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Sampling in low oxygen aquatic environments: The deviation from anoxic conditions

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Abstract

Studies of the impact of hypoxic or anoxic environments on both climate and ecosystems rely on a detailed characterization of the oxygen (O_2) distribution along the water column. The former trivial separation between oxic and anoxic conditions is now often redefined as a blurry concentration range in which both aerobic and anaerobic processes might coexist, both in situ and during experimental incubations. The O_2 concentrations during such incubations have often been assumed to be equal to in situ levels, but the concentration was rarely measured. In order to evaluate the actual oxygen concentration in samples collected from low-oxygen environments, a series of measurements were performed on samples collected in the Pacific oxygen minimum zones. Our results show a significant deviation from in situ anoxic conditions in samples collected by Niskin bottles where leakage from the bottle material resulted in O_2 concentrations of up to 1 μM. Subsequent sampling further increased the O_2 contamination. Sampling and analysis by Winkler method resulted in variable apparent concentrations of 2–4 μM. Two common procedures to avoid atmospheric contamination were also tested: allowing gentle overflow and keeping the sampling bottle submersed in a portion of the sampled water. Both procedures resulted in similar O_2 contamination with values of 0.5–1.5 μM when bottles were immediately closed and measurements performed with optical sensors, and 3–4 μM apparent concentration when analyzed by the Winkler method. Winkler titration is thus not suited for analysis of low-O_2 samples. It can be concluded that incubation under anoxic conditions requires deoxygenation after conventional sampling.

The characterization of low oxygen and anoxic environments has received increasing attention during the past few decades. The increased number of hypoxic events detected in the coastal areas and the expansion of the oceanic oxygen minimum zones (OMZs) (Horak et al. 2016; Stramma et al. 2008) place ocean deoxygenation as one of the most relevant problems caused by global warming (Breitburg et al. 2018; Sampaio et al. 2021). The distribution and extension of low oxygen environments has not only a direct impact in the abundance, diversity and distribution of macroorganisms (Breitburg et al. 2009; Vaquer-Sunyer and Duarte 2008), but also affect the composition and activity of the microbial community.

At the boundaries of the OMZs and other anoxic water masses, variations in the oxygen concentration of a few micromolar may cause large differences in microbial metabolism, pathways and rates. In the Eastern Tropical South Pacific (ETSP) OMZ, aerobic processes such as ammonium and nitrite oxidation were active at nanomolar O_2 concentrations, and 3–5 μmol O_2 L^{-1} sufficed to reach maximum rates (Bristow et al. 2016). On the other side, anaerobic processes in the same OMZ such as denitrification and anammox were inhibited at nanomolar oxygen concentrations, reaching complete inhibition at concentrations below 1–2 μmol O_2 L^{-1} (Dalsgaard et al. 2014), while others have found substantial anammox and denitrification rates at considerably higher O_2 concentrations (Bristow 2018; Kalvelage et al. 2011). The water layers in the OMZs where both aerobic and anaerobic processes might coexist (< 5 μmol O_2 L^{-1}) are often tens of meters in the upper oxycline and up to hundreds of meters in the lower oxycline (Tiano et al. 2014*a*). Estimates of global impacts of OMZs commonly assume an O_2 threshold when anaerobic processes are upscaled (Paulmier and Ruiz-Pino 2009), but due to the resolution of the historical oxygen concentration measurements, values of 5–20 μmol L^{-1} have often been assumed (Codispoti et al. 2001). Recent advances in sensor technology now makes it possible to measure O_2 in

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situ at few nanomolar detection limit and resolution (Larsen et al. 2016; Revsbech et al. 2011), and the same sensor technologies now show that low O_2 samples for laboratory incubations are O_2 contaminated during sampling and analysis. Although the oxygen contamination during the ex situ incubation of OMZs samples were previously quantified (De Brabandere et al. 2012; Lehner et al. 2015), the initial oxygen contamination produced by the sampling procedure remains poorly characterized.

Besides OMZs, other stratified aquatic environments such as freshwater lakes, fjords, and marine basins can also develop anoxic bottom waters. Unlike open ocean OMZs, euxinia is commonly present in these environments, showing certain accumulation of free sulfide (van Vliet et al. 2020). The potential O_2 contamination from the sampling procedure might be considered negligible under these conditions as the reduced compounds can consume the oxygen (Ingvorsen and Jorgensen 1979) and the O_2 contamination during sampling could pass unnoticed. However, the presence of small amounts of oxygen significantly increase some microbial processes such as methane and sulfide oxidation (Jørgensen et al. 1991; Milucka et al. 2015; Steinle et al. 2017) while the generation of oxidized sulfur compounds (elemental sulfur, thiosulfate, sulfite…) might enhance reducing microbial processes (Hayes et al. 2006). As a consequence, the measured rates of microbial processes in euxinic environments might be altered by the uncharacterized intrusion of oxygen during sampling.

