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# Comparison of freezing and pasteurization as long-term preservation methods for nutrient samples collected from inshore coastal waters

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**Abstract**
Within the framework of the EuroGO-SHIP Project, an experiment on the long-term preservation of nutrients in seawater samples was conducted in May 2023 using high-salinity, low-nutrient continental shelf waters from the Gulf of Trieste. Sample comparison included filtered and non-filtered samples, preserved by both freezing and pasteurization techniques. The results indicated that neither of these methods is individually ideal for the long-term (6- and 12-month) preservation of seawater samples, although freezing is less affected by experimental biases than pasteurization. Syringe filtration (0.22 $\mu$m pore size MCE filters) can cause the breakage of plankton cells and the release of nitrogen into the samples, whereas pasteurization can cause the remineralization of dissolved organic phosphorus and the release of phosphorus and silicate from marine particulate matter. Experimental results indicated that the best preservation method should be chosen depending on the biogeochemical characteristics of the marine system studied.

**Keywords**
Dissolved inorganic nutrients; Filtration; Pasteurization; Storage; Determination; Best practices

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## 1. Introduction

Studies on availability and ratios of dissolved inorganic macro-nutrients are of basic importance in understanding marine systems, particularly since the findings that their dynamics in seawater is modulated by the activity of marine biota (Richards, 1958; Redfield et al., 1963). There is a large volume of published literature for their manual or automated chemical analytical determination at both micro- and nano-molar concentrations, like those typically found in the world oceans, and these methods have been published over the last 70-years (Strickland and Parsons, 1972; Grasshoff et al., 1999; Crompton, 2006; Hydes et al., 2010; Becker et al., 2020; see references therein). In parallel, but to a much lesser extent, the methods for the preservation of nutrient samples have also been investigated, although it is widely recognized that wherever possible, the best laboratory practice is to analyze seawater samples at sea, and as shortly after they are collected during oceanographic field surveys as possible.

Freezing is commonly suggested as a method for long-term preservation (up to 2 years) of nutrient samples (Dore et al., 1996; Chapman and Mostert, 1990; Clementson and Wayte, 1992; Segura-Noguera et al., 2011). This method has been suggested to be improved by rapid freezing (Macdonald and McLaughlin, 1982) or cryogenic freezing (−80°C; Rho et al., 2022) procedures. Freezing can also be coupled with a preliminary filtration process of the seawater to stabilize the samples by removing any microbial biomass (Dore et al., 1996). The freezing method is simple, and it does not require the introduction of chemicals into the sample, hence avoiding problems of contamination or modification of the seawater matrix, although it needs the availability of low-temperature laboratory facilities for sample storage close to the point of sampling. The main point of debate about this method is a possible underestimation of dissolved silicate concentration, due to its polymerization when the samples are frozen and its incomplete redissolution when the samples are thawed before the analysis, especially when this nutrient is at high concentrations (Burton et al., 1970; MacDonald and McLaughlin, 1982; MacDonald et al., 1986; Sakamoto et al., 1990). For a quantitative recovery of silicate, it was recommended to thaw frozen samples overnight, in the

dark and at room temperature (Dore et al., 1996) or to defrost them in a heating bath (45 min., 50°C) and then cool them back to room temperature for a further 45 minutes before analysis (Becker et al., 2020; Rho et al., 2022).

Pasteurization (single heating) or more rarely tyndallization (multiple heating) have also been proposed as simple methods for the preservation of nutrient seawater samples, when the shipping of frozen packages is problematic (Aminot and Kérouel, 1997a, 1998; Daniel et al., 2012). The pasteurization technique (80°C, 2 hours; Daniel et al., 2012) was proposed as an improvement of the autoclaving method (120°C, 3 hours), which was used for the preparation of Reference Material for Nutrients in Seawater (RMNS) in early intercomparison laboratory experiments. In those experiments, autoclaving showed good stability for the nitrate and nitrite nutrients, but changes for the ammonium, phosphate, and silicate concentrations were observed, possibly due to the hydrolysis of organic matter, glass bottle leakage, or precipitation (Aminot and Kérouel, 1997b; Aoyama et al., 2007; Felgentreu et al., 2018). Despite these issues, pasteurization was previously used and applied to the preservation of natural seawater samples for nitrate and nitrite analyses (Aminot and Kérouel, 1998). In intercomparison experiments, it was mostly used to stabilize RMNS, which does not reflect the characteristics of natural seawater samples as it is prepared using aged low-nutrient seawater, filtered, pH-adjusted, and then spiked with nutrient standard solutions (Daniel et al., 2012).

Other methods used to stabilize nutrient samples involve their spiking with chemicals or poisoning compounds: acidification (Kotlash and Chessman, 1998), alkalinization (Wong et al., 2017), poisoning with chloroform (Gardolinsky et al., 2001), and poisoning with Mercury (II) Chloride (Kirkwood, 1992; Kattner, 1999; Kim et al., 2025). However, these methods are usually not recommended because of the possible contamination of the samples, and the results are not reliable for all the nutrient species. There are environmental hazards with using Mercuric Chloride, and the modifications induced in the seawater matrix and the need to restore the original pH of the samples prior to laboratory analyses when using acidification or alkalinisation (Grasshoff et al., 1999; Becker et al., 2020; Kim et al., 2025).

In this study, freezing (FR) and pasteurization (PA) were reconsidered for comparison as methods for long-term (up to up to one year) preservation of seawater samples for the determination of the nutrients collected in continental shelf waters (Gulf of Trieste; Northern Adriatic Sea). Despite the availability of several scientific studies on these two preservation methods, which are probably the most widely used globally for nutrient preservation, there have been no actual direct comparison experiments carried out previously. This experiment was specifically addressed to evaluate the performance of these methods with natural seawater samples that have different salinity, nutrient concentration, and organic matter availability compared to oceanic waters and laboratory-produced reference materials (Daniel et al., 2012, Aminot and Keruel, 1998, Aoyama et al., 2007) that have been mostly used to date. Moreover, experimental biases due to the filtration of the samples and possible interferences of the natural marine particulate matter were also analyzed as factors that can alter the performance of FR and PA methods.

Preservation methods of natural nutrient samples should be chosen depending on the major characteristics of the marine ecosystems: trophic level, nutrient availability, and adaptation of plankton communities to high or low ambient temperatures (Becker et al., 2020; Kim et al., 2025). This study provided more specific insights into their real applicability in field oceanographic surveys carried out in temperate coastal zones.

A recent revision of the best practices for nutrient analysis in marine systems was carried out in the framework of the International SCOR working group #147 (Towards comparability of global nutrient data (COMPONUT); https://scor-int.org/group/147/), which provided an updated GO-SHIP repeat hydrography nutrient manual (The Global Ocean Ship-based Hydrographic Investigations Program GO-SHIP; http://www.go-ship.org/) that covers all the aspects of nutrient analysis, from sampling to storage and determination (Hydes et al., 2010; Becker et al., 2020). As a part of the EuroGO-SHIP Project (HORIZON EUROPE no. 101094690; https://eurogo-ship.eu/), the applicability of these preservation methods in the specific conditions encountered in European Coastal Zones has been further assessed through pilot activities (EuroGO-SHIP Deliverables 3.3, 3.4, and 3.5) that include the present study.

## 2. Material and methods

### 2.1 Field activity and sampling

Nutrient (nitrate, $NO_3^-$; ammonium, $NH_4^+$; nitrite, $NO_2^-$; dissolved reactive phosphorus, $PO_4^{3-}$; and silicic acid $Si(OH)_4$) samples were collected between 9 and 30 May 2023 at six stations in the Gulf of Trieste (GoT), as part of the coastal water monitoring of the Environmental Protection Agency of Friuli Venezia Giulia Region (ARPA FVG; https://www.arpa.fvg.it/), using the cabin cruiser 'Folaga'. Sampling stations were located at different sites in the GoT (Figure 1), which are variably affected by atmospheric forcings and by the circulation of fresher waters originating from the discharges of the main rivers in the area (Cozzi et al., 2020).

Weather and marine conditions at the time of the sampling were assessed by visual observations and using meteorological probes mounted on the boat. Measured parameters included: air temperature (°C), humidity (%), cloud cover (number of eighths), wind speed and direction ($m\,s^{-1}$; degrees North), and wave height and direction (cm; degrees North).

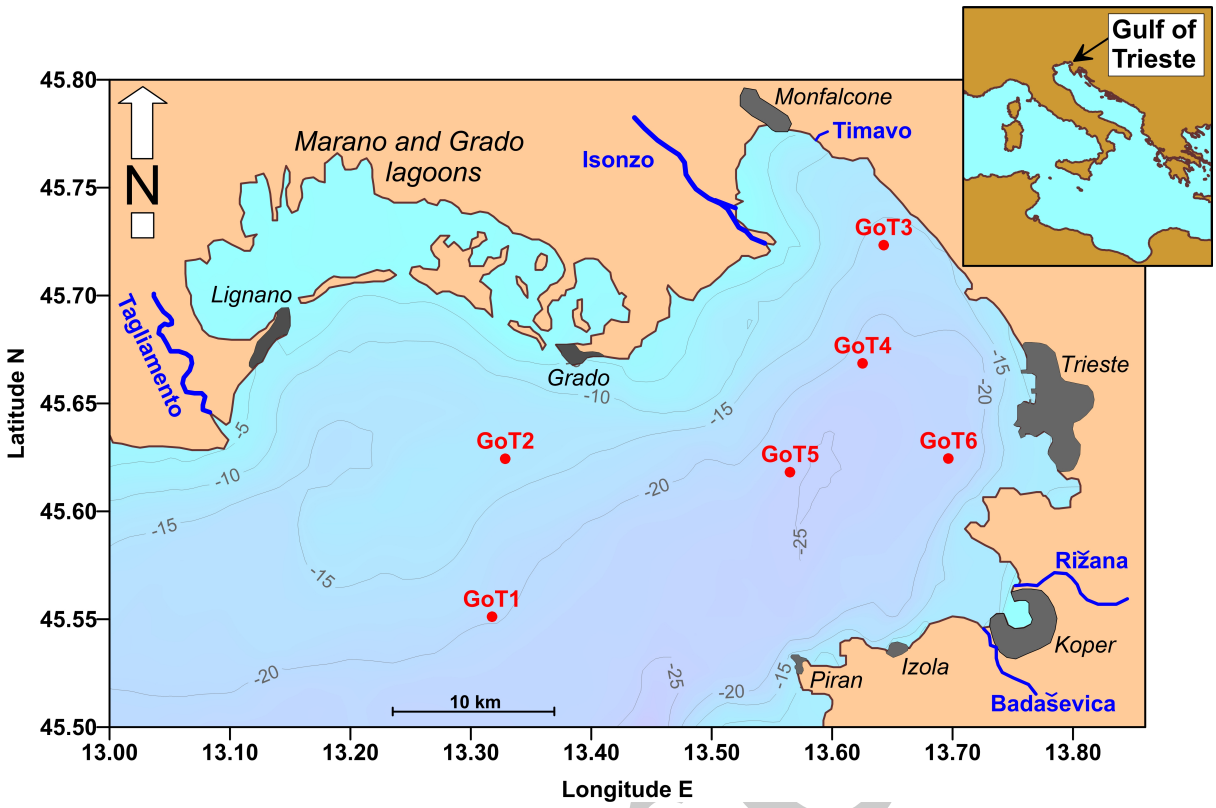


**Figure 1.** Sampling stations (GoT1 to GoT6) in the Gulf of Trieste, Italy. The bathymetry (m) of this coastal zone, the main urban settlements (grey), and river courses (blue) are also shown.

