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Original Article

Impacts of sample storage time on estimates of phytoplankton abundance: how long is too long?

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ABSTRACT

Phytoplankton play a key role in marine ecosystems, making the accurate quantification of functional groups/species, using standardized microscopy techniques, essential in many research efforts. In this study we aimed to evaluate the effects of (a) storage time on the abundance of microphytoplankton in samples fixed with acid Lugol's solution; (b) storage time on the abundance of pico- and nanophytoplankton in frozen microscopy slides; (c) storage time on the abundance of pico- and nanophytoplankton in fixed refrigerated samples and (d) storage protocol (refrigerated versus frozen) on the abundance of pico- and nanophytoplankton. Microphytoplankton were analyzed using inverted microscopy, and pico- and nanophytoplankton using epifluorescence microscopy. Results indicate storage time negatively impacted the abundances of all phytoplankton size classes; however, effects were group-specific. For accurate abundance estimates, we suggest that samples fixed with acid Lugol's should be analyzed within 30 and 180 days, for dinoflagellates/total microphytoplankton and diatoms, respectively. For picoeukaryotes, glutaraldehyde fixed samples should be kept refrigerated for up to 4 days, and slides should be prepared immediately before observation. It is recommended that authors specify the exact lag times between sample collection, fixation and analysis, to allow the comparability of phytoplankton datasets across different studies and/or monitoring programs.

KEYWORDS: lugol, glutaraldehyde, fixatives, inverted microscopy, epifluorescence microscopy

INTRODUCTION

Phytoplankton play a key role in marine food webs and act as important indicators of environmental status, valuable for marine ecosystem management (McQuatters-Gollop *et al.*, 2017; Tweddle *et al.*, 2018). Thus, phytoplankton have been the subject of extensive research efforts aiming to better understand variability patterns and environmental drivers (Calbet and Landry, 2004; Wu *et al.*, 2021; Baer *et al.*, 2023). Such efforts are of upmost importance in tackling the impacts of current threats on marine ecosystems, including climate change and anthropogenic eutrophication (Zhou *et al.*, 2022; Thompson and Carstensen, 2023), and imply a need for accurate quantification of phytoplankton metrics. For phytoplankton species forming harmful algal blooms (HABs), accurate estimates of abundance are also critical to define appropriate HAB management and mitigation strategies (Andersen and Throndsen, 2004; Liu *et al.*, 2023).

In the context of phytoplankton analysis, and despite the emergence of a wide range of automated technologies for plankton imaging (Olson and Sosik, 2007; Owen *et al.*, 2022; Agarwal *et al.*, 2023), one of the most common tools to quantify and identify phytoplankton functional groups or species is microscopy (McQuatters-Gollop *et al.*, 2017). For microphytoplankton (>20 μm) analysis, the most frequently used microscopic technique is brightfield inverted microscopy (Utermöhl, 1958). For pico- (<2 μm) and nanophytoplankton (2–20 μm), epifluorescence microscopy, using autofluorescence or secondary staining with proflavine (Haas, 1982) or primulin (Bloem *et al.*, 1986), is the most common strategy. Yet, most phytoplankton studies have typically relied on the Utermöhl method, using inverted microscopy (Andersen and Throndsen, 2004; Soares *et al.*, 2011), and this procedure is the standardized method in the European Union (EN 15204 2006; Muñiz *et al.*, 2020). However, epifluorescence microscopy not only enables the quantification of smaller cells but also allows distinction between autotrophic and heterotrophic cells (Havskum *et al.*, 2004; Seoane *et al.*, 2011). As such, studies that combine the use of both microscopic techniques have been suggested as beneficial (Domingues *et al.*, 2008), since a more accurate and comprehensive understanding of the whole phytoplankton community can be obtained (Domingues *et al.*, 2021).

A vital stage in any study addressing phytoplankton quantification is sample fixation, and subsequent storage prior to microscopic analysis. Depending on the aim of the study and the microscopic technique used, different fixatives are added to the sample to stop biological activity, ensure sample quality over time and minimize cell degradation and morphological change (Sournia, 1978). For microphytoplankton analysis, the most common fixatives are formaldehyde and Lugol's iodine solution (Andersen and Throndsen, 2004; Zarauz and Irigoien, 2008). However, Lugol's solution tends to be used more frequently due to its advantages for human health (lower

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toxicity), cell visualization (improved due to cell staining) and cell concentration (increased cell density and settling velocity) (Andersen and Throndsen, 2004; Williams *et al.*, 2016). For pico- and nanophytoplankton analysis, based on epifluorescence microscopy, glutaraldehyde is the preferred fixative, since the staining caused by Lugol interferes with the cell autofluorescence, a key feature to distinguish autotrophic from heterotrophic cells (Booth, 1987). Typical procedures to prevent the loss of autofluorescence in pico- and nanophytoplankton include sample filtration, staining and slide mounting (hereafter designated as slide preparation) within 24 h after sample collection and fixation. Slides are then frozen (approximately −20°C) until analysis is possible (Bloem *et al.*, 1986; Havskum *et al.*, 2004; Domingues *et al.*, 2021).

