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Influence of the conservation mode of seawater for dissolved organic carbon analysis

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Abstract :

Marine dissolved organic matter (DOM) is one of the largest exchangeable organic carbon reservoir on the planet. The main proxy to track the distribution of DOM in the aquatic environments remains dissolved organic carbon (DOC). Thereby the optimal protocol for long-term DOC preservation in seawater samples must be defined. In this context, we monitored bulk DOC concentrations and its size class distribution in filtered seawater samples during yearlong experiments. With different conservation mode, we tested two types of commonly used materials (borosilicate brown glass and high-density polyethylene, HDPE) and three conditioning protocols (untreated, acidified at pH 2 and frozen at −20 °C). Offshore samples collected along the entire water column of the Pacific Ocean and stored in HDPE bottles were also analysed after 2 years of storage at pH of 2 and compared to frozen samples. Results demonstrated that bulk DOC concentrations can be accurately determined in untreated samples for one month and for years in frozen samples as well as in acidified samples, when samples are stored in acid cleaned HDPE bottles or flame sealed glass ampoules. Storage in brown glass vials with Bakelite caps seems more uncertain. The study of the size class distribution of DOC reveals the possibility to study DOM for 1 month in filtered samples with no additional treatment and for years in frozen samples when stored in acid cleaned HDPE bottles. Significant changes in DOC size fractionation were observed when samples were acidified. The high molecular weight (HMW) compounds and the humic substances from the upper 1000 m were significantly degraded at pH 2, incorporating DOC in the low molecular weight (LMW) fractions. These experiments provide preservation guidelines for future studies that aim either to study bulk DOC or the chemical properties marine DOM. It is recommended to store seawater in HDPE vials at −20 °C for DOM study, or at pH 2 for bulk DOC measurements.

Highlights

► HDPE bottles are the best containers for DOM preservation. ► Freezing (−20 °C) seawater is recommended for a study based on chemical properties of DOC. ► Acidification (pH 2) of seawater is only recommended for bulk DOC measurements.

Keywords : Dissolved organic carbon, Seawater analysis, DOM preservation, Size exclusion chromatography

1. Introduction

Dissolved organic carbon (DOC) is the largest organic carbon pool in the ocean (Hansell et al., 2009). In last decades, its distribution and composition have increasingly being measured in the marine environment notably to determine its sources and sinks. These measurements range from the global (quantification by concentrations, Carlson & Hansell, 2015; Hansell & Carlson, 1998) to the specific scale (molecular characterization, Hertkorn et al., 2013; Nebbioso & Piccolo, 2013). The quality of these different types of analysis can only be guaranteed by a sampling under perfectly clean conditions as well as an optimal preservation of the seawater samples. Therefore, a conditioning is a mandatory to avoid changes in composition or microbial ~~respiration~~ decomposition of DOC (Walker et al., 2017). Different preservation mode of the seawater samples were specified in oceanic studies for DOC analysis. After a filtration to remove the particulate material, the seawater is often stored in flame sealed pre-combusted glass ampoules (Dittmar, 2008) prior to the analysis by a total organic carbon (TOC-V) analyser (Sohrin & Sempéré, 2005). Chen et al. (2016) studied the effect of freezing-thawing cycles on DOM fractionation and aromaticity. It was shown that the times of freeze-thaw cycles and freezing time does not significant impact these properties. However, freezing-thawing induce a quenching of fluorescent proteins (Chen et al., 2016). Acidification of seawater samples is also used for DOC preservation (Calleja et al., 2013; Gasol et al., 2009; Griffith et al., 2012; Ruiz-Halpern et al., 2014). Hydrochloric acid (HCl) is used by the National Science Foundation-sponsored DOC Consensus Reference Materials (CRMs) to preserve reference DOC waters. Deep seawater reference (DSR) samples from *Hansell* research laboratory are usually used worldwide to validate the DOC measurements. This acidified material ensure accurate DOC measurements for up to two years. Nevertheless the use of HCl could induce hydrolysis and/or molecular changes in DOC (Walker et al., 2017) but the long-term effect of acidic storage of DOM composition remains unclear. To our knowledge, the difference between freezing or acidification has never been studied for a long-term DOC preservation.

Furthermore, the emergence of programs like BioGeoScapes that focus on both inorganics and organics, will be a challenge to preserve samples in material inert for both organic and inorganic (trace metals) phase. The purpose of this study is to show the potential analytical bias on DOC measurements depending on the choice of the type of container and the conditioning of the seawater. We have studied the effects of acidification (at pH 2) and freezing (at -20°C) in both borosilicate brown glass and high-density polyethylene (HDPE) vials for long-term DOC

preservation. On the one hand, it was done at the local level on a coastal seawater. On the other hand, the offshore area was also studied for the sake of representativeness of the water column and applicability to international campaigns.

The size-exclusion chromatography (SEC) coupled to an organic carbon detector (OCD) (Huber et al., 2011) was adapted to seawater using limited volumes (Dulaquais et al., 2018) and has shown its applicability on the water column of the open ocean (Fourrier et al., 2022). In addition to the quantification of the global DOC concentrations, this coupling allows an access to its fractionation (Huber et al., 2011). The main interest of this tool is that no sample extraction or purification are necessary prior to measurements. The fractionation is done within columns by size and polarity and is an indicator of the quality and the reactivity of the DOC. According to the size-reactivity continuum in oceanic waters (Benner & Amon, 2015), a DOC of high molecular weight (HMW) is more bioavailable and faster mineralised than a low molecular weight (LMW) one (Fourrier et al., 2022). The SEC coupled to an OCD is an ideal analytical tool for long-term monitoring of optimal conditions for DOC preservation.

2. Experimental Section

2.1. Seawater sampling

For preservation tests, the sampling was done in the coastal zone of the Bay of Brest (48°21'21.4 "N; 4°33'52.3"W) in October 2019. A seawater sample of 2 L was collected using a pole, at high tide at the surface (0.5 m) directly into an acid cleaned and three time sample-rinsed bottle of high-density polyethylene (HDPE) (NALGENE®). Within the hour after collection, the water was filtered on a precombusted (4h at 400°C) GF/F filter with a porosity of 0.7 µm (Ø 47 mm, Whatman®) placed on a polypropylene (NALGENE®) filter holder.

For long term preservation test an offshore station was sampled in the Western Tropical South Pacific (WTSP) Ocean (20° 24.431′ S; 166° 35.675′ W). The samples were collected during the GEOTRACES TONGA (shallow hydroThermal sOurces of trace elemeNts: potential impacts on biological productivity and the bioloGicAl carbon pump, GEOTRACES GPpr14) onboard the R/V *L'Atalante* in November 2019. Sampling was carried out using a trace metal clean polyurethane powder-coated aluminum frame rosette (TMR) equipped with twenty-four 12 L Teflon-lined GO-FLO bottles (General Oceanics) and attached to a Kevlar® wire. The cleaning protocols of all the sampling equipment followed the guidelines of the GEOTRACES Cookbook (http://www.geotraces.org). After recovery, the TMR was directly transferred into a

clean container equipped with a class 100 laminar flow hood. Samples were then taken from the filtrate of particulate samples (collected on acid cleaned polyethersulfone filters, 0.45 µm supor) and aliquots collected into acid cleaned and sample-rinsed HDPE 125 mL and 30 mL bottles (NALGENE®).

2.2. Conservation parameters

The water used was of ultra-pure quality (resistivity >18.2 MΩ.cm, MilliQ Element, Millipore®). The coastal sample was dispatched in two types of bottles. Part of the sample was placed in 60 mL borosilicate brown glass bottles with a Bakelite cap with unglued Teflon-lined septum (Schott DURAN®) and another part in 125 mL HDPE (NALGENE®) bottles. Glass bottles were cleaned with a pH 2 solution (hydrochloric acid, HCl, 0.01 M, Suprapur®, >99%) before to be calcinated (4h at 400°C). All the HDPE bottles (NALGENE®) used in this study were acid cleaned according to the GEOTRACES cleaning procedure.

For the coastal sample three preservation procedures were tested for both the glass and HDPE bottles: *(a)* simple filtration at 0.7 µm, double bagged and stored upright in the dark, *(b)* same as *(a)* + acidification at pH 2 using HCl suprapure grade, Merck ®; *(c)* same as *(a)* + frozen at -20°C. DOC measurements were operated the day of collection, one day after, each week during one month, after three months and finally one year after the sampling of the seawater. For procedures *(a)* and *(b)* a unique sample bottle was used all along the experiment. For procedure *(c)* six different single-use bottles (30 ml) were used to avoid freezing/thawing cycles potential artefacts. Figure 1 summarizes the experimental design implemented in this study.

In the case of the offshore station, two batches of eleven samples were collected in HDPE bottles. Before analysis, one batch was kept for 2 years following procedure *(b),* the other was stored 6 month according procedure *(c)*.

The eight samples in flame-sealed ampoules, only used here for an intercomparison (and not in the context of conservation tests) between DOC concentrations determined by TOC-V and SEC were taken from a classical rosette equipped with twenty-four 12 L Niskin bottles. The samples were filtered under low vacuum (< 50 mm Hg) through 25 mm glass fiber filters (porosity 0.7 µm, GF/F Whatmann) and transferred into 10 mL glass ampoules. The filtrates were then acidified with 20 µL of H_2SO_4 (95% – 98%, Sigma Aldrich), then the ampoules were flame sealed and stored at 4°C until the analysis.

