Combining benzalkonium chloride addition with filtration to inhibit dissolved inorganic carbon alteration during the preservation of seawater in radiocarbon analysis

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Abstract. Benzalkonium chloride (BAC) addition has shown great promise as a disinfectant for measuring $\delta^{13}C$ and ^{14}C of
dissolved inorganic carbon (DIC) in freshwater samples. However, it was reported that the effectiveness of BAC to prevent
10   DIC change was reduced for the use of seawater samples. The present study aimed to evaluate the effectiveness of adding
BAC as a disinfectant in carbon isotopic analyses of DIC in seawater samples. We compared the efficacy of BAC addition,
filtration (0.22 μm PTFE or 0.2–0.45 μm PES filters), and a combination of BAC addition and filtration in preventing DIC
alterations caused by biological activity. The combined procedure was effective in preserving seawater, although this
assessment was based on results from a single seawater sample. The ^{14}C concentration of samples treated with both BAC
15   addition and filtration exhibited minimal changes, ranging from 0.2–0.4 pMC over 41 weeks, despite the addition of sugar
included to increase biological activity several-fold. Although the complete elimination of biological effects may be
challenging with the combined method, the observed changes remained within practical limits. Concerns about CO_2
contamination during sample filtration were also addressed and found to be negligible. These results suggest that combining
filtration and BAC addition is an effective method for suppressing biological DIC alterations in ^{14}C analysis, even in seawater
20   samples.

1 Introduction

Radiocarbon (^{14}C) analysis of dissolved inorganic carbon (DIC) in seawater plays a vital role in the elucidation of seawater
circulation and atmospheric-ocean CO_2 exchange (Matsumoto, 2007; Mcnichol et al., 2022). For global understanding of ocean
water behaviors, it needs analysing samples from various regions over long timeframes, ensuring high-quality and consistent
25   analysis is crucial for maintaining data integrity and comparability across different oceans (Key et al., 2002; Anderson, 2020;
Olsen et al., 2020). Standard Operating Procedures (SOPs) for the analysis of seawater have been developed to define the
protocols and analytical methods necessary to meet these requirements (Dickson et al., 2007; Abrams, 2013). The SOPs
recommend that water samples collected for CO_2-related analyses such as DIC, total alkalinity, and CO_2 fugacity be treated
with a mercuric chloride ($HgCl_2$) solution to prevent biological activity that may alter the carbon distribution in the sample
30   container before analysis. However, the ecological toxicity of $HgCl_2$ poses significant challenges. Additionally, the use of
$HgCl_2$ in water samples can lead to uncertainties in the analytical results, as mercury interacts with dissolved organic matter
in the water, forming complexes that reduce the total alkalinity, potentially complicating the analysis (Mos et al., 2021).
Argentino et al. (2023) reported alternations in DIC concentration and $\delta^{13}C$ values in marine pore water samples from methane
seepage areas treated with $HgCl_2$. Given these environmental and practical concerns, alternative preservation methods that
35   avoid the use of mercury are increasingly desirable.

The methods have been proposed for the preservation of water samples without the use of $HgCl_2$. They include refrigeration,
filtration, and the addition of non-toxic or less toxic preservatives (Aucour et al., 1999; Doctor et al., 2008; Ascough et al.,
2010; Takahashi et al., 2019b; Wilson et al., 2020; Mos et al., 2021; Takahashi and Minami, 2022). Chemical sterilization

40 methods have been explored, such as adding acids or alkalis to prevent microbial activity in samples intended for the analysis of gases other than CO_2, such as methane (Magen et al., 2014). However, altering the pH of water samples is not suitable for DIC analysis, as DIC concentrations are highly sensitive to pH changes.

The addition of benzalkonium chloride (BAC) has shown great promise as a disinfectant for measuring $\delta^{13}C$ and ^{14}C of DIC in freshwater samples (Takahashi and Minami, 2022; Takahashi et al., 2019b), and for dissolved CH_4 concentrations in swamp water (Osaka et al., 2024). BAC is one of the quaternary ammonium compounds (QACs), a major product of cationic

45 surfactants, and is widely used as a disinfectant (Kuo, 1998; Mcdonnell and Russell, 1999). QACs penetrate cell membranes and disrupt both the physical and biochemical properties of cells (Gilbert and Moore, 2005; Wessels and Ingmer, 2013). As most of the bioavailable fraction of QAC in environmental waters can be reduced by sewage treatment plants (Deleo et al., 2020), the ecotoxicological hazard posed by QACs is far lower than that of mercury. Takahashi et al. (2019b) investigated alterations in DIC concentrations and $\delta^{13}C$ values in several natural waters (seawater, groundwater, river, pond, and brackish

