Dear Mario:

We have revised the manuscript considering the comments made by the referees. Our response to the referees is coloured in blue; the resulting changes in the paper are highlighted in red.

We are submitting the revised manuscript including a version with tracked changes so that it is clear what amendments we have made (including a few language clarifications).

Best regards
Jan Kaiser on behalf of all authors

Response to comments by Referee #1

We would like to thank the anonymous referee for their comments. The comments of the referee are reproduced below in black font colour; our response is in blue.

The authors tested if BAC is a suitable alternative chemical for the preservation of seawater samples for the measurement of oxygen to carbon ratios. They concluded that this is possible for the preservation of samples with low Chl a up to 3 days.

We are determining the oxygen-to-argon ratios, not oxygen-to-carbon ratios (see also the 4th comment-reply pair below). We concluded that BAC *could* be a suitable alternative to $HgCl_2$, but not without further testing because its efficacy may depend on the Chl a concentration and the composition of the microbial community.

We have revised the abstract to make it clear that our results apply to the samples tested, but not necessarily to other seawater samples. This now reads: "BAC at a concentration of 50 mg dm^{-3} inhibited microbial activity for at least three days in samples tested with chlorophyll a (Chl a) concentrations up to 1 mg m^{-3}." (lines 20-22).

The advantage of BAC compared to $HgCl_2$, which is commonly used, is that it is less hazardous and the disposal of the waste is less expensive. This might be a small advantage but the careful use of $HgCl_2$ is not dangerous for an experienced person.

We entirely agree that $HgCl_2$ is not dangerous to experienced users; we routinely use it ourselves. However $HgCl_2$ does pose a hazard to the environment and therefore indirectly to human health. Mercury bioaccumulates in the food chain and has long environmental persistence. Adverse health effects due to mercury intake through the food chain have been documented in animals and humans (cf. Minamata disease). We therefore believe it is important to investigate alternative, less environmentally hazardous preservatives. The environmental and health issues have also been recognised by Referee #2.

We have rephrased the second sentence of the abstract as "Mercuric chloride ($HgCl_2$) is routinely used for such preservation, but its handling and subsequent disposal incur environmental risks and significant expense." (l. 15-14)
We have also replaced "during handling" in the conclusions with "and the environment" to make it clear where the greatest risks lie. This now reads "Therefore, BAC, which poses fewer risks to human health and the environment and does not require expensive waste disposal, could be used as a viable alternative to $HgCl_2$ for short-term preservation of samples prior to MIMS analysis." (l. 240)

The preservation of seawater samples with $HgCl_2$ is more reliable especially when you are not sure that samples can be analyzed within the short time frame. The authors themselves recommend "further tests with BAC on a case basis because of cross-reactions especially under higher Chl a concentrations." It is unrealistic that you can test during field studies which method would be the best. Then you need to know, for example, the Chl a and nutrient concentration. Therefore, you have to use the safest method which is $HgCl_2$ preservation.

We agree with the referee that $HgCl_2$ is more reliable than BAC, and we state this in the paper. BAC did not prove to be an effective replacement for $HgCl_2$ and for the time being, there is no alternative to the use of $HgCl_2$ for our purposes.

No changes necessary.

I don't think that it is necessary to publish this technical study as a full paper. It is helpful as a discussion paper, and the method may be briefly explained if it is used for a scientific study of the oxygen to carbon ratio.

We think that it is important to publish this technical comment. There is a strong motivation to replace $HgCl_2$ with more environmentally benign alternatives, in particular in remote, sensitive and pristine environments such as the polar regions. We have shown that BAC is unlikely to be useful for long-term storage, but that there may be situations where it be used instead of $HgCl_2$. These are important conclusions and the comments from the referees have led to improvements of the paper that will be incorporated in the revised version we intend to submit. We were not measuring oxygen-to-carbon ratios, but oxygen-to-argon ratios.

We have added the sentence "There is therefore a strong motivation to find less hazardous alternatives" (l. 17) to the abstract and the sentence "These are strong incentives to find more environmentally benign alternatives to the use of $HgCl_2$, in particular in remote, sensitive and pristine environments such as polar regions." to the end of the first paragraph of the introduction (l. 48-49).

Overall, the study was well performed and the paper is clearly written.

Thank you.

No changes necessary.

It would be helpful to know more about the samples such as nutrient concentrations, the influence of salinity because the method may be better used in estuarine and coastal regions which are closer to the lab.

This is a very good point, and will be useful for potential users of BAC to know and complement the information provided in Table 1 one of the paper. See table below for salinities (Fishwick, 2014) and nutrient concentrations (Woodward et al., 2015) at the time of sampling at L4.

Date	*S*	$c(NO_3^-+NO_2^-)$ / µM	$c(SiO_4^{4-})$ / µM	$c(PO_4^{3-})$ / µM
8 Feb 2010	34.90	8.6	4.8	0.6
19 Apr 2010	35.06	3.3	0.5	0.3
17 May 2010	35.04	0.1	0.4	0.1

We have added the above information on salinities and nutrient concentrations to Table 1.

It would be good to know if bacterial cells are inactive and dead by treatment with $HgCl_2$.

We are testing whether $HgCl_2$ and BAC halt oxygen consumption by the cells, and we clearly show that treatment with $HgCl_2$ halts oxygen consumption by the cells. Therefore to the extent that oxygen consumption can be related to this, the cells are inactive or dead after treatment with $HgCl_2$.

No changes necessary.

The dilution effect can be simply calculated and don't need to be assumed.

