* spp. *Laboea strobila* and *Tontonia* spp., can be distinguished in Lugol's samples because of their characteristic morphology. Thus, based only on *L. strobila* and *Tontonia* spp. counts, mixotrophic abundance in Lugol's ranged from 0 to 75 cells l^{-1}, while

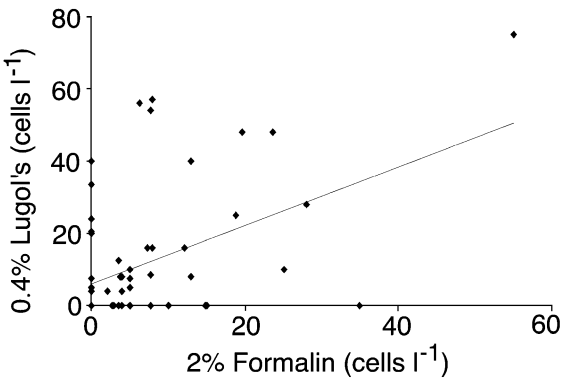

Fig. 3. *L. strobila* and *Tontonia* spp. abundance in Lugol's solution plotted against abundance in formalin ($n=61$).

their abundance in formalin ranged from 0 to 55 cells l^{-1}. Lugol's mean counts were higher than for formalin by a factor of >2, however, no relationship could be established between Lugol's and formalin abundances; this is likely due to the small number of cells present in the 250 ml samples (from 0 to <20 cells, see Fig. 3) which was not statistically useful. Thus, in 34 out of a total of 61 samples, *L. strobila* and *Tontonia* spp. were found in either Lugol's or formalin but not in both fixatives (Fig. 3). Furthermore, total mixotrophic abundance ranged from 0 to 302 cells l^{-1} in formalin fixed samples where chlorophyll fluorescence of *Strombidium* spp. could be also distinguished. Thus, the *p*-value of the paired *t*-test showed that there is a statistically significant difference of ciliate abundance in formalin compared to Lugol's fixation ($p < 0.0001$, $n = 92$). Conversion of numbers of mixotrophs—*L. strobila* and *Tontonia* spp. in Lugol's and *L. strobila*, *Tontonia* spp. and *Strombidium* spp. in formalin fixed samples—into biomass, resulted in similar values in the two kinds of treatments and ranged from 0 to ~ 1.2 μg l^{-1}. It is clear that total mixotroph abundance and biomass were severely underestimated in both treatments for different reasons: in formalin because of severe cell loss and in Lugol's because of chlorophyll masking.

Tintinnus spp. was abundant during the spring cruise. The total lorica abundance present in the samples ranged from 20 to 1076 and from 0 to 992 cells l^{-1} in formalin and Lugol's samples, respectively. On average, 60% of the loricas were full in both treatments. The abundance of full loricas was similar in Lugol's and formalin (Fig. 4) and the paired *t*-test did not show any statistically significant difference between the two treatments ($p > 0.05$, $n = 24$).

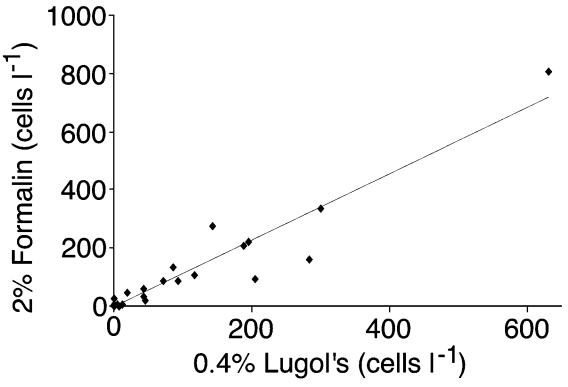

Fig. 4. *Tintinnus* spp. (full loricas) abundance in Lugol's solution plotted against abundance in formalin $r^2 = 0.896$, $p < 0.0001$ ($n = 24$).

4. Discussion

The taxonomic composition of the ciliate community sampled from the NE Atlantic study area was dominated by members of the family Strombidiidae (*Strombidium* spp., *L. strobila*, *Tontonia* spp.) as has already been recorded in previous studies conducted in the same area (Burkill et al., 1993; Stoecker et al., 1994b; Quevedo and Anadón, 2001). Additionally, the abundance and biomass were within the ranges previously reported for the northeastern Atlantic Ocean (Burkill et al., 1993; Stoecker et al., 1994b).