The sampling stations ranged in depth from 15.5 to 25.4 m (Table 1). CTD profiles were acquired at each station using an IDRONAUT CTD system equipped with pressure (dbar), temperature (°C), conductivity (mS $cm^{-1}$), dissolved oxygen concentration (DO; mg $l^{-1}$), pH, photosynthetic active radiation (PAR; %), chlorophyll *a* (Chl *a*; $\mu g\ l^{-1}$), and turbidity (Turb.; Nephelometric Turbidity Units) sensors. Practical salinity (Sal.) and the saturation of dissolved oxygen in seawater ($DO_{sat}$; %) were calculated according to the UNESCO standard EOS-80.

**Table 1.** Timing of field activity and position of the sampling stations in the Gulf of Trieste (May 2023).

| Station | Date (dd–mmm–yyyy) | Start time UTC (hh:mm) | End time UTC (hh:mm) | Long. E [dec] | Lat. N [dec] | Bottom depth [m] | Sampling depths [m] |
|---|---|---|---|---|---|---|---|
| GoT1 | 09-May-2023 | 10:15 | 10:30 | 13.3176 | 45.5511 | 21.0 | 1.0<br>18.5 |
| GoT2 | 09-May-2023 | 11:00 | 11:10 | 13.3286 | 45.6244 | 15.5 | 1.0<br>12.5 |
| GoT3 | 25-May-2023 | 07:00 | 07:50 | 13.6429 | 45.7234 | 15.7 | 1.0<br>13.5 |
| GoT4 | 25-May-2023 | 08:10 | 08:22 | 13.6253 | 45.6686 | 22.7 | 1.0<br>20.5 |
| GoT5 | 30-May-2023 | 08:32 | 09:18 | 13.5651 | 45.6182 | 25.4 | 1.0<br>23.5 |
| GoT6 | 30-May-2023 | 11:25 | 11:42 | 13.6966 | 45.6244 | 22.5 | 1.0<br>20.0 |

Water samples were collected using 5 litre horizontal NISKIN sampling bottles taken at 1 m below the sea surface and 2 m above the bottom. It is important to note that the collection of all subsamples that are needed to replicate nutrient analyses cannot be carried out on board when the operations at sea are performed with small boats. For this reason, the seawater was drawn from the NISKIN bottle and placed in 5-litre HDPE tanks, previously washed and cleaned with laboratory detergent, HCl 10%, and then zero-grade laboratory water (MILLIPORE Direct 8 system; Merck Inc.; https://www.merckmillipore.com/). The tanks with the sample waters were then kept refrigerated and in the dark until the collection of all subsamples, which was carried out over the course of 12 hours in the laboratories of CNR ISMAR Trieste.

## 2.2 Laboratory methods and analysis

For this experiment, nutrient samples were divided into four groups: filtered (f), non-filtered (nf), and for storage after pasteurization (PA) and freezing (FR). In the laboratory, the tanks with the sample waters were gently shaken to homogenize the samples. The subsamples for nutrient analysis were drawn from these tanks using a 60 ml clean plastic syringe with a 3-way automatic valve and with a 47 mm plastic filter holder in the case of the collection of the f-samples. For the filtration, Mixed Cellulose Esters (MCE) membrane filters 0.22 $\mu$m pore size were used (Fisherbrand, Cod. 16202772; https://www.fishersci.co.uk/). The subsamples were collected both in 125 ml-HDPE bottles and in 30 mL-borosilicate glass vials with Teflon caps, previously washed with (1) laboratory detergent, (2) HCl 10%, and (3) zero-grade laboratory water (MILLIPORE Direct 8 system). HDPE bottles were used for the analysis of all inorganic nutrients ($NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, and $PO_4^{3-}$), whereas the borosilicate vials were used to replicate the analysis of $NH_4^+$ in PA samples (as according to Daniel et al., 2012), to check for possible contamination of the samples by the plastic bottles during their pasteurization and storage at room temperature.

The freezing of subsamples was performed immediately at −28°C using a scientific freezer. The efficiency of the freezer can be considered constant as its auto-defrost function was turned off (Rho et al., 2022). The pasteurization was performed at 80°C for 2 hours (Daniel et al., 2012) and, afterwards, the subsamples were cooled at room temperature, sealed in Polyethylene (PE) bags, and stored in the dark. The subsamples were collected in sufficient numbers to perform the analyses at the beginning of the experiment (month 0) and after a medium (month 6) and long (month 12) term storage periods (Table 2). All subsamples were collected in triplicate. Overall, 504 subsamples (432 HDPE bottles, 72 borosilicate bottles) were collected from 12 tanks (i.e., 6 sampling stations, 2 depths for each station).

The nutrient concentrations fixed as initial values in this experiment (month 0) were those of the seawater in the tanks just before being split into the subsamples in the laboratory, as until this step of the experiment, each group of subsamples drawn from the same tank was homogeneous. Moreover, considering that immediate nutrient analyses were not possible in concomitance with the subsampling procedure, the subsamples for the determination of the initial concentrations of the nutrients were also quickly frozen and analyzed within one week after their collection. This procedure was preferred to exclude any fast alteration (from hours to a few days) in nutrient concentration in month 0 subsamples due to a possible interior biological activity, as they both contained (unfiltered) and not contained (filtered) living particulates (Becker et al., 2020).

**Table 2.** Replicates of the subsamples collected in each tank for nutrient analysis at the months 0, 6, and 12.

| Storage months | Filtration 0.22 $\mu$m | Bottles type | Preservation method | Replicates no. | Analyses |
|---|---|---|---|---|---|
| 0 | Yes | 125 ml-HDPE | – | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 0 | No | 125 ml-HDPE | – | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 6 | Yes | 125 ml-HDPE | Freezing | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 6 | Yes | 125 ml-HDPE | Pasteurization | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 6 | Yes | 30 ml glass | Pasteurization | 3 | $NH_{4+}$ |
| 6 | No | 125 ml-HDPE | Freezing | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 6 | No | 125 ml-HDPE | Pasteurization | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 6 | No | 30 ml glass | Pasteurization | 3 | $NH_4^+$ |
| 12 | Yes | 125 ml-HDPE | Freezing | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 12 | Yes | 125 ml-HDPE | Pasteurization | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 12 | Yes | 30 ml glass | Pasteurization | 3 | $NO_3^-$ |
| 12 | No | 125 ml-HDPE | Freezing | 3 | $NO_3^-$, $NO_2^-$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 12 | No | 125 ml-HDPE | Pasteurization | 3 | $NO_3^-$, $NO_{2-}$, $NH_4^+$, $Si(OH)_4$, $PO_4^{3-}$ |
| 12 | No | 30 ml glass | Pasteurization | 3 | $NH_4^+$ |

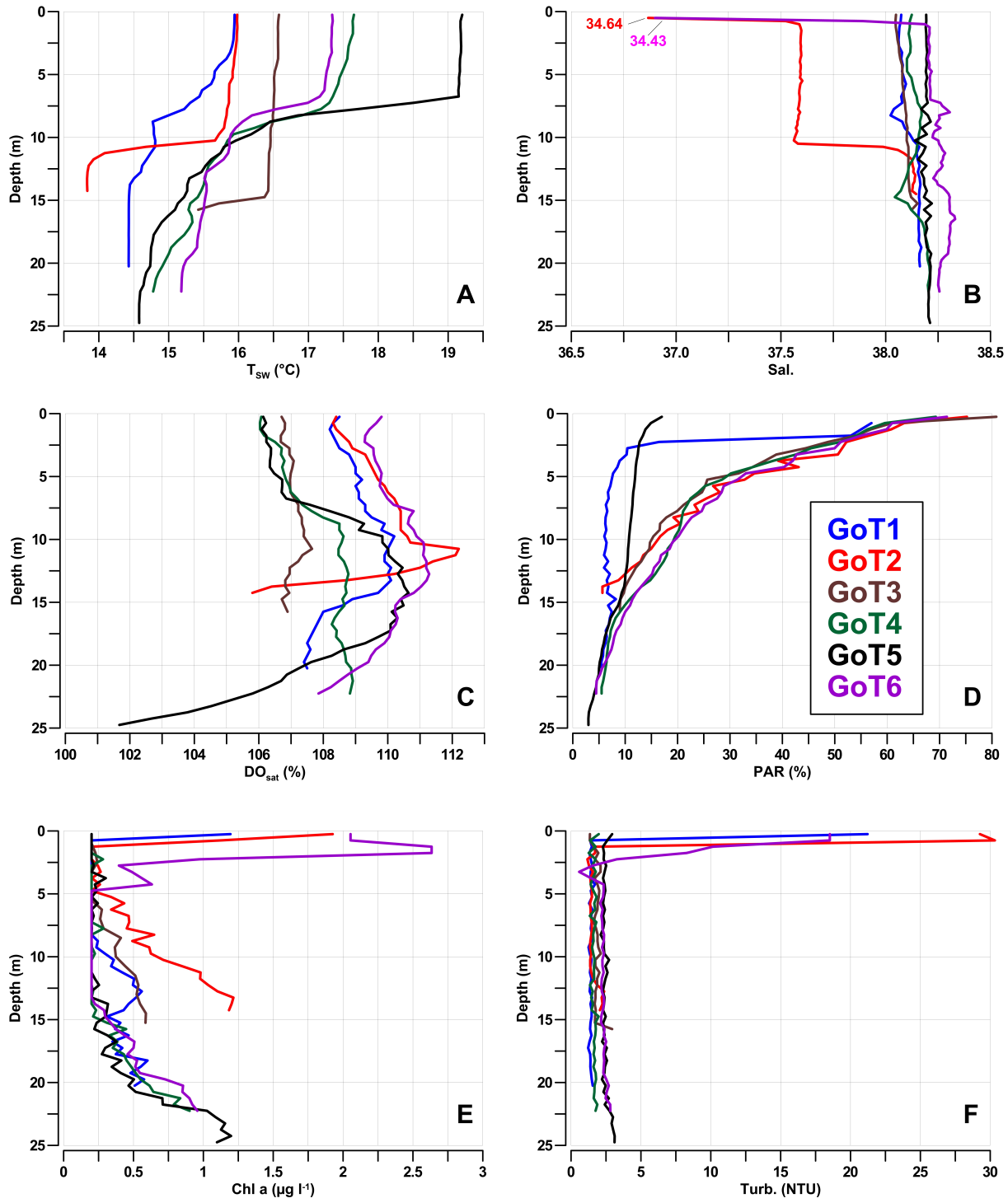


**Figure 2.** CTD profiles of seawater temperature ($T_{SW}$; °C), salinity (Sal.), dissolved oxygen saturation ($DO_{sat}$; %), photosynthetically active radiation (PAR; %), chlorophyll *a* (Chl *a*; $\mu g\ l^{-1}$), and turbidity (Turb.; NTU) in the sampling stations.