50 waters) exposed to BAC and beet sugar for about 60 days. They observed that DIC concentrations and $\delta^{13}C$ values in freshwater samples remained unaltered throughout the preservation period. In contrast, salty water samples exhibited DIC changes exceeding the analytical error beyond 15 days. Takahashi and Minami (2022) performed a similar assessment of ^{14}C and DIC concentrations in seawater and groundwater. They observed constant ^{14}C and DIC concentrations after 30 days of preservation in groundwater samples, while seawater samples experienced increases in both ^{14}C and DIC concentrations over time. These

55 studies suggest a common trend: seawater samples treated with BAC remain unaltered for a few days but begin to show changes after one or two weeks. Gloël et al. (2015) examined the impact of BAC addition on the Ar/O_2 ratio in dissolved gases in seawater. They reported that seawater samples treated with BAC initially showed values identical to those preserved with $HgCl_2$ for the first 3 or 4 days, but changes emerged after 8–17 days. García et al. (2001) found that 50% of the primary biodegradation of BACs, including benzyl dimethyl tetradecyl ammonium chloride (BAC-C_{14}) and benzyl dimethyl hexadecyl

60 ammonium chloride (BAC-C_{16}), was completed by marine bacterial populations in 8 to >15 days, respectively. Although they did not identify the specific microorganisms responsible for this degradation, their findings align with the observation that BAC's effectiveness in seawater does not persist long term.

Gloël et al. (2015) noted that the factor diminishes the effectiveness of BAC in seawater over time is likely spores, that is resistant to heat and sterilization. They are highly durable cells that form and lie dormant when bacterial growth conditions

65 deteriorate. Then, as conditions improve because the effectiveness of BAC diminishes, possibly due to interaction with something in the seawater, they can resume growth. The process responsible for reducing BAC's efficacy is unclear, but previous studies mentioned above have suggested that it likely occurs 1–2 weeks after BAC addition to seawater samples. A key factor may be the presence of bacterial spores, but as spores exist universally in both seawater and freshwater (Brown, 2000), their presence alone may cannot fully explain the reduced effectiveness of BAC in seawater compared to freshwater.

70 However, a more practical approach would be to focus on removing the spores present in the water sample. Wilson et al. (2020) demonstrated that filtering water samples to 0.2 μm effectively preserves water for 66 days for $\delta^{13}C$ measurement of DIC. Spores are primarily produced by aerobic *Bacillus* species and anaerobic *Clostridium* species (Brown, 2000), and these rod-shaped bacteria range from approximately 0.2–1 μm in diameter and 1–10 μm in length. As spores are relatively large, they can be easily removed by filtration.

75 This study aimed to determine an effective method for preventing DIC changes caused by biological activity in seawater samples using BAC addition. We evaluated the effectiveness of BAC treatment alone, filtration alone, and the combined use of BAC and filtration for measuring $\delta^{13}C$ and ^{14}C of DIC in both freshwater and seawater samples.

2 Materials and procedures

2.1 Natural water samples

80   This study used two types of water: seawater (SW) and groundwater (GW). The SW was collected from the sea surface of the Pacific coast at the Nagoya Port, located in Nagoya City, Aichi Prefecture, Japan. This site is located near the estuaries of three rivers: the Nikko, Shinkawa, and Shonai rivers, in a tidal flat region. The chemical composition of the SW (Table S1) indicated that the SW was slightly diluted by river water. While SW can be considered as a brackish water sample, but it is treated as a coastal seawater sample in this study. The GW was obtained from a well of 80 m deep in Tsukuba City, Ibaraki Prefecture,

85   Japan (Table S1). The DIC and ^{14}C concentrations of groundwater taken from the same well were reported to be 20.3 mg·L^{-1} and 21.3 ± 0.1 percent Modern Carbon (pMC, Stuiver and Polach, 1977), respectively (Takahashi et al., 2019a; Takahashi and Minami, 2022). For the SW, the expected values of DIC and ^{14}C concentrations were approximately 24 mg·L^{-1} and ~100 pMC, respectively. At the time of sampling, neither disinfectant treatment nor filtration was applied to any of the natural water samples.

90   To promote microbial activity and detect even minor changes in DIC that might result from biological processes, beet sugar powder was added in the sample water at a concentration of 2 g·L^{-1} before the preservation of the sample. As beet sugar contains a high ^{14}C concentration of 103.3 ± 0.7 pMC (Takahashi and Minami, 2022), any ^{14}C changes resulting from the microbial decomposition of beet sugar to DIC might be undetectable in SW samples because of the similar ^{14}C levels between beet sugar and SW. To address this, NaHCO$_3$ solution (1 mol·L^{-1} solution, Kanto Chemical Co. Inc., Japan), which has a low

95   ^{14}C concentration of ~0.7 pMC and a high δ^{13}C value of −3.8‰ (Takahashi et al., 2021), was added to the samples at a rate of 2.5 mL·L^{-1} of SW and 2 mL·L^{-1} of GW, effectively doubling the DIC concentration in both water types. The addition of NaHCO$_3$ also helped to clarify the δ^{13}C changes in DIC due to beet sugar decomposition, as beet sugar exhibits a low δ^{13}C value of −26.2‰ (Takahashi and Minami, 2022). Unless otherwise stated, beet sugar and NaHCO$_3$ solution were added to all water samples.