In order to explore the oxygen contamination produced during sampling in anoxic environments, we performed a series of measurements in the Eastern Tropical North and South Pacific (ETNP and ETSP) OMZs as well as in the laboratory. By the use of STOX sensors (Revsbech et al. 2011) and high-sensitivity optodes (Lehner et al. 2015), we quantified the increase in measured oxygen concentration introduced by (1) the collection of the sample using standard Niskin bottles; (2) the transfer of the water sample from the Niskin bottle to gas-tight containers under various conditions (under N_2 atmosphere, with gentle overflow and in submerged containers); and (3) a standard Winkler O_2 determination procedure.

Material and procedures

Cruises locations, sampling, and in situ data collection

Data and samples were collected during three oceanographic cruises in the ETNP and ETSP OMZs. Samplings in the ETSP were performed during the activities of research dedicated to the minimum of oxygen in the Eastern Pacific (AMOP) cruise (February 2014) on board the R/V L'Atalante (IFREMER, France) off Peru. Another two samplings were performed in the ETNP off the Mexican coast: OC1705, May 2017, on board of the R/V Oceanus (Oregon State University) and SR1805, May 2018, on board of the R/V Sally Ride (Scripps Institution of Oceanography). During the three cruises, ships were equipped with a Seabird SBE CTD system and a SBE-43 oxygen sensor. The SBE-43 oxygen sensors were calibrated before the cruises by the manufacturer (Seabird). Seawater samples were collected using Niskin bottles mounted on a CTD rosette with volumes of 12 L for R/V L'Atalante, 10 L for R/V Oceanus, and 20 L for R/V Sally Ride.

High-resolution oxygen profiles were carried out using custom-made STOX sensors mounted on an in situ STOX electronics unit (UNISENSE A/S, Denmark) that was connected to the Seabird CTD as described previously (Revsbech et al. 2011; Tiano et al. 2014*a*). STOX sensor signals were analyzed and calibrated as described previously (Tiano et al. 2014*a*). Based on the information obtained by the STOX sensors, different depths inside the anoxic core were selected for sampling. Up-cast STOX data were used to define the in situ oxygen concentration of samples collected by the Niskin bottles. In situ STOX sensors were not available during the AMOP cruise and depths were selected based on the information of the SBE-43 sensor.

Oxygen in collected samples: Concentration inside the Niskin bottle and Winkler determination

The oxygen concentrations in the Niskin bottles were measured during the AMOP cruise (Table 1). Samples from the anoxic core at depths between 150 and 400 m were collected in 12-L Niskin bottles. Once the rosette arrived on board, the bottles were dismounted from the rosette and oxygen concentrations were measured using STOX sensors. For that purpose, a short (ca. 10 cm) piece of Tygon tubing (6 mm i.d.) was connected to the Niskin bottle and water flow was simultaneously directed toward two STOX sensors by the use of a glass Y-piece. Sensors tips were inserted inside the glass of the Y-piece to avoid oxygen contamination from the atmosphere. After 2 min of flow, a minimum of three STOX cycles (1 min each) were recorded.

Immediately after STOX readings, samples for Winkler oxygen determination were collected from the same bottle. From each Niskin bottle, three 160-mL Winkler flasks were filled, trying to minimize contamination by extensive initial rising and gentle overflow. Winkler reagents were added and flasks were closed and kept in darkness for 24 h prior to analysis (Carpenter 1965). The potential contamination caused by the replacement of the headspace in the Niskin bottle with air was minimized by collecting less than 50% of the total Niskin volume. Some contamination was thus possible, but the high reproducibility of the Winkler replicates in the sequentially collected samples suggests no significant impact. Dissolved oxygen titration was performed on board applying an autotitrator, a 20-mL burette and potentiometric end-point determination (reproducibility of 0.9% $\pm$ 0.1%) (Granéli and Granéli 1991).

Sample collection and oxygen measurements: Overflow vs. submerged bottles

Samples were collected during cruise SR1805 off Mexico (May 2018) from the anoxic layers at 65 m at the coastal

Table 1. Oxygen concentration (in μmol O_2 L^{-1}) measured in the same Niskin bottle using STOX sensors and Winkler titration during the AMOP cruise in the ETSP off Peru. All samples were collected in the core of the OMZ in water layers apparently anoxic. All data are means ± standard deviation (SD), determined from the corresponding triplicates.

Station_Cast	Date	Depth (m)	*T* (°C)	STOX	Winkler
am11_01	04 Feb 2014	400	8.95	0.83 ± 0.14	4.07 ± 0.02
am11_14	06 Feb 2014	400	8.83	0.67 ± 0.07	3.21 ± 0.08
am13_03	07 Feb 2014	300	10.43	0.96 ± 0.07	2.56 ± 0.11
am25_02	14 Feb 2014	282	12.54	0.6 ± 0.08	2.85 ± 0.05
am25_02	14 Feb 2014	200	11.86	0.56 ± 0.24	3.27 ± 0.02
am25_11	16 Feb 2014	288	13.74	0.45 ± 0.06	2.87 ± 0.02
am25_11	16 Feb 2014	150	13.29	0.25 ± 0.06	3.99 ± 0.01
am25_11	16 Feb 2014	80	11.92	0.46 ± 0.07	4.47 ± 0.04
am28_02	17 Feb 2014	300	13.45	0.47 ± 0.12	3.82 ± 0.02
am28_02	17 Feb 2014	153	12.71	0.39 ± 0.05	3.2 ± 0.06
am28_02	17 Feb 2014	100	10.63	0.66 ± 0.09	3.23 ± 0.04
am28_16	19 Feb 2014	300	12.80	0.49 ± 0.09	5.02 ± 0.02
am28_16	19 Feb 2014	200	12.29	0.58 ± 0.18	6.43 ± 1.42
am28_16	19 Feb 2014	150	10.85	0.47 ± 0.14	4.12 ± 0.04

station PS3. A piece of 50-cm Tygon tubing piece was connected to the Niskin bottle upon arrival on deck and water was transferred into six Winkler bottles (65 mL) by two standard procedures:

1. Gentle overflow: tubing was inserted inside the bottles and water was left to overflow for several volumes before closing the bottles with a solid glass stopper.
2. Submerged bottles: one beaker (0.5 L) was quickly filled up with water from one Niskin while Winkler bottles were kept submerged. Water flowing from another Niskin bottle collected at the same depth was used to overflow the submerged Winkler bottles by several volumes before closing with a solid glass stopper while submerged.

Winkler bottles were equipped with O_2 optode sensing spots and oxygen concentration was measured after 10 min by luminescence measuring oxygen sensor (LUMOS) technology (Lehner et al. 2015).

In order to exclude the oxygen contamination from Niskin bottle or chemical interferences, the same experiment was repeated in the laboratory using demineralized water. Water was deoxygenated by gentle bubbling with N_2 (g) for 20–30 min in a 5-L glass bottle. Winkler bottles (65 mL) were filled up with the artificial anoxic water following the same two procedures described above. Oxygen concentrations in the collected samples were measured in the Winkler bottles with LUMOS optodes (Lehner et al. 2015) or trace oxygen optodes (PyroScience GmbH). Samples for the determination of oxygen concentration by Winkler titration were also collected in 270-mL glass bottles, following the procedures described above of gentle overflow and submerged vessels. Immediately after collection, Winkler reagents were added to the bottles exposed to air (filled by overflow) or in submerged conditions using micropipettes and closed with glass stoppers. The bottles were kept submerged in water and analyzed after 24 h following the procedures described by Labasque et al. (2004).

Results and discussion

Sample collection in anoxic layers: Oxygen contamination from Niskin bottles

The standard procedure for sample collection in oceanography is by use of Niskin bottles, commonly attached to a rosette for multiple sample collection and controlled by the CTD unit. These bottles, normally made of polyvinyl chloride (PVC), are closed by the action of an elastic mechanism localized inside the bottle, commonly made of silicone or rubber tubing and more recently by polytetrafluoroethylene-coated (commercially known as Teflon) or epoxy-coated springs. These polymers have certain oxygen permeability and solubility (Robb 1968) and the often long resting periods on deck between casts ensure equilibration of these materials with the atmosphere. After closing of the bottle, the O_2 dissolved in the polymers is equilibrating with the O_2 concentration in the enclosed water. Sampling of anoxic water masses induces the largest oxygen gradient between the polymers and the confined water mass and oxygen thus leaks into the confined water sample.

We performed STOX measurements of O_2 contamination in the Niskin bottles during two cruises to the Pacific OMZs (AMOP and OC1705). In both cases and with two different approaches, O_2 contamination values in water samples collected at anoxic water depths were similar. Oxygen concentrations measured in a direct flow from the Niskin bottle ranged from 0.25 to 0.96 μM, being on average 0.56 ± 0.18 μM

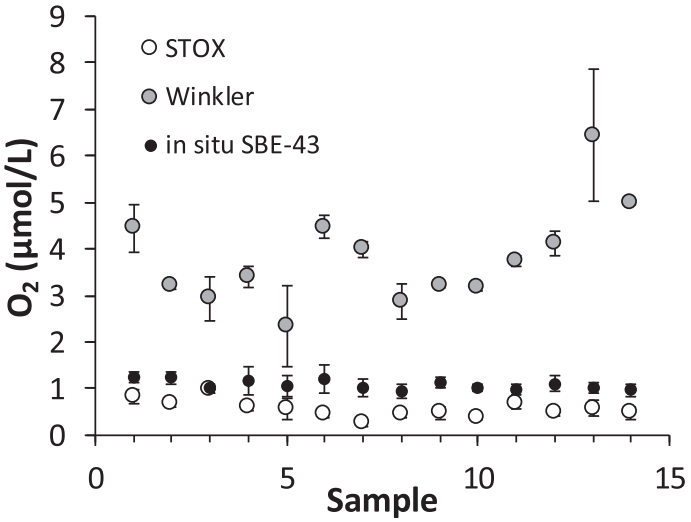

Figure 1. Oxygen concentration measured in the same Niskin bottle using STOX sensors and Winkler titration during the AMOP cruise at the ETSP off Peru. All samples were collected in apparently anoxic water layers. Data measured *in situ* with the SBE43 oxygen sensor mounted on the CTD are shown for comparison. Bar graph in the right panel shows mean ± satandard deviation (SD) for each sample.

