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

J. Gloël[1,2], C. Robinson[1], G. H. Tilstone[2], G. Tarran[2], and J. Kaiser[1]

[1]Centre for Ocean and Atmospheric Sciences, School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich, UK
[2]Plymouth Marine Laboratory, The Hoe, Plymouth, UK

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Correspondence to: J. Kaiser (j.kaiser@uea.ac.uk)

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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 significant risks and expense. Benzalkonium chloride (BAC) has been used previously for freshwater samples. Here, we assess BAC as a less hazardous alternative microbial inhibitor 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 seawater with chlorophyll *a* (Chl *a*) concentrations up to 1 mg m^{-3}, possibly longer when Chl *a* concentrations were lower. 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.

1 Introduction

Marine fieldwork often requires water samples to be collected by ship and returned to the shore-based laboratory for chemical analysis. For longer-term storage over weeks to years, 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,

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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.

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

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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 from the Western English Channel Observatory time series station L4 (Smyth et al., 2010), approximately 13 km southwest of Plymouth, UK (50°15.00′ N 4°13.02′ W; http://www.westernchannelobservatory.org.uk/). Samples were maintained at in situ temperature (8–10 °C) 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

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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 °C. 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.

2.1 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

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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. 1–3). 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.

2.2 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 °C. Chlorophyll *a* (Chl *a*) concentrations were measured using a Turner fluorometer (Welschmeyer, 1994).

2.3 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 min

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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 h 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 % after 2 days and by 1.0 % after 7 days. This suggests that BAC was as effective as $HgCl_2$ at inhibiting the production/consumption of O_2 in these particular low Chl *a* concentration-seawater samples for up to 7 days.

The Chl *a* concentration in TS2 samples was 1.0 g m^{-3}. Heterotrophic bacterial number concentration was 6.9×10^5 cm^{-3}. $\Delta(O_2/Ar)$ of the untreated samples decreased by 1 % after 1 day and by 5 % 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 % after 8 days. This

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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 3 % decrease of the $\Delta(O_2/Ar)$ of the untreated sample after 4 days and an 8 % 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 0.3 % lower 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 0.2 % lower than the other samples throughout the time series. This decrease appears even at time zero, presumably due to a dilution effect caused by the larger volume of BAC solution used.

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,

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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 during handling 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. We would also recommend further tests with BAC on a case-by-case basis because its

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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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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)	c (Chl a) (mg m^{-3})	c (O_2) (mmol m^{-3})	Δ (O_2)	$\Delta(O_2/Ar)$	Cell number concentration (cm^{-3})
TS1	8 Feb 2010	8.2	0.4	292.4	−0.4 %	−0.2 %	not analysed
TS2	19 Apr 2010	9.0	1.0	311.5	+8.1 %	+6.7 %	6.9×10^5
TS3	17 May 2010	10.2	0.6	315.4	+12.3 %	+9.5 %	6.8×10^5

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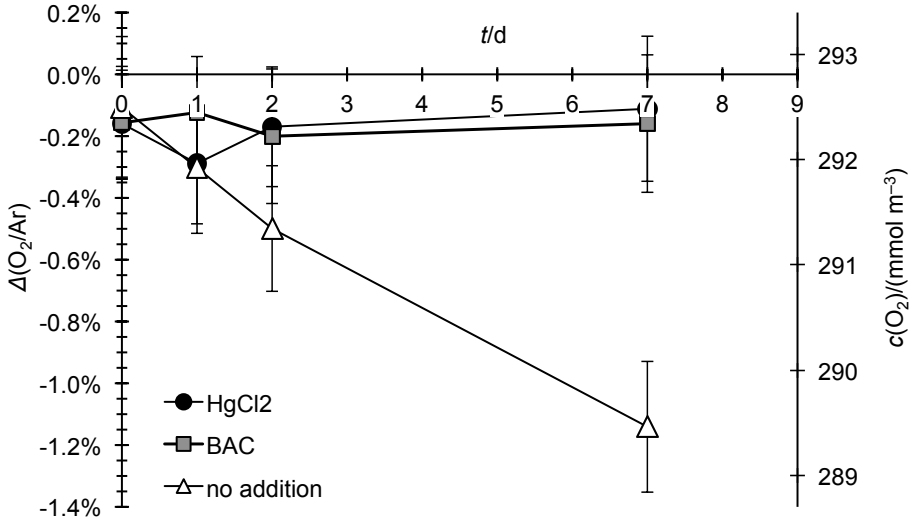

Figure 1. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentration during TS1 (February 2010), for samples without treatment (white triangle), BAC-treated (grey square) and $HgCl_2$-treated (black circle). Error bars include the day-to-day calibration uncertainty.