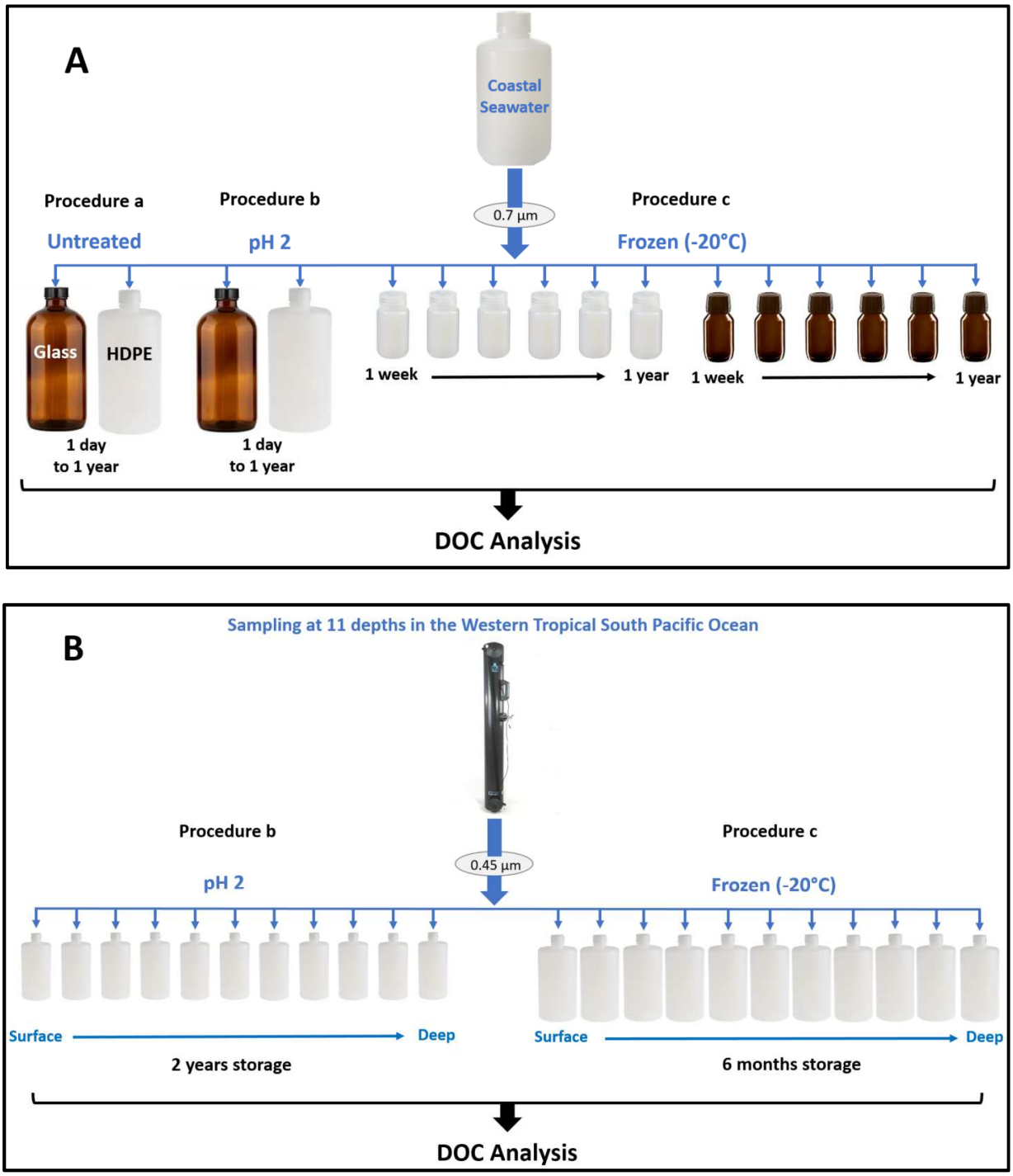

Figure 1. Diagram of the conditions of the experiment on conservation modes of the coastal seawater (**A**) and WTSP seawater (**B**) prior to DOC measurements.

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2.3. DOC analysis

An hour before DOC determination, aliquots of samples were directly poured, under a laminar flow hood (class 100), from the sample bottle into a pre-combusted glass vial. The concentrations of DOC were then measured by SEC coupled with an OCD (DOC-Labor®, Karlsruhe, Germany). All the chemicals (mobile/acid phases) for SEC were prepared following the protocol from Huber et al. (2011). The data treatment was also done in the same way as these authors. The calibration of the OCD was performed as Dulaquais et al. (2018) which adapt DOC measurement by SEC for marine waters. The SEC device, equipped with two chromatographic columns (250 mm × 20 mm, TSK HW-50S, 3000 theoretical plates, Toso, Japan), permits the separation of DOM into five fractions of organic compounds with an optimal resolution. These fractions were described in order of retention as biopolymers that refers to high molecular weight compounds (BP, > 10 kDa), humic substances (HS, 0.5 – 10 kDa), BP by-products (or building-blocks, BB, 0.3 – 0.5 kDa, Fourrier et al., 2022), low molecular weight (LMW) acids (< 0.3 kDa) and LMW neutrals (< 0.3 kDa). LMW monoprotonic acids are small-degradated HS and LMW neutral compounds are small hydrophilic compounds. The respective composition of each DOC fraction are described in detail in Huber et al. (2011) and Dulaquais et al. (2018b). The detection limits, the reproductibility and the repeatability of the SEC-coupled carbon detector for a seawater matrix were detailed by Dulaquais et al. (2018b). The same apparatus was employed for the present study. All the DOC concentrations measured within each fraction of each sample largely exceeded the limits of detection determined by Dulaquais et al. (2018b) for marine waters. Deep seawater reference (DSR) samples used to validate the DOC measurements were provided by the *Hansell* research laboratory (DOC_{DSR} = 43.2 ± 1.7 µmol L^{-1}; n = 5; consensus value of lot #10–18: 43 – 45 µmol L^{-1}). All DOC measurements on natural samples were performed in single shot.

3. Results and discussion

3.1. Influence of the conservation mode on a coastal seawater

The initial total DOC concentration on the day of sampling (T_0) was 64.6 ± 1.9 µMC. Results of the one-year sample conservation experiment display different pattern depending ~~of~~ on the procedure and bottles (Figure 2). After one year of storage in HDPE bottles, the DOC concentration increases up to 122.8 ± 3.7 µMC for procedure *(a)* (Figure 2A), slightly increase to 69.6 ± 2.1 µMC for procedure *(b)* (Figure 2C), and was statistically unchanged (61.9 ± 1.9

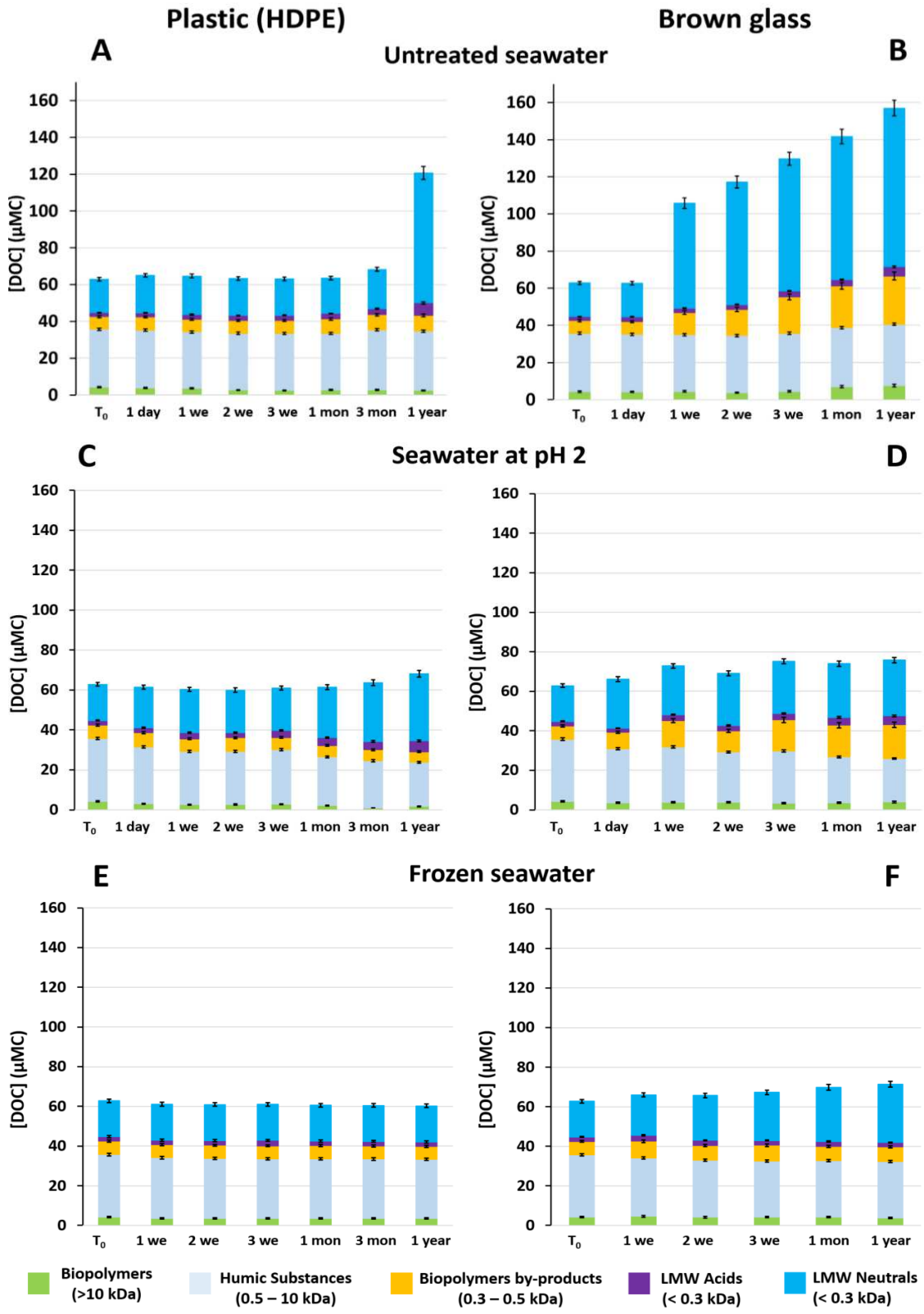

Figure 2. Conservation tests of DOC operational fractions defined by SEC for a coastal surface water (Bay of Brest - 2019) as a function of time and container type.