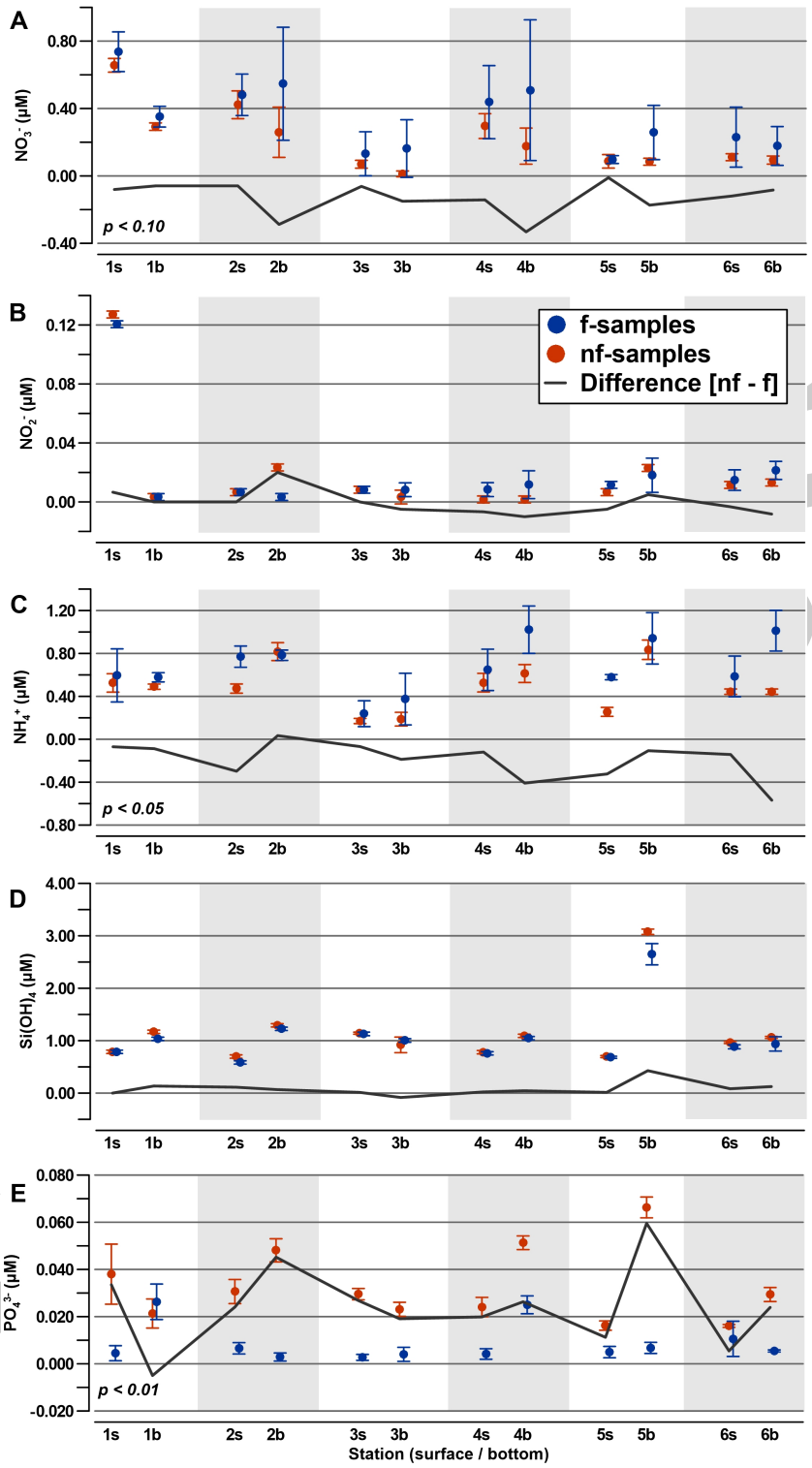


**Figure 3.** Initial (month 0) nutrient concentrations ($\mu$M) in non-filtered (nf; red) and filtered (f; blue) seawater samples collected at the surface (s) and bottom (b) in the stations GoT1–GoT6 (mean and standard deviation). The grey line indicates the difference between these two concentrations. The significance (p; U-Test) of the difference between f- and nf-samples is also shown.

Before laboratory analysis, FR samples were thawed overnight at room temperature and in the dark to avoid losses of $Si(OH)_4$ due to silica polymerization (Sakamoto et al., 1990). Silica polymerization can, however, be con-

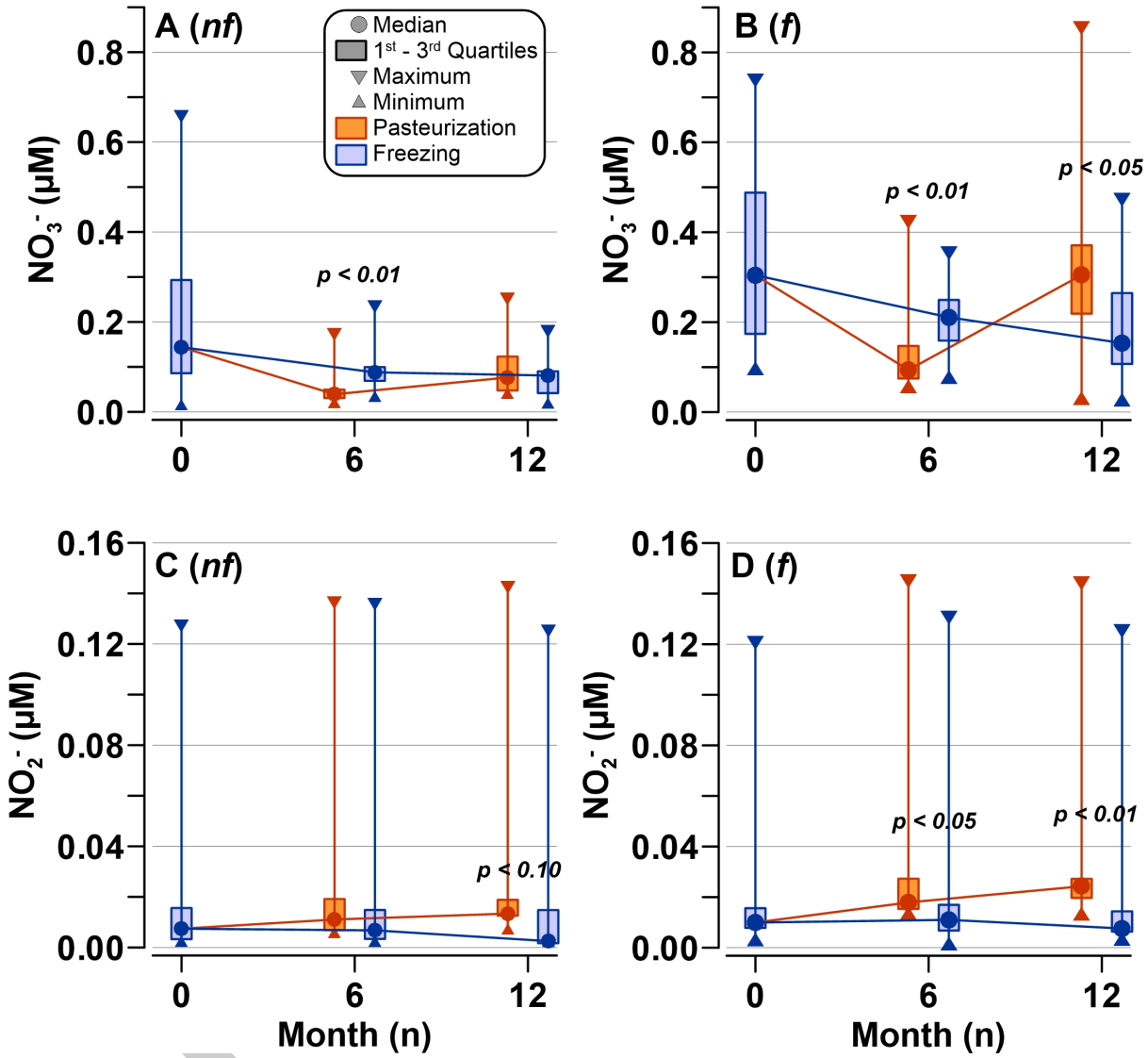


**Figure 4.** A box-and-whisker plot of the concentrations ($\mu$M) of $NO_3^-$ (A, B) and $NO_2^-$ (C, D) in surface and bottom waters of all the stations in the GoT after 0, 6, and 12 months of storage in f/nf and PA/FR samples. The significance (p; U-Test) of the difference between each couple of PA and FR groups of samples is also shown.

**Table 3.** Meteo-marine conditions during the operation at sea in the sampling stations.

| Station | T air [°] | Humidity [%] | Clouds (eighths) | Wind speed [m $s^{-1}$] | Wind direction [degree N] | Sea force (Beaufort scale) | Wave [cm] | Wave direction [degree N] |
|---|---|---|---|---|---|---|---|---|
| GoT1 | 20.0 | 72.0 | 2/8 | 4.5 | 50 | 2–3 | 30–40 | 50 |
| GoT2 | 21.0 | 67.0 | 2.0 | 3.5 | 30 | 2 | 30–40 | 50 |
| GoT3 | 22.3 | 56.2 | 1/8 | 6.9 | 70 | 2 | 50 | 100 |
| GoT4 | 21.6 | 65.0 | 1/8 | 6.8 | 45 | 2 | 50 | 60 |
| GoT5 | 19.8 | 70.7 | 4/8 | 3.9 | 70 | 2 | 40 | 70 |
| GoT6 | 24.2 | 47.9 | 4/8 | 5.4 | 65 | 2 | 40 | 65 |

**Table 4.** Physical-chemical characteristics of seawater at the depths of collection of nutrient samples. Temperature ($T_{SW}$; °), salinity (Sal.), dissolved oxygen saturation ($DO_{sat}$; %), photosynthetically active radiation (PAR; %), chlorophyll *a* (Chl *a*; $\mu$g $l^{-1}$), and turbidity (Turb.; NTU).

| Station | Depth [m] | $T_{SW}$ [°C] | Cond. [mS $cm^{-1}$] | Sal. | $DO_{sat}$ [%] | DO [mg $l^{-1}$] | pH unit | PAR [%] | Chl *a* [$\mu$g $l^{-1}$] | Turb. NTU |
|---|---|---|---|---|---|---|---|---|---|---|
| GoT1 | 1.0 | 15.94 | 47.25 | 38.07 | 108.3 | 8.47 | 8.17 | 66.5 | 0.20 | 1.38 |
| | 18.5 | 14.43 | 45.76 | 38.16 | 107.5 | 8.66 | 8.18 | 6.0 | 0.48 | 1.39 |
| GoT2 | 1.0 | 15.98 | 46.77 | 37.59 | 108.3 | 8.49 | 8.17 | 59.7 | 0.82 | 1.45 |
| | 12.5 | 13.84 | 45.10 | 38.13 | 110.6 | 9.02 | 8.16 | 10.6 | 1.10 | 2.09 |
| GoT3 | 1.0 | 16.57 | 47.90 | 38.05 | 106.68 | 8.25 | 8.08 | 58.7 | 0.20 | 1.74 |
| | 13.5 | 16.43 | 47.82 | 38.11 | 106.82 | 8.28 | 8.10 | 10.6 | 0.55 | 1.69 |
| GoT4 | 1.0 | 17.64 | 49.12 | 38.12 | 106.04 | 8.03 | 8.13 | 58.6 | 0.22 | 1.64 |
| | 20.5 | 14.88 | 46.29 | 38.21 | 108.80 | 8.69 | 8.14 | 6.1 | 0.61 | 1.58 |
| GoT5 | 1.0 | 19.18 | 50.88 | 38.19 | 106.22 | 7.81 | 8.09 | 14.0 | 0.21 | 2.47 |
| | 23.5 | 14.58 | 45.96 | 38.20 | 104.16 | 8.37 | 8.09 | 3.2 | 1.13 | 2.95 |
| GoT6 | 1.0 | 17.34 | 48.89 | 38.19 | 109.43 | 8.33 | 8.13 | 60.6 | 2.11 | 21.07 |
| | 20.0 | 15.23 | 46.72 | 38.26 | 109.22 | 8.66 | 8.14 | 5.9 | 0.84 | 2.50 |

sidered negligible in this experiment due to the low initial concentrations of this nutrient in the samples (0.6–3.5 $\mu$M). The determination of inorganic nutrients was carried out following standard colorimetric methods applied to automated Flow-Segmented Analyzers (Grasshoff et al., 1999; Hydes et al., 2010; Becker et al., 2020).