100   ### 2.2 ^{14}C concentration and δ^{13}C measurements

CO$_2$ extraction from water samples for the measurement of ^{14}C concentration and δ^{13}C values was performed using the ReCEIT method (Takahashi et al., 2021). This method, which extracts CO$_2$ without bubbling, is particularly well-suited for BAC-added samples, which tend to foam. An approximate DIC concentration was calculated from the volume of water treated and the CO$_2$ extracted. The CO$_2$ gas was reduced to graphite (Kitagawa et al., 1993) for analysis by accelerator mass spectrometry (AMS),

105   following the removal of sulfide gas using the Sulfix reagent (8–20 mesh, Kishida Chemical Co., Ltd., Japan) as necessary. The ^{14}C concentrations were measured using a 3 MV AMS (Model 4130-AMS, HVEE, Netherlands) at the Institute for Space–Earth Environmental Research, Nagoya University, Japan (Nakamura et al., 2000) and a 1 MV AMS (4110Bo-AMS-3, HVEE, Netherlands) at the Korea Institute of Geoscience and Mineral Resources (KIGAM), Korea (Hong et al., 2010). Corrections for isotopic fractionation were performed using the ^{13}C/^{12}C ratio measured by AMS. The standard deviations for ^{14}C

110   measurements were 0.02–0.04 pMC for waters with concentrations below 1 pMC, and less than 0.8% of ^{14}C concentration for waters above 10 pMC when measured at the Nagoya University. The precision of the quantitative analysis of carbon was better than 3%, and the background was below 2 × 10^{-15} at KIGAM. The δ^{13}C values of CO$_2$ gas extracted by the ReCEIT procedure were determined via isotope-ratio mass spectrometry (IRMS) with a dual inlet system (Delta-V Advantage, Thermo Fisher Scientific, Inc., USA) at the Geological Survey of Japan.

115   ### 2.3 Filtration and BAC addition

Filtration can introduce microbubbles, potentially leading to gas dissemination or atmospheric gas exchange. However, contamination, rather than gas dissemination, is the primary concern in the ^{14}C analysis. The potential for contamination during

filtration was investigated by comparing the ^{14}C concentrations of NaHCO$_3$ solutions before and after filtrations. The assessments were carried out using a polyether sulfone (PES) disk filter (25 mm diameter, 0.22 µm pore size), and a glass fiber

120 (GF) disk filter (25 mm diameter, 1.0 µm pore size) attached to the syringe. These samples were designated NaHCO$_3$-unfiltered, NaHCO$_3$-PES, and NaHCO$_3$-GF, respectively. Filtration was performed under atmospheric conditions, exposing the sample to atmospheric CO$_2$. The filtrates from the PES and GF filters were collected in beakers and injected into reaction containers for the ReCEIT method. PES and PTFE filters have relatively high filtration resistances, increasing filtration time, while GF filters allow faster water passage, potentially reducing filtration time.

125 To compare the efficacy of the various treatments, the following six was evaluated: (1) no filtration, no BAC addition (Control), (2) BAC addition alone (BAC), (3) filtration through a PTFE filter (PTFE), (4) filtration through a PES filter (PES), (5) PTFE filtration with BAC addition (PTFE+BAC), and (6) PES filtration with BAC addition (PES+BAC). Changes in ^{14}C and δ^{13}C values across three preservation periods were compared for SW and GW samples treated with these methods. SW and GW were preserved in 125 mL glass vials sealed with butyl rubber septa coated with Teflon, and aluminium caps for 14, 28, and

130 285 days, and 14, 28, and 126 days, respectively. Water was injected into pre-evacuated glass vials using a needle attached to a syringe. In cases where filtration was tested, a disk filter was additionally attached to the syringe. The vials and butyl rubber septa were sterilized by heating at 450°C for 6 h and by autoclaving, respectively. BAC (10% solution, FUJIFILM Wako Pure Chemical Co., Japan) was added to the water samples at a concentration of 0.01% before injection into the vials. The BAC used in this study primarily consisted of benzyl dimethyl dodecyl ammonium chloride (BAC-C$_{12}$) and benzyl dimethyl

135 tetradecyl ammonium chloride (BAC-C$_{14}$).