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µMC) for procedure *(c)* (Figure 2E) (p value < 0.05). For samples stored in brown glass bottles, the final concentration reach 160.9 ± 4.8 µMC for procedure *(a)* (Figure 2B) and increase up to 78.4 ± 2.4 µMC and 73.2 ± 2.2 µMC for procedure *(b)* and *(c)* respectively (Figure 2D & F). Among all the results, the DOC concentration the closest to T_0 after one year of storage was found in the HDPE bottle that suffer from procedure *(c)* (difference of - 4.1 % ± 5.8 after 1 year of storage).

In the seawater sample that suffer from procedure *(a)*, stored in HDPE bottle, there was no change in DOC concentration during the first month of the preservation experiment. We measured a gain of 5.6 ± 0.2 µMC of DOC after 3 months of conservation and up to 58.2 µMC of DOC after 1 year of storage. For the glass bottle, the DOC increment was even more marked starting during the first month and concentrations increased by a factor 2.5 after a year of storing. The 0.7 µm GF/F filter we used for a pre-treatment in this study does not retain small planktonic cells, heterotrophic bacteria and viruses (Lee et al. 1995; Suttle et al., 1991; Taguchi & Laws, 1988), thereby the production of DOC was unexpected and a decrease of DOC was instead expected. This increase of DOC along with time is probably resulting from the ~~non-sealing of the caps~~ lack of seal between the Bakelite stopper and the neck of the glass bottle that may induced biological fertilization/contamination and subsequent organic matter production through aerosols deposition. In absence of any further treatment, we suggest not to store filtered samples more than one day in glass bottles closed with Bakelite caps with unglued Teflon-lined septum and no more than 1 month in HDPE (NALGENE®) bottles.

Walker et al. (2017) studied the differences in DOC concentrations between two modes of storage (frozen versus acidified) of a coastal seawater as a function of time. Their work conducted with samples stored in brown glass bottles (1 L) with polytetrafluorethylen (PTFE) caps did not monitored the changes with time from the initial state but compared variability between the two storage procedures that are equivalent to procedure *(b)* and *(c)* in this study. Walker et al. (2017) reported a difference of approximately 2.2 ± 0.2 µMC between these two types of storage after 380 days with systematic higher concentration in frozen samples compared to acidified samples. In this work, we estimated the difference between procedures (b) and (c) to be 5.2 ± 4.6 µMC under the same conditions (glass bottles). Our results contrast with those of Walker et al. (2017) since we did not see loss of DOC but instead gain of DOC potentially suggesting that long acid storing of seawater in glass bottles with Bakelite caps (instead of PTFE caps, whose choice would certainly have been wiser) may be a source of DOC

contamination. This could be due to an elution of DOC from Bakelite caps, resulting on the alteration of the soft plastic seal or the phenoplast (or resins) of the Bakelite. No flexible plastic, however stable it may be, can be insensitive/resistant to contact with seawater (oxidizing milieu) and moreover at low pH (acid milieu).

After one year of storage under acidic conditions (Figure 2C&D), DOC increase in HDPE and glass bottles were of 5.0 ± 4 µMC and 13.8 ± 4.3 µMC respectively. For the glass bottle, the DOC increment alongside with time may be associated with to the deterioration of the seal and the Bakelite cap of the glass bottle by acidic vapour. Regarding the HDPE bottle, the DOC increase only occurred after one year but was unexpected considering the results of the long-term experiment storage presented in the next section (see 3.2). Seawater is an oxidizing environment and combined with HCl, even at low concentration (pH 2), it may slightly altered the HDPE bottle increasing DOC. Nevertheless there was no significant DOC input during the first three month of storage (Figure 2c) and the final DOC statistical increase (1 µMC for 1SD) represented only 1.5% of the initial DOC concentration. Our result suggest that storage of a coastal seawater sample in HDPE bottles at pH 2 can provide good DOC data, at least for 3 months of storage (Figure 2C).

The experiment provide further information regarding the modification of DOM under acidic environment. Long-term storage of DOM in acidic conditions change the size fractionation of DOC (Figure 2C). Precipitation of HS under acidic conditions is often invoked to explain the decrease of DOC with time (Walker et al., 2017). It was clear from our result that HS and BP (e.g. HMW DOM) fractions decreased with time of conservation in line with previous hypothesis. However in the same time LMW compounds increased keeping DOC concentration relatively stable in the HDPE bottle. Thereby our result suggest a hydrolysis of these compounds into LMW compounds rather than their precipitation from the dissolved to the particulate phase. Interestingly the size fractionation of the acidified samples changed differently between the HDPE and glass bottles (Figure 2C & D). High loss of biopolymers and high increase of LMW neutrals occurred in the HDPE bottles whereas biopolymers kept statistically constant and building blocks tends to increase in the glass bottle (p value < 0.05). Because both sample suffer the same procedure *(b)* we suggest that surface reaction taking place on the wall of the bottle are different between HDPE and glass bottles with no further explanation.

The differences of bulk DOC and of its size fractionation compared to T_0 were the lowest for the frozen samples (- 2.7 ± 3.8 µMC for the HDPE bottle, Figure 2E; + 8.6 ± 4.1 µMC for the glass bottle, Figure 2F). When stored in HDPE bottles there was no significant changes in DOC and size fractionation of DOM indicating that procedure (c) using HDPE bottle permits an excellent long-term preservation of DOM. Differently for the glass bottle with Bakelite cap there was a gain (+ 13.3 ± 1.3%) in the LMW neutrals fraction. These latter increase could again been explained by alteration (contraction and then expansion) of the seal and/or cap itself. Historically, Bakelite screw caps with Teflon-lined septum were often used in order to analyse DOC or humic properties in aquatic environments (Robinson & Novak, 1994; Rostan & Cellot, 1995) including estuarine, coastal (Dulaquais et al., 2018b), and oceanic water (Sharp et al., 1993) for the storage in glass vials. Sharp et al. (1993) already advised to abandon the use of glue between the Teflon seal and the bottom of the Bakelite cap. Our results suggest to no longer use this kind of stopper, even unglued. These are also in line with Sheyer et al. (2021), who point out that these stoppers become brittle rather quickly. Moreover there was a significant DOC decrease in the HS fraction (- 9.4 ± 1.2 µMC) for the glass bottles frozen suggesting a precipitation of HS during the freezing-thawing procedure. Because both samples in the HDPE and glass bottles suffer the same freezing-thawing procedure, the precipitation of HS seems enhanced by the glass material and imply surface reaction (adsorption processes and/or flocculation) on the wall of the glass bottles during freezing-thawing steps.

Overall, our conservation experiments suggest using acid cleaned HDPE bottles rather than glass bottles with Bakelite caps. In HDPE bottles, DOC can be accurately measured for 1 month the filtered sample is kept double bagged, in the dark, and for at least a year when acidified or frozen. Regarding the study of DOM, the best preservation procedure is the freezing of the sample.

3.2. Long-term conservation of oceanic samples

During offshore campaign, the preservation of samples for DOC determination often implies its filtration through glass fiber filters (porosity ~ 0.7 µm, GF/F Whatmann) an acidification with H_2SO_4 and a conservation in flame-sealed ampoules stored at 4°C (Alperin & Martens, 1993). For DOM study, samples are often extracted onboard using the solid phase extraction procedure of Dittmar et al., (2008). Methanol extracts are then again flame sealed in glass ampoules and frozen until analysis (Osterholtz et al., 2021). For the trace metal community, the

filtration of samples onto polyethylsulfone filters and their acidification after their collection in acid clean HDPE bottles is commonly used for sample preservation during offshore campaign. Onboard samples for trace metals and organics analyses are often collected from different rosettes, filtered with different filter types placed on different filter holders. It results in increasing the number of casts and handling time onboard for each stations.