Thank you for this suggestion. For the BAC×4 experiments, 1 cm^3 of BAC solution was added to 0.5 dm^3 of sample. We cannot calculate the dilution effect because we did not measure the oxygen concentration of the BAC solution. However, we note that in order to explain the initial 0.2 % drop in $c(O_2)$, the oxygen concentration of the BAC solution would have to have been near 0 which is unlikely. We therefore cannot fully explain the decrease in oxygen concentration associated with the BAC×4 addition. However, this does not invalidate any of our conclusions because, after this initial drop, the BAC×4 time series shows the same relative trend as with respect to the initial concentration as the BAC and BAC×2 time series.

We have added these considerations to the text, which now reads "This may be partially due to a dilution effect caused by the larger volume of BAC solution used, but in order to explain the initial 0.2 % drop in $c(O_2)$, the oxygen concentration of the BAC solution would have to have been near 0, which is unlikely. We therefore cannot fully explain the decrease in oxygen concentration associated with the BAC×4 addition. However, this does not invalidate any of our conclusions because, after this initial drop, the BAC×4 time series shows the same relative trend with respect to the initial concentration as the BAC and BAC×2 time series." (l. 189-194).

The use of SI units (cm^3, dm^3, etc.) is of course correct but quite unusual for this kind of papers.

The manuscript preparation guidelines of Ocean Science recommend that "wherever possible, SI units should be used."

No changes necessary.

There are some other uncertainties as also mentioned by the authors which would be good to be tested.

Based on our experiments, we rule out BAC as a universal alternative to $HgCl_2$, in particular for applications where long-term storage is required such as for oxygen triple isotope analyses. Other researchers may want to explore the use of BAC further, but – as stated in the paper – we would recommend further testing under the relevant conditions. This is outside the scope of the present paper.

No changes necessary.

Response to comments by Referee #2

We would like to thank the anonymous referee for their comments. The comments of the referee are reproduced in black font colour; our response is in blue.

The main objective of this study, which is written in the form of a technical note, is to test if Benzalkonium chloride (BAC) was as effective as Mercuric chloride ($HgCl_2$) in the prevention of microbial activity. Microbial activity was evaluated as production/consumption of O_2 during short term incubations. O_2 time course experiments were monitored by O_2/Ar ratios with membrane inlet mass spectrometry (MIMS). Authors worked with natural samples that were collected at different times and had different autotrophic and heterotrophic compositions"

The reasons to replace $HgCl_2$ with BAC are extremely relevant from an ecological, environmental and health point of view, and I agree with the authors that it is necessary to reduce the use of $HgCl_2$ due to its toxic nature.

Thank you.

No changes necessary.

However, in order to instigate changes in the accepted methodology established by the scientific community, a new technique/method must be presented with irrefutable evidence, and my opinion is that this research is still very limited and does not provide reliability. There are several reasons for this conclusion:

We would agree with the referee that further testing was required if it was our aim to establish BAC as a universal alternative for $HgCl_2$. However, based on our experiments, we cannot recommend the universal replacement of $HgCl_2$ with BAC, in particular for applications where long-term storage is required such as for oxygen triple isotope analyses. During both TS2 and TS3, the samples preserved with BAC showed significant changes in the O_2 concentration, after 8 and 17 days, respectively.

We have revised the abstract to make it clear that our results apply to the samples tested, but not necessarily any other seawater sample. This now reads: "BAC at a concentration of 50 mg dm^{-3} inhibited microbial activity for at least three days in samples tested with chlorophyll a (Chl a) concentrations up to 1 mg m^{-3}." (l. 20-22).

Apparently, BAC had very short term effectiveness, and therefore does not correspond with the concept of preservation. It seems to work as a short-term microbial inhibitor. In the case that the process takes place over a period of a few days, the effect of BAC should be monitored hourly.

We agree that BAC may be useful if short-term storage only was required – probably up to 3 days, but this would leave little safety margin, so 24 h may be a more conservative upper duration. Hourly measurements will add little useful information because it would be impracticable to plan the analyses with just hours as safety margin to spare.

No changes necessary.

Since the authors were evaluating O_2 evolution (respiration and photosynthesis), authors should test the effect of BAC addition on nutrient and dissolved organic matter pools under the specific conditions in which incubation was carried out (e.g. light and darkness).

We are unsure what the reviewer means here. The samples were not incubated samples, we were not determining respiration and photosynthesis. They were in situ water samples that were collected and needed to be preserved until later analysis for oxygen-

to-argon ratio. The samples were treated with BAC and then stored in darkness until analysis. Photosynthesis could not occur in darkness.
The referee might be suggesting that if BAC was used as a preservative after incubation, the preservative effect might be affected by the incubation conditions. However, we were testing the use of BAC for preservation of samples at the time of collection, for the purpose of measuring oxygen-to-argon ratios and oxygen triple isotope measurements to derive net and gross biological production from air-sea gas exchange fluxes; both of which are techniques not requiring incubations (Kaiser et al., 2005; Kaiser, 2011).
Any residual respiration that is not completely inhibited by the preservative also changes nutrient and dissolved inorganic carbon (DIC) pools, following the stoichiometry of the dissolved and particulate organic matter pools with respect to oxygen (Anderson and Sarmiento, 1994). BAC would therefore not be suitable for the preservation of nutrients or DIC either.

We have added a sentence to the conclusions to indicate that nutrient and dissolved organic and inorganic carbon concentrations are likely to change as well if respiration is not completely inhibited: "Any respiration that is not completely inhibited by the preservative also changes nutrient and dissolved organic and inorganic carbon concentrations, following the stoichiometry of the dissolved and particulate organic matter pools with respect to oxygen (Anderson and Sarmiento, 1994), which would mean that BAC is not suitable for these parameters either." (l. 244-248).

