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An improved method for the determination of dissolved nitric oxide (NO) in seawater samples

H. E. Lutterbeck and H. W. Bange

Department, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

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Correspondence to: H. E. Lutterbeck (hlutterbeck@geomar.de)

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Abstract

Nitric oxide (NO) is a short-lived intermediate of the oceanic nitrogen cycle, however, due to its high reactivity, measurements of dissolved NO in seawater are rare. Here we present an improved method to determine NO concentrations in discrete seawater samples. The set-up of our system consisted of a chemiluminescence NO analyser connected to a stripping unit. The limit of detection for our method was 5 pmol NO in aqueous solution which translates into 0.25 nmol L^{-1} when using a 20 mL seawater sample volume. Our method was applied to measure high resolution depth profiles of dissolved NO during a cruise to the eastern tropical South Pacific Ocean. Our method is fast and comparably easy to handle thus it opens the door for deciphering the distribution of NO in the ocean and it facilitates laboratory studies on NO pathways.

1 Introduction

Nitric oxide (NO) is a short-lived intermediate of various microbial processes of the nitrogen cycle (see e.g. Thamdrup, 2012). Molecular analysis and lab culture experiments showed that various kind of bacteria are able to metabolize NO, e.g. ammonium-oxidizing bacteria (Lipschultz et al., 1981), nitrite-oxidizing bacteria (Freitag and Bock, 1990), methanotrophic bacteria (Yoshinari, 1985) and denitrifying bacteria (Firestone et al., 1979). However, it is still unclear which processes are responsible for the occurrence of NO in natural environments. Although ammonium- and nitrite-oxidizing bacteria can produce NO, there is no evidence for NO as an intermediate during nitrification. A study which compared mathematical models with the results from a laboratory-scale waste water sludge reactor showed that denitrification indeed could be a dominating process of NO release (Kampschreur et al., 2007). This pathway has been investigated in great detail and therefore its enzymatic NO production and the subsequent reduction of NO to nitrogen (N_2) are well understood (Zumft, 1997). Another process where NO is probably involved as an intermediate is anammox (Strous et al., 2006; Kartal et al.,

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2011). The latest discovery was the enzymatic dismutation of NO resulting in the microbial production of oxygen (O_2) used to oxidize methane under anaerobic conditions (Ettwig et al., 2010, 2012). Additionally, NO can be produced in the ocean surface layer by the photochemical reduction of dissolved nitrite (NO_2^-) (Zafiriou and True, 1979; Olasehinde et al., 2010).

In summary, there are various potential microbial NO production/consumption pathways in the ocean. Unfortunately, our knowledge about the oceanic NO distribution and the major pathways of NO is very poor. There are only a few published NO concentration measurements available (Bange, 2008) because a reliable and easy to use method to determine dissolved NO at in-situ concentrations in seawater samples is missing.

Gaseous and dissolved NO is a very reactive and, thus short-lived molecule because it is a free radical. Its occurrence is predominantly depending on the presence of O_2 because it is rapidly oxidized to nitrogen dioxide (NO_2) (Lewis and Deen, 1994). Therefore, the determination of low concentrations of dissolved NO at in situ conditions is challenging. A summary of the existing methods for the determination of NO is given by Hetrick and Schoenfisch (2009). The published methods for measurement of dissolved NO in seawater are listed in Table 1. The detection limits range from 0.0015 to 140 nmol L^{-1}. (Please note that the fluorometric detection of NO as described by Olasehinde et al., 2009 is suitable only for formation rates of NO from NO_2^-.) The sensor of Schreiber et al. (2008) was developed for sediments but works in seawater samples, as well. The chemiluminescence system of Zafiriou and McFarland (1980) consisted of an NO analyser connected to a stripping unit and is, thus, similar to the set-up described here. The method by Zafiriou and McFarland (1980) is the only one so far which was applied on board to measure NO depth profiles during a cruise (Ward and Zafiriou, 1988). However, the required intensive cleaning of the Niskin bottles prior to the CTD/rosette casts together with the fact that each depth was sampled with a separate cast resulted in a time-consuming and unhandy sampling procedure.

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Therefore, it was the aim of this study to develop an improved (easy to handle) method for the detection of dissolved NO in discrete seawater samples at in situ concentrations in order to decipher its role in the oceanic nitrogen cycle.

2 Material and methods

2.1 Instrumental set-up

The set-up of our system consisted of a NO analyser connected via a 4-way gas stream selecting valve to a stripping unit and to the gas cylinders for reference gas and carrier gas. A schematic diagram of the system set-up is shown in Fig. 1.