Filters for treatments (3) and (4) were pre-washed with preserved water but not sterilized. Thus, additional assessments were conducted using sterilized PES disk filters (25 mm diameter, 0.2 µm and 0.45 µm pore sizes, GVS Japan). GW samples, with or without filtration, were preserved in 34 mL glass vials for 6, 14, and 28 days. These treatments were labelled GW-Control2, GW-PES2 (0.2 µm), and GW-PES2 (0.45 µm), respectively. δ^{13}C values from initial water samples and preserved samples

140 were compared for three different periods, using randomly selected vials measured by GC-IRMS (Delta-V Advantage with Gas Bench II, Thermo Fisher Scientific, Inc., USA) at the Geological Survey of Japan (Takahashi et al., 2019b). As these samples were not analyzed for ^{14}C, NaHCO$_3$ solution to lower the initial ^{14}C concentration was not added. Other procedures were same as treatments (3) and (4).

3 Results and discussion

145 **3.1 Background on the ^{14}C concentration and δ^{13}C in the filtration treatment process**

The ^{14}C concentrations of NaHCO$_3$-unfiltered, NaHCO$_3$-PES, and NaHCO$_3$-GF were consistent within the error range (Fig. 1, Table S2). This suggests that any increase in ^{14}C due to CO$_2$ contamination during filtration was not a significant concern. In contrast, the δ^{13}C values showed a very slight decrease for NaHCO$_3$-PES and NaHCO$_3$-GF (Fig. 1, Table S2). This slight change in δ^{13}C is assumed to be caused by atmospheric CO$_2$ contamination or CO$_2$ degassing from the NaHCO$_3$ solution during

150 filtration. If atmospheric CO$_2$ contamination caused the δ^{13}C shift in the NaHCO$_3$ solution, the ^{14}C concentration would vary according to the amount of atmospheric CO$_2$ contamination. Assuming atmospheric CO$_2$ has a δ^{13}C value of −10‰ and a ^{14}C concentration of 100 pMC, the ^{14}C concentrations of NaHCO$_3$-PES and two NaHCO$_3$-GF could be calculated as 3.6 ± 0.2 pMC, 4.5 ± 0.2 pMC, and 2.4 ± 0.2 pMC, respectively. These calculated values do not match the measured ^{14}C concentrations, indicating that the change in δ^{13}C is not caused by atmospheric CO$_2$ contamination.
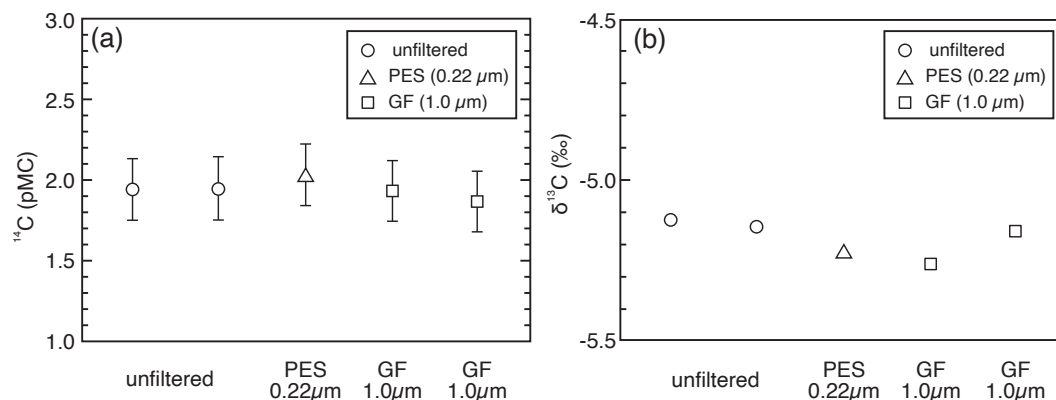
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Figure 1: Comparisons of ^{14}C (a) and δ^{13}C (b) among the unfiltered and filtered solutions of 1 mmol·L^{-1} of NaHCO$_3$. PES: filterd by PES disk filter (25 mm in diameter, 0.22 μm in pore size), GF: filtered by GF disk filter (25 mm in diameter, 1.0 μm in pore size).

The δ^{13}C of DIC would change due to isotope fractionation associated with degassing. When DIC and gaseous CO$_2$ are in isotopic equilibrium, the δ^{13}C of DIC is typically higher than that of gaseous CO$_2$ (Zhang et al., 1995). As carbon with a lower δ^{13}C value is removed as CO$_2$ during degassing, the δ^{13}C of the remaining DIC in the solution would gradually increase. However, the measured δ^{13}C showed the opposite trend, indicating that the change in δ^{13}C is not due to CO$_2$ degassing from the NaHCO$_3$ solution. Thus, the two predictions—that δ^{13}C changes were caused by atmospheric CO$_2$ contamination or by degassing—were rejected. Therefore, it was concluded that the δ^{13}C change is not due to filtration but rather to some other unidentified process.