Previous studies conducted with marine or freshwater species from natural populations or cultures on a restricted number of samples (1–12, Table 2) reported cell densities for formalin fixed samples from 28 to ~ 100% (mean = 70 ± 22%) compared to Lugol's solution fixed samples (Table 2). Based on 110 samples, the present study established a statistically significant relationship of 2:1 for Lugol's and formalin samples (Fig. 2). Replicate counts of samples treated with the same fixative are in the order of 12–17% (Sime-Ngando et al., 1990; Thouvenot et al., 1999).

Ohman and Snyder (1991) argued that there is no systematic trend between concentration of acid Lugol's solution and cell densities although experiments conducted by Stoecker et al. (1994a) showed significantly higher oligotrich counts in concentrated (≥ 10%) rather than in dilute (≤ 2%) acid Lugol's solution. A previous study in the north Atlantic (Stoecker et al., 1994a) reported a lower mean difference between 10% Lugol's and 2% formalin samples (36%) than in our samples (50%). Different sampling methodology could be a probable explanation for the discrepancy between our data and that of Stoecker et al. (1994a) in that the latter reported results from a lower number of samples (12 samples, see Table 2) and from only one sampling site and period, whereas our data set was from different sampling sites and periods. Differences in cell loss could be also interpreted as differences in physiological and nutritional

Table 2
Comparison of Lugol's and formalin fixation on the abundance of aloricate ciliates

Species	System	Fixatives	F/L	*N* (*n*)	Source
Total ciliates	Lake Aydat	1% L, 2% uF	76	1 (3)	Sime-Ngando et al. (1990)
Strombidium sp.	culture	2% L, 1% F	~ 100 stationary phase, L>F exponential phase (% not given)	9–11	Ohman and Snyder (1991)
Aloricate ciliates	S. Brazilian Coast	1% L, 1% bF	91	8	Suzuki et al. (1991)
Aloricate ciliates	N. Atlantic culture	10% L, 2% bF	64	12	Stoecker et al. (1994a)
S. spiralis		2%, 5%, 10% L,	91, 99, 83	(3)	
S. capitatum		2% bF	85, 71, 64		
Aloricate ciliates	Plymouth Sound	0.4% L, 1% bF	52	1 (3)	Leaky et al. (1994)
Balanion sp.	culture	0.4% L, 1% bF	28	1 (3)	
S. epidemum	culture	0.4% L, 1% bF	35	1 (3)	
Aloricate ciliates	NE Atlantic	0.4% L, 2% bF	50	110	This study

Ratios expressed as percentages. *N* (*n*): number of samples (replicates per fixative), L = Lugol's, F = formalin, uF/bF = unbuffered/buffered formalin.

states of aloricate ciliates (Ohman and Snyder, 1991; Leaky et al., 1994).

Mean cell volumes were greater in formalin fixed samples compared to Lugol's for most taxa and size-classes identified in our samples. Previously reported results showed the same trend between the two fixatives (Table 3). In these studies, cell volume in Lugol's ranged from 42% to 94% (mean 69 ± 14%) for formalin-treated cells. In our study, the range was from 78% to 109% (mean 89 ± 11%) which corresponds to the higher limits of values previously reported for aloricate ciliates. Previous studies have

Table 3
Comparison of Lugol's solution and formalin fixation on the mean cell volume of ciliated protozoa

Species	System	Fixatives	Fixed/Live	L/F	*N* (*n*)	Source
L. strobila, Strombidium spp.,	Culture	2% L	78	76	20–50	Putt and Stoecker (1989)
S. spiralis		2% bF	103			
Strombilidium spiralis	Culture	L	64	80	20–30/replicate (3)	Choi and Stoecker (1989)
		1% F	80			
Strombidium acutum	Culture	2% L	74	90	20–30/replicate (3)	
		1% F	83			
Strombidium sp.	Culture	1% L	69	77/79	973–1035	Ohman and Snyder (1991)
		2% L	64	71/74		
		1% bF or uF	87/90			
S. spiralis	Culture	2%, 5%, 10% L	ND	74, 66, 59	90–93 (3)	Stoecker et al. (1994a)
S. capitatum		2% bF		61, 56, 40		
Halteria grandinella	Culture	1% L	63	70	300 (3)	Wiackowski et al. (1994)
		2% uF	90			
Colpidium kleini	Culture	1% L	80	64	300 (3)	
		2% uF	124			
Urotricha armata	Culture	1% L	89	94	210 (3)	
		2% uF	95			
Balanion sp.,	Plymouth	0.4% L	ND	69	max 50 cells/taxon	Leaky et al. (1994)
Strombidium epidemum	Sound	1% bF		42	1 sample (triplicate)	
Laboea strobila,	NE Atlantic	0.4% L	ND	75–119	8–240 cells/taxon	This study
Lohmaniella spp.,		2% bF			22 samples	
Tontonia spp.,						
Strombidium spp.						