It is important to state the time of day at which the samples were analyzed, because the O_2 cycle depends on light (for photosynthesis) , Are the samples taking a the same time in each experiment?

We're not sure what the reviewer means here because the purpose of preserving the sample is to arrest any metabolic activity. Samples were collected around 0830 local time (in the morning) and the samples were stored in darkness. Even the untreated samples did not show any signs of net oxygen accumulation due to photosynthesis.

We have added information on sampling time and storage conditions to the first paragraph of the experimental methods section: "Surface water (5 m) was collected at around 0830 local time from the Western English Channel Observatory time series station L4 (Smyth et al., 2010), approximately 13 km southwest of Plymouth, United Kingdom (50° 15.00' N 4° 13.02' W; http://www.westernchannelobservatory.org.uk/). Samples were maintained in darkness at in situ temperature (8-10 ºC) in 30 dm^{-3} carboys whilst being transported to the shore-based laboratory within 3 h of collection." (l. 88-90)

It is not sufficient to monitor an experiment lasting from 7-16 days only 4 times, especially as there is a poor understanding of the behavior of BAC

We consider the number of measurements sufficient for the purposes of our study. The oxygen concentration determinations are very precise. In principle, a single negative result (i.e. a change in oxygen concentration) is sufficient to show that BAC is not suitable as an alternative to $HgCl_2$. Replicate samples showed reproducible behaviour; further measurements are unlikely to invalidate the conclusion that BAC is at best a short-term replacement for $HgCl_2$.

No changes necessary.

The statistical analysis is not appropriate and the experimental setup is not clear; authors reported duplicate and three treatment s. It is not possible to calculate and ANOVA test. I would suggest that each treatment should be repeated a minimum of 3 times.
The authors did not explain what kind of statistical analysis was performed or how the combined errors in each treatment are estimated;

An ANOVA test is not required because we are only comparing two time series (BAC vs. $HgCl_2$). As explained in the methods section, the repeatability of duplicate samples analysed in a single day was 0.02 %. Any change greater than 2 times the repeatability (2σ, i.e. 0.04 %) is considered to be a statistically significant difference ($p < 0.05$) for samples analysed on a given day. In other words, if the BAC-treated samples differ by more than 0.04 % from the $HgCl_2$ samples, this is considered to be statistically significant (coverage factor of 2, as commonly used in analytical sciences). In practice, the BAC-treated samples were 2.5 % lower in O_2/Ar than the $HgCl_2$-treated ones after day 8 in TS2, and 0.4 % lower after day 17 in TS3. The statistical significance could be formally calculated using a *t*-test (two tailed, two degrees of freedom), giving *p* values of 0.0025 and 6.4×10^{-5}.
Furthermore, we have estimated the day-to-day reproducibility of O_2/Ar analysis including calibration errors as 0.2 %. This estimate is the result of formal error propagation. In practice, it is actually an overestimate as the relative standard deviation for the $HgCl_2$-samples over the time course of the experiment shows: It varies between 0.05 and 0.08 %, indicating that calibration and sample analysis errors co-vary. So, even without analysis of the $HgCl_2$-control samples, the BAC treated samples changed by $>12\sigma$ during TS2 and $>2\sigma$ during TS3 relative to the initial sample, indicating high statistical significance.

We already explain our statistical treatment in the methods section. For clarification, we have added the sentence "Direct comparison of BAC-treated and $HgCl_2$-treated samples after the same storage period therefore gives the most reliable indication of the relative efficacy of both preservatives." (l. 139-140).
We have also included the uncertainties of concentration changes in the results and discussion section and increased the number of significant figures reported (l. 164-185). For example, the changes with respect to the initial concentration during TS1 are now reported as "However, $\Delta(O_2/Ar)$ of untreated samples decreased by (0.4±0.2) % after 2 days and by (1.0±0.2) % after 7 days."
We also added *p* values to indicate the statistical significance of the changes in the BAC-treated samples with respect to the $HgCl_2$-treated samples at the same time points. Note that the *p* values for TS2 and TS3 were transposed in the response to the referees and miscalculated due to rounding errors. However, the correct values of $p = 1.6 \times 10^{-5}$ for TS2 and $p = 0.0056$ for TS3 still indicate highly significant differences. We report these conservatively as $p < 0.001$ and $p < 0.01$. None of these changes affect the conclusions of the paper.

it is very difficult to determine the errors associated with sampling such as avoid contamination by oxygen (since atmospheric levels of this gas is high).

Since all sample bottles were filled from the same reservoir, these errors will be the same for each treatment and will not affect the comparison between the different treatments. Also, the oxygen saturations of the samples were –0.4 %, +8.1 % and +12.3 %, i.e. near or slightly above equilibrium with the atmosphere. If anything, they may have suffered a small degree of outgassing, but considering that the initial concentrations agree for all treatments in each time series, this error is negligible.

No changes necessary.

Finally, most of coastal areas have Chl-a > 1 mg m-3, thus, why BAC is less efficient in productive waters, the added dose was not enough?

Higher chlorophyll concentrations are likely to be associated with higher organic matter and bacterial cell concentrations, but the fact that there was no clear difference in the response during TS3 in relation to concentration of BAC suggests that the dose was sufficient to halt the oxygen consumption of the initial bacterial population. However, BAC is not effective against spores, which could have been responsible for the O_2/Ar decrease after day 17 in TS3.

We are already speculating on the role of spores (l. 217), therefore no changes are necessary.

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Could benzalkonium chloride be a suitable alternative to mercuric chloride for preservation of seawater samples?