The identification and enumeration of phytoplankton using epifluorescence and inverted microscopy requires a high degree of analyst skill and taxonomic expertise and is usually a time-consuming process (Menden-Deuer *et al.*, 2020). Therefore, samples normally cannot be analyzed immediately after sampling and fixation, particularly when several samples must be processed, for example during oceanographic cruises or manipulation experiments (Domingues *et al.*, 2021). For microphytoplankton analysis, samples fixed with Lugol's solution are often referred to be suitable for long-term storage that can exceed a year (Andersen and Throndsen, 2004). Lugol's fixed samples are kept in dark conditions, and recent studies report storage temperatures that range from cold (4°C) (Mäki *et al.*, 2017; Muñiz *et al.*, 2020) to room temperature (Williams *et al.*, 2016; Sweet *et al.*, 2022; Liu *et al.*, 2023). For pico- and nanophytoplankton analysis, slide preparations are often kept frozen at −20°C (Domingues *et al.*, 2021; Chin *et al.*, 2022; Safi *et al.*, 2023) or in some cases at −80°C (Albin *et al.*, 2022; Latasa *et al.*, 2022) and can be stored for several months (Booth, 1987; Havskum *et al.*, 2004), or occasionally even years (Parsons *et al.*, 2021), until analysis is possible. Furthermore, some studies do not provide specific information on the sample storage time, implicitly assuming no temporal change over time (Liu *et al.*, 2023).

Sample storage, along with the use of fixatives, inevitably introduces artifacts, causing changes in several phytoplankton metrics (e.g. cell abundance, morphology, surface area, volume, autofluorescence). These artifacts may vary depending on analytical methods used (e.g. microscopy, flow cytometry), phytoplankton taxon, fixative type/composition, addition of buffers or cryoprotectants, preservation temperature and preservation time (Klein Breteler, 1985; Sato *et al.*, 2006; Zarauz and Irigoien, 2008; Katano *et al.*, 2009; Mukherjee *et al.*, 2014; Williams *et al.*, 2016; Naik and Anil, 2017). Some studies have previously addressed the effects of sample storage on plankton size structure (Zarauz and Irigoien, 2008; Liu *et al.*, 2023) or on phytoplankton cell surface area/volume, throughout periods up to 1 year, for different fixatives (Montagnes *et al.*, 1994; Menden-Deuer *et al.*, 2001; Mukherjee *et al.*, 2014; Yang *et al.*, 2017). However, only a limited number of studies addressed the effects of storage time on the abundance of specific groups (Katano *et al.*, 2009; Naik *et al.*, 2010) or whole microphytoplankton communities (Hällfors *et al.*, 1979; Williams *et al.*, 2016). In the case of pico- and nanophytoplankton, the influence of slide preparation storage time on abundance estimates using epifluorescence microscopy was investigated in earlier studies (Bloem *et al.*, 1986; Booth, 1987; Hall, 1991), whereas more recent reports evaluated the effects of sample storage time on abundances obtained from flow cytometry analysis (Troussellier *et al.*, 1995; Pan *et al.*, 2005; Marie *et al.*, 2014). To the best of our knowledge, the impact of storage time for the evaluation of pico- and nanophytoplankton based on epifluorescence microscopy after proflavine staining (Haas, 1982) was not yet been reported. Knowledge of the effects of sample preservation and storage time is crucial to inform and improve analytical protocols and increase the accuracy of phytoplankton data. This information should be further considered in phytoplankton research, namely monitoring programs, to enable data comparability between studies (Williams *et al.*, 2016).

This study aimed to evaluate the effects of storage time on the abundances of phytoplankton using the standard protocols of sample fixation and microscopic analyses applied to different size classes. The specific objectives of our study were to evaluate (a) the effects of storage time on the abundance of microphytoplankton in samples fixed with acid Lugol's solution, analyzed using inverted microscopy; (b) the effects of storage time on the abundance of pico- and nanophytoplankton in frozen (−20°C) microscopy slides, prepared after sample fixation with glutaraldehyde, proflavine staining and filter mounting, analyzed using epifluorescence microscopy; (c) the effects of storage time on the abundance of pico- and nanophytoplankton in fixed refrigerated samples (4°C), analyzed using epifluorescence microscopy and (d) the effects of storage protocol (refrigerated fixed samples versus frozen microscopy slides) on the abundance of pico- and nanophytoplankton, using epifluorescence microscopy. We anticipate that increased lag times between sample collection and sample analysis will lead to lower phytoplankton abundances, for all phytoplankton size classes, due to increased cell degradation over time. We also expect that a short storage of fixed samples under refrigeration conditions will provide higher abundances of pico- and nanophytoplankton than storage of frozen microscopy slides, due to increased cell damage.

MATERIALS AND METHODS

Sampling and experimental strategy

Sampling was conducted in the Ria Formosa coastal lagoon, in October 2020. Sub-surface water samples were collected using a 5 L plastic bottle and transported under dark and cold conditions to the laboratory.