Conservation of frozen filtered seawater samples in HDPE bottles provides an accurate picture of the initial DOC and DOM content for at least 1 year (Figure 2 E). Moreover Fourrier et al., (2022) demonstrated that the determination of DOC in samples collected following a trace metal clean protocol and kept frozen in HDPE bottles provide similar results (statistically tested) to those from samples filtered on GF/F filters and stored in flame sealed ampoules. However, it remains unclear if samples collected during a trace metal cruise (e.g. GEOTRACES) and stored acidified can be used for the study of organics. To determine if long-term storage of acidified samples in HDPE bottles alters DOC concentrations and DOM size class distributions we analysed samples stored 2 years and half in HPDE bottles acidic conditions and compared to those frozen in eleven samples collected all along the water column in the Western Tropical South Pacific Ocean during the TONGA expedition. Results (Figure 3)

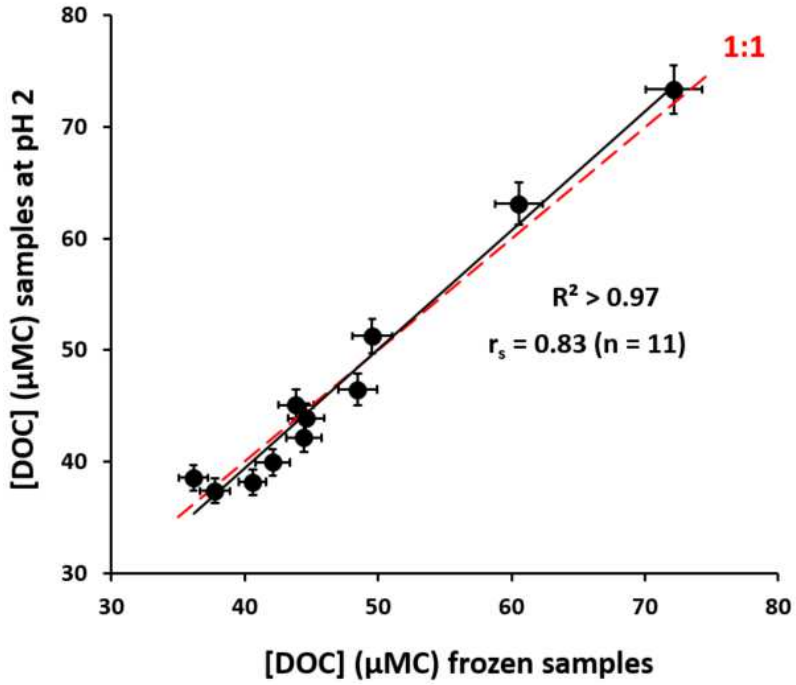

Figure 3. Comparison of dissolved organic carbon (DOC) concentrations (µMC) measured by SEC for a unique station of the TONGA campaign (2019) for two preservation modes (freezing and pH 2) of seawater samples in HDPE vials. r_s corresponds to the Spearman correlation coefficient.

297 demonstrate that DOC concentrations were statistically identical within paired samples at each
298 depth ($R^2 > 0.97$; Spearman coefficient $r_s = 0.83$; n = 11). Moreover, the DOC measured in this
299 acidified samples were also statistically identical to those measured in samples filtered on GF/F
300 filters and stored acidified in flame-sealed glass ampoules ($R^2 > 0.97$; $r_s = 0.92$; n = 8). These
301 results evidence that bulk DOC concentrations can be measured independently of the storage
302 method tested here (freezing or pH 2 in HDPE bottle, acidified in flame-sealed glass ampoules).
303 In both frozen and acidified samples were typical of those reported in the South Pacific gyre
304 (Swan et al., 2009). Marked changes were however, for the size-class distribution of DOC after
305 long-term conservation in acidic conditions (Figure 4).
306
307 In agreement with the previous tests presented for a surface coastal water, storage of samples
308 by freezing at -20°C in HDPE vials DOC size class fractionation in the frozen sample can be
309 considered as the reference or initial state (Figure 4A). The vertical shapes of the different DOC
310 fractions are relatively identical along the water column between the two modes of conservation
311 but the contribution of almost all the fraction to the global DOC is changing after a long-term
312 storage at pH 2 (Figure 4B). The concentration of humic carbon to total DOC decreased by 11.5
313 ± 1.2 % at pH 2 along the water column (deep blue axis, Figure 4C). The loss was even more
314 dramatic for the BP fraction with an average loss of 70% for this fraction (deep blue axis, Figure
315 4C). As described previously, acid hydrolysis or depolymerization of BP and HS could explain
316 a decrease in their DOC concentrations over time. Organic carbon incorporates most abundantly
317 into the LMW neutrals fraction (11.2 ± 1.4 % gain in contribution to total DOC, deep blue axis,
318 Figure 4C) but BBs increase from a contribution to DOC of 9.6 ± 0.8 % to 13.6 ± 1.1 % at the
319 studied station. The increase in concentration (2.0 ± 1.0 µMC, black axis) of the BBs fraction
320 was of the same magnitude that of the loss of BP. This result reflects the incorporation of
321 hydrolyzed carbon of HMW size fraction (BP) further supporting to consider this operationally
322 define fraction as BP by-products (Fourrier et al., 2022). After two years of acidic storage the
323 LMW acids fraction slightly increase (+ 17.1 ± 0.8 %, deep blue axis, Figure 4C). This increase
324 does not seem to be significant both in the surface and in the deep water samples (Figure
325 4A&B). Interestingly both surface and deep samples were affected by long-term storage in
326 acidic conditions (Figure 4), indicating that both labile and refractory DOC can be hydrolysed
327 at pH 2. This experiment strengthen the choice of working with the frozen samples rather than
328 in an acidified environment for the study of the size fractionation of oceanic DOC.

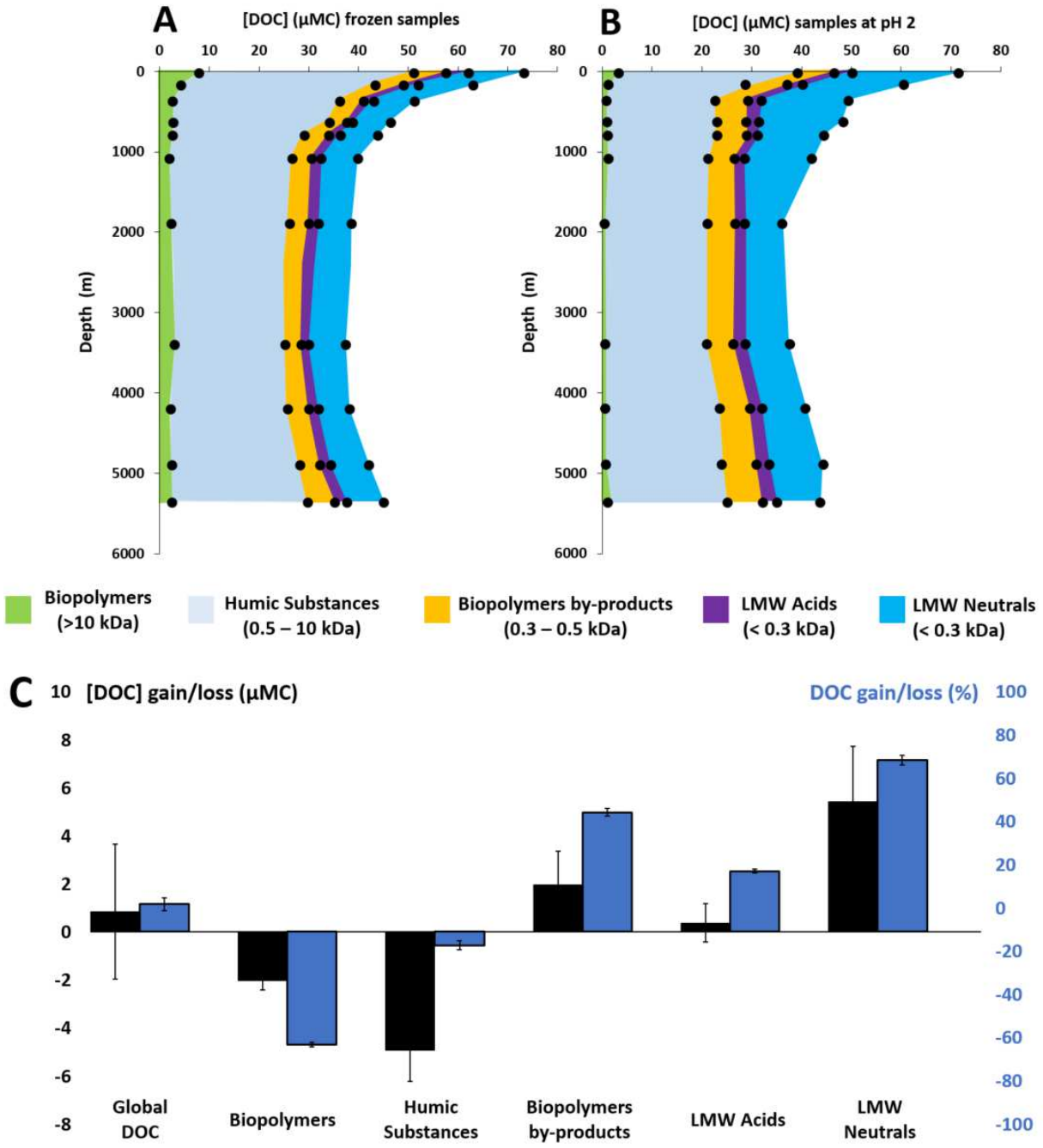

Figure 4. Conservation tests of DOC operational fractions defined by SEC for an unique station of the TONGA campaign (2019). These measurements were made two years after sample collection, conditioning and storage in HDPE vials. The vertical profiles (**A** & **B**) visualize the partitioning of DOC fractions (µMC) along the water column (m) and the diagram (**C**) estimates the average gain/loss of DOC in each fraction with regard to frozen samples along the water column (black axis in µMC and deep blue axis in %).

4. Conclusions

For bulk DOC concentrations measurements, acidified seawater samples (pH 2, HCl) can be analysed in the same way as a frozen sample in HDPE vials or a glass ampoules, without risking

an analytical bias and over the long term. Thereby our results demonstrate that a sample collected for trace metal determination and stored for years can be used for DOC measurement.

However, each DOC fraction comprising its own chemical properties, the acidification of the samples does not seem to be wise for an in-depth study of the size fractionation of DOC. We thus recommend the freezing for a study based on chemical properties of DOC. HDPE bottles has shown themselves as ideal containers, as no contamination were observed in DOC.