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Abstract

Instrumental equipment unsuitable or unavailable for fieldwork as well as lack of ship space can necessitate the preservation of seawater samples prior to analysis in a shore-based laboratory. Mercuric chloride ($HgCl_2$) is routinely used for such preservation, but its handling and subsequent disposal incur environmental risks and significant expense. There is therefore a strong motivation to find less hazardous alternatives. Benzalkonium chloride (BAC) has been used previously as microbial inhibitor for freshwater samples. Here, we assess the use of BAC for marine samples prior to the measurement of oxygen-to-argon (O_2/Ar) ratios, as used for the determination of plankton net community production. BAC at a concentration of 50 mg dm^{-3} inhibited microbial activity for at least three days in samples tested with chlorophyll *a* (Chl a) concentrations up to 1 mg m^{-3}. BAC concentrations of 100 and 200 mg dm^{-3} were no more effective than 50 mg dm^{-3}. With fewer risks to human health and the environment, and no requirement for expensive waste disposal, BAC could be a viable alternative to $HgCl_2$ for short-term preservation of seawater samples, but is not a replacement for $HgCl_2$ in the case of oxygen triple isotope analysis, which requires storage over weeks to months. In any event, further tests on a case-by-case basis should be undertaken if use of BAC was considered, since its inhibitory activity may depend on concentration and composition of the microbial community.

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1 Introduction

Marine fieldwork often requires water samples to be collected by ship and returned to a shore-based laboratory for chemical analysis. Mercuric chloride ($HgCl_2$) has routinely been used to inhibit microbial activity, which would otherwise alter the concentrations of oxygen (O_2), inorganic carbon (DIC) or inorganic nutrients (Emerson et al., 1991; Kattner, 1999; Dickson et al., 2007). However, the use of $HgCl_2$ has significant disadvantages including its human toxicity, bioaccumulation, long environmental persistence and the expensive disposal of hazardous mercury-containing wastewater. $HgCl_2$ is highly toxic to aquatic organisms, and is efficiently transferred through the food chain, accumulating in top predators such as fish (Morel et al., 1998). Consumption of mercury-contaminated fish can cause gut irritation and kidney damage in humans (Langford and Ferner, 1999). Hence, mercury-containing laboratory waste requires costly disposal to avoid it entering watercourses and wastewater treatment plants. These are strong incentives to find more environmentally benign alternatives to the use of $HgCl_2$, in particular in remote, sensitive and pristine environments such as polar regions.

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Benzalkonium chloride (alkyldimethylbenzylammonium chloride, BAC) has been used as a less hazardous alternative to $HgCl_2$ for freshwater preservation. It is a quaternary ammonium compound, widely used as a disinfectant in hospitals and an antiseptic, preservative and algicide in the food, ophthalmic, pharmaceutical and horticultural industries (Wessels and Ingmer, 2013). BAC is classified according to EU Directives 67/548/EEC and 1999/45/EC as harmful when in contact with skin and if swallowed and very toxic to aquatic organisms. Release to the environment should be avoided; however, the preservative effect of BAC can be neutralized by the emulsifiers polysorbate 80 and lecithin (Block, 2001). Kuo (1998) used BAC to preserve freshwater samples for carboxylic acid analysis and achieved effective preservation for up to 30 days using a concentration of 30-50 mg dm^{-3}.

$HgCl_2$ and BAC have different mechanisms of inhibiting microbial activity. Mercury binds to the thiol-groups of amino acids and therefore inhibits enzyme activity (Langford and Ferner, 1999). BAC is a cationic surfactant that physically permeates the cytoplasmic membrane causing its disruption, release of cytoplasmic constituents, precipitation of cell contents and cell death (Wessels and Ingmer, 2013; Ferreira et al., 2011).

The aim of this study was to test if BAC was as effective as $HgCl_2$ in preventing microbial activity. The target application was the preservation of marine samples for measurement of O_2/Ar ratios and oxygen triple isotopes used to determine plankton net and gross community

production (Craig and Hayward, 1987; Quay et al., 2012). Measurements of O_2/Ar ratios with membrane inlet mass spectrometry (MIMS) are usually made by immediate and continuous analysis of seawater from the underway sampling system on scientific research ships (Kaiser et al., 2005; Hamme et al., 2012). However, sampling in coastal areas may be conducted on small vessels or ships-of-opportunity without mass-spectrometric facilities to analyse samples on board. Similarly, laboratory studies of O_2 respiration or production may require arresting biological activity at defined time points and subsequent batch analysis of all samples together. These discrete samples have to be preserved until analysis, usually within a few days, and $HgCl_2$ has previously been used for this purpose (Holtappels et al., 2014; Kana et al., 2006). In contrast, weeks, months and, occasionally, years (Hendricks et al., 2005) may elapse before oxygen triple isotope samples are analysed. The effectiveness of BAC as an alternative preservative to halt microbial production or consumption of O_2 in seawater samples was therefore assessed.

2 Experimental methods

Surface water (5 m) was collected at around 0830 local time from the Western English Channel Observatory time series station L4 (Smyth et al., 2010), approximately 13 km southwest of Plymouth, United Kingdom (50° 15.00' N 4° 13.02' W; http://www.westernchannelobservatory.org.uk/). Samples were maintained in darkness at in situ temperature (8-10 ºC) in 30 dm^{-3} carboys whilst being transported to the shore-based laboratory within 3 h of collection.