The carrier gas (N_2) and the reference gas (1000 ppb NO in N_2) were connected to a two channel mass flow controller with mixing chamber (HTK Hamburg GmbH, Hamburg, Germany) to ensure a constant gas flow rate of 1 L min^{-1} and to enable the calibration of the detector signal (see Sect. 2.4.1). The mass flow controller, in turn, was connected to the gas stream selecting valve. This 4-way valve allowed to switch between two modes of gas flow: Mode A enabled the direct measurement of the reference gas and carrier gas and mode B allowed detection of the gas stream after going through the stripping unit.

The sample and stripping vials were connected to the gas line by needles (diameter 1.2 mm) pushed through the respective septa. Two inline filters (Whatman Solvent IFD, 0.2 µm, GE Healthcare UK Limited, Buckinghamshire, England) were installed to remove aerosols from the gas stream. Between the 4-way valve and the NO analyser a needle valve was installed to reduce pressure variations. For a detailed description of the measurement procedure see Sects. 2.2 (samples) and 2.4 (standards).

For detection, we used a chemiluminescence NO analyser (model 42i-TL, Thermo Fisher Scientific Inc, Waltham, MA, USA) with a detection range from 0 to 1000 ppb. In the reaction chamber of the analyser, NO reacts with ozone (O_3) generated by an O_3 generator and produces NO_2 in an excited state (NO_2^*). By relaxation to the ground

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state the NO_2^* emits a photon. The emitted light passes an optical filter to remove interferences from other compounds and is detected by a photomultiplier. The signal recording was done with the open source software PuTTY 0.62 (http://filehippo.com/de/download_putty/11216/).

2.2 Sample handling

Sampling took place with a commonly used Conductivity Pressure Depth sensor, equipped with a Niskin bottle rosette (CTD/rosette) and with a pump CTD (pCTD) system (Strady et al., 2008) during the Meteor cruise M93 from 6 February to 11 March 2013 to the Eastern Tropical South Pacific off Peru (Callao, Peru to Panama City, Panama). Seawater samples were taken bubble free in 20 mL brown glass vials, closed with rubber plugs and crimped with aluminium caps. Directly after sampling all samples were stored in a cooling box (~ 6 °C) until they were measured. From each water depth three to six replicates were taken. From the CTD/rosette all samples were taken as soon as possible, after the CTD was back on the ship's working deck and were measured within one hour. The samples from the pCTD were taken as soon as the target depth was reached and were measured immediately within a few minutes after sampling.

For the measurement, the 4-way valve was switched to mode A to enable the connection of the sample vial by the needles. In the next step the 4-way valve was switched to mode B to reroute the gas flow through the stripping unit. The water of the sample was pushed with the carrier gas into the stripping vial. The stripping vial had a larger volume (50 mL) as the sample vial to allow purging of the sample. The dissolved NO was stripped from the sample by N_2 and transported with the carrier gas stream into the analyser. The sample stayed connected stripping unit (mode B) until the detector signal came back to the baseline. Then the 4-way valve was switched to mode A and the next sample was connected.

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2.3 Experiments

For sample storage experiments we took 18 samples from the pCTD at two stations from the oxygen minimum zone (OMZ) at depths between 60 and 90 m and stored nine of them at room temperature (RT, ~ 24 °C) and nine at ~ 6 °C in the dark. For the time series, triplicates per temperature were measured in various time steps.

For NO_2^- addition tests we added 20 µL of a 20 mmol L^{-1} sodium nitrite ($NaNO_2$) aqueous solution to about 100 samples taken at different stations and depths; this corresponds to a concentration of 20 µmol L^{-1}, in addition to the natural concentration already present in the sample. Samples were stored for different time periods, between some minutes and some hours in warm (~ 24 °C) and cold (~ 6 °C) environments and then measured like normal samples. Additionally we stored control samples without NO_2^- addition under the same conditions.

2.4 Calibration

2.4.1 Detector calibration

To calibrate the detector signal the carrier gas (N_2) was blended with the reference gas (1000 ppb NO in N_2) by the mass flow controller (see above). The resulting NO mixing ratios covered the whole detection range of the NO analyser (0 to 1000 ppb).

2.4.2 Gas standard injection

Discrete volumes of reference gas ranging from 0.5 to 10 mL were injected with gas tight syringe (series A-2, Valco Instruments Company Inc., Houston, TX, USA) into the empty stripper. Two different reference gases with concentrations of 1000 ppb NO and 10 ppm NO were used.