(mean ± SD, $n = 14$, Fig. 1; Table 1). Similarly, oxygen concentration in water samples collected from the Niskin bottles into glass bottles under a N_2 (g) atmosphere ranged from 0.39 to 2.08 μM, being on average 0.81 ± 0.15 μM (mean ± SD, $n = 4$, Table 2). In spite of following the same procedure during several days, in one of the samplings (07 May 2017, Table 2) oxygen contamination values in the glass bottles were much higher, ranging from 0.87 to 4.45 μM, being on average 2.24 ± 1.41 μM (mean ± SD, $n = 5$).

The amount of oxygen dissolved in the polymers might depend of the history of the Niskin bottle before the sample confinement and thus it will be highly variable and unpredictable. Oxygen contamination values in the Niskin bottles showed a significant and negative correlation with temperature ($p < 0.01$, Spearman's rank correlation), with higher oxygen contamination values at the lowest in situ temperatures. In addition, colder waters are usually located at greater depths increasing the time between the confinement of the water sample and the collection of samples on deck. The O_2 contamination thus also exhibited a positive correlation with depth, but the correlation was not significant ($p = 0.055$, Spearman's rank correlation).

Besides these factors, other unpredictable events might significantly increase the contamination inside the bottles, such as the presence of air bubbles inside the bottle. Bubbles can be trapped inside the Niskin bottles, being imprisoned or attached in the closing mechanism, lids, walls, and so on. After the closing of the bottle, the oxygen in the bubble can dissolve in the sampled water, increasing the oxygen concentration. For instance, the high oxygen contamination measured in one of the samplings (07 May 2017, Table 2), could be caused by the presence of one single air bubble with a diameter of 6 mm at 100 m, increasing the oxygen concentration by 1.2 μM. Although a 6-mm diameter bubble might be unlikely, smaller bubbles might be more frequent and they also equilibrate faster, providing an unpredictable contamination of the sampled water.

The average contamination in the Niskin bottles of 0.56 ± 0.18 μM is similar to the accuracy of the Winkler method when analyzing air-equilibrated samples (Carpenter 1965; Labasque et al. 2004), and it might be unnoticeable when measurements in oxygenated water masses are performed. Contrary, for the anoxic water masses of the OMZ core or other euxinic environments, the increase in O_2 concentration might significantly change chemical and microbial transformations in the water samples. Some of the most relevant microbial processes have been shown to be highly sensitive to low O_2 concentrations, and processes like denitrification and anammox may be partly or totally inhibited (Dalsgaard et al. 2014) at submicromolar O_2 levels. On the other side, aerobic processes, such as ammonium and nitrite oxidation (Bristow et al. 2016), methane oxidation (Milucka et al. 2015; Steinle et al. 2017), or aerobic respiration (Garcia-Robledo et al. 2016; Gong et al. 2016), are highly efficient at low oxygen concentrations, with Km values below or similar to the O_2 contaminations measured in this study. The unavoidable oxygen contamination of the collected samples thus most likely result in wrong interpretations or estimations of microbial metabolism in the anoxic core of the OMZs if the

Table 2. Oxygen concentration (in μmol O_2 L^{-1}) measured in samples collected from Niskin bottles in full glass bottles using LUMOS sensors during the OC1705 cruise in the ETNP off Mexico. All samples were collected in the core of the OMZ in apparently anoxic water layers. Vigorous N_2 flow was applied inside the glass sampling bottles during the filling procedure to prevent air contact and oxygen contamination. Values are means ± SD, determined from the *N* replicates.

Date	St.	Lon. (°E)	Lat. (°N)	Depth (m)	*T* (°C)	[O_2]	*N*
06 May 2017	6	255.099	18.900	100	13.94	0.94 ± 0.30	7
07 May 2017	6	255.099	18.900	100	14.02	2.24 ± 1.41	5
10 May 2017	8	255.103	18.198	110	13.78	0.81 ± 0.58	7
11 May 2017	8	255.103	18.198	105	14.03	0.67 ± 0.32	7

collected samples are investigated without prior deoxygenation. Even with prolonged N_2 or He bubbling of water in a large container, and transfer with procedures optimized to minimize atmospheric contamination it was, however, very difficult to get final O_2 concentrations inside the custom-made all-glass bottles of less than 100 nM (Tiano et al. 2014*b*). To obtain samples much below this O_2 concentration it is apparently necessary to bubble with strictly O_2-free gas like helium directly within the glass container (Lehner et al. 2015), which is only possible with a gas headspace. By this approach, it is possible to reach O_2 concentrations below a detection limit of 1 nM (Sun et al. 2021).

Using an intensive bubbling deoxygenation procedure, other gases are also removed along with oxygen. Some gases of general interest in OMZs, such as methane or nitrous oxide, are added subsequently for transformation studies (Bourbonnais et al. 2021; Thamdrup et al. 2019). CO_2 is also partially removed, which can modify the final pH of the sample. However, CO_2 (g) concentration in seawater is relatively low as compared to the bicarbonate pool in the pH range of OMZ seawater (Hernandez-Ayon et al. 2019) and the change in both total CO_2 content (carbonate + bicarbonate + carbon dioxide) and pH could be considered negligible for most studies. We measured a drop of 0.1–0.2 pH units after 30 min of vigorous bubbling of coastal seawater with N_2 (g) in laboratory tests (data not shown). However, longer deoxygenation procedures could change these values, so specific validations are required if the main objective of the study is on pH-sensitive compounds.