An initial experiment was conducted to assess whether the addition of $HgCl_2$ or BAC solution interfered with the analysis of O_2/Ar ratios by MIMS. The seawater sample was distributed into six replicate 0.5 dm^3 glass bottles with ground glass stoppers. Two samples each were treated with $HgCl_2$, BAC or left untreated. The bottle stopper was replaced ensuring no headspace remained and secured with rubber bands (Dickson et al. 2007). For the samples treated with $HgCl_2$, 0.2 cm^3 of saturated $HgCl_2$ solution (76 g dm^{-3}), corresponding to 15 mg $HgCl_2$, was added to the sample, giving a final concentration of 30 mg dm^{-3}. This is the recommended concentration and addition volume (0.02-0.05 % of the sample volume) for inorganic nutrients (Kirkwood, 1992), DIC and total alkalinity (TA) samples (Dickson et al., 2007). For the samples treated with BAC, 0.25 cm^3 of a 1 g dm^{-3} solution was added to the sample, giving a final concentration of 50 mg dm^{-3} BAC as suggested by Kuo (1998). The samples were analysed immediately using MIMS.

The first time series experiment (TS1, see Table 1 for an overview of the water composition at the time of sampling) was conducted with water collected from L4 in February 2010 to test whether BAC was as efficient as $HgCl_2$ at preserving samples for 7 days. Replicate 0.5 dm^3 samples were prepared as described above and one third each treated with $HgCl_2$, BAC or left untreated. The bottles were stored underwater in the dark at 15 ºC. Samples from each treatment were analysed immediately, and then again after 1, 2 and 7 days.

A second time series experiment (TS2) was undertaken in April 2010 at the time of the spring phytoplankton bloom when chlorophyll *a* concentrations were higher. Again, replicate 0.5 dm^3 samples were prepared, treated and stored as described above. Samples from each treatment ($HgCl_2$, BAC, no addition) were analysed immediately and after 1, 3 and 8 days.

Finally, a third time series experiment (TS3) was undertaken in May 2010 to test the efficiency of increased concentrations of BAC (BAC×2: 100 mg dm^{-3} and BAC×4: 200 mg dm^{-3}) over a 17 day-period. Again, replicate 0.5 dm^3 samples were prepared, treated and stored as described above. Treatments were $HgCl_2$, BAC, BAC×2, BAC×4 and no addition, and samples of each of the treatments were analysed immediately and after 2, 4 and 17 days.

O_2/Ar ratios

O_2/Ar ratios were analysed using MIMS (Kaiser et al., 2005). The system was operated continuously: When not running a seawater sample, MilliQ water was circulated. Sample water was pumped through a Teflon AF membrane (*Random Technologies*) using a peristaltic pump. The membrane was held under vacuum at a constant temperature of 15 °C in a water bath. The gas from the membrane then flowed into a quadrupole mass spectrometer (*Pfeiffer Vacuum Prisma*). Its flight tube was held at 70 °C using heating tape. The flow of water was maintained at (38±1) cm^3 min^{-1}. Equilibrated water standards were prepared containing artificial seawater of salinity 35.1 at 15 °C, and were run before and after the samples to account for any drift in the MIMS output over the approximately 2 h taken for analysis of all samples and standards. Results are reported as biological oxygen supersaturations, $\Delta(O_2/Ar)$, with respect to air-equilibrated water (Kaiser et al., 2005). Drift was generally <0.1 %, and 0.15 % at most. Possible reasons for a drift would be a temperature change in the laboratory or a change of water flow. Each sample was analysed for seven minutes. The repeatability based on the analysis of duplicate samples was 0.02 %, on a given day. Any change greater than 2 times the repeatability (i.e. 0.04 %) is considered to be a statistically significant difference for samples analysed on a given day.

However, for comparison of samples analysed on different days of the time series, the calibration uncertainty needs to be taken into account, which is 0.2 % (error bars in Figs. 1 to 3). Any change greater than 2 times this uncertainty (i.e. 0.4 %) with respect to the initial O_2/Ar ratio is considered to be statistically significant.

Direct comparison of BAC-treated and $HgCl_2$-treated samples after the same storage period therefore gives the most reliable indication of the relative efficacy of both preservatives.

Chlorophyll *a* concentration

Water samples (0.1 dm^3) were filtered through 25 mm (nominal pore size 0.7 µm) glass-fibre filters (GF/F) and extracted in acetone/water (volume ratio 9:1) overnight at 4 ºC. Chlorophyll *a* (Chl *a*) concentrations were measured using a Turner fluorometer (Welschmeyer, 1994).

Heterotrophic bacteria

Heterotrophic bacterial number concentration was determined by analytical flow cytometry. Scattered light and fluorescence intensity were measured on a FACSort flow cytometer (Becton Dickinson, Oxford, UK) with log amplification on a four-decade scale with 1024-channel resolution (Tarran et al., 2006). Samples were analysed for 1 minute at a flow rate of 0.055 cm^3 min^{-1}, determined using Beckman Coulter Flowset fluorospheres in a 1:10 dilution. Each 0.5 cm^3 sample was stained for 1 hour with SYBR Green mixed with potassium citrate solution (Marie et al. 1997) prior to analysis. Data were analysed with the program WinMDI 2.9 (Joseph Trotter, SCRIPPS Research Institute). We assumed a coefficient of variation (standard deviation / mean) for bacterial number concentration of 5 % (Šantić et al., 2007).

3 Results and discussion

$HgCl_2$ is known to be a suitable preservative for seawater samples prior to mass spectrometric measurement of dissolved O_2 (e. g., Hendricks et al., 2005), whereas BAC is not routinely used in this way. We therefore tested whether the addition of BAC altered the seawater O_2 concentration or interfered with the MIMS analysis for O_2/Ar. $\Delta(O_2/Ar)$ of two replicate samples to which BAC had been added was not significantly different from $\Delta(O_2/Ar)$ of two replicate samples to which $HgCl_2$ had been added. Therefore, BAC did not interfere with the accurate determination of O_2/Ar.