Nitrate, nitrite, ammonium, and silicate samples were analyzed using an OI-Analytical Flow-Segmented Autoanalyzer (Flow Solution III; https://www.ysi.com/oi-analytical). The determination of $NO_3^-$ + $NO_2^-$ was performed by the reduction of $NO_3^-$ to $NO_2^-$ in an Open Tubular Cadmium Reactor (OTCR) with a following diazotization of $NO_2^-$ with sulfanilamide and naphthylethylene-diamine to form an azo dye, with absorbance determined at $\lambda = 540$ nm. The determination of $NO_2^-$ was performed by the same method without the preliminary reduction of $NO_3^-$ in the OTCR. The concentration of nitrate was calculated by subtracting $NO_2^-$ from ($NO_3^-$ + $NO_2^-$). The determination of $NH_4^+$ was performed by an indophenol blue reaction, with absorbance measured at $\lambda = 640$ nm. The determination of $Si(OH)_4$ was performed by the molybdenum blue reaction at $\lambda = 660$ nm, suppressing the interference of any orthophosphate by the addition of oxalic acid.

The determination of ultra-low concentrations of $PO_4^{3-}$ in seawater samples ($< 0.138\ \mu$M) was performed by a manual method based on phospho-molybdenum complex reaction at pH <1, using a Varian Cary 50 Spectrophotometer equipped with a PCB-1500 Peltier cryothermostatic system (T = 37°C). To achieve the highest precision, the absorbance ($\lambda = 882$ nm) was measured in large cylindrical glass colorimetric cells with a light path of 100 mm.

For all the nutrients, the total system blank and the calibration of the analytical colorimetric reactions were determined in each run of subsamples by analysing, respectively, zero-grade laboratory water and artificial nutrient standards prepared in a 35 g/l NaCl solution. The precision of nutrient determinations was estimated by the recalculation of the concentrations of daily standards, applying daily calibration curves, and obtaining the following standard deviation values: $NO_3^- = 0.04\ \mu$M, $NO_2^- = 0.002\ \mu$M, $NH_4^+ = 0.10\ \mu$M, $Si(OH)_4 = 0.02\ \mu$M, and $PO_4^{3-} = 0.003\ \mu$M. The Limit of Detection (LOD) of these analytical methods was estimated by residual standard deviation of the linear regressions performed as daily calibration for $NO_3^- = 0.01\ \mu$M, $NO_2^- = 0.002\ \mu$M, $NH_4^+ = 0.05\ \mu$M, and $Si(OH)_4 = 0.01\ \mu$M. For $PO_4^{3-}$, it was estimated by the standard de-

viation of blank measurements (= 0.002 $\mu$M; Shrivastava and Gupta, 2011). In this experiment, the standards for the analytical procedure were prepared in artificial seawater, this salinity reflecting that of the samples to be analyzed. However, it should be noted that for laboratories that prepare their standards using distilled/zero-grade laboratory water, then that water should actually be made up as a 0.2 g $l^{-1}$ NaCl solution, which will stop potential ammonium absorption onto borosilicate glass surfaces (Kérouel and Aminot, 1997).

### 2.3 Data processing

For each subsample, nutrient concentration was expressed as the mean and standard deviation of three replicates. The coefficient of variation (CV; %) was also calculated as a normalized measure of the dispersion of concentration values of the replicates. The distribution of concentration data in each group of subsamples after storage is shown in Figures 4–7 as box-and-whisker plots, indicating the median, the quartiles, and the range of variation.

For each nutrient, the significance of the differences between each pair of filtered ($fPA_{t=i}$ vs. $fFR_{t=i}$, for i = 6 and 12 months) and non-filtered ($nfPA_{t=i}$ vs. $nfFR_{t=i}$, for i = 6 and 12 months) sample groups, preserved by both freezing and pasteurization, was assessed by the non-parametric Mann Whitney U-Test at three levels of probability: $p < 0.01$, $p < 0.05$ and $p < 0.10$ (Figures 4–6). The significance of the changes in nutrient concentration after 6 and 12 months of storage compared to their initial values was also assessed by Mann-Whitney U-Test and shown, together with other ancillary data and figures, in the Supplementary Materials (Table S2).

## 3. Results

### 3.1 Meteorological and hydrological conditions during the sampling

In May 2023, meteorological conditions in the GoT were characterized by highly variable winds and by the transit of several weather systems through the area. During the sampling, the air temperature was in the range 19.8–24.2°C, with partial cloud cover and winds blowing prevalently from the eastern sector. These conditions caused the presence of rather high waves from the east and south-east sectors (Table 3).

The hydrological structure of the water column was strongly influenced by these meteorological conditions. A wind-driven mixing of the upper surface layer of the water column generated a layer of constant water temperature down to 8–12 m of depth, and sometimes reaching the bottom, as in the case of the station GoT3 (Figure 2A). Offshore winds can cause the upwelling of deep shelf waters along the eastern coast of the GoT, increasing the salinity in the water column. This process was observed at most of the sampling stations, where salinity values ranged from 38.09 to 38.33 (Figure 2B). Low-salinity water (Sal. = 37.59) was found only at the station GoT2 down to a depth of 10 m, while at the stations GoT2 and GoT6, we observed thin layers of fresher waters at the surface (down to salinities of 34.64 and 34.43, respectively).

The water column was slightly oversaturated by Dissolved Oxygen (DO) at levels that, however, did not indicate the presence of large phytoplankton blooms (Figure 2C; 101–112%). At the same time, strong oxygen consumption in the bottom waters was not observed, indicating that heterotrophic conditions typical of the summer were not yet established in the Gulf. Chlorophyll *a* showed two peaks at the surface and in the deeper layer (Figure 2E), where photosynthesis is usually reduced by the scarce penetration of the light (Figure 2D). The turbidity was not high, except for the surface waters in the stations GoT1, GoT2, GoT6 (Figure 2F). Considering a median turbidity of these samples equal to 1.72 NTU, the Total Suspended Matter can be estimated ~ 5 mg $l^{-1}$.

Since the samples for nutrient analysis were collected at 1 m of depth and also at about 2 m above the sea floor, their physical-chemical characteristics can be defined as detailed in (Table 4). Almost all nutrient samples were characterized by high salinity, slight DO oversaturation, high pH, and low Chl *a* and Turbidity values. The main exception was the upper sample at the station GoT6, where higher values of Chl *a* and Turbidity were observed. These characteristics indicated that the sampling involved high salinity and clear shelf waters not affected by large phytoplankton blooms or by a direct runoff of riverine waters. Weather conditions and river discharges are becoming highly variable in the region of the GoT, because of the climatic changes, and they are already having observable impacts on the biogeochemistry of these coastal waters (Cozzi et al., 2020).

### 3.2 Nutrient analysis at the beginning of the experiment

The analysis of the nutrients at the beginning of the experiment was carried out on f- and nf-samples. The filtration of seawater samples is mandatory in turbid or eutrophic coastal waters directly affected by river discharges, as the natural particulate matter interferes with the measurement of the absorbance of the samples and rapidly degrades the performance of the analytical systems (Grasshoff et al.,1999; Becker et al., 2020). However, in this case, the coastal waters collected in the GoT were in oligotrophic conditions, and they were not affected by large river plumes (Table 4). For this reason, the analysis of nf-samples was carried out similarly to f-samples, without specific analytical problems.

Considering the homogeneity of the physical-chemical characteristics of the samples, systematic differences were not observed among sampling stations or between surface and bottom depths. However, the filtration had distinct effects on the nutrient concentrations (Figure 3). For

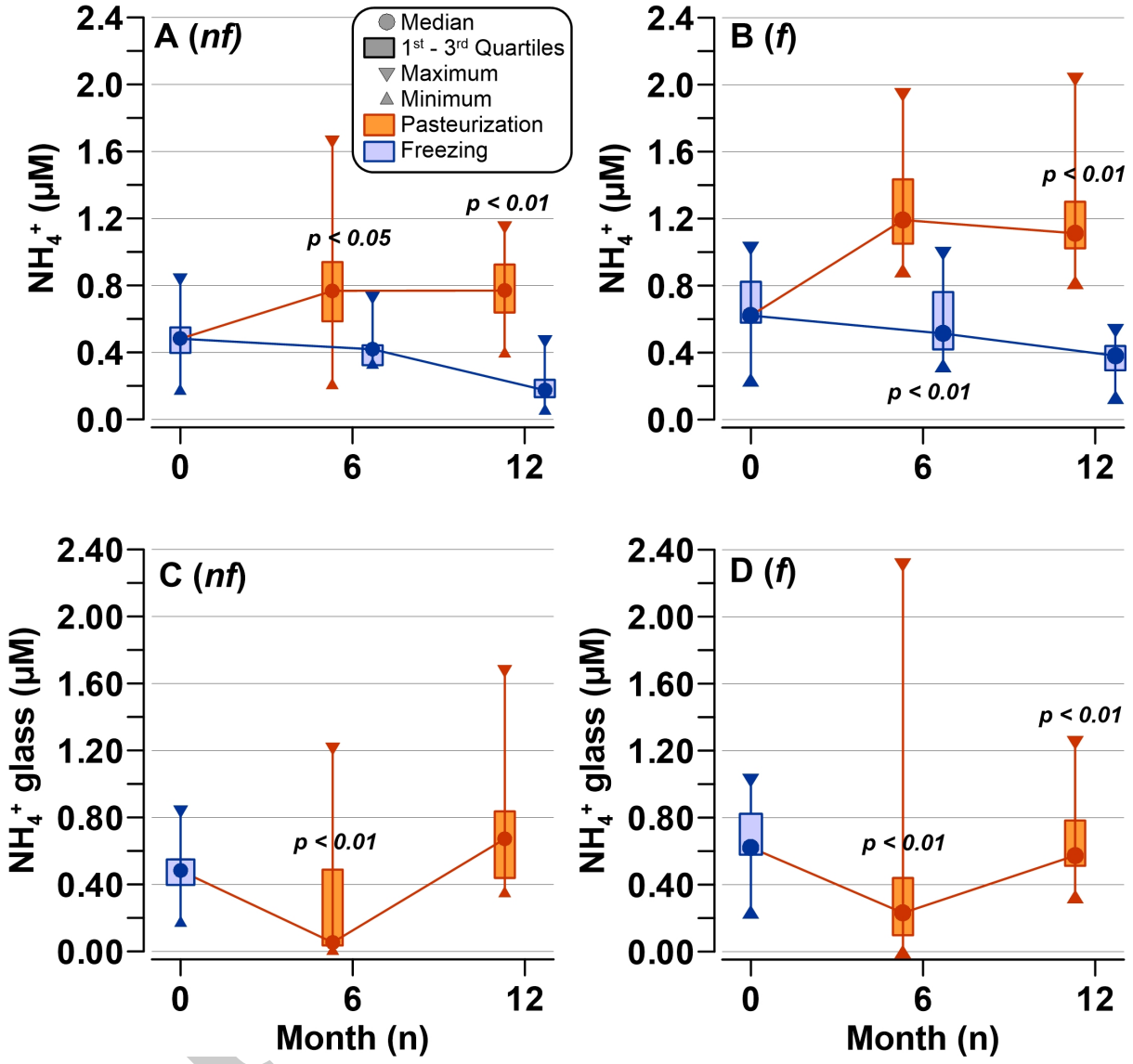


**Figure 5.** A box-and-whisker plot of the concentration ($\mu$M) of $NH_4^+$ in HDPE bottles (A, B) and glass borosilicate vials (C, D) in surface and bottom waters of all stations in the GoT after 0, 6, and 12 months of storage in f/nf and PA/FR samples. The significance (p; U-Test) of the difference between each couple of PA and FR groups of samples (A, B) and between the samples stored in HDPE bottles and glass borosilicate vials (C, D) is also shown.