The oxygen contamination might be removed by processing the sample after collection. However, it should be noted that this procedure does not guarantee the conservation of reduced compounds (sulfide, iodine, and so on), commonly present in anoxic waters. The oxygen contamination (up to 3–4 μM according to our measurements) might oxidize some of the reduced compounds initially present in the hypoxic/anoxic water and modify the chemical composition of the sample. Oxidized compounds such as elemental sulfur, thiosulfate, sulfite, or iodate are the substrate for different microbial processes (Hayes et al. 2006; Moriyasu et al. 2020; van Vliet et al. 2020) and the generation of such compounds during the sampling might result in the overestimation of microbial processes. Although this impact was not studied in detail in the present study, sampling in euxinic environments should consider the indirect effects of oxygen contamination by interpretation of the results.

Sample collection in anoxic layers: Overestimation by Winkler measurements

Measurement by the Winkler method is the gold standard for the determination of oxygen concentrations in the ocean. Besides their use for monitoring the oxygen distribution, they are also used for the calibration of sensors mounted on CTDs and other autonomous platforms such as moorings, buoys, and so on. The method is relatively simple and provides high accuracy and precision in seawater samples (Carpenter 1965). However, by all modifications of the method (Carpenter 1965; Helm et al. 2012; Labasque et al. 2004; Langdon 2010), the determined O_2 concentrations may deviate strongly from actual values in low O_2 samples, and in addition come contamination problems by sampling.

As discussed before, collecting low-O_2 samples by Niskin bottles increases the oxygen concentration of the bottled water with 0.5–1 μmol L^{-1}. During the AMOP cruise, oxygen concentrations were measured in samples from the same bottles by Winkler, and values ranged from 2.56 to 6.43 μmol L^{-1}, with an average of 3.79 ± 1.03 μmol L^{-1} (mean $\pm$ SD, $n = 14$, Table 1; Fig. 1). The titrations thus resulted in apparent O_2 concentrations that were 1.60–5.85 μmol L^{-1} higher than the actual values, with an average increase of 3.23 ± 1.08 μmol L^{-1} (mean $\pm$ SD, $n = 14$).

Measurement by Winkler in low O_2 waters has two important sources or error: Gas exchange during the manipulation of the samples/addition of reagents (excluding the oxygen added with reagents, taken into account by calculations), and interference from chemical compounds in the sample (Broenkow and Cline 1969; Langdon 2010; Wong and Li 2009). Some reducing compounds such as sulfide decrease the oxygen concentration measured (Broenkow and Cline 1969). However, most OMZs are characterized by lack of sulfide at relevant concentrations (Cutter et al. 2018; Schunck et al. 2013). On the other hand, nitrite and iodate increase the apparent oxygen concentration measured (Broenkow and Cline 1969; Wong 2012; Wong and Li 2009) and might be found at relevant concentrations in OMZs.

Iodate concentration in the oceans is in the range of 0.35–0.45 μmol L^{-1}, and might eventually increase to concentrations of 0.75 μmol L^{-1} in coastal areas or in layers with recent interaction with the sediment (Evans et al. 2020; Moriyasu et al. 2020; Wong and Li 2009). The interference corresponds to 1.5 mol of O_2 per mole of iodate (Wong and Li 2009). Consequently, the overestimation range in the open oxic ocean environment might be between 0.52 and 0.63 μmol O_2 L^{-1}. In the anoxic layers of the OMZ, iodate is biologically consumed and samples collected in anoxic waters of the ETNP OMZ and Arabian Sea OMZ have iodate concentrations below 0.2 μmol L^{-1} (Evans et al. 2020; Farrenkopf and Luther III 2002) that could increase the apparent oxygen concentration measured by up to 0.3 μmol O_2 L^{-1}. Iodate could thus only contribute by 5–18% to the apparent increase in oxygen concentration measured with Winkler method.

One mole of nitrite gives an interference equivalent to 0.25–0.5 mol of oxygen (Wong 2012), and as NO_2^- in the anoxic core may be present at concentrations up to 7 μmol L^{-1} (Peters et al. 2016), nitrite could potentially cause all the overestimation by our Winkler titrations (Broenkow and Cline 1969). We therefore conducted a series of laboratory determinations with nitrite, iodate and O_2 free demineralized water (Fig. 2). The Winkler determinations were in this case