Approximately 30 min after sample collection, the water sample was divided into subsamples that were fixed with acid Lugol's solution, for inverted microscopy, and with glutaraldehyde for epifluorescence microscopy (see below for further details on fixative preparation and final concentrations). For all subsamples, fixatives were added to the bottles/flasks already containing the water sample, those were then immediately homogenized after fixative addition to reduce the risk of sub-optimal fixative levels in the cells. Samples fixed with Lugol's were stored in 1 L plastic bottles, under dark conditions, in temperatures ranging from 15 to 20°C (Hawkins *et al.*, 2005; Williams *et al.*, 2016; Liu *et al.*, 2023), and analyzed using inverted microscopy after 7, 14, 30,

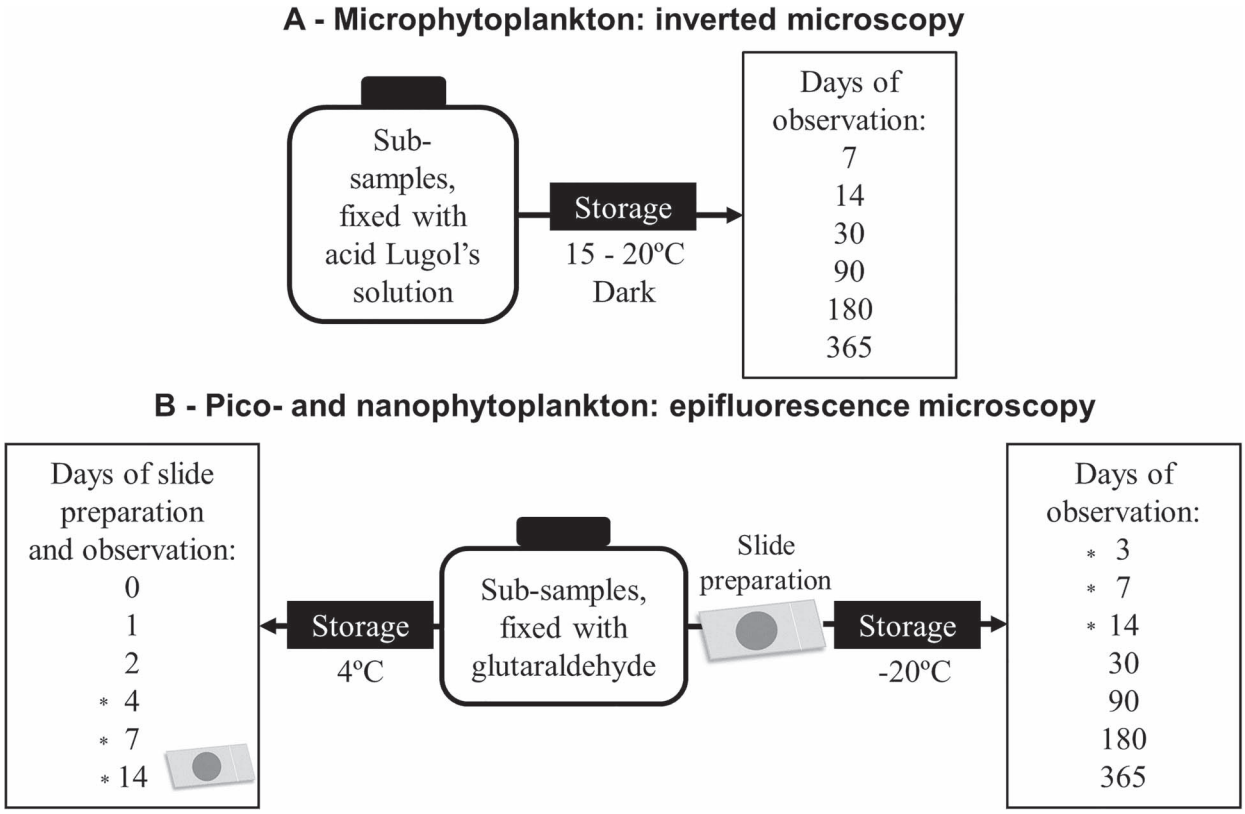

Fig. 1. Schematic representation of the strategies used to test the effects of storage time and protocol on the abundance of (A) microphytoplankton (>20 μm), analysed using inverted microscopy, and (B) pico- (<2 μm) and nanophytoplankton (2–20 μm) analysed using epifluorescence microscopy. (*) indicates the days used to determine the effects of storage protocol on pico- and nanophytoplankton abundance.