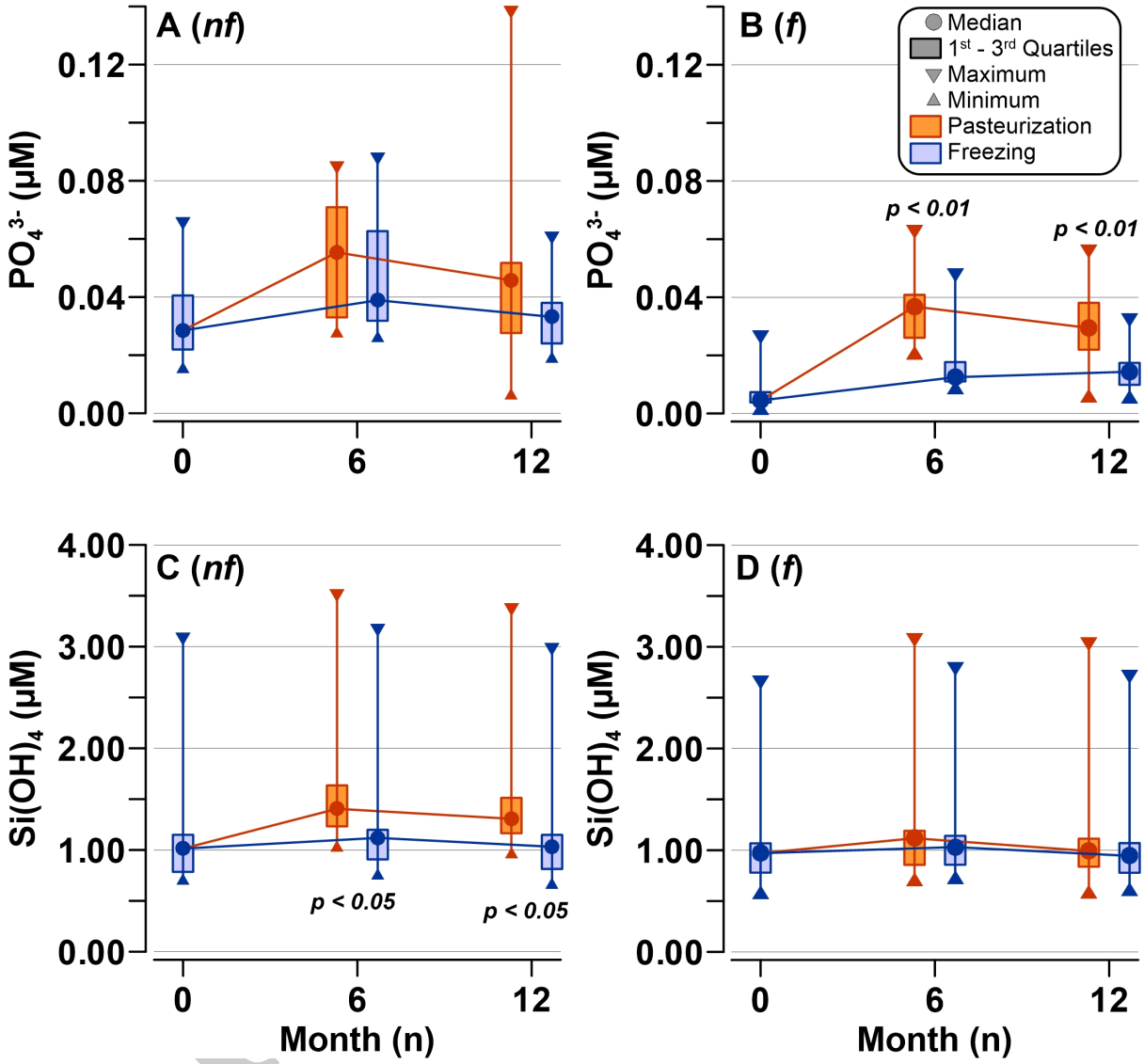


**Figure 6.** A box-and-whisker plot of the concentrations ($\mu$M) of $PO_4^{3-}$ (A, B) and $Si(OH)_4$ (C, D) in surface and bottom waters of all stations in the GoT after 0, 6, and 12 months of storage in f/nf and PA/FR samples. The significance (p; U-Test) of the difference between each couple of PA and FR groups of samples is also shown.

$NO_3^-$, nf-samples showed in several cases lower and more constant concentrations compared to f-samples, which resulted in an overall negative difference between these two groups of samples described by a $p < 0.10$ (Figure 3A). For $NO_2^-$, the two series of samples showed similar concentrations, with both positive and negative differences that, however, were not significant, being mostly close to the LOD of the method (Figure 3B). For $NH_4^+$, the difference between nf- and f-samples was negative, similar to the $NO_3^-$, but with a higher level of confidence ($p < 0.05$; Figure 3C). The concentration of $Si(OH)_4$ in nf-samples was higher than in f-samples only in one case, but the difference for all other samples was close to zero and not significant for the whole group of data (Figure 3D). The filtration had a large effect for $PO_4^{3-}$, which showed almost always higher concentrations in nf-samples compared to f-samples with a high level of confidence ($p < 0.01$; Figure 3E).

### 3.3 Nutrient preservation

Nutrient analysis after 6 and 12 months was performed on all four groups of the samples (f/nf and PA/FR), to assess the efficiency of the methods of preservation in the presence and absence of the natural marine particulates. The results are shown in Figures 4–6 and Tables S1 and S2 for statistical analyses. Considering the homogeneous characteristics of the samples at the initial time (Table 4), the data were primarily analyzed altogether, and the differences after 6 and 12 months were mainly ascribed to the effect of the storage. The variability of nutrient concentrations at each station, as well as in surface and bottom water samples, is shown in the Supplementary Materials, and they are discussed as necessary.

The concentration of $NO_3^-$ showed significant decreasing trends during the storage in FR samples, both for filtered and non-filtered samples (Figure 4A,B, Table S2). PA samples showed a decrease in concentrations after six months and an increase after twelve months, especially in the case of the f-samples (Figure 4B). This oscillation was the result of the combination of two opposite processes: a decrease of $NO_3^-$ concentration in the stations characterized by the highest $NO_3^-$ levels (GoT1, 2 and 4) and an increase of $NO_3^-$ levels after 12 months in the stations (GoT3, 5, and 6) that were almost depleted in $NO_3^-$ at the beginning of the experiment (Figures S1–S6). At months 6 and 12, the differences in $NO_3^-$ concentration in the FR and PA sample groups were often significant at the levels of $p < 0.01$ and $p < 0.05$ (Figure 4A,B). The dispersion of the data in each of these groups (i.e., quartiles and ranges of the values; Table S1) further indicated a frequent decrease in the variability of $NO_3^-$ concentrations from the beginning of the experiment, suggesting that possible partial homogenization of the samples can occur compared to the initial conditions during storage.

The concentration of $NO_2^-$ in most of the samples was very low and often close to the LOD of the analytical method, although some elevated values were present in each batch of the samples (Figure 4C,D). $NO_2^-$ concentration showed a slight decrease in FR samples and an increase in PA samples with the storage, but none of these temporal changes were significant compared to the initial concentrations at levels of $p < 0.10$ (Table S2). However, as a result of these opposite trends, PA samples showed significantly higher concentrations than FR samples, in particular after 12 months of storage (Figure 4C,D). The most constant $NO_2^-$ concentrations were observed in the case of nfFR samples (Figures S1–S6).

The concentration of $NH_4^+$ in HDPE bottles showed a significant decrease ($p < 0.01$) after 12 months of storage in FR samples (Table S2), with the only exceptions being the stations GoT3 and GoT5 at the surface, where the concentration of $NH_4^+$ was low at the beginning of the experiment (Figures S3, S5). By contrast, PA samples showed clear increases compared to the initial concentrations and compared to FR samples, both in f- and nf-samples, already after 6 months (Figure 5A,B). $NH_4^+$ samples stored in glass borosilicate vials showed oscillating concentrations after 6 and 12 months of storage, and values often significantly lower than those of PA samples stored in HDPE bottles (Figure 5C,D).

Ultra-low concentrations of $PO_4^{3-}$ were measured in all the samples collected in the GoT (0.002–0.138 $\mu$M). This condition allows the analysis of samples investigating the effects of sample preservation when the concentration of $PO_4^{3-}$ is lower than that of dissolved and particulate organic phosphorus. Frozen samples showed the best stability during storage, although some significant changes were observed with time (Table S2). It is also noticeable that nf-samples (Figure 6A) often had higher concentrations than f-samples (Figure 6B) for both FR and PA treatments, indicating a possible overestimation of $PO_4^{3-}$ concentration due to the presence of marine particulate phosphorus. Moreover, fPA samples also had concentrations significantly higher than fFR samples (Figure 6B), suggesting an additional increasing effect of $PO_4^{3-}$ concentration due to the pasteurization technique. The concentration of $Si(OH)_4$ was comparatively stable after storage for 6 and 12 months, as this nutrient was the least affected by problems of preservation, using both treatment methods (Figure 6C,D, Table S2). However, nfPA samples showed significantly higher concentrations than nfFR samples at months 6 and 12 ($p < 0.05$), suggesting a possible increase in dissolved silicates due to the dissolution of particulate silica during the pasteurization again.

The variations of the reproducibility of nutrient analysis were assessed by the Coefficient of Variation (CV; %) calculated for each triplicate sample, as CV represents a normalized standard deviation for each set of analytical results (Figures 7 and 8).

For $NO_3^-$, the values of CV at 6 months were similar to the initial values for fFR and nfFR samples, but they