Aknowledgments

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Influence of the conservation mode of seawater for dissolved organic carbon analysis

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Abstract

Marine dissolved organic matter (DOM) is one of the largest exchangeable organic carbon reservoir on the planet. The main proxy to track the distribution of DOM in the aquatic environments remains dissolved organic carbon (DOC). Thereby the optimal protocol for long-term DOC preservation in seawater samples must be defined. In this context, we monitored bulk DOC concentrations and its size class distribution in filtered seawater samples during yearlong experiments. With different conservation mode, we tested two types of commonly used materials (borosilicate brown glass and high-density polyethylene, HDPE) and three conditioning protocols (untreated, acidified at pH 2 and frozen at -20°C). Offshore samples collected along the entire water column of the Pacific Ocean and stored in HDPE bottles were also analysed after 2 years of storage at pH of 2 and compared to frozen samples. Results demonstrated that bulk DOC concentrations can be accurately determined in untreated samples for one month and for years in frozen samples as well as in acidified samples, when samples are stored in acid cleaned HDPE bottles or flame sealed glass ampoules. Storage in brown glass vials with Bakelite caps seems more uncertain. The study of the size class distribution of DOC reveals the possibility to study DOM for 1 month in filtered samples with no additional treatment and for years in frozen samples when stored in acid cleaned HDPE bottles. Significant changes in DOC size fractionation were observed when samples were acidified. The high molecular weight (HMW) compounds and the humic substances from the upper 1000 meters were significantly degraded at pH 2, incorporating DOC in the low molecular weight (LMW) fractions. These experiments provide preservation guidelines for future studies that aim either to study bulk DOC or the chemical properties marine DOM. It is recommended to store seawater in HDPE vials at -20°C for DOM study, or at pH 2 for bulk DOC measurements.

1. Introduction

Dissolved organic carbon (DOC) is the largest organic carbon pool in the ocean (Hansell et al., 2009). In last decades, its distribution and composition have increasingly being measured in the marine environment notably to determine its sources and sinks. These measurements range from the global (quantification by concentrations, Carlson & Hansell, 2015; Hansell & Carlson, 1998) to the specific scale (molecular characterization, Hertkorn et al., 2013; Nebbioso & Piccolo, 2013). The quality of these different types of analysis can only be guaranteed by a sampling under perfectly clean conditions as well as an optimal preservation of the seawater samples. Therefore, a conditioning is a mandatory to avoid changes in composition or microbial decomposition of DOC (Walker et al., 2017). Different preservation mode of the seawater samples were specified in oceanic studies for DOC analysis. After a filtration to remove the particulate material, the seawater is often stored in flame sealed pre-combusted glass ampoules (Dittmar, 2008) prior to the analysis by a total organic carbon (TOC-V) analyser (Sohrin & Sempéré, 2005). Chen et al. (2016) studied the effect of freezing-thawing cycles on DOM fractionation and aromaticity. It was shown that the times of freeze-thaw cycles and freezing time does not significant impact these properties. However, freezing-thawing induce a quenching of fluorescent proteins (Chen et al., 2016). Acidification of seawater samples is also used for DOC preservation (Calleja et al., 2013; Gasol et al., 2009; Griffith et al., 2012; Ruiz-Halpern et al., 2014). Hydrochloric acid (HCl) is used by the National Science Foundation-sponsored DOC Consensus Reference Materials (CRMs) to preserve reference DOC waters. Deep seawater reference (DSR) samples from *Hansell* research laboratory are usually used worldwide to validate the DOC measurements. This acidified material ensure accurate DOC measurements for up to two years. Nevertheless the use of HCl could induce hydrolysis and/or molecular changes in DOC (Walker et al., 2017) but the long-term effect of acidic storage of DOM composition remains unclear. To our knowledge, the difference between freezing or acidification has never been studied for a long-term DOC preservation.

Furthermore, the emergence of programs like BioGeoScapes that focus on both inorganics and organics, will be a challenge to preserve samples in material inert for both organic and inorganic (trace metals) phase. The purpose of this study is to show the potential analytical bias on DOC measurements depending on the choice of the type of container and the conditioning of the seawater. We have studied the effects of acidification (at pH 2) and freezing (at -20°C) in both borosilicate brown glass and high-density polyethylene (HDPE) vials for long-term DOC

preservation. On the one hand, it was done at the local level on a coastal seawater. On the other hand, the offshore area was also studied for the sake of representativeness of the water column and applicability to international campaigns.

The size-exclusion chromatography (SEC) coupled to an organic carbon detector (OCD) (Huber et al., 2011) was adapted to seawater using limited volumes (Dulaquais et al., 2018) and has shown its applicability on the water column of the open ocean (Fourrier et al., 2022). In addition to the quantification of the global DOC concentrations, this coupling allows an access to its fractionation (Huber et al., 2011). The main interest of this tool is that no sample extraction or purification are necessary prior to measurements. The fractionation is done within columns by size and polarity and is an indicator of the quality and the reactivity of the DOC. According to the size-reactivity continuum in oceanic waters (Benner & Amon, 2015), a DOC of high molecular weight (HMW) is more bioavailable and faster mineralised than a low molecular weight (LMW) one (Fourrier et al., 2022). The SEC coupled to an OCD is an ideal analytical tool for long-term monitoring of optimal conditions for DOC preservation.

2. Experimental Section

2.1. Seawater sampling

For preservation tests, the sampling was done in the coastal zone of the Bay of Brest (48°21'21.4 "N; 4°33'52.3"W) in October 2019. A seawater sample of 2 L was collected using a pole, at high tide at the surface (0.5 m) directly into an acid cleaned and three time sample-rinsed bottle of high-density polyethylene (HDPE) (NALGENE®). Within the hour after collection, the water was filtered on a precombusted (4h at 400°C) GF/F filter with a porosity of 0.7 µm (Ø 47 mm, Whatman®) placed on a polypropylene (NALGENE®) filter holder.

For long term preservation test an offshore station was sampled in the Western Tropical South Pacific (WTSP) Ocean (20° 24.431′ S; 166° 35.675′ W). The samples were collected during the GEOTRACES TONGA (shallow hydroThermal sOurces of trace elemeNts: potential impacts on biological productivity and the bioloGicAl carbon pump, GEOTRACES GPpr14) onboard the R/V *L'Atalante* in November 2019. Sampling was carried out using a trace metal clean polyurethane powder-coated aluminum frame rosette (TMR) equipped with twenty-four 12 L Teflon-lined GO-FLO bottles (General Oceanics) and attached to a Kevlar® wire. The cleaning protocols of all the sampling equipment followed the guidelines of the GEOTRACES Cookbook (http://www.geotraces.org). After recovery, the TMR was directly transferred into a

clean container equipped with a class 100 laminar flow hood. Samples were then taken from the filtrate of particulate samples (collected on acid cleaned polyethersulfone filters, 0.45 µm supor) and aliquots collected into acid cleaned and sample-rinsed HDPE 125 mL and 30 mL bottles (NALGENE®).

2.2. Conservation parameters

The water used was of ultra-pure quality (resistivity >18.2 MΩ.cm, MilliQ Element, Millipore®). The coastal sample was dispatched in two types of bottles. Part of the sample was placed in 60 mL borosilicate brown glass bottles with a Bakelite cap with unglued Teflon-lined septum (Schott DURAN®) and another part in 125 mL HDPE (NALGENE®) bottles. Glass bottles were cleaned with a pH 2 solution (hydrochloric acid, HCl, 0.01 M, Suprapur®, >99%) before to be calcinated (4h at 400°C). All the HDPE bottles (NALGENE®) used in this study were acid cleaned according to the GEOTRACES cleaning procedure.

For the coastal sample three preservation procedures were tested for both the glass and HDPE bottles: *(a)* simple filtration at 0.7 µm, double bagged and stored upright in the dark, *(b)* same as *(a)* + acidification at pH 2 using HCl suprapure grade, Merck ®; *(c)* same as *(a)* + frozen at -20°C. DOC measurements were operated the day of collection, one day after, each week during one month, after three months and finally one year after the sampling of the seawater. For procedures *(a)* and *(b)* a unique sample bottle was used all along the experiment. For procedure *(c)* six different single-use bottles (30 ml) were used to avoid freezing/thawing cycles potential artefacts. Figure 1 summarizes the experimental design implemented in this study.

In the case of the offshore station, two batches of eleven samples were collected in HDPE bottles. Before analysis, one batch was kept for 2 years following procedure *(b),* the other was stored 6 month according procedure *(c)*.

The eight samples in flame-sealed ampoules, only used here for an intercomparison (and not in the context of conservation tests) between DOC concentrations determined by TOC-V and SEC were taken from a classical rosette equipped with twenty-four 12 L Niskin bottles. The samples were filtered under low vacuum (< 50 mm Hg) through 25 mm glass fiber filters (porosity 0.7 µm, GF/F Whatmann) and transferred into 10 mL glass ampoules. The filtrates were then acidified with 20 µL of H_2SO_4 (95% – 98%, Sigma Aldrich), then the ampoules were flame sealed and stored at 4°C until the analysis.