The Chl *a* concentration in TS1 samples was 0.4 mg m^{-3} (Table 1). $\Delta(O_2/Ar)$ in replicate samples to which BAC was added were not significantly different from samples to which $HgCl_2$ was added and both stayed constant over the seven days of the experiment (Fig. 1). However, $\Delta(O_2/Ar)$ of untreated samples decreased by (0.4±0.2) % after 2 days and by (1.0±0.2) % after 7 days. This suggests that BAC was as effective as $HgCl_2$ at preserving the O_2 concentration in these particular low Chl *a* concentration-seawater samples for up to 7 days.

The Chl *a* concentration in TS2 samples was 1.0 mg m^{-3}. Heterotrophic bacterial number concentration was 6.9×10^5 cm^{-3}. $\Delta(O_2/Ar)$ of the untreated samples decreased by (0.8±0.2) % after 1 day and by (5.2±0.2) % after 8 days of storage, indicating O_2 consumption in these samples (Fig. 2). $\Delta(O_2/Ar)$ of $HgCl_2$-treated samples remained constant over the 8 days. $\Delta(O_2/Ar)$ of BAC-treated samples remained constant and not significantly different from $HgCl_2$-treated samples for 3 days, but decreased by (2.3±0.2) % after 8 days and were at that time (2.35±0.03) % lower ($p < 0.001$, two-tailed *t*-test) than the $HgCl_2$-treated samples. This suggests that the time over which BAC is effective at preserving seawater samples decreases with increasing Chl *a* concentration.

The Chl *a* concentration in TS3 samples was 0.6 mg m^{-3}. Heterotrophic bacterial number concentration was 6.8×10^5 cm^{-3}. $\Delta(O_2/Ar)$ showed similar results to TS2, with a (2.6±0.2) % decrease of the $\Delta(O_2/Ar)$ of the untreated sample after 4 days and a (7.7±0.2) % decrease after 17 days (Fig. 3). $\Delta(O_2/Ar)$ of samples containing BAC remained constant and not significantly different from the samples containing $HgCl_2$ for 4 days; by 17 days they were (0.34±0.03) % lower ($p < 0.01$) than the samples containing $HgCl_2$ (Fig. 3). BAC×2 (100 mg dm^{-3}) and BAC×4 (200 mg dm^{-3}) were no more effective as preservatives than BAC (50 mg dm^{-3}), and there was no significant difference in the temporal evolution of $\Delta(O_2/Ar)$ in samples containing BAC, BAC×2, BAC×4. $\Delta(O_2/Ar)$ of the BAC×4 samples were (0.19±0.03) % lower than the other samples throughout the time series. This decrease appears even at time zero. This may be partially due to a dilution effect caused by the larger volume of BAC solution used, but in order to explain the initial 0.2 % drop in $c(O_2)$, the oxygen concentration of the BAC solution would have to have been near 0, which is unlikely. We therefore cannot fully explain the decrease in oxygen concentration associated with the BAC×4 addition. However, this does not invalidate any of our conclusions because, after this initial drop, the BAC×4 time series

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shows the same relative trend with respect to the initial concentration as the BAC and BAC×2 time series.

Analysis of green fluorescence and side scatter determined by flow cytometry during TS3 enabled an assessment of the effect of $HgCl_2$ and BAC on heterotrophic bacterial number concentration (Fig. 4). The bacterial cell number concentration in the sample which had not been treated increased from 7.2 to 11.2 × 10^5 cm^{-3} in the first 2 days, before decreasing to 2.4 × 10^5 cm^{-3} after 17 days, presumably due to a combination of grazing and nutrient limitation. Number densities in samples treated with $HgCl_2$ remained relatively constant, from 6.5 × 10^5 cm^{-3} at time 0 to 5.2 × 10^5 cm^{-3} on day 17. However, since $\Delta(O_2/Ar)$ barely changed (Fig. 3), the cells must have been inactive or dead. The number concentration in samples treated with BAC declined immediately on addition of BAC to 1.5 × 10^5 cm^{-3}, decreasing to less than 0.2 × 10^5 cm^{-3} within 2 days and to less than 0.1 × 10^5 cm^{-3} after 17 days. This is consistent with the mode of toxicity of BAC: disruption of the cell membrane and release of the cell contents. However, it is not consistent with the decrease in $\Delta(O_2/Ar)$ seen after 17 days in the BAC-treated samples (Fig. 3). BAC is not effective against bacterial spores (Block, 2001), so it is possible that viable bacterial cells in the sample were killed immediately, leaving spores to become viable after a few days. BAC can be a carbon and energy source for some bacteria (Oh et al., 2013) and acquired bacterial resistance to BAC has also been recorded (Wessels and Ingmer, 2013). However, if any of these suggestions were the case, then the bacterial number concentration would have increased after day 4 alongside the decrease in $\Delta(O_2/Ar)$. An alternative possibility is that the low bacterial number concentration derived from flow cytometric analysis is due to interference between BAC and the SYBR Green stain. SYBR Green staining is not recommended for use with surfactants (http://tools.lifetechnologies.com/content/sfs/manuals/td004.pdf), hence bacterial cells could have been inhibited by BAC for up to 4 days, but then recovered to continue to consume O_2. This would reduce $\Delta(O_2/Ar)$, but the cells would not be counted by the staining and counting procedure. It is also possible that the decrease in $\Delta(O_2/Ar)$ after 4 days was due to the growth of microzooplankton rather than bacteria. Characterisation of the mode of toxicity of BAC on each component of the plankton community is beyond the scope of this study; rather, we focussed on ascertaining the time scale over which seawater samples could be preserved prior to analysis.

