

Effects of Lugol's fixation on the size structure of natural nano–microplankton samples, analyzed by means of an automatic counting method

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*Accurate abundance and biomass measurements are essential steps for determining the role of nano–microplankton in the microbial food web. Owing to practical constraints, traditional microscope analysis of nano–microplankton requires preservation; but preservatives alter plankton samples and bias the measurements. The majority of studies on the effects of preservation have been based on cell cultures. However, new automatic counting systems offer the possibility to investigate the effect of fixatives on large numbers of natural samples. In the present study, cell counts of live and 1% Lugol's preserved samples were compared at 115 stations located in the Bay of Biscay. Additionally, the effect of different Lugol's concentration (1 and 5%) was studied. Analyses were performed with the FlowCAM (see Sieracki *et al.* in *An imaging-in-flow system for automated analysis of marine microplankton. Mar. Ecol. Progress Ser.*, **168**, 285–296, 1998), using plankton samples directly collected from the field. The results show that the analysis of natural samples preserved with a single fixative biases the abundance and biomass estimates of different size ranges of the nano- and microplankton, not only in the large sizes. This is due to changes in cell abundance, especially in the nanoplankton size range, and to the formation of aggregates.*

INTRODUCTION

Accurate abundance and biomass measurements are essential steps for determining the role of plankton in the microbial food web. However, obtaining reliable estimates for the diverse trophic components of marine plankton is not an easy task.

Estimates of biomass of nanoplankton (2–20 μm ESD, equivalent spherical diameter) and microplankton (20–200 μm ESD) have been based traditionally on cell counts and microscopic measurements of cell size. However, microscope counting requires fixation, and available techniques for optimal preservation and enumeration of nano- and microplankton are taxon specific. As such, the selection of methods is determined

by the objectives of the individual study and consideration of the target taxa (Gifford and Caron, 2000). This consideration implies that, for accurate enumeration of the whole nano–microplankton compartment, counting several sub-samples fixed in different ways is needed. However, because of practical limitations, generally nano–microplankton sub-samples are preserved (and subsequently counted) with a limited number of preservatives (most often a single one, Lugol's iodine).

Lugol's iodine (Thronsdén, 1978) is a widely used fixative and is recommended commonly for preserving ciliates and flagellates (Thronsdén, 1978; Leakey *et al.*, 1994; Karayanni *et al.*, 2004). However, it has been shown to introduce artifacts, including changes in cell size,

reduction in the abundance of cells detected and a failure to preserve certain taxa (Leakey *et al.*, 1994; Stoecker *et al.*, 1994). Artifacts induced by Lugol's fixation have been studied extensively in ciliates (Leakey *et al.*, 1994; Stoecker *et al.*, 1994; Modigh and Castaldo, 2005); likewise in some dinoflagellate and diatom species (Montagnes *et al.*, 1994; Mender-Deuer *et al.*, 2001). However, fewer studies have estimated the effect of Lugol's fixation on nanoplankton (Klein Breteler, 1985; Choi and Stoecker, 1989; Montagnes *et al.*, 1994). The majority of these studies used cell cultures, to estimate the effects of fixation on individual taxa. But it must be remembered that the composition of natural plankton assemblages is relatively unknown, especially for smaller nanoplankton; and therefore, the extrapolation of the effects of fixation on cell cultures to natural plankton communities can result in inadequate data and conclusions.

In the last few decades, image or optical properties-based automatic systems have experienced a rapid development, allowing *in situ* enumeration of a wide size range within the same sample (Sieracki *et al.*, 1998; Benfield *et al.*, 2007; Olson and Sosik, 2007). Image analysis techniques, combined with automatic recognition algorithms, are a promising approach to meet the requirements of higher resolution studies (Culverhouse *et al.*, 2003; Blaschko *et al.*, 2005; Hu and Davis, 2006; Benfield *et al.*, 2007). Through rapid counting, such systems offer the possibility of investigating the effect of fixatives on large numbers of natural samples.

The aim of this study was to evaluate the effect of Lugol's fixation on samples of natural plankton communities collected in the field, covering a broad size range, from nanoplankton to microplankton. The analysis was performed by means of an automatic image analysis-based instrument: the FlowCAM (Sieracki *et al.*, 1998).