90, 180 and 365 days (Fig. 1A). Bottles containing samples fixed with Lugol's were kept inside well-closed cardboard boxes to ensure darkness and placed in a protected shelf within the laboratory, as it is a common practice. Glutaraldehyde fixed samples were stored, in dark glass flasks, using two distinct storage protocols: (1) "frozen microscopy slides" and (2) "refrigerated fixed samples" (Fig. 1B). The first protocol or the "frozen microscopy slides" protocol consisted in sample filtration, staining and filter mounting, i.e. slide preparation, within a maximum period of 24 h after sample fixation. Slide preparations were then stored in the freezer (−20°C) until analysis (Tsuji and Yanagita, 1981; Hall, 1991; Domingues *et al.*, 2021) after 3, 7, 14, 30, 90, 180 and 365 days of storage. For the second protocol or the "refrigerated fixed samples" protocol, fixed samples were kept under refrigerated conditions (4°C) until analysis after 0, 1, 2, 4, 7 and 14 days of storage, with sample filtration, staining and slide preparation occurring immediately before the analysis. For all microscopy techniques and storage protocols, microscopic analyses at each time-step were made in triplicate.

Phytoplankton abundance and composition

Inverted microscopy was used to determine the composition and abundance of microphytoplankton (>20 μm) (Utermöhl, 1958). Subsamples for microphytoplankton analyses were fixed with acid Lugol's solution, prepared by dissolving 100 g of potassium iodate in 1 L of distilled water, adding 50 g of crystalline iodine and finally adding 100 mL acetic acid (2 mL of Lugol added to 1 L bottles: final concentration in the sample of ~0.2%; Throndsen, 1978). Samples were settled in sedimentation chambers (50 mL), and observed using a Zeiss Axio Observer S1 inverted microscope, at 400× magnification. Acid Lugol's solution was chosen since it is considered to better preserve microflagellates and diatoms (Hällfors *et al.*, 1979; Williams *et al.*, 2016), dominant components of phytoplankton assemblages in the Ria Formosa coastal lagoon (Barbosa, 2010), than other Lugol's solutions (neutral or alkaline solutions). Lugol's fixed samples were checked regularly to guarantee the presence of a brown "tea-like" color. There was no need to add more Lugol's solution, since the color of the samples remained stable throughout the storage time.

Epifluorescence microscopy was used to determine the composition and abundance of pico- (<2 μm) and nanophytoplankton (2–20 μm) (Haas, 1982). Subsamples were fixed with glutaraldehyde (Merck, glutaraldehyde solution for electron microscopy 25%) at 2% final concentration (160 μL of glutaraldehyde added to 20 mL of sample), stained with proflavine, and filtered (5 mL) onto black polycarbonate membrane filters (Whatman, nominal pore diameter = 0.4 μm). Slide preparation was made using glass slides and non-fluorescent immersion oil (Cargille type A) under dark conditions, to minimize loss of fluorescence. Analysis was done using a Zeiss Axio Imager A1 epifluorescence microscope, at 1000x magnification.

For both methods, a minimum of 50 random visual fields, at least 400 cells in total and 100 cells of the most common taxon were enumerated. Assuming cells were randomly distributed, the counting precision was ±10% (Venrick, 1978). All microscopic analyses were made by the same analyst to avoid artifacts associated with variability in taxonomic expertise between analysts (Culverhouse *et al.*, 2003; Muñiz *et al.*, 2020).

Data analysis

Potential trends in phytoplankton abundance, for different phytoplankton size classes and functional groups, with storage time were evaluated using linear regression analysis. Data were log transformed whenever nonlinear trends were obtained to provide linear responses. The effects of storage time for both fixed samples and/or frozen microscopy slides on phytoplankton abundance, for different phytoplankton size classes and functional groups, were statistically tested using a one-way analysis of variance (ANOVA). One-way ANOVA was also used to test the effects of storage protocol on pico- and nanophytoplankton by comparing the abundances between frozen microscopy slides and refrigerated fixed samples, for similar storage times, up to 14 days. Data normality and homogeneity of variances were tested using Shapiro–Wilk and Levene's tests, respectively. Data were log transformed whenever normality conditions were not met. Tukey post hoc tests were used to evaluate significant differences between different storage days. Effect sizes were assessed using omega-square statistics (ω^2) to quantify the strength of the differences observed (Olejnik and Algina, 2003). Statistical analyses were conducted with IBM SPSS Statistics 22 (Field, 2013) and GraphPad Prim 5, considering a significance level of 0.05.

RESULTS

Effects of storage time on microphytoplankton abundance—Lugol-fixed samples

Total abundance of microphytoplankton in Lugol's fixed samples showed a significant declining trend along the 1-year storage period ($F = 20.01$, $r^2 = 0.55$, $P < 0.001$), with a reduction of ~51% comparing the first and last measurements. Significant differences in microphytoplankton abundance were only detected after 30 days of sample storage ($P < 0.001$, $\omega^2 = 0.76$), and for storage times longer than 90 days (reduction in abundance at 90 days, ~39%), abundance values stabilized (Fig. 2A).