4 Conclusions

Samples for accurate determination of O_2/Ar ratios, if not analysed immediately after collection, need to be preserved with an inhibitor of microbial activity. $HgCl_2$ reliably preserved samples for the maximum experimental time of 17 days. BAC was found to be an effective preservative for at least 3 days, for seawater samples containing Chl *a* concentrations of up to 1 mg m^{-3}. Therefore, BAC, which poses fewer risks to human health and the environment and does not require expensive waste disposal, could be used as a viable alternative to $HgCl_2$ for short-term preservation of samples prior to MIMS analysis. However, it is not effective as a replacement for $HgCl_2$ in oxygen triple isotope samples, which require longer-term storage over weeks to month, or even years. Any respiration that is not completely inhibited by the preservative also changes nutrient and dissolved organic and inorganic carbon concentrations, following the stoichiometry of the dissolved and particulate organic matter pools with respect to oxygen (Anderson and Sarmiento, 1994), which would mean that BAC is not suitable for these parameters either. We would also recommend further tests with BAC on a case-by-case basis because its mode of action and efficacy might be affected by cross-reactions with other seawater constituents, especially under higher Chl a concentrations.

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253 **Acknowledgements**

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258 INTERREG IVA 2 Mers Seas Zeeen Cross-border Cooperation Programme 2007-2013).

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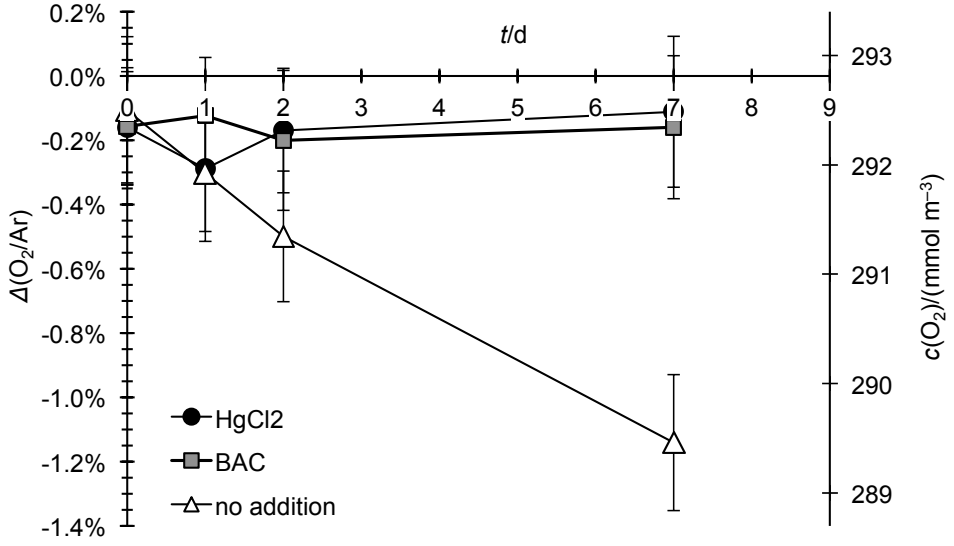
Table 1. Initial conditions of time series experiments. The oxygen supersaturation is defined as $\Delta(O_2) = c(O_2) / c_{sat}(O_2) - 1$. The biological oxygen supersaturation is defined as $\Delta(O_2/Ar) = [c(O_2)/c(Ar)] / [c_{sat}(O_2)/c_{sat}(Ar)] - 1$.

Expt.	Sampling date	θ/°C	S(PSS-78)	$c(NO_3^- + NO_2^-)$ / (mmol m^{-3})	$c(SiO_4^{4-})$ / (mmol m^{-3})	$c(PO_4^{3-})$ / (mmol m^{-3})	c(Chl *a*) / (mg m^{-3})	$c(O_2)$ / (mmol m^{-3})	$\Delta(O_2)$	$\Delta(O_2/Ar)$	Cell number density / cm^{-3}
TS1	08/02/2010	8.2	34.90	8.6	4.8	0.6	0.4	292.4	–0.4 %	–0.2 %	not analysed
TS2	19/04/2010	9.0	35.06	3.3	0.5	0.3	1.0	311.5	+8.1 %	+6.7 %	6.9×10^5
TS3	17/05/2010	10.2	35.04	0.1	0.4	0.1	0.6	315.4	+12.3 %	+9.5 %	6.8×10^5



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334 Figure 1. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentra-
335 tion during TS1 (February 2010), for samples without treatment (white triangle), BAC-treated
336 (grey square) and $HgCl_2$-treated (black circle). Error bars include the day-to-day calibration
337 uncertainty.

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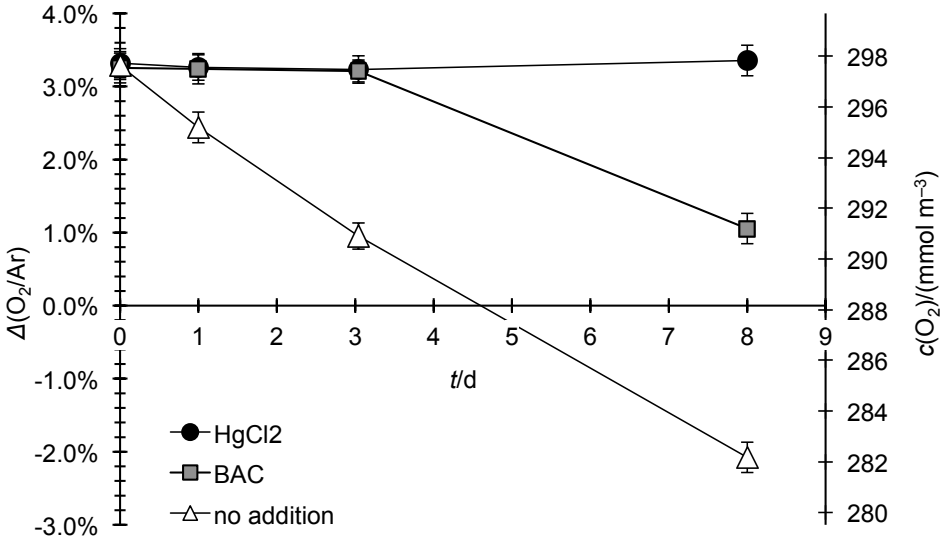