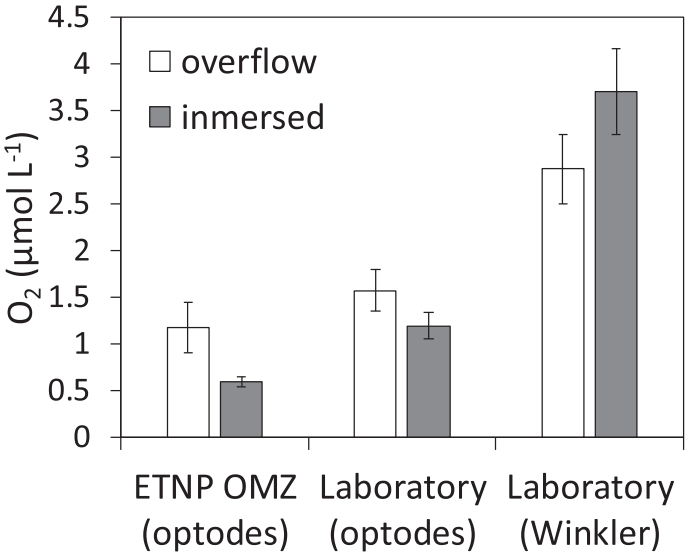

Fig 2. ETNP OMZ (optodes): Oxygen concentration in samples collected at anoxic water in the ETNP during the OC1705 cruise. Samples were collected directly from the Niskin bottle to glass Winkler bottles (60–65 mL total volume) allowing gentle overflow before the closing of the bottle with glass stoppers, or filling the bottles submerged in a beaker containing the sampled water to avoid atmospheric contamination ($n = 3$). Oxygen optode spots were preinstalled inside the bottles and oxygen was measured with LUMOS technology. Laboratory (optodes): The same procedure was repeated in laboratory, deoxygenating 5 L of distilled water by gentle N_2 (g) bubbling for 35–40 min and transferring the anoxic water by siphoning to the same Winkler bottles as used during the OC1705 cruise. The experiment was repeated to get $n = 12$. Laboratory (Winkler): Similar procedure was performed in the laboratory, collecting samples in 270-mL bottles and measuring O_2 concentration with Winkler determination as described in Labasque et al. (2004; $n = 7$). Bar graphs are means ± SE from the n replicates.

done with both bottles filled the usual way with Tygon tubing at the bottom of the bottles and allowing gentle overflow, and with submerged bottles. Under the well-controlled laboratory conditions, the determined O_2 concentrations were extremely similar to the O_2 concentrations measured by Winkler in Niskin bottle water (Table 1), indicating that nitrite and iodate were not the major factors causing the overestimation by Winkler observed with anoxic core samples. There was no statistically significant difference between the two types of bottle filling and the average determined concentration was $3.03 \pm 0.36\,\mu$M which is very close to the average O_2 concentration measured in water from Niskin bottles by Winkler ($3.81 \pm 0.28\,\mu$M).

To avoid the uncertainty introduced by Winkler on the difference between usual filling procedure and submerged bottles, the comparison was also conducted with Winkler bottles containing optode dots. Here, the field sampling directly from the Niskin bottle showed less contamination by submerged bottles than by bottles filled under air (ETNP OMZ, Fig. 2). The picture was not so clear when the experiment was repeated in the laboratory with a higher number of replicates (Laboratory, Fig. 2). It is thus not clear whether the filling of bottles while submerged constitutes a real advantage. The O_2 concentration in the water bath evidently affect the effectiveness of the submerged procedure, and the number of volume changes by flushing have an affect by both procedures.

Recommendations

O_2 contamination of anoxic or near-anoxic water by collection in PVC Niskin bottles and subsequent transfer into glass containers is unavoidable and O_2 is introduced at concentrations of 0.5–1.0 μM. Determination of the in situ O_2 concentration from such water samples is thus not possible, and by even very careful use of the Winkler technique, more O_2 is introduced so that in situ anoxic water appeared to contain 2–4 μM O_2. Determination of in situ O_2 concentrations must therefore be conducted by equipment that can actually measure in situ and Winkler data at low concentrations (< 30 μM) should not be used for calibration of in situ sensors to avoid significant errors (> 10%). Water sampling from anoxic environments introduces O_2 in the samples and deoxygenation of samples are mandatory if truly anoxic conditions are required. Deoxygenation of water samples by bubbling with O_2-free gas should preferably be conducted in all-glass containers in which the experiments are to be conducted, as a laboratory transfer of water leads to unpredictable O_2 contamination.

References

Bourbonnais, A., and others. 2021. Protocols for assessing transformation rates of nitrous oxide in the water column. Front. Mar. Sci. **8**: 293–310. doi:10.3389/fmars.2021.611937

Breitburg, D., and others. 2018. Declining oxygen in the global ocean and coastal waters. Science **359**: eaam7240. doi:10.1126/science.aam7240

Breitburg, D. L., D. W. Hondorp, L. A. Davias, and R. J. Diaz. 2009. Hypoxia, nitrogen, and fisheries: Integrating effects across local and global landscapes. Ann. Rev. Mar. Sci. **1**: 329–349. doi:10.1146/annurev.marine.010908.163754

Bristow, L. A., and others. 2016. Ammonium and nitrite oxidation at nanomolar oxygen concentrations in oxygen minimum zone waters. Proc. Natl. Acad. Sci. **113**: 10601–10606. doi:10.1073/pnas.1600359113

Bristow, L. A. 2018. Anoxia in the snow. Nat. Geosci. **11**: 226–227.