As the filtration in this assessment was performed under atmospheric conditions with CO$_2$ exposure, it was likely to cause carbon contamination. However, the identical ^{14}C concentrations (Fig. 1) suggest that a ^{14}C increase due to CO$_2$ contamination during filtration should not be considered a concern. Nonetheless, depending on the filter material or pore size, the water sample may not pass through unless the syringe is pressed forcefully, which can lead to contamination by the atmospheric CO$_2$ inside the syringe. When filtration was performed with a 1 mmol·L^{-1} NaHCO$_3$ solution and an equal volume of air inside the syringe using a PES filter (0.22 μm), the ^{14}C concentration of the NaHCO$_3$ solution was measured to increase by 0.7 pMC, rising to 4.6 pMC from an initial 3.9 pMC in our assessment. This ^{14}C increase is quantitatively reasonable, assuming a CO$_2$ concentration of 400 ppm and that the CO$_2$ inside the syringe fully dissolved into the NaHCO$_3$ solution. It is important to remove air bubbles in the syringe at the filtration.

3.2 ^{14}C concentration and δ^{13}C changes in natural water samples

The initial values of DIC concentrations, ^{14}C concentrations, and δ^{13}C values for SW mixed with NaHCO$_3$ solution were 43.2–43.8 mg·L^{-1}, 41.2–42.2 pMC, and −4.05 to −3.72‰, respectively (Table S3). For GW, these values were 53.7–59.1 mg·L^{-1}, 10.2–10.9 pMC, and −6.00 to −5.98‰ when mixed with NaHCO$_3$ solution, and 22.2–22.4 mg·L^{-1} and −7.74 to −7.69‰ when not mixed with NaHCO$_3$ solution (Table S3), respectively. After mixing with NaHCO$_3$, the ^{14}C concentrations in both SW and GW were approximately half or slightly less than half of their original concentrations.

The largest changes in ^{14}C and δ^{13}C during the preservation period were observed in the Control samples, with progressively smaller changes occurring in the order of filtration-only samples, BAC-only samples, and those treated with both filtration and BAC. The ^{14}C concentrations increased as the preservation period lengthened for SW-Control, GW-Control, SW-PES, GW-PES, SW-PTFE, GW-PTFE, and SW-BAC (Fig. 2). It is reasonable to assume that these large changes in ^{14}C concentration and δ^{13}C were caused by the DIC derived from beet sugar, given that beet sugar is more easily degraded. Given that DIC change during the preservation was enhanced by the incorporation of sugar, it is imperative to ascertain the impact of sugar

addition. It is anticipated that the effect will be more pronounced in instance where there is a paucity of organic matter and a greater prevalence of microorganisms in the water sample. The SW in this study was sampled in location at the tidal flat along

190 the Pacific coast, near the estuaries of major rivers. It can be reasonably assumed that water discharged from tidal flats will have higher concentrations of organic carbon, nutrient salts, and microbes than typical seawater (Sakamaki et al., 2006; Hu et al., 2016). Accordingly, the boost effect of SW in this study may be identical to or slightly smaller than 3.0 ± 1.4, as reported by Takahashi and Minami (2022) for the seawater sample sampled at Kashima Port on the Pacific coast. This seawater was not mixed with river waters and was not sampled from the tidal flat. The boost effect of groundwater sampled from the same