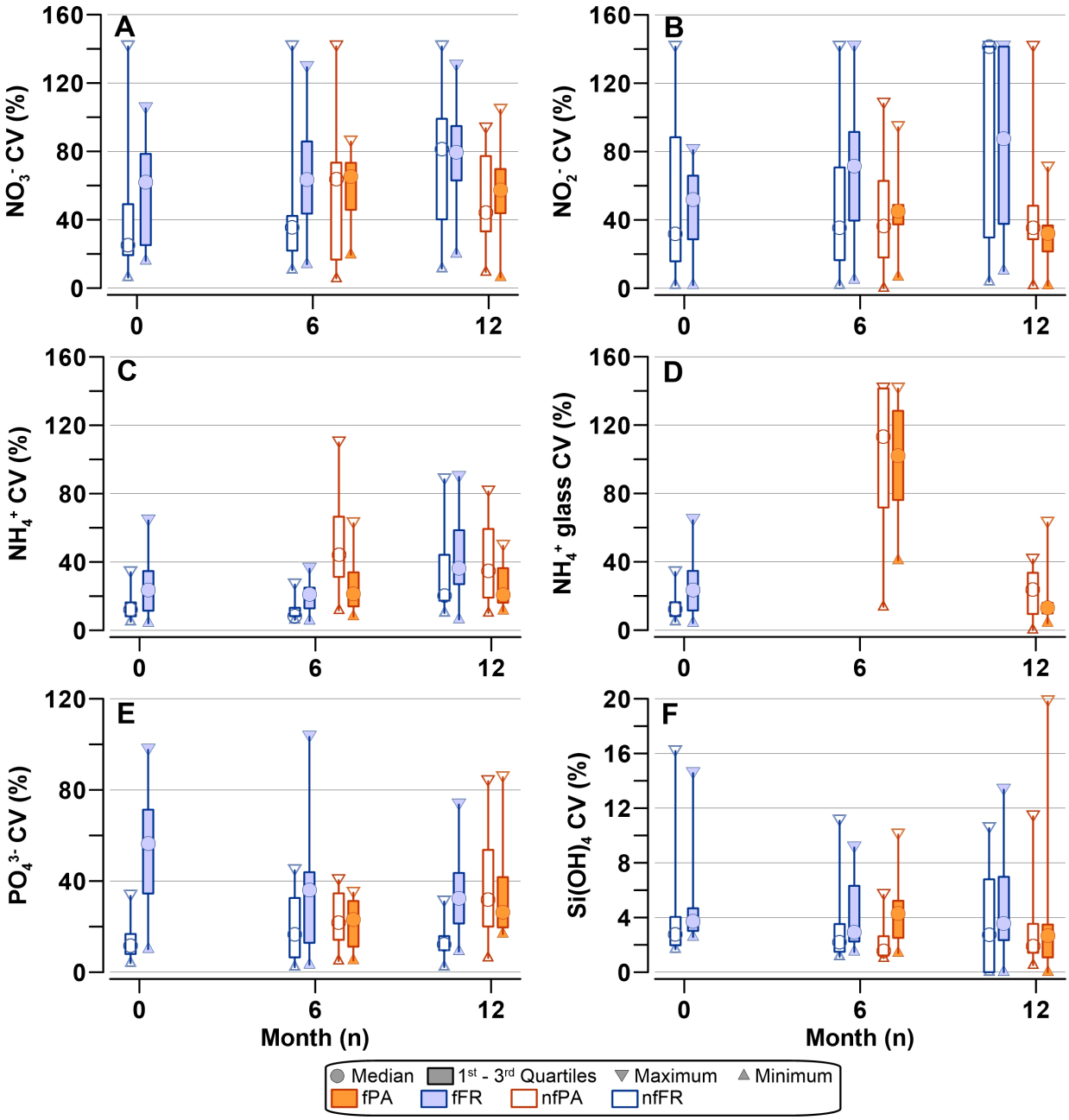


**Figure 7.** A box-and-whisker plot of the coefficients of variation (CV; %) for each triplicate nutrient sample across the four groups (nfPA, fPA, nfFR, fFR) after 0, 6, and 12 months. For $NH_4^+$, results are shown for both HDPE bottles (C) and borosilicate glass vials (D). Notice the different Y-scales in the plots of $PO_4^{3-}$ and $Si(OH)_4$.

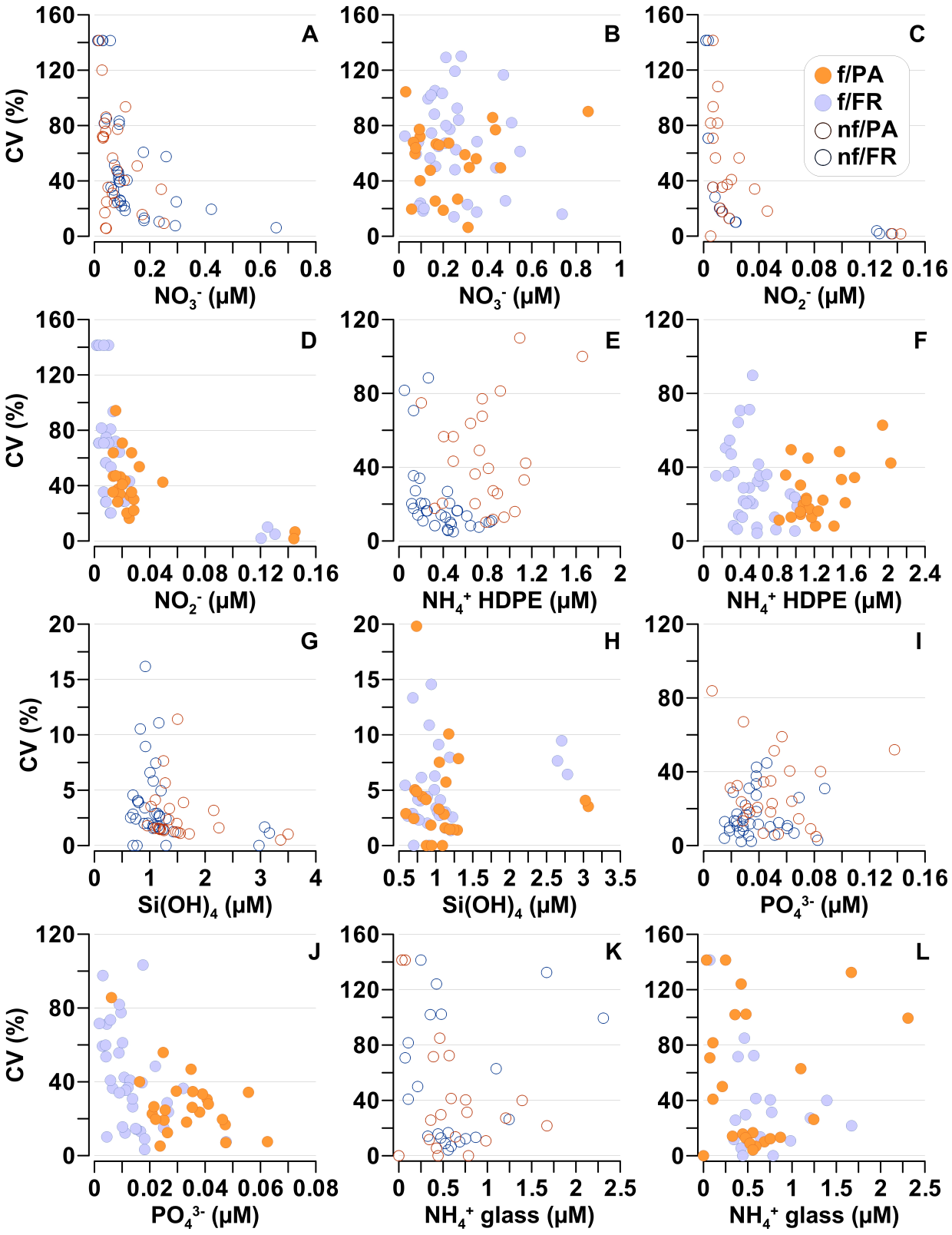


**Figure 8.** Coefficient of Variation (CV; %) vs the concentrations of the nutrients ($\mu$M) in f/nf and PA/FR samples. For $NH_4^+$, the plots show the results in HDPE bottles (E, F) and in borosilicate glass vials (K, J).

increased at 12 months. The nfPA samples showed an increase of CVs with storage, whereas fPA showed similar values over the whole period (Figure 7A). $NO_2^-$ showed trends similar to $NO_3^-$ with a worsening of the performance of the analysis in FR samples compared to PA samples at the 12-month sampling point (Figure 7B). However, this larger variability can be explained by the presence of several concentration values close to the LOD of the analytical method. The reproducibility of the analysis of $NH_4^+$ was rather constant in all groups of samples except for the fPA and nfPA samples stored in glass borosilicate bottles and analyzed after 6 months (Figure 7D). These two groups of data showed a higher variability that was not related to the presence of marine particulates and not confirmed in other groups of subsamples collected both in plastic and glass containers. For this reason, it was supposed that their poorer reproducibility might be mostly due to a random contamination of some subsamples rather than to systematic biases originating from the filtration or from the type of containers. For $PO_4^{3-}$, the lowest values of CV were obtained in nfFR samples, but also the other groups of samples showed a rather good reproducibility that is also favored by the high precision of the manual method of determination (Figure 7E). $Si(OH)_4$ showed the best results independently of the filtration and by the method of conservation of the samples, with CV often below 6% (Figure 7F).

The analysis of the relationship between the CV and the concentrations of the samples indicated that a primary factor influencing the reproducibility of these analytical methods is the initial level of the nutrient concentrations (Figure 8). An opposite relationship between CV and the concentration, following a non-exponential decay shape, was found in nf-samples for $NO_3^-$, $NO_2^-$, and $Si(OH)_4$ (Figure 8A,C,G), as well as in f-samples for $NO_2^-$, $Si(OH)_4$, and $PO_4^{3-}$ (Figure 8D,H,J). In these cases, CVs increased to 140% for N-nutrients, 100% for $PO_4^{3-}$, and 20% for $Si(OH)_4$ when the concentration levels of the nutrient were approaching the lowest values and the LOD of the analytical methods.

By contrast, variable results without a clear relationship between CV and concentration were found for $NO_3^-$ in f-samples (Figure 8B), for $NH_4^+$ in all the cases (Figure 8E,F,K,L), and for $PO_4^{3-}$ in nf-samples (Figure 8I). In these cases, it can be supposed that a variable release of $NO_3^-$ and $NH_4^+$ due to the cell breakage during the filtration and the hydrolysis of particulate phosphorus, as seen in Figure 3, might have contributed to an increase in the variability of the results.

## 4. Discussion

The water masses on the continental shelf of the Northern Adriatic are often affected by large discharges from the regional rivers, which strongly decrease the salinity and increase the concentration of N- and Si-nutrients (Cozzi and Giani, 2011; Grilli et al., 2020). Nutrient loads are mainly of anthropogenic origin, and they largely modulate the biological productivity of this marine region (Solidoro et al., 2009; Viaroli et al., 2018; Grilli et al., 2020), including the GoT (Cozzi et al., 2020). However, prolonged periods of atmospheric perturbations coupled with low runoff can enhance the circulation and mixing of shelf waters, leading to higher salinity values, scarce loadings of suspended matter, and low Chl *a* and nutrient concentrations, like those observed in this experiment (Table 4, Table S1). These hydrological conditions allowed the experiment to be conducted on low-nutrient seawater samples, where the weight of particulate and dissolved organic pools is, however, important compared to that of the dissolved inorganic nutrients.

### 4.1 Effects of marine particulate matter

The comparison between f- and nf-samples of seawater at month 0 indicated that the filtration could have distinct effects for each nutrient, since any other biological transformation can be excluded in these sub-samples thanks to their immediate freezing after collection (Figure 3). For $NO_3^-$ and $NH_4^+$, the higher and more variable concentrations measured in f-samples suggested a release of dissolved inorganic nitrogen in the samples that were syringe filtered before the analysis. This effect can be primarily caused by the breakage of phytoplankton cells on the filter and by the consequent release into the sample seawater of the interior cellular nitrogen pool. This finding stresses the importance of the utilization of low-pressure or low-vacuum filtrations to avoid cell breakage and sample contamination when membrane filters at low porosity are used for sample preparation (Becker et al., 2020). It was also observed that borosilicate glass microfiber filters have a higher loading capacity compared to membrane filters, which can make low-pressure filtrations easier (Lee et al., 1995). However, these filters do not have constant porosity (i.e., they cause an incomplete retention of picoplankton on the filter), and they must be carefully washed to avoid silicate contamination of the samples (Lee et al., 1995; Grasshoff et al., 1999).

For $PO_4^{3-}$, nf-samples had significantly higher concentrations than f-samples in almost all cases (Figure 3). This difference might originate from the dissolution and hydrolysis of labile particulate phosphorus to form $PO_4^{3-}$ during the analysis process, as the analytical method used for this nutrient (i.e., molybdenum blue reaction) includes the heating of the samples and the presence of strongly acidic conditions (Grasshoff et al., 1999). It was already observed that the concentration of dissolved reactive phosphorus can be overestimated in turbid and eutrophic estuarine waters because of the contribution of labile particulate phosphorus (Felgentreu et al., 2018). The present study shows that the effect of marine particulate matter is also not negligible in the high-salinity continental shelf waters

of the Gulf of Trieste, if they are characterized by low nutrient concentrations (Salvi et al., 1998; Lipizer et al., 2012).

### 4.2 Performance of sample preservation methods

The analyses of the preserved seawater samples after 6 and 12 months indicated that neither FR nor PA is an entirely suitable method for preventing statistically significant changes of nutrient concentration during the storage period, although FR showed less uncertainty compared to the PA method.