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2.4.3 Aqueous NO standard solutions

For preparation of aqueous NO standard solutions a 20 mL brown glass vial filled with 10 mL MilliQ water was purged with N_2 for one hour with a flow rate of 100 mL min^{-1} and then with pure NO or a reference gas (1 % NO in N_2), with a flow rate of 5 mL min^{-1} for two hours. Assuming a solubility of 1.94 ± 0.03 mmol L^{-1} atm^{-1} at 25 °C for NO (Zacharia and Deen, 2004 and references therein) the final concentrations of the solutions were 1.94 mmol L^{-1} and 19.4 µmol L^{-1} respectively. The standards were stored in the dark at RT.

For the actual measurements 20 mL MilliQ water were deoxygenated with N_2 for one hour at a flow rate of 150 mL min^{-1} in a 50 mL vial. Then the vial was connected to the stripping unit followed by an injection of varying volumes (in the range from 1 to 100 µL) of standard through the septum of the vial.

2.4.4 In situ NO formation from NO_2^- reduction

This calibration method is based on the in situ formation of NO by chemical reduction of NO_2^- with iodide (I^-) in an acidic aqueous medium (Cox, 1980). The preparation of the NO_2^- solution started with a stock solution of 1 mol L^{-1} $NaNO_2$ in MilliQ water followed by a two-step dilution series (100 µL in 100 mL MilliQ water) to get two NO_2^- standards with concentrations of 1 mmol L^{-1} and 1 µmol L^{-1}, respectively. They were stored in the dark at RT.

The reaction solution is made of two solutions: 11 mL glacial acetic acid were added to 100 mL MilliQ water yielding a 10 % acetic acid (with a concentration of 1.68 mol L^{-1}; Kester et al., 1994) and 3 g KI were dissolved in 100 mL MilliQ water to get a 3 % w/v KI solution (Garside, 1982).

Prior to a measurement, 1 mL of the KI solution and 1.5 mL 10 % acetic acid were mixed in a 50 mL vial and MilliQ water was added to a final volume of 20 mL. The vial was purged for 20 min with N_2 (flow rate 150 mL min^{-1}) to remove the O_2 and was then

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connected to the stripping unit. Then the NO_2^- solution was added via the septum as described above.

2.5 Data analysis

The recorded NO signals (i.e. peaks) were integrated manually with Origin (OriginLab, Northampton, MA). With the obtained peak areas from the standard measurements a linear calibration equation was calculated to convert the peak areas of sample measurements into concentrations.

The signal to noise ratio (S/N) was calculated by an equation of Stauffer (2008):

$$S/N = 2H/h_N \quad (1)$$

H stands for the height of the signal and h_N is the height of the baseline noise. For the limit of detection (LOD) the value of S/N was set to 3 and for the limit of quantification (LOQ) a value of 10 was used. NO signals below the detection limit were set to zero in further calculations (e.g. averaging of replicates).

3 Results and discussion

3.1 Method evaluation

3.1.1 Standard measurements

To assess the performance of our instrumental set-up, we calculated the LOD, LOQ and the SD (average from all measurements over the whole detection range) for the different gaseous and aqueous standards. The results are summarised in Table 2. Please note that the values are given as molar amounts of substance and not as concentrations in order to get volume independent numbers and thus a better comparability between the different kinds of standards described in Sects. 2.4.2–2.4.4. Concentrations for sample measurements are discussed in Sect. 3.1.2.

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For aqueous NO standards (see Sect. 2.4.3) the lowest detectable molar amount of NO was 5 pmol with a SD of 25 %. We observed that the aqueous NO solutions did not change within ten months. This indicates that the standard solutions are much longer stable than previously reported (Mesaros et al., 1997; Menon et al., 1990).

For in situ NO formation from NO_2^- reduction (see Sect. 2.4.4) the LOD was 10 pmol NO and the SD 3 %. The detection limit is higher than for aqueous NO solutions because NO is formed in situ and this results in broader peaks with lower peak heights. We observed no decrease of the NO_2^- concentration in the standards during our measurements and conclude that the NO_2^- solutions should be stable when kept in the dark. The ratio of peak area to concentration is similar for both the aqueous NO standards and the in situ formation of NO from NO_2^- reduction. Thus both standards can be used for calibration of aqueous samples.

Discrete gas standard measurements had a detection limit of 15 pmol NO. By cleaning the gas tight syringes after five measurements with 100 % ethanol the SD could be decreased from 65 to 10 %. We observed no influence of the injected volume between 0.5 and 10 mL on the detected NO. The stability of the used reference gases (one year) was given by the manufacturer.