195 well as the GW was reported to be 5.3 ± 1.8. As the exact boost effect is not measured in the present study, the increase in DIC due to sugar addition was not corrected through calculation. It is important to note that the described DIC change includes an increase of probably two or three times for SW and five times for GW caused by the addition of sugar.
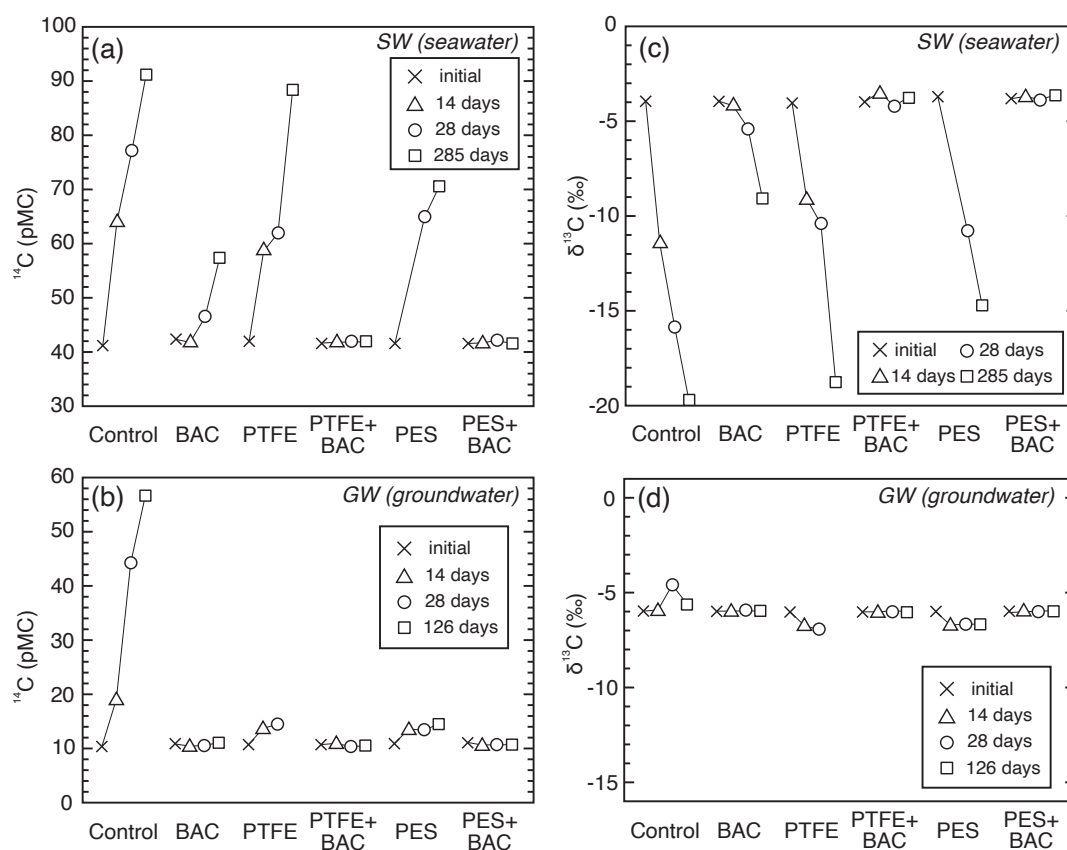
200 **Figure 2: Changes in ^{14}C and δ^{13}C during the preservation of SW and GW mixed with NaHCO$_3$ solution and beet sugar. Changes in DIC were augmented by the addition of beet sugar. (a) ^{14}C of SW, (b) ^{14}C of GW, (c) δ^{13}C of SW, (d) δ^{13}C of GW.**

3.3 Comparison of treatments

Filtration, BAC addition, and the combined treatment reduced the changes in DIC during preservation (Fig. 2). However, in

205 some cases, changes could not be completely reduced, depending on the type of treatment or the water used. In samples treated with BAC alone, the results matched those of previous studies (Takahashi et al., 2019b; Takahashi and Minami, 2022), where GW showed suppression of DIC changes, but SW showed changes of 15.1 pMC in ^{14}C concentration and -5.2‰ in δ^{13}C. This

confirms that BAC alone is not suitable for seawater samples and indicates that the seawater sample utilized in this investigation contains constituents, probably unique microorganisms, whose biological activity cannot be entirely suppressed by BAC addition, as reported in previous studies. In other words, if a treatment approach that negligible of the alteration in DIC using this sample can be identified, it is insight into the development of an efficacious procedure. Regardless of BAC addition, all treatments showed that GW exhibited smaller DIC changes than SW. The ^{14}C concentrations and δ^{13}C values were observed to be relatively constant in samples treated with both filtration and BAC: SW-PTFE+BAC, SW-PES+BAC, GW-BAC, GW-PTFE+BAC, and GW-PES+BAC. The ^{14}C and δ^{13}C changes were minimal, ranging from 0.4 to 0.6 pMC and −0.25 to +0.36‰ for SW-PTFE+BAC, 0.2 to 0.7 pMC and −0.01 to +0.19‰ for SW-PES+BAC, −0.4 to +0.1 pMC and +0.03 to +0.04‰ for GW-BAC, −0.1 to +0.4 pMC and +0.02 to +0.03‰ for GW-PTFE+BAC, and −0.4 to −0.1 pMC and +0.02‰ for GW-PES+BAC (Table S3).

The ^{14}C concentration and δ^{13}C value of filtered waters without BAC addition showed significant changes, although they were smaller than those in unfiltered samples. The ^{14}C changes of SW-Control, SW-PTFE, and SW-PES were 50.0 pMC, 46.4 pMC, and 29.2 pMC, respectively, while those of GW-Control, GW-PTFE, and GW-PES were 46.1 pMC, 18.3 pMC, and 3.6 pMC, respectively. DIC changes were smaller with PES filtration than PTFE for both SW and GW, despite using the same pore-size filter. This may be related to the fact that PTFE has higher resistance than PES, requiring more force during filtration.