METHODS

Sample collection and analysis

Samples were collected from 112 stations (in total) in the Bay of Biscay, during May 2004. A broad sampling grid, covering coastal, continental shelf and shelfbreak waters, was completed (Fig. 1). At every station, seawater samples for nano–microplankton measurements were collected at 3 m depth, using 1.5l Niskin bottles.

For 105 stations, one sub-sample was separated and analyzed immediately, without fixation. Another sub-sample of 125 mL was preserved with acid Lugol's solution (hereafter Lugol's) at a final concentration of 1%. Additionally, 14 stations were selected to study the effects of different concentrations of Lugol's. At these

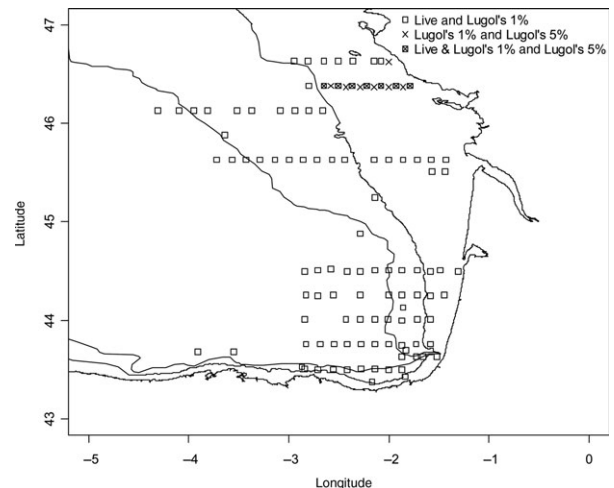


Fig. 1. Location of sampling stations. Different symbols correspond to the fixation treatments applied to the samples. 100 and 200 m isobaths are shown.

stations, two sub-samples of 125 mL were preserved with acid Lugol's, at final concentrations of 1% and 5, respectively. Seven of these stations were also analyzed without fixation (Fig. 1).

Acid Lugol's was prepared following the method described by Thronsdon (Thronsdon 1978). As such, 100 g of KI was dissolved in 1 L of distilled water and 50 g of iodine (crystalline) was dissolved in 100 mL of glacial acetic acid. The two solutions were mixed and any precipitates removed. 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). Samples were stored in amber glass bottles at ambient temperature, and analyzed in the laboratory 4 months after fixation.

The analysis of both fixed and live samples was undertaken with a FlowCAM (Sieracki *et al.*, 1998) following a standard procedure. The samples were analyzed in auto-trigger mode, in which the flow sample stream is sampled regularly by the imaging system, with no fluorescence measurements being taken (Sieracki *et al.*, 1998). Therefore, every particle (phytoplankton, zooplankton, aggregates, inorganic, and so on) ranging from 8 to 200 μm ESD was counted and imaged. For each sample, a maximum of either 2000 particles or 10 mL were analyzed. A $\times 4$ objective was used in the sample analysis and the instrument was calibrated using beads of a known size. Invalid pictures (i.e. bubbles, repeated images) were removed from the image database, through visual recognition. The biovolume of each cell was calculated from its ESD. Correction for shrinkage was applied to preserved cells: $\text{Volume}_{\text{live cells}} = 1.33 \times \text{Volume}_{\text{fixed cells}}$ (Montagnes *et al.*, 1994).

Particles measured by the FlowCAM were divided into three size groups: 8–20 μm (nanoplankton), 20–40 μm (small microplankton) and 40–200 μm ESD (large microplankton). Live, Lugol's 1% and Lugol's 5% biomass and abundance distributions were represented with box plots and compared using analysis of the variance (ANOVA).

RESULTS

Total plankton abundance ranged from 122 to 7842 ind. mL^{-1} in the live samples was analyzed. In the samples preserved with 1% Lugol's solution, abundance ranged from 213 to 3556 ind. mL^{-1} . Total biovolume ranged from 123 to 12 060, and from 372 to 9379 $\mu\text{m}^3 \text{L}^{-1}$ in the live and 1% Lugol's samples, respectively.