Diatoms (dominant species: *Cylindrotheca closterium*) and plastidic dinoflagellates (dominant species: *Prorocentrum micans*) represented the dominant functional groups in the sample, accounting for ~40% and 55% of total microphytoplankton abundance, respectively. Abundance of plastidic dinoflagellates in Lugol's fixed samples presented a significant decline over the 1-year storage period ($F = 14.97$, $r^2 = 0.50$, $P = 0.002$), with a reduction of ~44% between the first and last measurements, and a variability pattern like that described for total microphytoplankton abundance (reduction in abundance at 90 days, ~36%; $P = 0.003$, $\omega^2 = 0.66$) (Fig. 2B). Abundance of diatoms also revealed a significant decline throughout the storage period ($F = 29.21$, $r^2 = 0.68$, $P < 0.001$), with significant differences occurring only when comparing first and last measurements (reduction in abundance at 365 days, ~62%; $P = 0.003$, $\omega^2 = 0.68$). Yet, this declining trend continued until the end of the experiment (Fig. 2C).

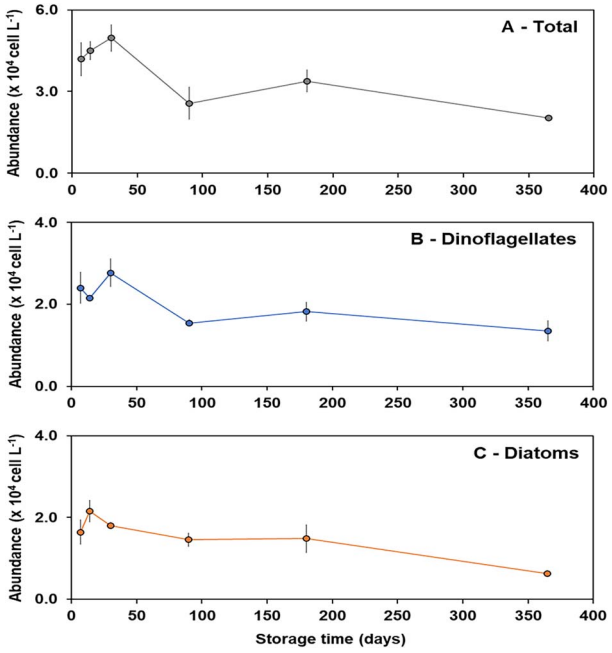

Fig. 2. Mean abundance ($n = 3$) of total microphytoplankton (A), plastidic dinoflagellates (B) and diatoms (C) for increasing storage times (days), for samples fixed with acid Lugol's and analysed using inverted microscopy. The vertical lines represent standard deviation.

Effects of storage time on pico- and nanophytoplankton abundance—"frozen microscopy slides" protocol

For slides stored in the freezer (−20°C), abundances of cyanobacteria (Fig. 3E) and cryptophytes (Fig. 3F) did not show significant changes along the 1-year storage period (Table I). However, the abundances of both eukaryotic picophytoplankton (Fig. 3G; $F = 27.99$, $r^2 = 0.60$, $P < 0.001$) and other plastidic nanoflagellates (Fig. 3H; $F = 18.9$, $r^2 = 0.50$, $P < 0.001$) declined significantly along the storage period (Table I). For both groups, significant differences started after 3 days of slide storage (picophytoplankton: $P < 0.001$, $\omega^2 = 0.93$; nanoflagellates: $P < 0.001$, $\omega^2 = 0.95$), with reductions of ~27% and 43% at 7 days, and abundances stabilizing for storage periods longer than 180 and 90 days for eukaryotic picophytoplankton and plastidic nanoflagellates, respectively (Fig. 3G-H).

Effects of storage time on pico- and nanophytoplankton abundance—"refrigerated fixed samples" protocol

For glutaraldehyde fixed samples stored under refrigerated conditions (4°C), abundances of both cyanobacteria (Fig. 3A) and cryptophytes (Fig. 3B) did not change significantly along the 14-day storage period (Table I). However, under 4°C, the abundances of eukaryotic picophytoplankton (Fig. 3C; $F = 97.72$, $r^2 = 0.86$, $P < 0.001$) and other plastidic nanoflagellates (Fig. 3D; $F = 44.99$, r^2 0.74, $P < 0.001$) showed significant linear declining trends during the storage period (Table I). For both eukaryotic picophytoplankton and plastidic nanoflagellates (Fig. 3 C-D), abundances until 4 days of sample storage were