Ratios expressed as percentages. ND: no data. *N* (*n*): number of cells counted (replicates per treatment) L = Lugol's, F = formalin, uF/bF = unbuffered/buffered formalin.

also considered the initial short-term effects of fixation (maximum of 2 months) while some of our samples were counted up to 6 months after fixation; thus, the smaller difference between formalin and Lugol's biovolumes might indicate long-term shrinkage effect in formalin samples. In experiments conducted by Wiackowski et al. (1994), *Halteria* sp. continued to shrink after 2 months of storage in formalin but its volume did not change after 2 months of storage in Lugol's solution. According to Stoecker et al. (1994a) and Wiackowski et al. (1994), it seems that the effect of fixatives was not the same for all morphotypes and size-classes. Here, for example, cells with conical morphology (*L. strobila*, *Strombidium* spp. 3 and *Tontonia* spp.) showed the greatest shrinkage in Lugol's solution compared to formalin. Within species, this effect was different for the 4 cell-volume categories identified.

Estimates of mixotrophic ciliate abundance and biomass have been usually based on Lugol's counts of species with very distinctive gross morphologies *Laboea* sp. and *Tontonia* spp. (Dolan and Marrasé, 1995; Dolan et al., 1999; Quevedo and Anadón, 2000). However, our results in formalin counts showed that ciliates not distinguishable in Lugol's such as *Strombidium* spp. are important in terms of abundance and biomass. Similar results were reported for the oligotrophic Mediterranean Sea (Perez et al., 2000; Pitta and Giannakourou, 2000; Pitta et al., 2001). Thus, it seems that neither Lugol's nor formalin give accurate estimates of mixotrophs. An important observation in the present study is that abundance and biomass varied by the same factor in the two treatments (Fig. 2), indicating a non-selective loss of cells in formalin. Thus, if we consider the Lugol's to formalin ratio of abundance to be 2:1, then estimates for mixotrophic ciliates could be based on formalin counts multiplied by a factor of 2. Overall, these results indicate a severe underestimation of mixotrophic abundance and biomass in studies conducted up to now. Further investigation in samples rich in mixotrophs is necessary in order to establish a relationship between Lugol's and formalin fixed samples.

Finally, regarding tintinnids, it is suggested that cells leave their loricas when disturbed (Jörgensen, 1924) and there are indications that fixatives may also cause dissolution of agglomerated material and loricas themselves after long-term storage (Dale and Dahl, 1987). In many studies, only intact (full) loricas were counted and recorded (Sime-Ngando et al., 1990; Leaky et al., 1994). However, it is not clear whether empty loricas were dead or alive before fixation. If we assume all empty loricas were dead before fixation, this may lead to an underestimation of tintinnid abundance and biomass. Our results indicate that in contrast to aloricate ciliates, full or empty *Tintinnus* spp. loricas did not vary significantly between the two treatments. These results are similar to Leaky et al. (1994) who only counted intact cells of tintinnids in samples from Plymouth Sound.

Accurate quantification of ciliate abundances depends on a firm understanding of the relative strengths and weaknesses of both Lugol's and formalin solutions. Lugol's is generally preferred over formalin as it minimizes cell loss, although its big disadvantage is that it masks chlorophyll fluorescence, which is crucial in understanding a mixotrophic regime. This study established a 50% cell loss in formalin ciliate counts. With carbon budget estimates of primary production consumption by ciliates based on formalin counts (where chlorophyll can be distinguished), this could lead to a 100% error if a correction is not applied. Finally, as both fixatives have their advantages and disadvantages, we recommend duplicate counts using both fixatives in order to estimate mixotroph numbers as this is a more accurate quantitative procedure.

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