Abundance and biovolume distributions of unpreserved and 1% Lugol's samples were significantly different (ANOVA, $P < 0.01$) in the three size ranges studied (Figs 2a and 3a; Table I). A total of 105 samples were used for these analyses (Fig. 1). A decrease in average abundance and biovolume induced by fixation can be observed in the nanoplankton size range (8–20 μm ESD), as well as in particles between 40 and 200 μm ESD. However, in the size range from 20 to 40 μm , the mean abundance (and biovolume) of particles preserved with 1% Lugol's was higher than that in the live samples (Figs 2a and 3a). A significant linear

relationship ($P < 0.01$) is observed for cell counts on live and 1% Lugol's, for the three size groups ($r^2 = 0.63$, 0.56 and 0.37) (Fig. 4a; Table II). In samples preserved with Lugol's, small particles (8–20 μm ESD) and large microplankton (40–200 μm ESD) were underestimated. In comparison, particles in the size range from 20 to 40 μm ESD were overestimated.

Visual examination of FlowCAM images revealed that diatom chains and ciliates larger than 20 μm ESD could be recognized in unpreserved samples. However, in samples fixed with Lugol's, only large diatom chains could be identified. Ciliates were affected due to the loss of cilia, high staining and shrinkage of cells (Fig. 5). Moreover, the formation of abundant aggregates in the preserved samples is notable, mostly within the size range from 20 to 40 μm ESD (Fig. 5).

A second ANOVA was performed (Table I), to examine the differences between the abundance and biovolume distributions of live samples and samples preserved with 5% Lugol's. Only seven samples were available for this comparison. Average abundances observed for 5% Lugol's preserved samples were significantly lower ($P < 0.05$) in the nanoplankton size range; and significantly higher ($P < 0.005$) for particles sized between 20 and 40 μm ESD. For larger microplankton, differences were not significant (Fig. 2b). The same trend was observed in biovolume distributions (Fig. 3b).

Cell counts on live and on 5% Lugol's preserved samples showed a significant linear relationship

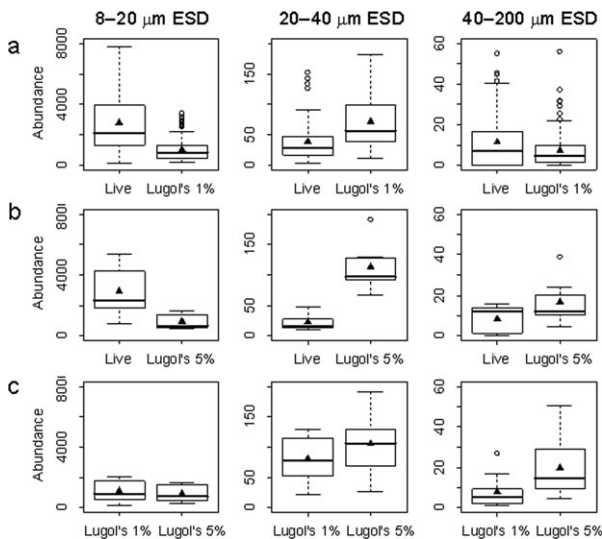


Fig. 2. Boxplots showing the distributions of particle abundance (particle mL^{-1}), for the different ANOVAs described in Table I: (a) samples without fixation and preserved with 1% Lugol's; (b) samples without fixation and preserved with 5% Lugol's; (c) samples preserved with 1 and 5% Lugol's. The black triangle represents the mean value of the distribution. All boxplots performed for the three size groups studied: 8–20 μm ESD; 20–40 μm ESD and 40–200 μm ESD.