The changes in δ^{13}C of the GW-PES2 as the sample filtered through a sterile filter were consistent with those of GW-Control2 as the unfiltered sample, except for a slight change of GW-PES2 (0.2 µm) after 6 days (Table 1). This suggests that DIC changes of the filtered samples shown in Fig. 2 were not caused by the microorganisms derived from the filter. While using a 0.2 µm filter reduces the number of microorganisms compared with a 0.45-µm filter, once some slip through, the difference between filters may disappear as the microorganisms proliferate. Wilson et al. (2020) reported that filtration alone is sufficient to prevent DIC changes due to its biological activity. However, our results showed that this method was insufficient, as DIC changes could not be ignored for SW-PTFE, SW-PES, and GW-PES2 (0.2 µm) with preservation periods longer than 14 days, although this study confirmed that filtration reduces DIC changes in water samples during preservation (Fig. 2, Table 1). This was especially the case for GW-PES2 (0.45 µm) samples, where sugar addition increased biological activity. Sugar addition may have artificially triggered microbial growth, resulting in DIC changes that would not have occurred otherwise. Without microbial growth triggers, filtration may be more effective, but DIC changes were smaller with SW-BAC compared to SW-PTFE or SW-PES. Therefore, BAC addition is more effective than filtration alone in reducing DIC changes, although it has the disadvantage of not being able to use the sample for other analyses.

Table 1: Initial values of DIC concentrations and δ^{13}C values for GW-Control2, GW-PES2 (0.2 µm), and GW-PES2 (0.45 µm), and the changes in δ^{13}C values during the preservation.

sample	DIC initial (mg·L⁻¹)	δ^{13}C initial (‰)	δ^{13}C change from initial (‰)		
			6 days	*14 days*	*28 days*
GW-Control2	22.4	−7.74 ± 0.06	−1.88 ± 0.13	−2.15 ± 0.73	−2.07 ± 0.60
GW-PES2 (0.2 µm)	22.2	−7.70 ± 0.07	−0.06 ± 0.08	−2.05 ± 0.08	−1.99 ± 0.24
GW-PES2 (0.45 µm)	22.2	−7.69 ± 0.10	−2.28 ± 0.47	−2.09 ± 0.12	−2.20 ± 0.41

3.4 Combined treatment of BAC addition and filtration

The combined treatments, PTFE+BAC and PES+BAC, showed consistent ^{14}C concentrations within the analytical error during preservation for both SW and GW. Though slight δ^{13}C changes were observed in SW, but this δ^{13}C change seems to be negligible given the uncertainty and the change to be extremely small that it only became detectable through sugar-induced microbial activity magnification.

245   One possible explanation for the minimal DIC changes in the combined treatment may be due to effectiveness of BAC was
       enhanced by reducing the number of microorganisms through filtration. This explains why no DIC changes were observed in
       SW-PTFE+BAC and SW-PES+BAC during the preservation period. In contrast, the DIC change observed in SW-BAC may
       be caused by BAC being insufficient against the number of microorganisms present, however, the reason why the change was
       observed during the only second half of the preservation period cannot be explained. If microorganisms are killed by BAC,
250   reactivation should not occur in the second half of the preservation period. As mentioned in the Introduction, it has been
       suggested that the lower effectiveness of BAC in seawater may be due to spores (Gloël et al., 2015), that cannot be effectively
       inactivated by BAC. They are not significantly different in size from rod-shaped bacteria, the main spore-forming
       microorganisms (Brown, 2000), and range in size from 0.6–4 μm (Reponen et al., 2001). These larger microorganisms are
       likely to be removed by filtration. Our assessment indicated that filtration alone might allow some microorganisms to pass
255   through, but BAC can inactivate the vegetative cells of small microorganisms. Filtration removes larger spores that may cause
       DIC changes in seawater. The role of spores has not been fully verified, but this could explain why DIC in SW-BAC remained
       unchanged until 14 days and changed after 28 days.