For FR samples, the concentrations of $NO_3^-$ and $NH_4^+$ frequently decreased during the storage, both in f- and nf-samples. This trend was mainly due to the decrease in concentrations of these nutrients in the samples that had the highest initial concentrations, a process that suggests an incomplete shutdown of microbial activity in the samples and that also leads to an underestimation of the real variability of these parameters in the coastal marine environment. PA samples showed a similar decrease in $NO_3^-$ concentration after 6 months, but after 12 months the concentrations increased again, particularly in filtered samples of some stations (GoT3, 5, and 6) that were initially depleted in $NO_3^-$. Considering that fPA samples do not contain marine particulate matter, the increase in $NO_3^-$ concentration after 12 months of storage could be mainly ascribed to changes in the composition of the dissolved nitrogen pool in the samples.

For $NO_2^-$, the temporal changes of the concentration were not always significant during sample storage (Table S2), and they were negligible in determining the overall N-content of these samples, due to the very low concentration levels of this nutrient. However, PA samples showed higher concentrations than FR samples after 12 months of storage (Figure 4C,D). Considering that HDPE bottles, when properly washed, are considered reliable for the pasteurization and storage of $NO_3^-$ and $NO_2^-$ seawater samples (Daniel et al., 2012; Becker et al., 2020), this difference could again mainly be ascribed to changes in the composition of the dissolved nitrogen pool in the samples.

$NH_4^+$ showed oscillations in the samples stored in glass borosilicate vials, whereas it showed a net increase in the concentrations in HDPE bottles (Figure 5A,B). This difference suggests an additional problem of contamination by $NH_4^+$ due to the plastic containers during PA treatment at high temperature. The overall poor performance of both the FR and PA methods for the preservation of N-nutrient samples can be explained by considering that microbial oxidation and reduction of dissolved inorganic nitrogen compounds easily occur in seawater and that coastal waters always contain a large quantity of dissolved organic nitrogen and urea, which remain in the samples also after the filtration (Cozzi et al., 2014). Urea can be easily hydrolyzed to $NH_4^+$, and organic nitrogen compounds can also be transformed into dissolved inorganic nitrogen during storage, due to microbiological and physical-chemical transformations of the samples.

The concentration of $PO_4^{3-}$ in high-salinity shelf waters of the Northern Adriatic Sea is often low, making this marine system frequently in a P-limited condition (N/P > 50; Ivančić et al., 2021). The samples analyzed in this experiment also showed nanomolar levels of $PO_4^{3-}$ close to the LOD of the analytical method. However, important observations regarding the variability of $PO_4^{3-}$ were as follows: (i) higher $PO_4^{3-}$ concentrations in nf-samples compared to f-samples at the beginning of the experiment (Figure 8 3E), and as well as after storage both in the FR and PA samples (Figure 8 6A, versus 6B), and (ii) significantly higher concentration levels in fPA samples with respect to fFR samples after storage (Figure 8 6B). These differences suggested an increase in $PO_4^{3-}$ concentration due to the dissolution and remineralization of the natural pool of particulate phosphorus in the nf-samples, which can occur during the laboratory analysis due to the high temperature and acidic conditions used in the $PO_4^{3-}$ determination method (Grasshoff et al., 1999). On the other hand, the highest concentrations observed in fPA samples compared to fFR samples (Figure 8 6B) further suggest that the pasteurization treatment at high temperature can also induce the hydrolysis of labile dissolved organic phosphorus and polyphosphates in the samples, increasing the $PO_4^{3-}$ concentrations (Diaz et al., 2018), thus leading to an overestimation of this nutrient concentration. For these reasons, the PA method should be applied preferentially to filtered samples and/or to samples where the concentration of dissolved organic phosphorus is negligible compared to that of $PO_4^{3-}$ (Liang et al., 2023; Garcia et al., 2023).

The best results in terms of preservation of the samples were obtained for $Si(OH)_4$, both in the case of FR and PA methods. This result is easily favored by the reliability of the colorimetric method for the determination of this nutrient and by concentration levels in the samples (0.58–3.50 $\mu$M Si) being significantly higher than the LOD. However, slightly higher concentrations were found in nfPA compared to nfFR samples at the 6 and 12 month sampling times (Figure 8C), suggesting a possible increase in the concentration of $Si(OH)_4$ due to the dissolution of biogenic silica occurring during the pasteurization. This aspect should be further studied considering that it is known that the solubility of biogenic silica is affected by changes in pH and temperature (Van Cappellen et al., 2012).

### 4.3 Reproducibility of nutrient analyses

Data presented in this study further indicate that the reproducibility of nutrient analysis depends on (i) the analytical method used for the determination of each nutrient, (ii) the initial level of concentration in the samples, and (iii) the treatment and preservation method used.

The determination of $Si(OH)_4$ with flow-segmented automatic colorimetry and a highly sensitive manual method

for the determination of $PO_4^{3-}$ provided the best results in terms of reproducibility, independent of the procedures used for preservation of the samples (Figure 7). N-nutrients gave more variable results depending on the presence/absence of the marine particulate matter in the samples and on the method of preservation

The inverse relationship between the CVs and the concentration observed in several cases for the analyses of $NO_3^-$, $NO_2^-$, $Si(OH)_4$, and $PO_4^{3-}$ (Figure 8) indicated that the precision of nutrient determination commonly decreases when the concentration of the nutrient approaches the LOD of the method (Grasshoff et al., 1999). On the other hand, the larger variability of CVs for $NO_3^-$ and $NH_4^+$ in f-samples independent of the concentrations seems to confirm the effect of a variable release of dissolved nitrogen due to the cell breakage during filtration, as already observed at the beginning of the experiment (Figure 3). Similarly, the large variability of CVs for $PO_4^{3-}$ in nf-samples can be induced by the hydrolysis of particulate phosphorus.

Sample storage also had some effects on the reproducibility of the analysis. For $NO_3^-$ and $NH_4^+$, CV values slightly increase after 12 months in both fFR and nfFR samples compared to the initial values. PA samples also showed CVs similar to FR samples, suggesting that neither of these two methods leads to significantly better results. $Si(OH)_4$ showed lower values of CV that are probably due to the higher reliability of the laboratory analytical methods for its determination (Figure 7).

## 5. Conclusions

This study has analyzed the performance of two of the most commonly used methods of preservation of nutrient samples during long-term storage. High-salinity low-nutrient seawater samples were considered instead of artificial batches of nutrient-spiked reference seawater that was already carried out in past experiments (Aminot and Kérouel, 1998; Daniel et al., 2012). Moreover, this experiment was carried out using coastal monitoring facilities, which often make the preservation and processing of nutrient samples more difficult compared to oceanic cruises, where the samples can be analyzed soon after sampling on board the research vessels. The analysis of nutrient-depleted coastal waters highlights potential interferences of the particulate matter and of organic compounds on nutrient determination that can often be masked by their high concentration levels in oceanic waters. Despite freezing being less affected by experimental biases than pasteurization, neither of these two methods is perfect for long-term preservation of natural seawater samples for all the nutrients studied. The data presented here indicate that the efficiency of preservation varies among nutrients and is also a function of the presence/absence of particulates in the samples. For this reason, we recommend that nutrients should be analyzed within a short time after the sampling as is possible (Becker et al., 2020). If laboratory analyses in real-time are not possible, nutrient samples should be processed and stored correctly as soon as possible to reduce interior biogeochemical transformations during their handling in the field.

The following points were highlighted in this experiment:

1. Frozen samples can show a progressive decrease in the concentrations of the nutrients during long-term storage, whereas those treated with the pasteurization process can show variations in the concentrations over time. In both cases, these trends suggest that the microbial activity in the samples is not completely stopped by the treatment of the samples.

2. Freezing and Pasteurization are both effective methods for the preservation of $Si(OH)_4$ samples and for nutrients with low concentrations (i.e., $NO_2^-$ and $PO_4^{3-}$), where absolute changes in concentration are less important. They appear to be less effective for $NO_3^-$ and $NH_4^+$, easily due to possible transformations of N-nutrients within the samples.

3. Syringe filtration with MCE membrane filters (0.22 $\mu$m pore size) can cause the breakage of plankton cells and cause the increase of $NO_3^-$ and $NH_4^+$ concentrations in filtered samples when compared to non-filtered samples, in particular if the filters begin to clog because of the accumulation of the marine suspended matter.

4. Higher concentrations of $PO_4^{3-}$ were found in non-filtered samples compared to filtered samples at the beginning of the experiment, suggesting that the dissolution of the natural P particulate can increase the concentrations of this nutrient due to the conditions of high temperature and low pH that are used for the laboratory analysis. For this reason, the filtration of nutrient samples should be included in the sampling protocols in $PO_4^{3-}$ depleted coastal waters.

5. In filtered samples, pasteurization increases the concentration of $PO_4^{3-}$ with respect to frozen samples due to possible remineralization of dissolved organic phosphorus, indicating that this treatment is not suitable for the preservation of seawater samples where the concentration of dissolved organic phosphorus is not negligible with respect to that of $PO_4^{3-}$.

6. In non-filtered samples, there is evidence that the pasteurization treatment at high temperature can increase the concentrations of $Si(OH)_4$ and $PO_4^{3-}$ in the samples because of the dissolution and remineralization of biogenic silicon and of particulate phosphorus, respectively. This suggests that the pasteurization should be preferentially applied to filtered samples.

7. The reproducibility of the methods of nutrient determination varies inversely with the concentration levels, but it could also be decreased because of the release of dissolved nitrogen and phosphorus from the particulate matter.

8. Borosilicate glass containers should be preferred to HDPE bottles to store $NH_4^+$ samples treated with the method of pasteurization.

9. This study shows the results from a specific coastal system (Gulf of Trieste), during a period characterized by the presence of high-salinity and low-nutrient shelf waters. Other coastal, shelf, and open-ocean waters may well exhibit different characteristics and outcomes when comparing freezing and pasteurization as storage methods for dissolved inorganic nutrients.

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## CRediT authorship contribution statement

Stefano Cozzi: Conceptualization, Investigation, Data curation, Methodology, Formal analysis, Writing – original draft. E. Malcolm S. Woodward: Conceptualization, Supervision, Methodology, Formal analysis, Validation, Writing – review and editing.

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## Conflict of interest

None declared.

## Supplementary material

Supplementary material associated with this article can be found here.