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340 Figure 2. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentra-
341 tion during TS2 (April 2010), for samples without treatment (white triangle), BAC-treated
342 (grey square) and $HgCl_2$-treated (black circle). Error bars include the day-to-day calibration
343 uncertainty.

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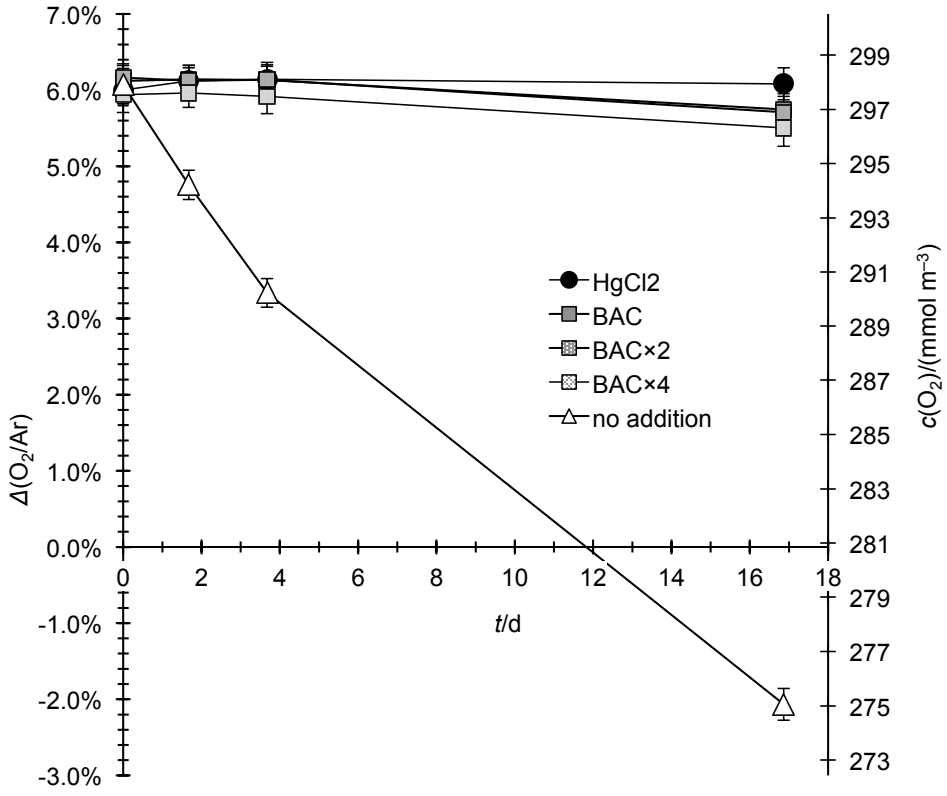

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346 Figure 3. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentra-
347 tion during TS3 (May 2010), for samples without treatment (white triangle) and treated with
348 BAC (grey square), BAC×2 (dark grey stippled square), BAC×4 (light grey stippled square)
349 or $HgCl_2$ (black circle). Error bars include the day-to-day calibration uncertainty.

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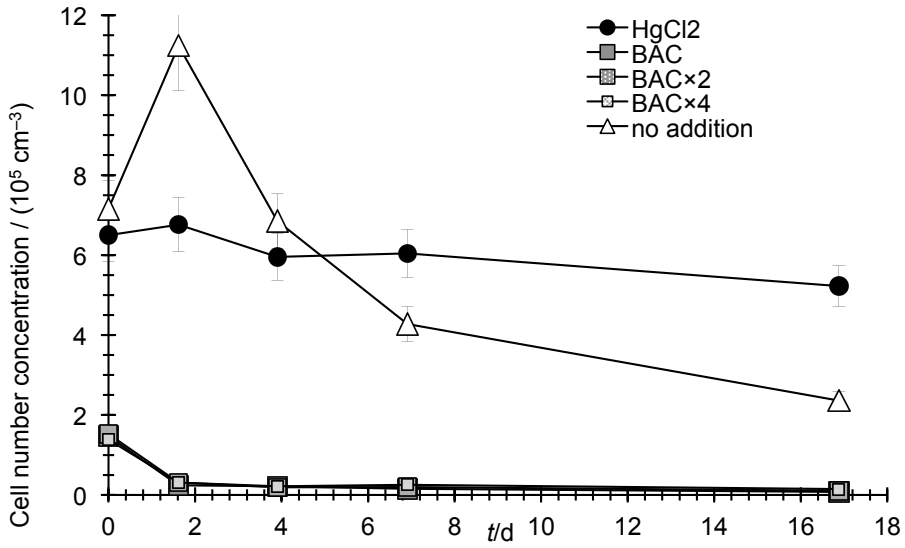

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352 Figure 4. Number concentration of heterotrophic bacteria in samples collected during TS3
353 treated with different concentrations of BAC, $HgCl_2$ and with no addition of preservative.

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