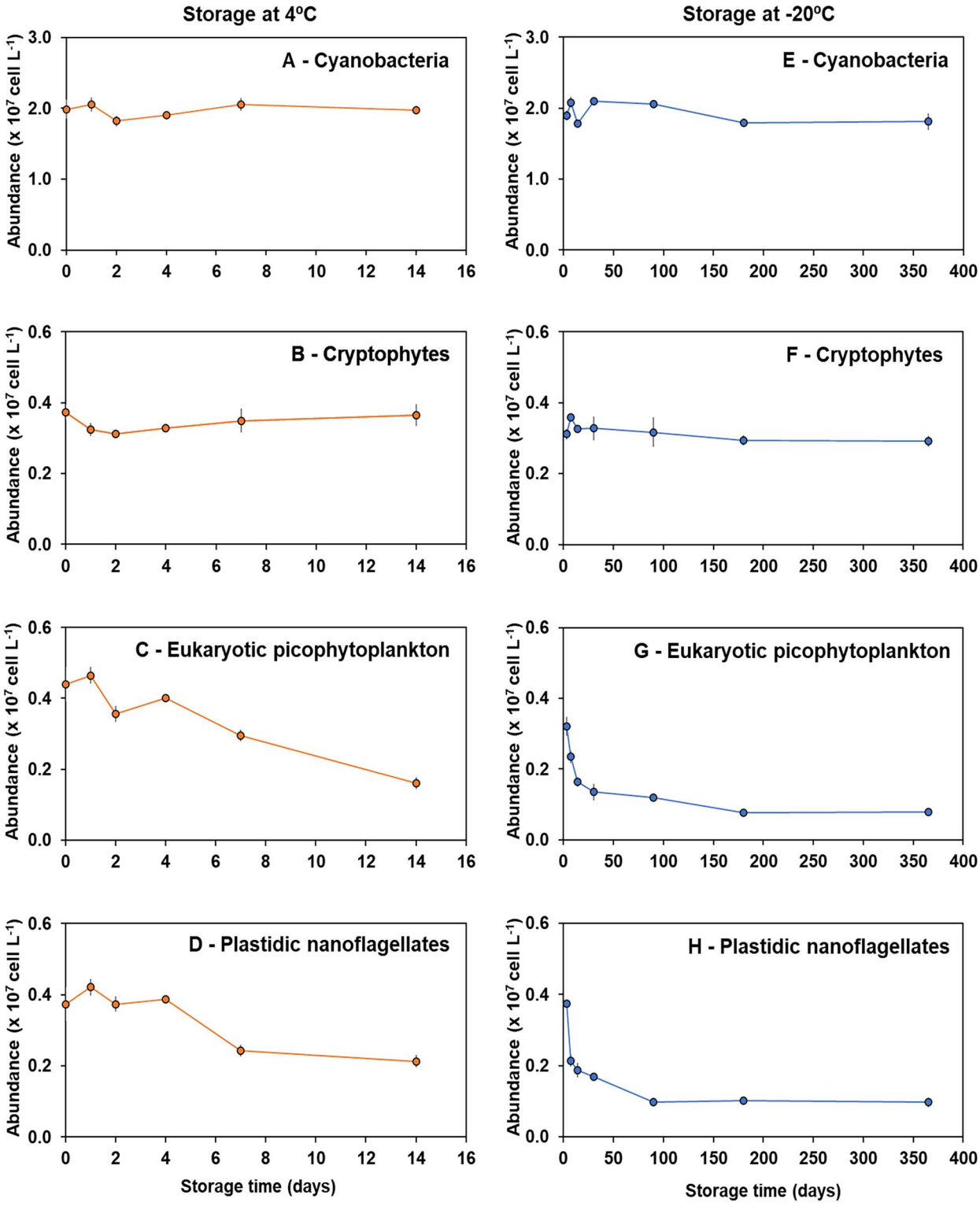

Fig. 3. Mean abundance ($n = 3$) of cyanobacteria (A, E), cryptophytes (B, F), eukaryotic picophytoplankton (C, G) and plastidic nanoflagellates (D, H) fixed with glutaraldehyde and analysed using epifluorescence microscopy for increasing storage time (days), following two different storage strategies: on the left panels are represented samples stored under refrigerated conditions (4ºC) and on the right panels are represented microscopy slides, prepared within 24 h of sample fixation, and stored under −20ºC. The vertical lines represent standard deviation.

Table I. One-way ANOVA output with *P*-values (P) and omega-squared values (ω^2) for the effects of storage time on the abundance of different phytoplankton size classes and groups, considering the different fixatives and storage protocols used.

Fixative	Size class	Groups	Storage protocol	Storage time	
				P	ω^2
Acid Lugol	Micro >20μm	Diatoms	15–20°C, dark	0.003	0.68
		Dinoflagellates	15–20°C, dark	0.003	0.66
		Total microphytoplankton	15–20°C, dark	<0.001	0.76
Glutaraldehyde	Nano 2–20μm	Plastidic nanoflagellates	4°C	<0.001	0.89
			−20°C	<0.001	0.95
		Cryptophytes	4°C	0.104	0.27
			−20°C	0.119	0.23
	Pico <2 μm	Cyanobacteria	4°C	0.157	0.21
			−20°C	0.001	0.67
		Eukaryotic picophytoplankton	4°C	<0.001	0.91
			-20°C	<0.001	0.93

similar, and decreased significantly thereafter (picophytoplankton: $P < 0.001$, $\omega^2 = 0.91$; nanoflagellates: $P < 0.001$, $\omega^2 = 0.89$).