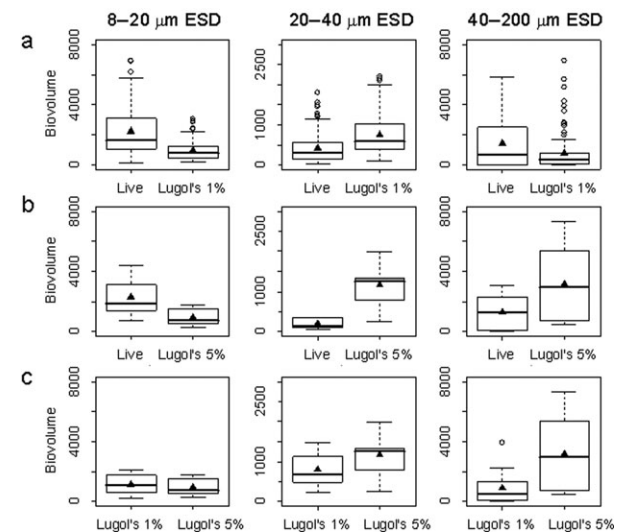


Fig. 3. Boxplots showing the distributions of particle biovolume ($\mu\text{m}^3 \text{L}^{-1}$), for different ANOVAs described in Table I: (a) samples without fixation and preserved with 1% Lugol's; (b) samples without fixation and preserved with 5% Lugol's; (c) samples preserved with 1 and 5% Lugol's. The black triangle represents the mean value of the distribution. All boxplots performed for the three size groups studied: 8–20 μm ESD; 20–40 μm ESD and 40–200 μm ESD.

Table I: Analysis of the variance (ANOVA) between the abundance and biomass of plankton of samples preserved with 1% Lugol's versus samples analyzed without fixation; samples preserved with 5% Lugol's versus samples analyzed without fixation and samples preserved with 5% Lugol's versus samples analyzed with 1% Lugol's

	N	8–20 μm ESD		20–40 μm ESD		40–200 μm ESD	
		F	P-value	F	P-value	F	P-value
Abundance							
Lugol's 1% versus live	105	78.63	<0.001***	36.06	<0.001***	7.27	<0.01**
Lugol's 5% versus live	7	8.8	<0.05*	32.16	<0.001***	2.79	0.12
Lugol's 5% versus Lugol's 1%	14	0.73	0.4	2.53	0.12	8.22	<0.01**
Biovolume							
Lugol's 1% versus live	105	57.17	<0.001***	28.56	<0.001***	10.03	<0.01**
Lugol's 5% versus live	7	12.34	<0.01**	30.06	<0.001***	3.14	0.092
Lugol's 5% versus Lugol's 1%	14	0.92	0.347	5.234	<0.05*	9.1	<0.01**

All ANOVAs performed for the three size groups studied: 8–20 μm ESD; 20–40 μm ESD and 40–200 μm ESD.
 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

($P < 0.005$) for the nanoplankton size range (Fig. 4b; Table II), with a high correlation coefficient ($r^2 = 0.96$). The slope ($b = 0.31$) revealed that cell counts were underestimated in Lugol's samples. The linear correlation for the small microplankton was also significant ($P < 0.05$). In this size group, cell counts of preserved samples were overestimated.

Finally, the differences between the effects of different concentrations of the preservative (1 and 5% Lugol's) were evaluated. Fourteen samples were considered for

this analysis. The differences in abundance distributions were significant ($P < 0.01$) only for microplankton larger than 40 μm in size (Fig. 2c; Table I). In terms of biovolume (Fig. 3c), differences were significant ($P < 0.05$) for the microplankton size range (20–200 μm ESD).

In the nanoplankton and small microplankton size ranges, cell counts for both of the fixatives showed a significant linear relationship ($P < 0.005$), with a high correlation ($r^2 = 0.93$ and 0.83 , respectively) (Fig. 4c; Table II).