3.1.2 Sample measurements

With a water volume of 20 mL the LOD as well as the LOQ for dissolved NO translates into concentrations of 0.25 and 1 nmol L^{-1}, respectively. By enlarging the sample volume the detection limit can be lowered. However, the peaks will get broader with larger volume thus the detection limit will not decrease in the same amount as the sample volume is increased. We observed, for example, that by increasing the sample volume from 20 to 80 mL the detection limit rose to 10 pmol detectable molar amount of NO but the detectable concentration decreased to 0.125 nmol L^{-1}.

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3.2 Interferences by other components

3.2.1 Hydrogen sulphide (H_2S)

During the cruise M93 we faced a sulfidic event close to the coast of Peru. Therefore, some of the samples contained H_2S which resulted in a strong negative detector signal (Fig. 2). A visible negative response of the NO analyser (i.e. stronger than the baseline noise of the instrument) was determined down to a concentration of about 80 nmol L^{-1} H_2S, but even lower H_2S concentrations could have an impact on the NO signal such as neutralisation of a positive NO signal. Tests with addition of $ZnCl_2$ (in order to precipitate H_2S as ZnS) showed that the negative peaks of H_2S vanished indeed, but the impact of $ZnCl_2$ addition on the NO concentration in the sample is unknown. It might be possible that NO is removed from the sample by chemical reduction. Some preliminary tests showed that $ZnCl_2$ can also increase the NO concentration, possibly a release from poisoned plankton.

3.2.2 Nitrite (NO_2^-)

NO can photochemically be produced from dissolved NO_2^- (Zafiriou and True, 1979; Olasehinde et al., 2010). As NO_2^- can be enhanced in the water column (especially in OMZs) we performed NO_2^- addition tests to find out if there is any light induced production of NO caused by our sample handling.

Our experiments showed no differences in NO concentrations between samples with and without NO_2^- addition. The addition of 1 mL of a 1 mmol L^{-1} $NaNO_2$ solution to 20 mL MilliQ water resulted only in a very small NO peak. Thus we conclude that a potential in situ production of NO from NO_2^- does not affect the measurement method described here.

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3.3 Sample handling

Two factors influenced the NO concentrations in the samples: the storage time (i.e. the time between sampling and the actual measurement of the sample) and the ambient O_2 concentrations. This is especially important for samples from the OMZ where slight changes in O_2 are expected to have a significant effect on dissolved NO (Lewis and Deen, 1994).

The storage experiments showed a decrease in NO concentrations over time with a stronger decline at room temperature compared to storage at ~ 6 °C (Fig. 3). The decrease of the NO concentrations may be explained by the well-known common effect of bottle consumption caused by pores in the glass vials and in the rubber stoppers. However, a stronger effect on the NO concentration is probably caused by diffusion of O_2 into the sample. De Brabandere et al. (2012) showed that O_2 contamination can be caused by diffusion of O_2 out of the rubber plugs. At room temperature the diffusion of 1 nmol O_2 into a water sample takes only a few seconds. As NO is very O_2 sensitive (Lewis and Deen, 1994) it can be assumed that this O_2 impurity resulted in a decrease of NO in the sample vials. It can also partly explain the enhanced decrease at room temperature compared to storage at ~ 6 °C. At higher temperatures the input of O_2 is faster and thus more NO could be degraded. Another reason for the temperature effect is a potential biological consumption, e.g. by denitrification and anammox, in the samples from the OMZ. As the metabolic activity is higher at room temperature compared to ~ 6 °C more NO could be used up.

A large impact on the NO concentrations in the samples had the choice of the water sampling system (Niskin bottles or pCTD). The scatter plot with our measurements from the Niskin bottles of the CTD/rosette (Fig. 4a) shows that the NO concentrations were mostly near or below the detection limit. Only a few samples showed NO concentrations of up to 2 nmol L^{-1}. Contrasting to this, samples from the pump CTD (Fig. 4b) showed a broad range of concentrations up to 10 nmol L^{-1}. This has been confirmed by direct comparison of both CTD systems on two stations (Fig. 4c and d). No change

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in NO concentrations with depth was evident from CTD/rosette casts whereas from the pCTD plausible shapes of the NO depth profiles were obtained.