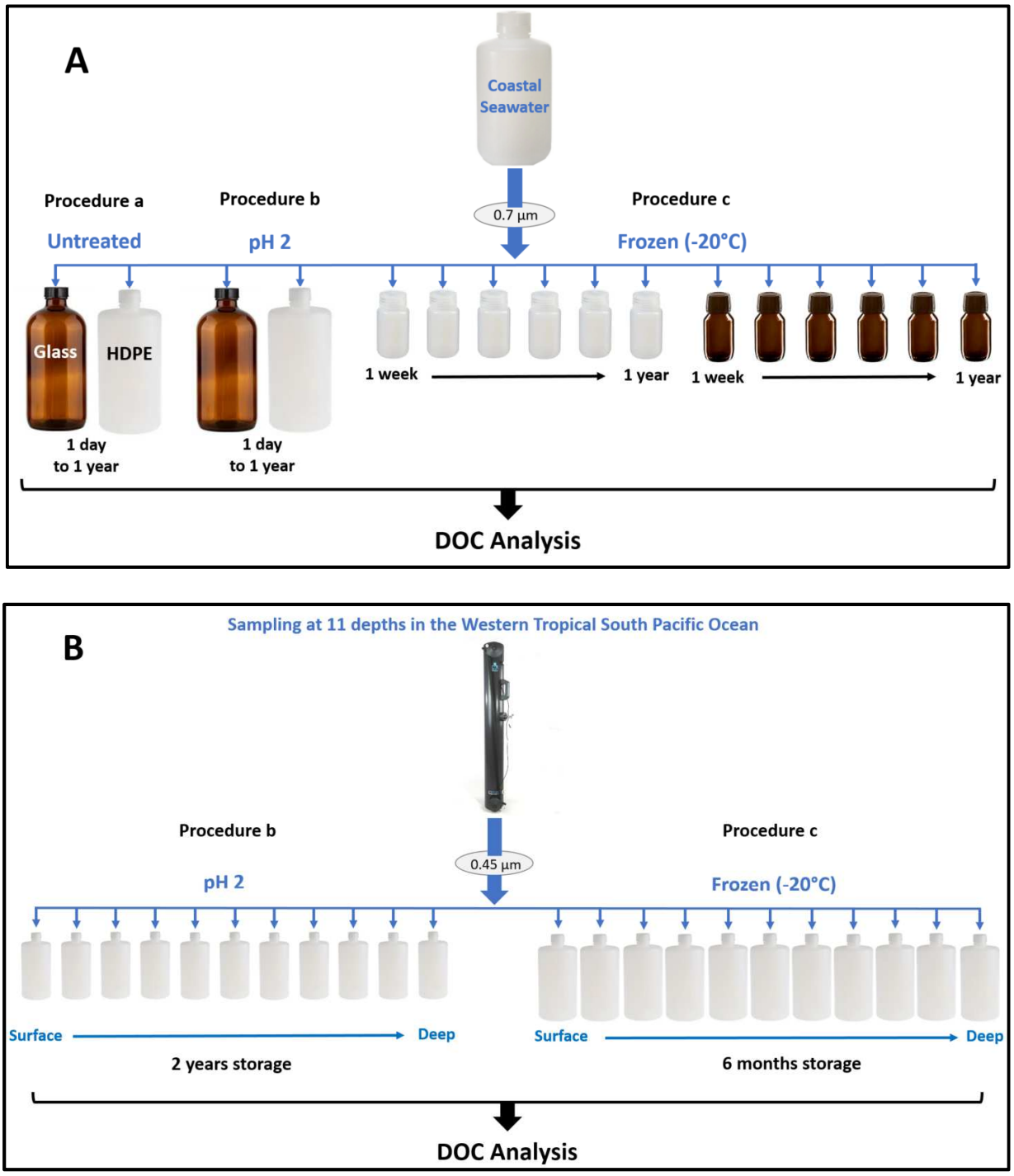

Figure 1. Diagram of the conditions of the experiment on conservation modes of the coastal seawater (**A**) and WTSP seawater (**B**) prior to DOC measurements.

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2.3. DOC analysis

An hour before DOC determination, aliquots of samples were directly poured, under a laminar flow hood (class 100), from the sample bottle into a pre-combusted glass vial. The concentrations of DOC were then measured by SEC coupled with an OCD (DOC-Labor®, Karlsruhe, Germany). All the chemicals (mobile/acid phases) for SEC were prepared following the protocol from Huber et al. (2011). The data treatment was also done in the same way as these authors. The calibration of the OCD was performed as Dulaquais et al. (2018) which adapt DOC measurement by SEC for marine waters. The SEC device, equipped with two chromatographic columns (250 mm × 20 mm, TSK HW-50S, 3000 theoretical plates, Toso, Japan), permits the separation of DOM into five fractions of organic compounds with an optimal resolution. These fractions were described in order of retention as biopolymers that refers to high molecular weight compounds (BP, > 10 kDa), humic substances (HS, 0.5 – 10 kDa), BP by-products (or building-blocks, BB, 0.3 – 0.5 kDa, Fourrier et al., 2022), low molecular weight (LMW) acids (< 0.3 kDa) and LMW neutrals (< 0.3 kDa). LMW monoprotonic acids are small-degradated HS and LMW neutral compounds are small hydrophilic compounds. The respective composition of each DOC fraction are described in detail in Huber et al. (2011) and Dulaquais et al. (2018b). The detection limits, the reproductibility and the repeatability of the SEC-coupled carbon detector for a seawater matrix were detailed by Dulaquais et al. (2018b). The same apparatus was employed for the present study. All the DOC concentrations measured within each fraction of each sample largely exceeded the limits of detection determined by Dulaquais et al. (2018b) for marine waters. Deep seawater reference (DSR) samples used to validate the DOC measurements were provided by the *Hansell* research laboratory (DOC_{DSR} = 43.2 ± 1.7 µmol L^{-1}; n = 5; consensus value of lot #10–18: 43 – 45 µmol L^{-1}). All DOC measurements on natural samples were performed in single shot.

3. Results and discussion

3.1. Influence of the conservation mode on a coastal seawater

The initial total DOC concentration on the day of sampling (T_0) was 64.6 ± 1.9 µMC. Results of the one-year sample conservation experiment display different pattern depending on the procedure and bottles (Figure 2). After one year of storage in HDPE bottles, the DOC concentration increases up to 122.8 ± 3.7 µMC for procedure *(a)* (Figure 2A), slightly increase to 69.6 ± 2.1 µMC for procedure *(b)* (Figure 2C), and was statistically unchanged (61.9 ± 1.9

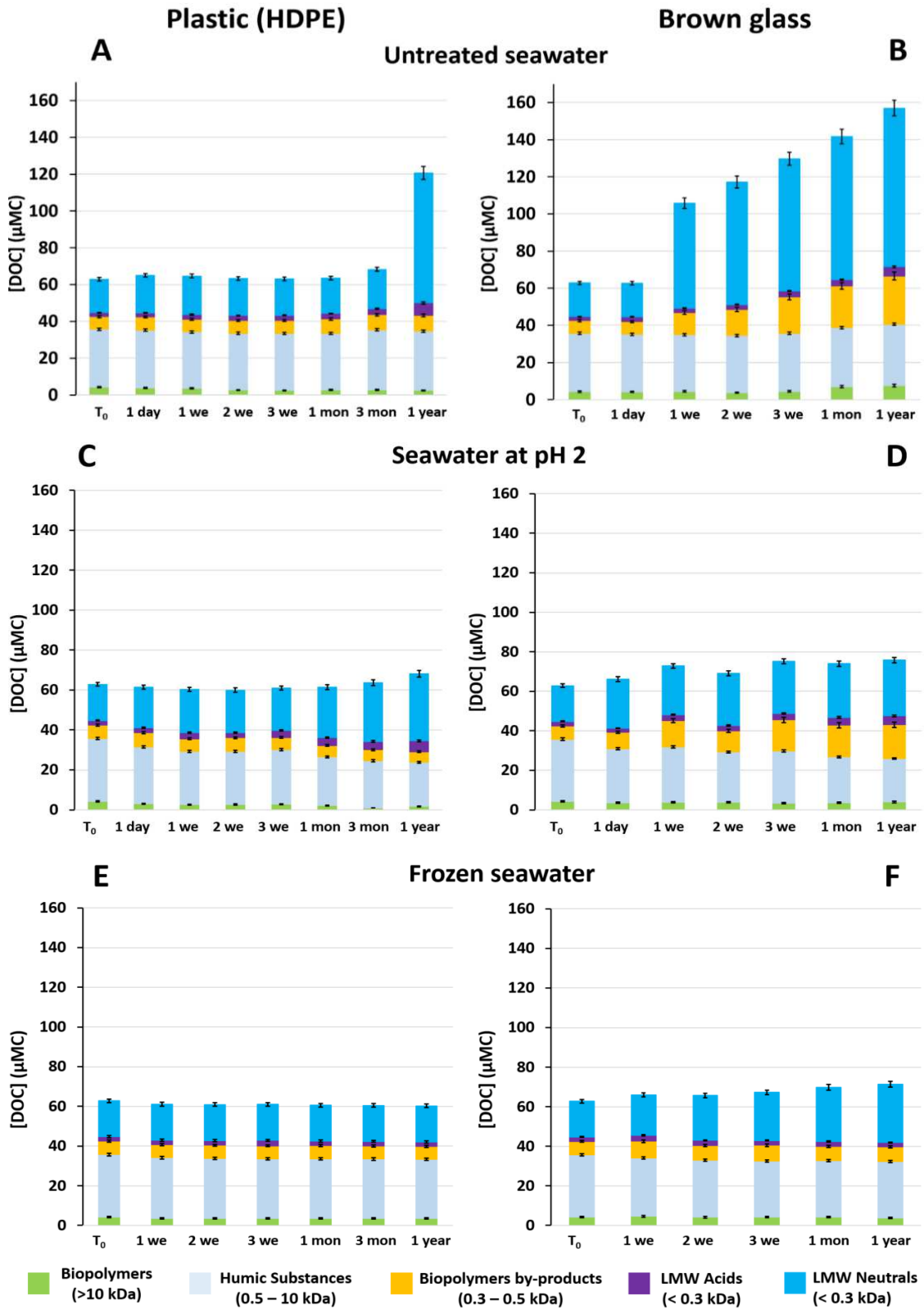

Figure 2. Conservation tests of DOC operational fractions defined by SEC for a coastal surface water (Bay of Brest - 2019) as a function of time and container type.