## References

Aminot, A., Kérouel, R., 1997a. *Assessment of heat treatment for nutrient preservation in seawater samples*. Anal. Chim. Acta 351(1–3), 299–309. https://doi.org/10.1016/S0003-2670(97)00366-8

Aminot, A., Kérouel, R., 1997b. *Reference material for nutrients for the QUASIMEME laboratory performance studies 1993–1996*. Mar. Pollut. Bull. 35, 78–83. https://doi.org/10.1016/S0025-326X(97)00125-2

Aminot, A., Kérouel, R., 1998. *Pasteurization as an alternative method for preservation of nitrate and nitrite in seawater samples*. Mar. Chem. 61, 203–208. https://doi.org/10.1016/S0304-4203(98)00021-8

Aoyama, M., Becker, S., Dai, M., Daimon, H., Gordon, L. I., Kasai, H., Kérouel, R., Kress, N., Masten, D., Murata, A., Nagai, N., Ogawa, H., Ota, H., Saito, H., Saito, K., Shimizu, T., Takano, H., Tsuda, A., Yokouchi, K., Youenou, A., 2007. *Recent Comparability of Oceanographic Nutrients Data: Results of a 2003 Intercomparison Exercise Using Reference Materials*. Anal. Sci. 23, 1151–1154. https://doi.org/10.2116/analsci.23.1151

Becker, S., Aoyama, M., Woodward, E.M.S., Bakker, K., Coverly, S., Mahaffey, C., Tanhua, T., 2020. *GO-SHIP Repeat Hydrography Nutrient Manual: The Precise and Accurate Determination of Dissolved Inorganic Nutrients in Seawater, Using Continuous Flow Analysis Methods*. Front. Mar. Sci. 7, 581790. https://doi.org/10.3389/fmars.2020.581790

Burton, J.D., Leatherland, T.M., Liss, P.S., 1970. *The reactivity of dissolved silicon in some natural waters*. Limnol. Oceanogr. 15, 472–476

Chapman, P., Mostert, S.A., 1990. *Does freezing of nutrient samples cause analytical errors?* S. Afr. J. Marine Sci. 9, 239–247. https://doi.org/10.2989/025776190784378763

Clementson, L.A., Wayte, S.E., 1992. *The effect of frozen storage of open-ocean seawater samples on the concentration of dissolved phosphate and nitrate*. Water Res. 26, 1171–1176. https://doi.org/10.1016/0043-1354(92)90177-6

Cozzi, S., Cabrini, M., Kralj, M., De Vittor, C., Celio, M., Giani, M., 2020. *Climatic and anthropogenic impacts on environmental conditions and phytoplankton community in the Gulf of Trieste (northern Adriatic Sea)*. Water 12, 2652. https://doi.org/10.3390/w12092652

Cozzi, S., Giani, M., 2011. *River water and nutrient discharges in the Northern Adriatic Sea: current importance and long term changes*. Cont. Shelf Res. 31, 1881–1893. https://doi.org/10.1016/j.csr.2011.08.010

Cozzi, S., Mistaro, A., Sparnocchia, S., Colugnati, L., Bajt, O., Toniatti, L., 2014. *Anthropogenic loads and biogeochemical role of urea in the Gulf of Trieste*. Sci. Total Environ. 493, 271–281.

https://doi.org/10.1016/j.scitotenv.2014.05.148

Crompton, T.R., 2006. *Analysis of seawater: a guide for the analytical and environmental chemist*. Springer-Verlag, Berlin and Heidelberg GmbH & Co., ISBN-10 3-540-26762-X.

Daniel, A., Kérouel, R., Aminot, A., 2012. *Pasteurization: A reliable method for preservation of nutrient in seawater samples for inter-laboratory and field applications*. Mar. Chem. 128-129, 57–63. https://doi.org/10.1016/j.marchem.2011.10.002

Diaz, J.M., Holland, A., Sanders, J.G., Bulski, K., Mollett, D., Chou, C.-W., Phillips, D., Tang, Y., Duhamel S., 2018. *Dissolved Organic Phosphorus utilization by phytoplankton reveals preferential degradation of Polyphosphates over Phosphomonoesters*. Front. Mar. Sci. 5, 380. https://doi.org/10.3389/fmars.2018.00380

Dore, J.E., Houlihan, T., Hebel, D.V., Tien, G., Tupas, L., Karl, D.M., 1996. *Freezing as a method of sample preservation for the analysis of dissolved inorganic nutrients in seawater*. Mar. Chem. 53, 173–185. https://doi.org/10.1016/0304-4203(96)00004-7

Felgentreu, L., Nausch, G., Bitschofsky, F., Nausch, M., Schulz-Bull, D., 2018. *Colorimetric chemical differentiation and detection of phosphorus in eutrophic and high particulate waters: advantages of a new monitoring approach*. Front. Mar. Sci. 5, 212. https://doi.org/10.3389/fmars.2018.00212

Garcia, H.E., Bouchard, C., Cross, S.L., Paver, C.R., Wang, Z., Reagan, J.R., Boyer, T.P., Locarnini, R.A., Mishonov, A.V., Baranova, O., Seidov, D., Dukhovskoy, D., 2024. *World Ocean Atlas 2023, Volume 4: Dissolved Inorganic Nutrients (phosphate, nitrate, silicate)*. A. Mishonov (Tech. Ed). NOAA Atlas NESDIS 92. https://doi.org/10.25923/39qw-7j08

Gardolinski, P.C.F.C, Hanrahan, G., Achterberg, E.P., Gledhill, M., Tappin, A.D., House, W.A., Worsfold, P.J., 2001. *Comparison of sample storage protocols for the determination of nutrients in natural waters*. Water Res. 35, 3670–3678. https://doi.org/10.1016/S0043-1354(01)00088-4

Grasshoff, K., Kremling, K., Ehrhardt, M., 1999. *Methods of seawater analysis*. 3rd edn. (completely revised and extended edition), Wiley-VCH Verlag GmbH, Weinheim. https://doi.org/10.1002/9783527613984

Grilli, F., Accoroni, S., Acri, F., Bernardi Aubry, F., Bergami, C., Cabrini, M., Campanelli, A., Giani, M., Guicciardi, S., Marini, M., Neri, F., Penna, A., Penna, P., Pugnetti, A., Ravaioli, M., Riminucci, F., Ricci, F., Totti, C., Viaroli, P., Cozzi, S., 2020. *Seasonal and interannual trends of oceanographic parameters over 40 years in the northern Adriatic Sea in relation to nutrient loadings from EMODnet Chemistry data portal*. Water 12, 2280. https://doi.org/10.3390/w12082280

Hydes, D.J., Aoyama, M., Aminot, A., Bakker, K., Becker, S., Coverly, S., Daniel, A., Dickson, A.G., Grosso, O., Kérouel, R., van Ooijen, J., Sato, K., Tanhua, T., Woodward, E.M.S., Zhang, J.Z., 2010. *Determination of dissolved nutrients (N, P, Si) in seawater with high precision and inter-comparability using gas-segmented continuous flow analysers. GO-SHIP Repeat Hydrography Manual: A collection of expert reports and guidelines*. IOCCP rep. no. 14, ICPO publ. ser. no. 134, Ver. 1.

Ivančić, I., Kraus, R., Najdek, M., Cozzi, S., 2021. *Ecological importance of alkaline phosphatase activity in changing marine environmental conditions*. Water 13, 2750. https://doi.org/10.3390/w13192750

Kattner, G., 1999. *Storage of dissolved inorganic nutrients in seawater: poisoning with mercuric chloride*. Mar. Chem. 67, 61–66. https://doi.org/10.1016/S0304-4203(99)00049-3

Kérouel, R., Aminot, A., 1997. *Fluorometric determination of ammonia in sea and estuarine waters by direct segmented flow analysis*. Mar. Chem. 57, 265–275. https://doi.org/10.1016/S0304-4203(97)00040-6

Kirkwood, D.S., 1992. *Stability of solutions of nutrient salts during storage*. Mar. Chem. 38, 151–164. https://doi.org/10.1016/0304-4203(92)90032-6

Kim, M.S., Choi, M.S., Rhee, T.S., 2025. *Comparative assessment of preservation methods for major nutrients in polar seawater*. Mar. Chem. 272, 104546. https://doi.org/10.1016/j.marchem.2025.104546

Kotlash, A.R., Chessman, B.C., 1998. *Effects of water sample preservation and storage on nitrogen and phosphorus determinations: implications for the use of automated sampling equipment*. Water Res. 32, 3731–3737. https://doi.org/10.1016/S0043-1354(98)00145-6

Lee, S., Kang, Y.-C., Fuhrman, J.A., 1995. *Imperfect retention of natural bacterioplankton cells by glass fiber filters*. Mar. Ecol. Prog. Ser. 119, 285–290. https://doi.org/10.3354/meps119285

Liang, Z., Letscher, R.T., Knapp, A.N., 2023. *Global patterns of surface ocean dissolved organic matter stoichiometry*. Glob. Biogeochem. Cycles 37, e2023GB007788. https://doi.org/10.1029/2023GB007788

Lipizer, M., De Vittor, C., Falconi, C., Comici, C., Tamberlich, F., Giani, M., 2012. *Effects of intense physical and biological forcing factors on CNP pools in coastal waters (Gulf of Trieste, Northern Adriatic Sea)*. Estuar. Coast. Shelf Sci. 115, 40–50. https://doi.org/10.1016/j.ecss.2012.03.024

Macdonald, R.W., McLaughlin, F.A., 1982. *The effect of storage by freezing on dissolved inorganic phosphate, nitrate, and reactive silicate for samples from coastal and estuarine waters*. Water Res. 16, 95–104. https://doi.org/10.1016/0043-1354(82)90058-6

Macdonald, R.W., McLaughlin, F.A., Wong, C.S., 1986. *The storage of reactive silicate samples by freezing*. Limnol. Oceanogr. 31, 1139–1142.

https://doi.org/10.4319/lo.1986.31.5.1139

Redfield, A.C., Ketchum, B., Richards, F.A., 1963. *The influence of organisms on the composition of seawater*. In: Hill, M. (Ed.), *The Sea*. Interscience, New York, 26–77.

Rho, T., Son, P., Choi, S.-H., Kang, D.-J., 2022. *Cryogenic freezing: A reliable preservation method of samples for seawater nutrient analysis*. Limnol. Oceanogr.: Methods 20, 543–552. https://doi.org/10.1002/lom3.10503

Richards, F.A., 1958. *Dissolved silicate and related properties of some western North Atlantic and Caribbean waters*. J. Mar. Res. 17, 449–465.

Sakamoto, C.M., Friederich, G.E., Codispoti, L.A., 1990. *MBARI procedures for automated nutrient analyses using a modified Alpkem Series 300 Rapid Flow Analyzer*. Monter. Bay Aquar. Res. Inst. Tech. Rep. 9:84.

Segura-Noguera, M., Cruzado, A., Blasco, D., 2011. *Nutrient preservation, analysis precision and quality control of an oceanographic database of inorganic nutrients, dissolved oxygen and chlorophyll a from the NW Mediterranean Sea*. Sci. Mar. 75, 321–339. https://doi.org/10.3989/scimar.2011.75n2321

Salvi, C., Melis, R., Celio, M., Faganeli, J., 1998. *Suspended matter in the Gulf of Trieste (northern Adriatic Sea) during the occurrence of macroaggregates in 1991*. Boll. Geofis. Teor. Appl. 39, 219–241.

Shrivastava, A., Gupta, V., 2011. *Methods for the determination of limit of detection and limit of quantitation of the analytical methods*. Chronicles Young Sci. 2, 1. https://doi.org/10.4103/2229-5186.79345

Solidoro, C., Bastianini, M., Bandelj, V., Codermatz, R., Cossarini, G., Melaku Canu, D., Ravagnan, E., Salon, S., Trevisani, S., 2009. *Current state, scales of variability, and trends of biogeochemical properties in the northern Adriatic Sea*. J. Geophys. Res. 114, C07S91. https://doi.org/10.1029/2008JC004838

Strickland, J.D.H., Parsons, T.R., 1972. *A Practical Handbook of Seawater Analysis*. Fish. Res. Board Canada Bull., Ottawa, 167 pp.

Van Cappellen, P., Dixit, S., van Beusekom, J., 2012. *Biogenic silica dissolution in the oceans: Reconciling experimental and field-based dissolution rates*. Glob. Biogeochem. Cy. 16(4), 1075. https://doi.org/10.1029/2001GB001431

Viaroli, P., Soana, E., Pecora, S., Laini, A., Naldi, M., Fano, E.A., Nizzoli, D., 2018. *Space and time variations of watershed N and P budgets and their relationships with reactive N and P loadings in a heavily impacted river basin (Po River, Northern Italy)*. Sci. Total Environ. 639, 1574–1587. https://doi.org/10.1016/j.scitotenv.2018.05.233

Wong, G.T.F., Li-Tzu Hou, L., Li, K.-Y., 2017. *Preservation of seawater samples for soluble reactive phosphate, nitrite, and nitrate plus nitrite analyses by the addition of sodium hydroxide*. Limnol. Oceanogr.: Methods 15, 320–327. https://doi.org/10.1002/lom3.10160