Effects of storage protocol on pico- and nanophytoplankton abundance—"frozen microscopy slides" versus "refrigerated fixed samples"

The two different storage protocols, microscopy slides stored frozen (−20°C) versus glutaraldehyde-fixed samples stored refrigerated (4°C), were compared, for similar storage times (3–4, 7 and 14 days of storage). For cyanobacteria (Fig. 3A, E), cryptophytes (Fig. 3B, F), and other plastidic nanoflagellates (Fig. 3D, H), no significant differences were detected between storage protocols after 4, 7 and 14 days of storage. However, the abundance of eukaryotic picophytoplankton after 3 and 7 days of slide storage, at −20°C, was significantly lower than that observed in refrigerated samples, stored during periods up to 4 ($P < 0.006$) and 7 days ($P < 0.03$), respectively. No differences were detected between storage protocols for 14-days storage periods.

DISCUSSION

Our study showed that increased sample and/or slide storage time significantly decreased the estimates of abundance for all phytoplankton size classes, analyzed using standard fixation and microscopy protocols (Utermöhl, 1958; Haas, 1982). However, within phytoplankton size classes, the effects of storage time were group-specific. Apparent increases in phytoplankton abundance in fixed samples along the storage period, also detected in other studies (Sato *et al.*, 2006; Marie *et al.*, 2014; Williams *et al.*, 2016), were likely a result of inherent variability of the analytical methods used.

Effects of sample storage time on microphytoplankton abundance

Overall, the significant decreases in the abundance of diatoms, dinoflagellates and the whole microphytoplankton community in Lugol-fixed samples, during the 1-year storage period, agreed with previous studies using similar fixation and observation protocols (Hällfors *et al.*, 1979; Naik *et al.*, 2010; Williams *et al.*, 2016). Results suggest that cell degradation during long-term storage may represent a problem even if phytoplankton handbooks usually refer that well-preserved samples may be kept for several months or even years (Andersen and Throndsen, 2004). In fact, these results indicate that for more accurate abundance estimates, the storage time of samples fixed with acid Lugol's solution (at low concentrations, ~0.2%) should be under 30 days, for analysis of total microphytoplankton and dinoflagellates. For diatoms, storage times until 180 days could still provide accurate estimates of abundance.

Any recommendations with regard to maximum sample storage times should however consider the fixative used and its respective concentration, as well as phytoplankton species composition, since storage impacts may differ from species to species (Hällfors *et al.*, 1979; Mukherjee *et al.*, 2014; Williams *et al.*, 2016; Yang *et al.*, 2017). Hällfors et al. (1979), for example, reported a quicker decline in the abundance of weakly silicified diatoms in samples fixed with formaldehyde or neutral Lugol's, and relatively stable abundances over storage periods up to 1 year for samples fixed with acid Lugol's. Throndsen (1978), however, indicated that acid Lugol's solution could dissolve silica in long-term storage. Williams et al. (2016) reported no effects of storage time on the abundances of *Ditylum brightwellii*, *Karenia mikimotoi* and *Prorocentrum lima* in samples fixed with acid Lugol's, for periods up to 225 days. In contrast, for the coccolithophore *Coccolithus pelagicus*, a significant decline in abundance occurred after only 15 days of sample storage, for samples preserved with acid and neutral Lugol's solution (Williams *et al.*, 2016). Further, for the dinoflagellate *Karlodinium veneficum*, storage times shorter than 15 days were recommended for Lugol's fixed samples (Naik *et al.*, 2010). Other storage conditions, such as storage under refrigerated conditions (4–5°C), could influence cell long-term preservation; however, to the best of our knowledge studies that report how this will influence storage periods are still lacking. Improving sample fixation protocols may enable longer storage periods. For example, the combination of buffered glutaraldehyde and paraformaldehyde as fixative allowed storage times up to 50 days without significant declines in the abundance of the raphidophyte *Chattonella* spp. (Katano *et al.*, 2009).

Effects of sample and slide storage time, and storage protocol on pico- and nanophytoplankton abundance

The influence of storage time on the abundance of pico- and nanophytoplankton, for both fixed refrigerated samples and frozen microscopy slides, strongly varied between different functional groups. Abundances of cyanobacteria and cryptophytes remained stable along the tested storage periods, indicating that no relevant losses of autofluorescence or cell degradation occurred for glutaraldehyde fixed samples refrigerated at 4°C for 14 days, or slides frozen at −20°C during a storage period up to 1 year. This relative reduced response to storage contrasts with the loss of autofluorescence of nanoflagellates, reported for short storage periods (2 days) of glutaraldehyde fixed samples at ∼5°C (Bloem *et al.,* 1986). However, studies using flow cytometric analysis indicated no changes in the abundance of *Synechoccocus* and cryptophytes in glutaraldehyde fixed samples deep frozen into liquid nitrogen and stored at −80°C for periods up to 1 year (Marie *et al.,* 2014).

For eukaryotic picophytoplankton and other plastidic nanoflagellates, however, increased sample storage time was associated with severe declines in abundance for periods longer than 4 days, at 4°C. Rapid losses of eukaryotic picophytoplankton in samples fixed with glutaraldehyde were also reported after 1 week of storage at 5°C (Troussellier *et al.,* 1995). In our study, eukaryotic picophytoplankton and other plastidic nanoflagellates remained stable under refrigerated conditions longer than expected. Indeed, standard protocols recommend slide preparation within 24 h after sample collection and fixation, to avoid losses of autofluorescence and cell degradation (Bloem *et al.,* 1986; Havskum *et al.,* 2004; Domingues *et al.,* 2021).