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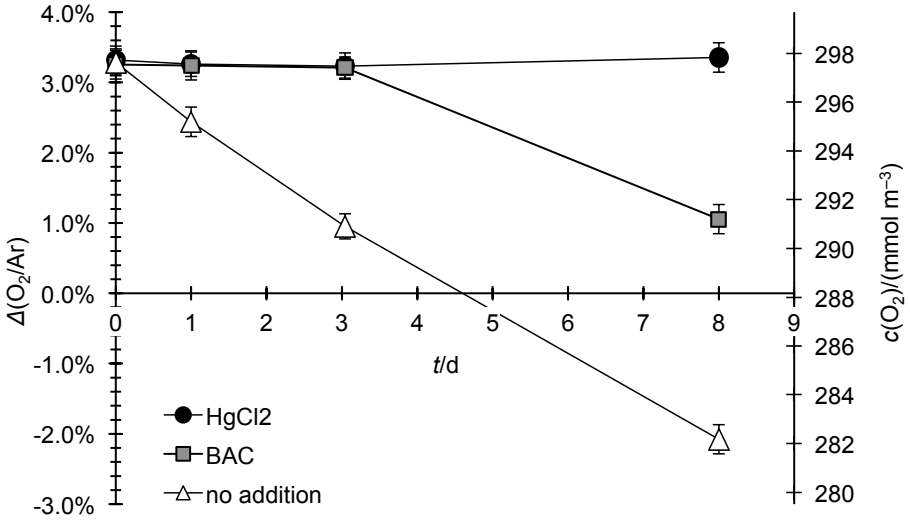

Figure 2. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentration during TS2 (April 2010), for samples without treatment (white triangle), BAC-treated (grey square) and $HgCl_2$-treated (black circle). Error bars include the day-to-day calibration uncertainty.

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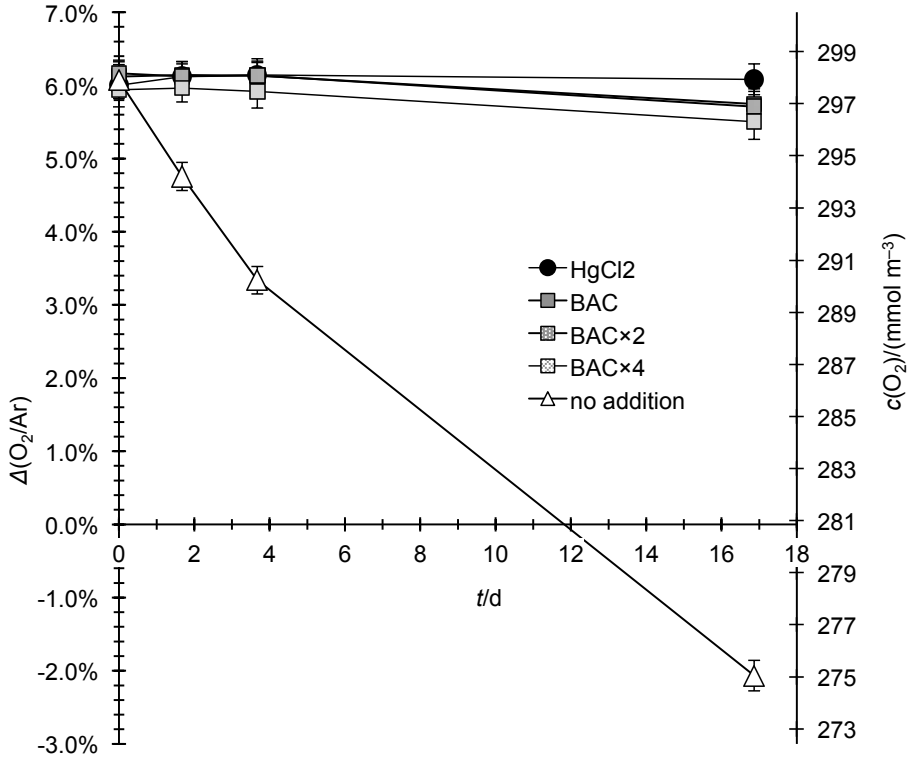

Figure 3. Biological oxygen supersaturation $\Delta(O_2/Ar)$ and corresponding oxygen concentration during TS3 (May 2010), for samples without treatment (white triangle) and treated with BAC (grey square), BAC × 2 (dark grey stippled square), BAC × 4 (light grey stippled square) or $HgCl_2$ (black circle).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

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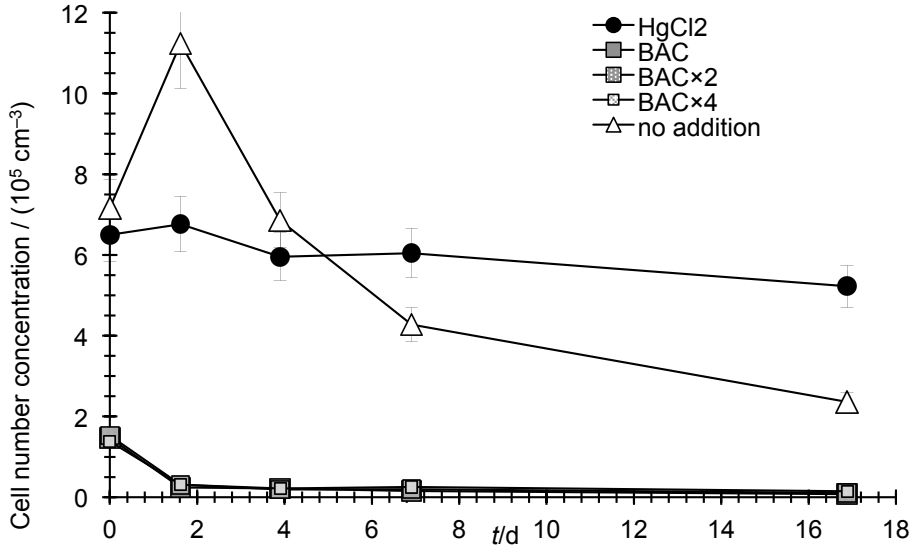

Figure 4. Number concentration of heterotrophic bacteria in samples collected during TS3 treated with different concentrations of BAC, $HgCl_2$ and with no addition of preservative.

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