       Certain bacteria can degrade QACs (García et al., 2001; Patrauchan and Oriel, 2003; Zhang et al., 2011; Oh et al., 2013). If
       the water sample contained microorganisms capable of degrading BAC, biological activity would not be inhibited, leading to
260   DIC changes. While BAC may kill most microorganisms, BAC-tolerant microorganisms could survive and recover, causing
       detectable DIC changes. This could explain the DIC change observed in SW-BAC. If BAC-tolerant microorganisms were
       removed by filtration, it would align with the lack of DIC changes in SW-PTFE+BAC and SW-PES+BAC. Microorganisms
       reported to adapt to BAC and cause biodegradation include *Pseudomonas* spp., *Aeromonas hydrophila*, *Salmonella enterica*,
       and *Klebsiella oxytoca* (Ferreira et al., 2011; Khan et al., 2015; Cui et al., 2023). These species are commonly found in aquatic
265   environments. These microorganisms are not originally tolerant to BAC but gradually adapt over long periods, such as several
       tens of days of exposure (Oh et al., 2013; Yang et al., 2023). Preservation in sealed vials without aeration for DIC analysis is
       unlikely to permit the adaptation of microorganisms to BAC due to insufficient exposure time. Thus, for BAC biodegradation
       to occur, microorganisms must be initially tolerant of BAC in the water sample. QACs in water are removed by microbial
       communities tolerant to them, often found in sewage treatment plants (Zhang et al., 2015; Deleo et al., 2020). Previous
270   biodegradation studies have isolated microbial communities from enriched cultures grown on BAC-based media or activated
       sludge (Chacón et al., 2023). Therefore, it cannot be ruled out that microorganisms tolerant to BAC might be present in estuary
       and coastal waters where sewage effluents mix. However. degradation of BAC, a refractory organic compound, only begins
       after readily decomposable organic matter is fully consumed (Zhang et al., 2011). If BAC-tolerant microorganisms were
       present, beet sugar would be consumed first, and the ^{14}C and δ^{13}C of DIC in preserved water samples would reflect beet sugar
275   more closely than BAC. Even in this case, the lack of DIC changes in the SW-PTFE+BAC and SW-PES+BAC samples
       indicates that filtration is effective at removing such microorganisms. When combining filtration and BAC addition, avoiding
       contamination with ambient carbon (atmospheric CO_2) during filtration is essential. A blank check with $NaHCO_3$ demonstrated
       that the ^{14}C background remained unchanged, confirming that filtration is a viable process when the necessary precautions are
       properly followed. As a consequence of ongoing technological advancements, the quantity of carbon required for ^{14}C
280   measurement by AMS is progressively decreasing (Minami et al., 2013; Ruff et al., 2010). The reduction in sample size
       facilitates filtration and minimizes background contamination, representing a favorable development for this combined
       procedure.

4 Conclusions

       This study assessed several treatments aimed at suppressing changes in DIC during sample preservation as an alternative to
285   HgCl₂ addition. We found that the combination of filtration and BAC addition effectively inhibited DIC changes due to

microbial activity during preservation. In water samples treated with this method, DIC changes were minimal, even when sugar was added to significantly enhance microbial activity. In practical analyses, such a boost from adding sugar would not occur, leading to the conclusion that the combined method of BAC addition and filtration is an effective procedure for inhibiting DIC changes caused by biological activity. The slight changes in DIC observed in BAC-supplemented seawater

290   samples may be attributed to microorganisms that BAC could not inactivate. They are presumed to be spores or BAC-tolerant microorganisms. The size of spores and spore-forming microorganisms varies among species, but they are generally large enough to be removed through filtration. Many microorganisms that adapt to BAC are also relatively large and can be removed by filtration.

DIC changes in seawater samples can be suppressed through a two-step process: first, filtration to remove spores or

295   microorganisms tolerant to BAC (if present), followed by BAC treatment to inactivate microorganisms that passed through filtration. It should be noted that smaller microorganisms may still pass through the filtration system. In some cases, the partial removal of microorganisms through filtration may not fully suppress the DIC changes, leading to microorganism recovery within a few days, resulting in DIC changes similar to those in the unfiltered samples. Therefore, careful consideration of the preservation period is necessary when using filtration alone to suppress DIC changes.

300   We recommend a combined treatment of filtration and BAC addition to suppress DIC changes during sample preservation, as it offers a safer alternative to $HgCl_2$. In this study, a 0.22 µm pore size filter was used to validate earlier findings. However, it is likely that a filter with a coarser pore size could also remove spores, given their size. Verifying the optimal pore size is an important next step. As observed in this study, using a very fine pore-size filter can slow sample flow, increase resistance, impair operability, and elevate blanks. Future research should include blank verifications of the combined technique and

305   further verification of whether the slight DIC change observed here can be detected in other water samples. It should also be noted that BAC in water may be removed primarily by adsorption onto sludge rather than by biodegradation (Zhang et al., 2015). Our preliminary result showed that the bactericidal efficiency of BAC was likely diminished in water sample containing muddy sediment. While filtration can remove sludge or sediment, caution is needed when applying the combined treatment to water samples containing large amounts of suspended material. In such cases, increasing the amount of BAC may be necessary

310   for effective treatment. The assessment of this combined procedure was conducted on a limited number of natural water samples, and further investigation into the optimal filter pore size and verification using other natural water samples is necessary. However, this procedure offers a practical and environmentally friendly alternative to conventional mercury-disinfected methods for water sample preservation in aquatic environments.

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