Storage of frozen slides is the most common storage protocol when using epifluorescence microscopy, also reported to be appropriate for long-term storage (Booth, 1987; Havskum *et al.,* 2004). However, for microscopy slides stored at −20°C, the declining trends for both eukaryotic picophytoplankton and other plastidic nanoflagellates were exponential over the 1-year storage period. Reported effects of storage time on the abundances of pico- and nanophytoplankton for fixed samples stored frozen are quite variable. Some studies indicate no changes in the abundance of eukaryotic picophytoplankton during long-term storage (Hall, 1991; Troussellier *et al.,* 1995), whereas Pan et al. (2005) detected significant declines after a 3-month storage for both cyanobacteria and picoeukaryotes.

When compared with abundance estimates for Day 0 in refrigerated samples, the exponential decline of other plastidic nanoflagellates started after 3 days of slide storage, whereas the decline of eukaryotic picophytoplankton started even earlier. In addition to cell degradation along the slide storage period, the freezing process may damage the membrane filters used (e.g. formation of water crystals), and could make difficult the detection and counting of small weakly autofluorescent phytoplankton cells, such as eukaryotic picophytoplankton, in relation to larger cells with stronger autofluorescence, such as cyanobacteria and nanoflagellates. Based on our results, to get more accurate estimates of picoeukaryote abundance, glutaraldehyde fixed samples should be, ideally, kept refrigerated (4°C) during a storage period up to 4 days, and slides should be prepared immediately before observation, without prior freezing. Nevertheless, the microscopic analysis of phytoplankton samples within 4 days of sample collection and fixation is often not easy to achieve, namely if many samples need to be processed (e.g. oceanographic cruises, experiments). Therefore, whenever the samples cannot be analyzed in <4 days, slide freezing should be the alternative. Even if the two storage protocols provide similar abundance estimates until a 14-day storage period, slide freezing will enable a longer sample stability. Yet, it is important to underline that this storage protocol will inevitably produce less accurate abundance estimates for eukaryotic picophytoplankton and nanoflagellates other than cryptophytes, with reductions higher than 40% for storage periods equal or longer than 7 days. These patterns clearly contrast with some literature recommendations, which suggest that frozen slide preparations may be analyzed over the course of several years (Booth, 1987).

In face of the significant impacts of sample and/or slide storage on phytoplankton abundance and regardless of the technique and storage protocol used, the exact lag times between sample collection, fixation and analysis should always be indicated. Additionally, information on fixation protocol, and changes in sample analyst, external factors that may influence phytoplankton, should be also provided (Culverhouse *et al.,* 2003; Williams *et al.,* 2016; Muñiz *et al.,* 2020). This information would enable the comparability of phytoplankton data across different studies (Williams *et al.,* 2016).

CONCLUSIONS

Storage time negatively influenced the estimates of abundance in all phytoplankton size classes; however, within size classes the effect of increasing storage times was group-specific. For samples fixed with acid Lugol's solution, significant decreases in the abundance of diatoms, dinoflagellates and the total microphytoplankton community occurred during the 1-year storage period, indicating that cell degradation may represent a problem during long-term storage. It is suggested that for more accurate abundance estimates of the total microphytoplankton community and dinoflagellates, samples fixed with acid Lugol's should, ideally, be analyzed within 30 days, while for diatoms storage times until 180 days could still provide accurate estimates of abundance. The influence of storage time on the abundance of pico- and nanophytoplankton, for both glutaraldehyde fixed samples refrigerated at 4°C and frozen (−20°C) microscopy slides, varied between functional groups. Abundances of cyanobacteria and cryptophytes remained stable along the tested storage periods, despite the storage protocol used. However, abundances of eukaryotic picophytoplankton and other plastidic nanoflagellates suffered severe declines for storage periods longer than 4 days in refrigerated samples, and for frozen slides the declining trends were exponential over the 1-year storage period. Further, for short storage times, up to 7 days, storage of fixed samples under refrigerated conditions provided higher estimates of eukaryotic picophytoplankton abundance than frozen microscopy slides, stored during similar periods. Based on these results, to get more accurate estimates of picoeukaryote abundance, glutaraldehyde fixed samples should preferably be kept refrigerated (4°C) during a storage period

up to 4 days, and slides should be prepared immediately before observation, without prior freezing. However, if this timeline is not viable, slide freezing should be preferred, since it will allow a longer sample stability, even though it may provide less accurate abundance estimates for some functional groups. Considering the significant impacts of sample and/or slide storage on phytoplankton abundance and regardless of the technique and storage protocol used, it is highly recommended that authors indicate the exact lag times between sample collection, fixation and analysis, since this information will ensure the comparability of phytoplankton datasets across different studies and monitoring programs.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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