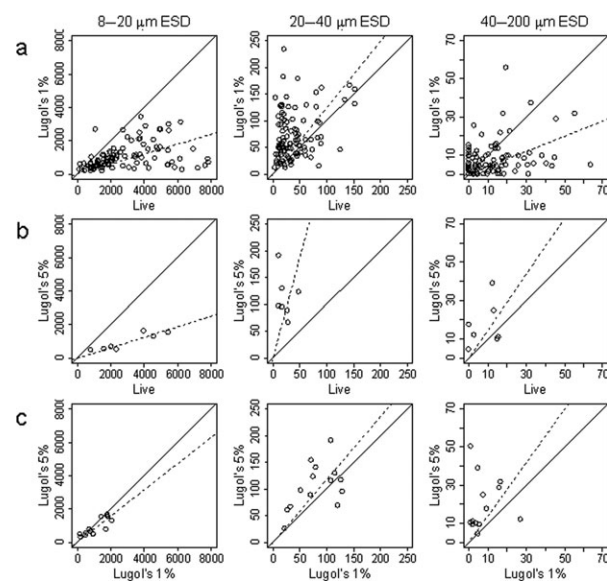


Fig. 4. Relationship between particle abundance (particle mL⁻¹) of (a) samples preserved with 1% Lugol's and samples analyzed without fixation; (b) samples preserved with 5% Lugol's and samples analyzed without fixation; (c) samples preserved with 5% Lugol's and samples analyzed with 1% Lugol's. The solid line indicates a 1:1 relationship. The dotted line represents the fitted linear regressions forced through the origin. All regressions performed for the three size groups studied: 8–20 μm ESD; 20–40 μm ESD and 40–200 μm ESD.

DISCUSSION

The data show that fixation with Lugol's significantly alters the estimates of plankton abundance and size in natural samples. A main effect of Lugol's fixation in the present study is a decrease in the abundance of nanoplankton (Fig. 4b). This factor may be due to their aggregation into larger particles, or to their disintegration into smaller units, beyond the detection range of the FlowCAM. The identification of cell losses of small cells due to fixation is particularly important because studies on the impact of fixation have focused traditionally on larger organisms, such as ciliates, diatoms and dinoflagellates (Leakey *et al.*, 1994; Montagnes *et al.*, 1994; Stoecker *et al.*, 1994; Mender-Deuer *et al.*, 2001). However, nanoplankton contributes significantly to primary production in many regions of the world's oceans (Li, 2002), and a failure to evaluate fixation-induced changes in this community could lead to important errors in the estimates of abundance and biomass.

Another significant effect of Lugol's fixation on plankton samples is the formation of aggregates. Aggregates may originate from the addition of fecal material (Stoecker, 1984), broken small cells, fragments

Table II: Linear regressions forced through the origin, between particle abundance (particle mL^{-1}) of: samples preserved with 1% Lugol's versus samples analyzed without fixation; samples preserved with 5% Lugol's versus samples analyzed without fixation and samples preserved with 5% Lugol's versus samples analyzed with 1% Lugol's

	N	8–20 μm ESD		20–40 μm ESD		40–200 μm ESD	
		Equation	R^2	Equation	R^2	Equation	R^2
Lugol's 1% versus live	105	$y = 0.3x$	0.63***	$y = 1.25x$	0.56***	$y = 0.4x$	0.36***
Lugol's 5% versus live	7	$y = 0.31x$	0.96***	$y = 3.76x$	0.56*	$y = 1.46x$	0.54*
Lugol's 5% versus Lugol's 1%	14	$y = 0.79x$	0.92***	$y = 1.18x$	0.85***	$y = 1.39x$	0.35*

All regressions performed for the three size groups studied: 8–20 μm ESD; 20–40 μm ESD and 40–200 μm ESD.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of larger cells and so on. Such particles are responsible for the higher abundance shown for preserved samples within the size range from 20 to 40 μm ESD (Fig. 2b). The organic carbon contained in these aggregates was once part of the plankton community, as it formed part of the energy transfer pathways in the pelagic food web (Roy *et al.*, 2000). However, in the analysis of preserved samples, aggregates bias the total estimate of biovolumes (and therefore biomass) and the size distribution of plankton. This is, on the one hand, because it is not possible to distinguish between naturally occurring aggregates and those created during fixation and, on the other hand, because the aggregates overlap in size with dinoflagellates, ciliates and diatoms (Fig. 5), which confounds the estimates of living and non-living particles when using automatic counting systems. Awareness of this has already been reported in other studies using automatic systems. Stoecker (Stoecker, 1984) determined that the similarity in size of microplanktonic algae and ciliate fecal aggregates made the

Coulter Counter inappropriate for use in determining the clearance or ingestion rates in ciliate-feeding experiments.