Broenkow, W. W., and J. D. Cline. 1969. Colorimetric determination of dissolved oxygen at low concentrations. Limnol. Oceanogr. **14**: 450–454. doi:10.4319/lo.1969.14.3.0450

Carpenter, J. H. 1965. The Chesapeake Bay institute technique for the Winkler dissolved oxygen method. Limnol. Oceanogr. **10**: 141–143. doi:10.4319/lo.1965.10.1.0141

Codispoti, L., and others. 2001. The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the

anthropocene? Scientia Marina **65**: 85–105. doi:10.3989/scimar.2001.65s285

Cutter, G. A., J. W. Moffett, M. C. Nielsdóttir, and V. Sanial. 2018. Multiple oxidation state trace elements in suboxic waters off Peru: In situ redox processes and advective/diffusive horizontal transport. Mar. Chem. **201**: 77–89. doi:10.1016/j.marchem.2018.01.003

Dalsgaard, T., and others. 2014. Oxygen at nanomolar levels reversibly suppresses process rates and gene expression in anammox and denitrification in the oxygen minimum zone off northern Chile. MBio **5**: e01966. doi:10.1128/mBio.01966-14

De Brabandere, L., B. Thamdrup, N. P. Revsbech, and R. Foadi. 2012. A critical assessment of the occurrence and extend of oxygen contamination during anaerobic incubations utilizing commercially available vials. J. Microbiol. Methods **88**: 147–154. doi:10.1016/j.mimet.2011.11.001

Evans, Z. C., and others. 2020. The role of water masses in shaping the distribution of redox active compounds in the Eastern Tropical North Pacific oxygen deficient zone and influencing low oxygen concentrations in the eastern Pacific Ocean. Limnol. Oceanogr. **65**: 1688–1705. doi:10.1002/lno.11412

Farrenkopf, A. M., and G. W. Luther III. 2002. Iodine chemistry reflects productivity and denitrification in the Arabian Sea: Evidence for flux of dissolved species from sediments of western India into the OMZ. Deep-Sea Res. II Top. Stud. Oceanogr. **49**: 2303–2318. doi:10.1016/S0967-0645(02)00038-3

Garcia-Robledo, E., S. Borisov, I. Klimant, and N. P. Revsbech. 2016. Determination of respiration rates in water with submicromolar oxygen concentrations. Front. Mar. Sci. **3**: 244. doi:10.3389/fmars.2016.00244

Gong, X., E. Garcia-Robledo, A. Schramm, and N. P. Revsbech. 2016. Respiratory kinetics of marine bacteria exposed to decreasing oxygen concentrations. Appl. Environ. Microbiol. **82**: 1412–1422. doi:10.1128/AEM.03669-15

Granéli, W., and E. Granéli. 1991. Automatic potentiometric determination of dissolved oxygen. Mar. Biol. **108**: 341–348. doi:10.1007/BF01344349

Hayes, M. K., G. T. Taylor, Y. Astor, and M. I. Scranton. 2006. Vertical distributions of thiosulfate and sulfite in the Cariaco Basin. Limnol. Oceanogr. **51**: 280–287. doi:10.4319/lo.2006.51.1.0280

Helm, I., L. Jalukse, and I. Leito. 2012. A highly accurate method for determination of dissolved oxygen: Gravimetric Winkler method. Anal. Chim. Acta **741**: 21–31. doi:10.1016/j.aca.2012.06.049

Hernandez-Ayon, J. M., and others. 2019. Dynamics of the carbonate system across the Peruvian oxygen minimum zone. Front. Mar. Sci. **6**: 617–633.

Horak, R. E. A., W. Ruef, B. B. Ward, and A. H. Devol. 2016. Expansion of denitrification and anoxia in the eastern tropical North Pacific from 1972 to 2012. Geophys. Res. Lett. **43**: 5252–5260. doi:10.1002/2016GL068871

Ingvorsen, K. & B. B. Jorgensen 1979. Combined measurement of oxygen and sulfide in water samples. 24: 390–393.

Jørgensen, B. B., H. Fossing, C. O. Wirsen, and H. W. Jannasch. 1991. Sulfide oxidation in the anoxic Black Sea chemocline. Deep Sea Res. A Oceanogr. Res. Pap. **38**: S1083–S1103. doi:10.1016/S0198-0149(10)80025-1

Kalvelage, T., and others. 2011. Oxygen sensitivity of Anammox and coupled N-cycle processes in oxygen minimum zones. PLoS One **6**: e29299. doi:10.1371/journal.pone.0029299

Labasque, T., C. Chaumery, A. Aminot, and G. Kergoat. 2004. Spectrophotometric Winkler determination of dissolved oxygen: Re-examination of critical factors and reliability. Mar. Chem. **88**: 53–60. doi:10.1016/j.marchem.2004.03.004

Langdon, C. 2010. Determination of dissolved oxygen in seawater by Winkler titration using the Amperometric technique. *In* E. M. Hood, C. L. Sabine, and B. M. Sloyan [eds.], The GO-SHIP repeat hydrography manual: A collection of expert reports and guidelines. International Ocean Carbon Coordination Project.

