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.

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.

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.

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.

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

Thank you.

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

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 $HgCl_2$.

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.

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."

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.

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.

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.

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.

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 nutrient or DIC either.

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.

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 $HgCl_2$.

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.

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 vials were filled from the same reservoir, these errors will be the same for each treatment. 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.

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.

References

Anderson, L. A., and Sarmiento, J. L.: Redfield ratios of remineralization determined by nutrient data analysis, Global Biogeochem. Cycles, 8, 65-80, 10.1029/93GB03318, 1994.

Fishwick, J.: CTD profiles (depth, pressure, temperature, salinity, potential temperature, density, fluorescence, transmissance, downwelling PAR, dissolved oxygen concentration) binned to 0.5 m and 0.25 m at sites L4 and E1 in the Western English Channel between January 2002 and December 2013, Journal, 10/vrp, 2014.

Kaiser, J., Reuer, M. K., Barnett, B., and Bender, M. L.: Marine productivity estimates from continuous oxygen/argon ratio measurements by shipboard membrane inlet mass spectrometry, Geophys. Res. Lett., 32, L19605, 10.11029/12005GL023459, 2005.

Kaiser, J.: Technical note: Consistent calculation of aquatic gross production from oxygen triple isotope measurements, Biogeosciences, 8, 1793-1811, 10.5194/bg-8-1793-2011, 2011.

Woodward, E., Harris, C., and Al-Moosawi, L.: Nutrient concentration profiles from long term time series at Station L4 in the Western English Channel from 2000 to 2014, Journal, 10/42f, 2015.