Changes in the size and shape of the cells (shrinkage, swelling and so on), in response to the use of Lugol's, have been reported extensively in the literature (Choi and Stoecker, 1989; Leakey *et al.*, 1994; Stoecker *et al.*, 1994; Montagnes *et al.*, 1994; Mender-Deuer *et al.*, 2001). In the present study, corrections for shrinkage have been applied to preserved samples, using the factor of 1.33 proposed by Montagnes *et al.* (Montagnes *et al.* 1994) for marine phytoplankton. However, specific shrinkage correction factors are difficult to extrapolate to natural plankton communities, where diverse autotrophic and heterotrophic organisms co-occur. Fixation effects are highly variable and may be a function of factors such as organism phylogenetic group, physiology or growth stage (Jerome *et al.*, 1993; Wiackowski *et al.*, 1994; Mender-Deuer *et al.*, 2001), which are often uncontrollable or unknown in natural plankton assemblages directly collected from the field. Strikingly, in estimates based on samples containing many species, cell volume changes induced by fixation have been described as negligible (Mender-Deuer *et al.*, 2001). At present, the lack of general factors which are valid for naturally occurring plankton communities makes it necessary to assume a certain degree of error when correcting for shrinkage natural plankton samples.

The concentration of acid Lugol's solution used does not appear to have a significant effect on nanoplankton and small microplankton abundance distribution (Figs 2c and 3c). However, abundance estimates of large microplankton fixed with 5% Lugol's are significantly higher than in samples fixed with 1% Lugol's (ANOVA; $P < 0.005$). This effect on large microplankton agree with the results obtained by Stoecker *et al.* (Stoecker *et al.*, 1994), who measured significantly higher ciliate cell counts in strong Lugol's (10 or 20%), rather than in 2% Lugol's. However, the influence of Lugol's concentration on cell densities is not clear; other authors

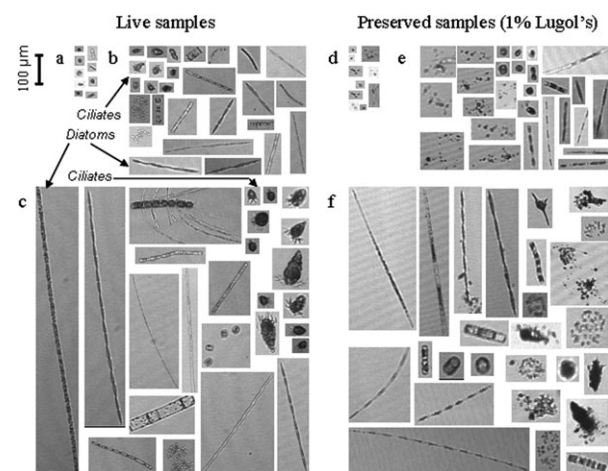


Fig. 5. Example of digital photographs recorded by the FlowCAM, for (a–c) live and (d, f, g) 1% Lugol's preserved samples. The images are divided into three size groups: (a, d) 7–20 μm ESD; (b, e) 20–40 μm ESD and (c, f) 40–200 μm ESD.

(Ohman and Snyder, 1991) have not found a systematic relationship between the concentrations of fixative and cell losses.

The preserved samples here were analyzed 4 months after collection; consequently, the storage time is also a factor to be considered, with potential effects on cell volume and cell abundance estimates. It has been described previously that cell losses in Lugol's preserved samples increase with the time of preservation (Sime-Ngando and Groliere, 1991; Stoecker *et al.*, 1994). Nevertheless, this pattern does not appear in the experiments undertaken by Ohman and Snyder (Ohman and Snyder, 1991), where changes in cell counts and cell size began to stabilize after 24 h. Only relatively small changes occurred after this time, with the last measurement being undertaken 1 month after preservation.

It has been found in the present study that the analysis of natural samples preserved with a single fixative biases the abundance and biomass estimates of different size ranges of the nano- and microplankton, not only on the large sizes. This is due to the changes in cell abundance, especially in the nanoplankton size range, and to the formation of aggregates. The use of semi-automatic counting systems that allow counting of a large number of samples in a short time without the need for preservation may contribute to improving biomass estimates in these size ranges.

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