One reason for the obvious difference between the two CTD systems is most likely the time effect as found in our sample storage experiments which might have occurred in the Niskin bottles as well. The seawater stays between 30 min and several hours in the Niskin bottles during the time of a CTD cast until sampling. During this time an O_2 contamination of 1 µmol L^{-1} can be induced (Alarcón and Ulloa, 2009). This O_2 entry into the bottles together with a comparably long CTD cast time may result in a strong decrease of NO already before the samples could be taken. For the pCTD system a diffusive O_2 input to the water while pumped up of only 20 nmol L^{-1} has been estimated (Canfield et al., 2010) resulting in a low O_2 contamination. The short residence time of the seawater in the tubing in combination with a smaller O_2 contamination might have led to comparable lower NO degradation and thus in turn to higher detectable NO concentrations in the samples taken from the pCTD.

Overall, NO samples are unstable after sampling so they have to be processed very fast. Thus, it is recommended to use a pCTD as sampling system and O_2 contamination should be reduce to a minimum (e.g. by using deoxygenated materials). However, it may be possible that NO concentrations are rather underestimated due to sampling time and delay until measurement.

4 Summary

Here we present an improved method to determine dissolved NO in discrete seawater samples. The set-up of our system consisted of a chemiluminescence NO analyser connected to a stripping unit. The lower limit of detection for our method was 5 pmol NO in aqueous solution which corresponds to 0.25 nmol L^{-1} when using a 20 mL seawater sample volume. Our method was applied to measure high resolution depth profiles of dissolved NO during a cruise to the eastern tropical South Pacific Ocean. One CTD cast (including sampling) can be processed in less than two hours. However, for the

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sampling we recommend using a pump CTD rather than a conventional CTD/rosette with Niskin bottles. In general, contamination by O_2 diffusion into the samples should be minimized by using appropriate materials. Dissolved H_2S interferes strongly with the NO detection, whereas the in situ production of NO from dissolved NO_2^- seems to be negligible.

The in situ formation of NO from NO_2^- reduction (with I^- in acidified aqueous medium) would also allow applying our set up for the measurements of dissolved NO_2^- (and nitrate) in seawater samples (Garside, 1982) down to very low concentrations.

The method for the determination of dissolved NO as described here is fast and comparably easy to handle thus it opens the door for comprehensive measurements of the distribution of oceanic NO and it facilitates laboratory studies on NO pathways.

Appendix:

Nitrogen (N_2, 99.999 %), reference gases (1000 ppb NO in N_2, 10 ppm NO in N_2, 1 % NO in N_2), and nitric oxide (NO, ≥ 99.5 %) were purchased from Air Liquide GmbH (Düsseldorf, Germany). Sodium nitrite ($NaNO_2$, ≥ 99.0 %, p.a.), zinc chloride ($ZnCl_2$, ≥ 98.0 %, p.a.) and acetic acide (100 %) were from Merck KGaA (Darmstadt, Germany). And potassium iodide (KI, ≥ 99.5 %, p.a.) was obtained from Carl Roth GmbH (Karlsruhe, Germany).

All tubings were from stainless steel or PTFE with a diameter of 1/4 and 1/8 inch. Valves, fittings and needles were also made of stainless steel.

Processing a measurement with our system is fast, easy and takes only a few minutes. Up to 20 measurements can be done within one hour.

The instrument showed a stable baseline and no drift over time for two years. For measurements in solution and at higher concentration ranges, it is important to include breaks and cleaning of the stripping unit between the measurements to prevent a less sensitive detection limit. The higher the NO concentrations are, the more often breaks and cleaning are needed to keep the baseline stable. The same applies for MilliQ water

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and reaction solution for aqueous standard measurements. The two liquids can be reused for several measurements. The number of possible measurements done with the same liquid depend on the concentration. With higher concentrations the liquid should be replaced more often to keep the baseline constant.

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Table 1. Overview of published methods for NO detection in seawater with the respective limit of detection (LOD) for each method.

Method	LOD [nmol L^{-1}]	Reference
Microelectrode	140	Zhang et al. (2003)
Microelectrode	42	Xing et al. (2005)
Microelectrode	30	Schreiber et al. (2008)
Fluorometric	0.0124*	Olasehinde et al. (2009)
Chemiluminescence	0.0015	Ward and Zafiriou (1988)
Chemiluminescence	0.25	this study

* LOD for the conversion product from the reaction of NO with the trapping compound.

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Table 2. Overview of the limit of detection (LOD), the limit of quantification (LOQ), the SD and the estimated stability time of the applied standards types.