625

µMC) for procedure *(c)* (Figure 2E) (p value < 0.05). For samples stored in brown glass bottles, the final concentration reach 160.9 ± 4.8 µMC for procedure *(a)* (Figure 2B) and increase up to 78.4 ± 2.4 µMC and 73.2 ± 2.2 µMC for procedure *(b)* and *(c)* respectively (Figure 2D & F). Among all the results, the DOC concentration the closest to T_0 after one year of storage was found in the HDPE bottle that suffer from procedure *(c)* (difference of - 4.1 % ± 5.8 after 1 year of storage).

In the seawater sample that suffer from procedure *(a)*, stored in HDPE bottle, there was no change in DOC concentration during the first month of the preservation experiment. We measured a gain of 5.6 ± 0.2 µMC of DOC after 3 months of conservation and up to 58.2 µMC of DOC after 1 year of storage. For the glass bottle, the DOC increment was even more marked starting during the first month and concentrations increased by a factor 2.5 after a year of storing. The 0.7 µm GF/F filter we used for a pre-treatment in this study does not retain small planktonic cells, heterotrophic bacteria and viruses (Lee et al. 1995; Suttle et al., 1991; Taguchi & Laws, 1988), thereby the production of DOC was unexpected and a decrease of DOC was instead expected. This increase of DOC along with time is probably resulting from the lack of seal between the Bakelite stopper and the neck of the glass bottle that may induced biological fertilization/contamination and subsequent organic matter production through aerosols deposition. In absence of any further treatment, we suggest not to store filtered samples more than one day in glass bottles closed with Bakelite caps with unglued Teflon-lined septum and no more than 1 month in HDPE (NALGENE®) bottles.

Walker et al. (2017) studied the differences in DOC concentrations between two modes of storage (frozen versus acidified) of a coastal seawater as a function of time. Their work conducted with samples stored in brown glass bottles (1 L) with polytetrafluorethylen (PTFE) caps did not monitored the changes with time from the initial state but compared variability between the two storage procedures that are equivalent to procedure *(b)* and *(c)* in this study. Walker et al. (2017) reported a difference of approximately 2.2 ± 0.2 µMC between these two types of storage after 380 days with systematic higher concentration in frozen samples compared to acidified samples. In this work, we estimated the difference between procedures (b) and (c) to be 5.2 ± 4.6 µMC under the same conditions (glass bottles). Our results contrast with those of Walker et al. (2017) since we did not see loss of DOC but instead gain of DOC potentially suggesting that long acid storing of seawater in glass bottles with Bakelite caps (instead of PTFE caps, whose choice would certainly have been wiser) may be a source of DOC

660 contamination. This could be due to an elution of DOC from Bakelite caps, resulting on the
661 alteration of the soft plastic seal or the phenoplast (or resins) of the Bakelite. No flexible plastic,
662 however stable it may be, can be insensitive/resistant to contact with seawater (oxidizing
663 milieu) and moreover at low pH (acid milieu).
664 After one year of storage under acidic conditions (Figure 2C&D), DOC increase in HDPE and
665 glass bottles were of 5.0 ± 4 µMC and 13.8 ± 4.3 µMC respectively. For the glass bottle, the
666 DOC increment alongside with time may be associated with to the deterioration of the seal and
667 the Bakelite cap of the glass bottle by acidic vapour. Regarding the HDPE bottle, the DOC
668 increase only occurred after one year but was unexpected considering the results of the long-
669 term experiment storage presented in the next section (see 3.2). Seawater is an oxidizing
670 environment and combined with HCl, even at low concentration (pH 2), it may slightly altered
671 the HDPE bottle increasing DOC. Nevertheless there was no significant DOC input during the
672 first three month of storage (Figure 2c) and the final DOC statistical increase (1 µMC for 1SD)
673 represented only 1.5% of the initial DOC concentration. Our result suggest that storage of a
674 coastal seawater sample in HDPE bottles at pH 2 can provide good DOC data, at least for 3
675 months of storage (Figure 2C).
676
677 The experiment provide further information regarding the modification of DOM under acidic
678 environment. Long-term storage of DOM in acidic conditions change the size fractionation of
679 DOC (Figure 2C). Precipitation of HS under acidic conditions is often invoked to explain the
680 decrease of DOC with time (Walker et al., 2017). It was clear from our result that HS and BP
681 (e.g. HMW DOM) fractions decreased with time of conservation in line with previous
682 hypothesis. However in the same time LMW compounds increased keeping DOC concentration
683 relatively stable in the HDPE bottle. Thereby our result suggest a hydrolysis of these
684 compounds into LMW compounds rather than their precipitation from the dissolved to the
685 particulate phase. Interestingly the size fractionation of the acidified samples changed
686 differently between the HDPE and glass bottles (Figure 2C & D). High loss of biopolymers and
687 high increase of LMW neutrals occurred in the HDPE bottles whereas biopolymers kept
688 statistically constant and building blocks tends to increase in the glass bottle (p value < 0.05).
689 Because both sample suffer the same procedure *(b)* we suggest that surface reaction taking place
690 on the wall of the bottle are different between HDPE and glass bottles with no further
691 explanation.
692

693 The differences of bulk DOC and of its size fractionation compared to T_0 were the lowest for
694 the frozen samples (- 2.7 ± 3.8 µMC for the HDPE bottle, Figure 2E; + 8.6 ± 4.1 µMC for the
695 glass bottle, Figure 2F). When stored in HDPE bottles there was no significant changes in DOC
696 and size fractionation of DOM indicating that procedure (c) using HDPE bottle permits an
697 excellent long-term preservation of DOM. Differently for the glass bottle with Bakelite cap
698 there was a gain (+ 13.3 ± 1.3%) in the LMW neutrals fraction. These latter increase could
699 again been explained by alteration (contraction and then expansion) of the seal and/or cap itself.
700 Historically, Bakelite screw caps with Teflon-lined septum were often used in order to analyse
701 DOC or humic properties in aquatic environments (Robinson & Novak, 1994; Rostan & Cellot,
702 1995) including estuarine, coastal (Dulaquais et al., 2018b), and oceanic water (Sharp et al.,
703 1993) for the storage in glass vials. Sharp et al. (1993) already advised to abandon the use of
704 glue between the Teflon seal and the bottom of the Bakelite cap. Our results suggest to no longer
705 use this kind of stopper, even unglued. These are also in line with Sheyer et al. (2021), who
706 point out that these stoppers become brittle rather quickly. Moreover there was a significant
707 DOC decrease in the HS fraction (- 9.4 ± 1.2 µMC) for the glass bottles frozen suggesting a
708 precipitation of HS during the freezing-thawing procedure. Because both samples in the HDPE
709 and glass bottles suffer the same freezing-thawing procedure, the precipitation of HS seems
710 enhanced by the glass material and imply surface reaction (adsorption processes and/or
711 flocculation) on the wall of the glass bottles during freezing-thawing steps.
712
713 Overall, our conservation experiments suggest using acid cleaned HDPE bottles rather than
714 glass bottles with Bakelite caps. In HDPE bottles, DOC can be accurately measured for 1 month
715 the filtered sample is kept double bagged, in the dark, and for at least a year when acidified or
716 frozen. Regarding the study of DOM, the best preservation procedure is the freezing of the
717 sample.
718

719 **3.2. Long-term conservation of oceanic samples**
720
721 During offshore campaign, the preservation of samples for DOC determination often implies
722 its filtration through glass fiber filters (porosity ~ 0.7 µm, GF/F Whatmann) an acidification
723 with H_2SO_4 and a conservation in flame-sealed ampoules stored at 4°C (Alperin & Martens,
724 1993). For DOM study, samples are often extracted onboard using the solid phase extraction
725 procedure of Dittmar et al., (2008). Methanol extracts are then again flame sealed in glass
726 ampoules and frozen until analysis (Osterholtz et al., 2021). For the trace metal community, the

727 filtration of samples onto polyethylsulfone filters and their acidification after their collection in
728 acid clean HDPE bottles is commonly used for sample preservation during offshore campaign.
729 Onboard samples for trace metals and organics analyses are often collected from different
730 rosettes, filtered with different filter types placed on different filter holders. It results in
731 increasing the number of casts and handling time onboard for each stations.
732
733 Conservation of frozen filtered seawater samples in HDPE bottles provides an accurate picture
734 of the initial DOC and DOM content for at least 1 year (Figure 2 E). Moreover Fourrier et al.,
735 (2022) demonstrated that the determination of DOC in samples collected following a trace
736 metal clean protocol and kept frozen in HDPE bottles provide similar results (statistically
737 tested) to those from samples filtered on GF/F filters and stored in flame sealed ampoules.
738 However, it remains unclear if samples collected during a trace metal cruise (e.g.
739 GEOTRACES) and stored acidified can be used for the study of organics. To determine if long-
740 term storage of acidified samples in HDPE bottles alters DOC concentrations and DOM size
741 class distributions we analysed samples stored 2 years and half in HPDE bottles acidic
742 conditions and compared to those frozen in eleven samples collected all along the water column
743 in the Western Tropical South Pacific Ocean during the TONGA expedition. Results (Figure 3)