Larsen, M., and others. 2016. In situ quantification of ultra-low O_2 concentrations in oxygen minimum zones: Application of novel optodes. Limnol. Oceanogr. Methods **14**: 784–800. doi:10.1002/lom3.10126

Lehner, P., and others. 2015. LUMOS—a sensitive and reliable Optode system for measuring dissolved oxygen in the Nanomolar range. PLoS One **10**: e0128125. doi:10.1371/journal.pone.0128125

Milucka, J., and others. 2015. Methane oxidation coupled to oxygenic photosynthesis in anoxic waters. ISME J. **9**: 1991–2002. doi:10.1038/ismej.2015.12

Moriyasu, R., Z. C. Evans, K. M. Bolster, D. S. Hardisty, and J. W. Moffett. 2020. The distribution and redox speciation of iodine in the eastern tropical North Pacific Ocean. Global Biogeochem. Cycl. **34**: e2019GB006302. doi:10.1029/2019GB006302

Paulmier, A., and D. Ruiz-Pino. 2009. Oxygen minimum zones (OMZs) in the modern ocean. Prog. Oceanogr. **80**: 113–128. doi:10.1016/j.pocean.2008.08.001

Peters, B. D., and others. 2016. Vertical modeling of the nitrogen cycle in the eastern tropical South Pacific oxygen deficient zone using high-resolution concentration and isotope measurements. Global Biogeochem. Cycles **30**: 1661–1681. doi:10.1002/2016GB005415

Revsbech, N. P., B. Thamdrup, T. Dalsgaard, and D. E. Canfield. 2011. Construction of STOX oxygen sensors and their application for determination of O_2 concentrations in oxygen minimum zones, p. 325-341. *In* M. G. Klotz [ed.], Methods in enzymology: Research on nitrification and

related processes, Vol. 486, Part A. Methods in enzymology. Elsevier Academic Press Inc.

Robb, W. L. 1968. Thin silicone membranes-their permeation properties and some applications. Ann. N. Y. Acad. Sci. **146**: 119–137. doi:10.1111/j.1749-6632.1968.tb20277.x

Sampaio, E., and others. 2021. Impacts of hypoxic events surpass those of future ocean warming and acidification. Nat. Ecol. Evol. **5**: 311–321. doi:10.1038/s41559-020-01370-3

Schunck, H., and others. 2013. Giant hydrogen sulfide plume in the oxygen minimum zone off Peru supports Chemolithoautotrophy. PLoS One **8**: e68661. doi:10.1371/journal.pone.0068661

Steinle, L., and others. 2017. Effects of low oxygen concentrations on aerobic methane oxidation in seasonally hypoxic coastal waters. Biogeosciences **14**: 1631–1645. doi:10.5194/bg-14-1631-2017

Stramma, L., G. C. Johnson, J. Sprintall, and V. Mohrholz. 2008. Expanding oxygen-minimum zones in the tropical oceans. Science **320**: 655–658. doi:10.1126/science.1153847

Sun, X., C. Frey, E. Garcia-Robledo, A. Jayakumar, and B. B. Ward. 2021. Microbial niche differentiation explains nitrite oxidation in marine oxygen minimum zones. ISME J. **15**(5):1317–1329. doi:10.1038/s41396-020-00852-3

Thamdrup, B., and others. 2019. Anaerobic methane oxidation is an important sink for methane in the ocean's largest oxygen minimum zone. Limnol. Oceanogr. **64**: 2569–2585. doi:10.1002/lno.11235

Tiano, L., E. Garcia-Robledo, T. Dalsgaard, A. H. Devol, B. B. Ward, O. Ulloa, D. E. Canfield, and N. Peter Revsbech. 2014a. Oxygen distribution and aerobic respiration in the north and south eastern tropical Pacific oxygen minimum zones. Deep-Sea Res. Part I-Oceanogr. Res. Pap. **94**: 173–183. doi:10.1016/j.dsr.2014.10.001

Tiano, L., E. Garcia-Robledo, and N. P. Revsbech. 2014b. A new highly sensitive method to assess respiration rates and kinetics of natural planktonic communities by use of the switchable trace oxygen sensor and reduced oxygen concentrations. PLoS One **9**: e105399. doi:10.1371/journal.pone.0105399

van Vliet, D. M., and others. 2020. The bacterial sulfur cycle in expanding dysoxic and euxinic marine waters. Environ. Microbiol. **23**(6):2834–2857.

Vaquer-Sunyer, R., and C. M. Duarte. 2008. Thresholds of hypoxia for marine biodiversity. Proc. Natl. Acad. Sci. **105**: 15452–15457. doi:10.1073/pnas.0803833105

Wong, G. T. F., and K.-Y. Li. 2009. Winkler's method overestimates dissolved oxygen in seawater: Iodate interference and its oceanographic implications. Mar. Chem. **115**: 86–91. doi:10.1016/j.marchem.2009.06.008

Wong, G. T. F. 2012. Removal of nitrite interference in the Winkler determination of dissolved oxygen in seawater. Mar. Chem. **130–131**: 28–32. doi:10.1016/j.marchem.2011.11.003

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Conflict of Interest

None declared.

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