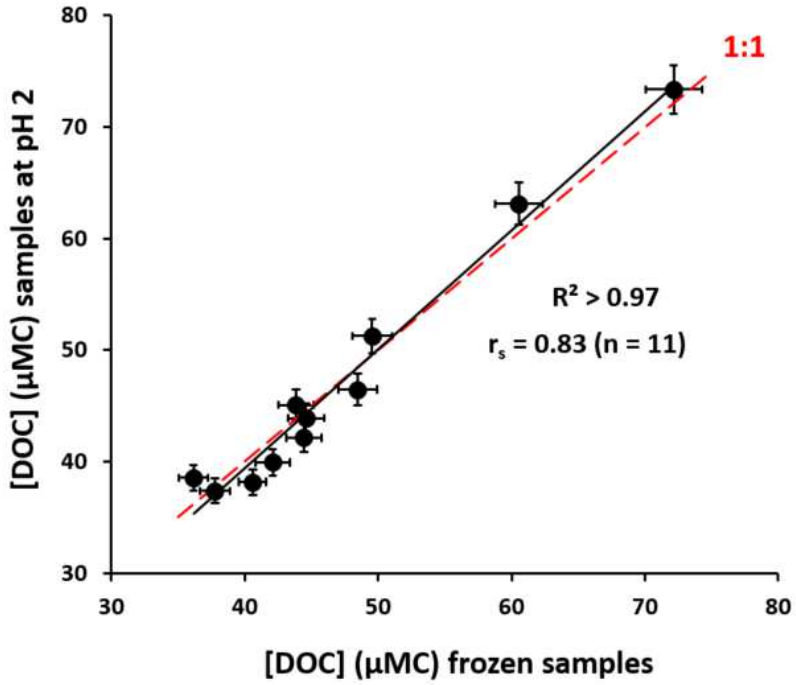

Figure 3. Comparison of dissolved organic carbon (DOC) concentrations (µMC) measured by SEC for a unique station of the TONGA campaign (2019) for two preservation modes (freezing and pH 2) of seawater samples in HDPE vials. r_s corresponds to the Spearman correlation coefficient.

demonstrate that DOC concentrations were statistically identical within paired samples at each depth ($R^2 > 0.97$; Spearman coefficient $r_s = 0.83$; n = 11). Moreover, the DOC measured in this acidified samples were also statistically identical to those measured in samples filtered on GF/F filters and stored acidified in flame-sealed glass ampoules ($R^2 > 0.97$; $r_s = 0.92$; n = 8). These results evidence that bulk DOC concentrations can be measured independently of the storage method tested here (freezing or pH 2 in HDPE bottle, acidified in flame-sealed glass ampoules). In both frozen and acidified samples were typical of those reported in the South Pacific gyre (Swan et al., 2009). Marked changes were however, for the size-class distribution of DOC after long-term conservation in acidic conditions (Figure 4).

In agreement with the previous tests presented for a surface coastal water, storage of samples by freezing at -20°C in HDPE vials DOC size class fractionation in the frozen sample can be considered as the reference or initial state (Figure 4A). The vertical shapes of the different DOC fractions are relatively identical along the water column between the two modes of conservation but the contribution of almost all the fraction to the global DOC is changing after a long-term storage at pH 2 (Figure 4B). The concentration of humic carbon to total DOC decreased by 11.5 ± 1.2 % at pH 2 along the water column (deep blue axis, Figure 4C). The loss was even more dramatic for the BP fraction with an average loss of 70% for this fraction (deep blue axis, Figure 4C). As described previously, acid hydrolysis or depolymerization of BP and HS could explain a decrease in their DOC concentrations over time. Organic carbon incorporates most abundantly into the LMW neutrals fraction (11.2 ± 1.4 % gain in contribution to total DOC, deep blue axis, Figure 4C) but BBs increase from a contribution to DOC of 9.6 ± 0.8 % to 13.6 ± 1.1 % at the studied station. The increase in concentration (2.0 ± 1.0 µMC, black axis) of the BBs fraction was of the same magnitude that of the loss of BP. This result reflects the incorporation of hydrolyzed carbon of HMW size fraction (BP) further supporting to consider this operationally define fraction as BP by-products (Fourrier et al., 2022). After two years of acidic storage the LMW acids fraction slightly increase (+ 17.1 ± 0.8 %, deep blue axis, Figure 4C). This increase does not seem to be significant both in the surface and in the deep water samples (Figure 4A&B). Interestingly both surface and deep samples were affected by long-term storage in acidic conditions (Figure 4), indicating that both labile and refractory DOC can be hydrolysed at pH 2. This experiment strengthen the choice of working with the frozen samples rather than in an acidified environment for the study of the size fractionation of oceanic DOC.

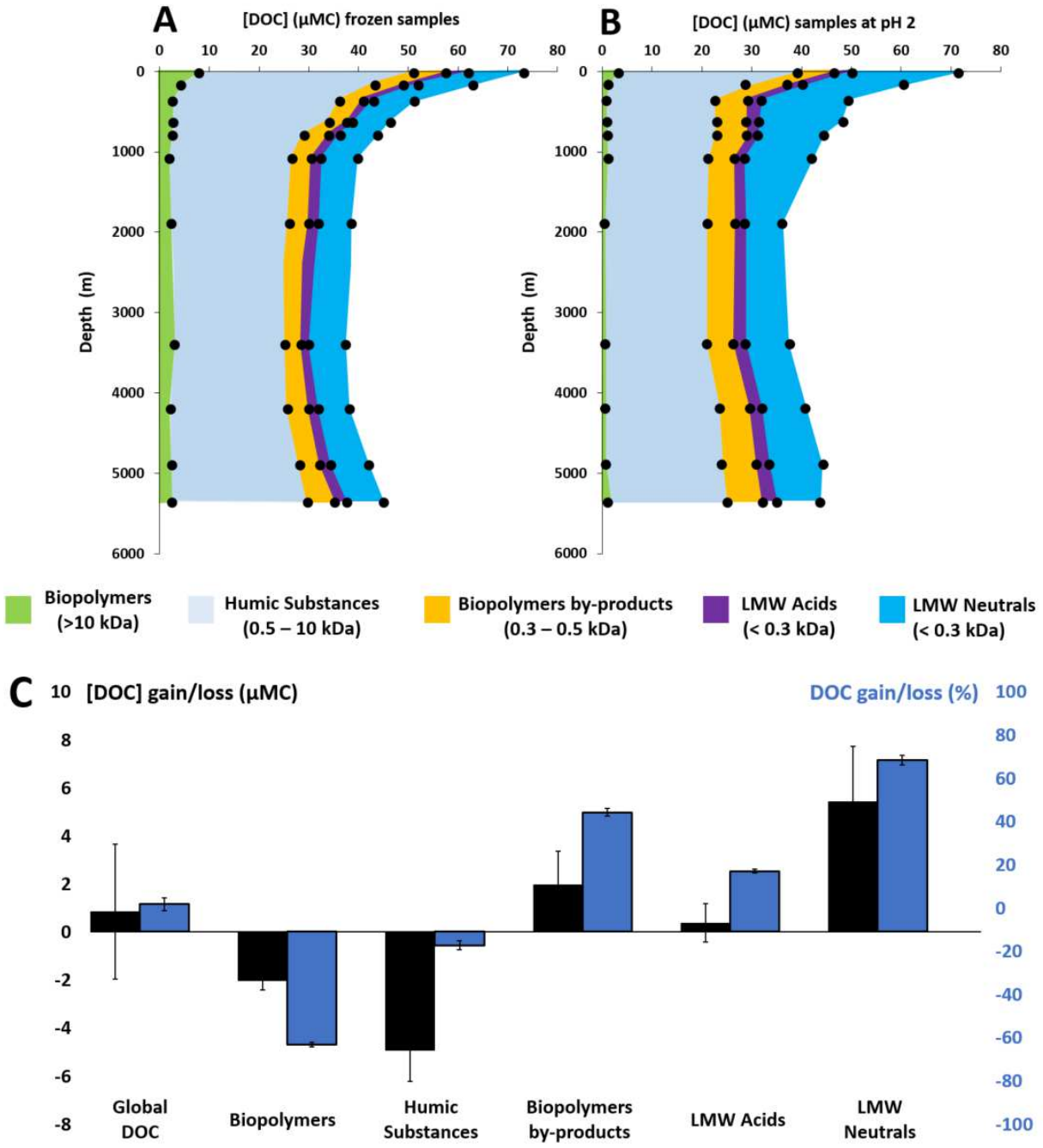

Figure 4. Conservation tests of DOC operational fractions defined by SEC for an unique station of the TONGA campaign (2019). These measurements were made two years after sample collection, conditioning and storage in HDPE vials. The vertical profiles (**A** & **B**) visualize the partitioning of DOC fractions (µMC) along the water column (m) and the diagram (**C**) estimates the average gain/loss of DOC in each fraction with regard to frozen samples along the water column (black axis in µMC and deep blue axis in %).

4. Conclusions

For bulk DOC concentrations measurements, acidified seawater samples (pH 2, HCl) can be analysed in the same way as a frozen sample in HDPE vials or a glass ampoules, without risking

an analytical bias and over the long term. Thereby our results demonstrate that a sample collected for trace metal determination and stored for years can be used for DOC measurement.

However, each DOC fraction comprising its own chemical properties, the acidification of the samples does not seem to be wise for an in-depth study of the size fractionation of DOC. We thus recommend the freezing for a study based on chemical properties of DOC. HDPE bottles has shown themselves as ideal containers, as no contamination were observed in DOC.

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