Standard	LOD [pmol]	LOQ [pmol]	SD [%]	Stability time
Aqueous NO standard solution	5	20	25	10 months
In situ NO formation from NO_2^- reduction	10	40	3	–
Reference gas	15	30	10	one year

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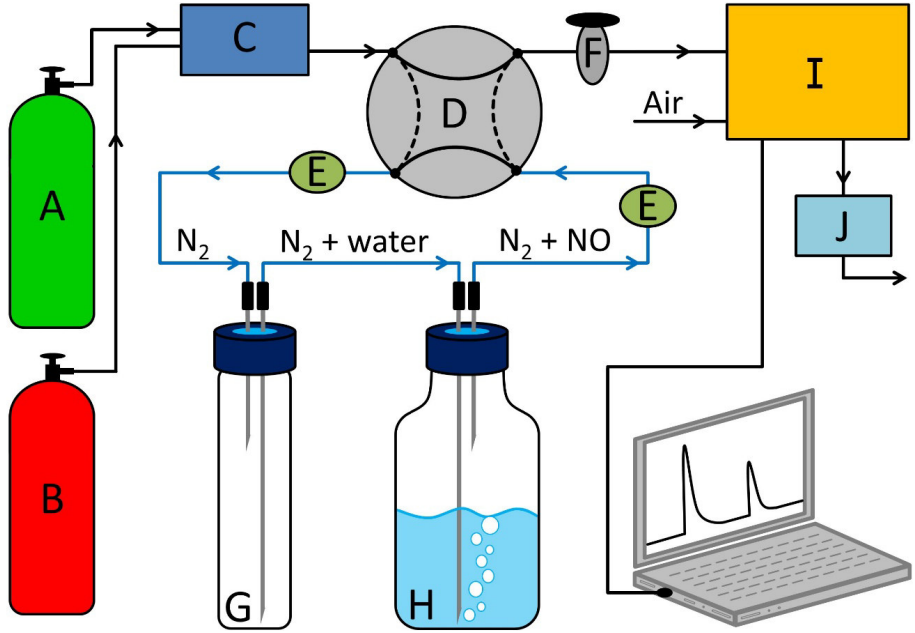

Figure 1. Schematic set-up of the developed measurement system consisting of an NO analyser connected to a stripping unit (blue lines). A: N_2 gas cylinder, B: reference gas cylinder, C: mass flow controller, D: 4-way valve (solid lines: mode A, dashed lines: mode B), E: inline filter, F: needle valve, G: sample vial, H: stripping vial filled with water, I: NO analyser, J: vacuum pump with vent.

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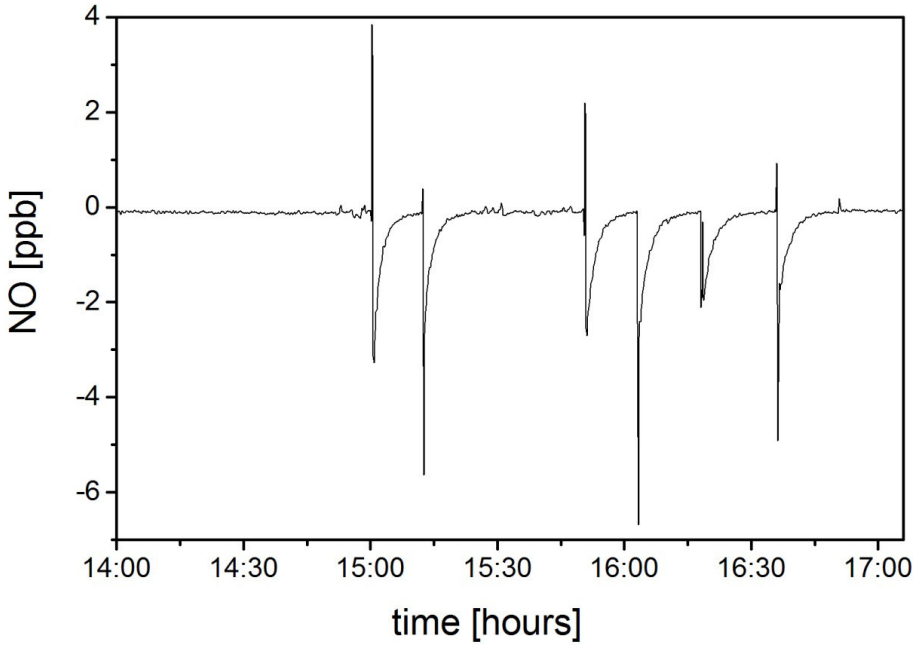

Figure 2. Detector signal for six seawater samples containing H_2S.

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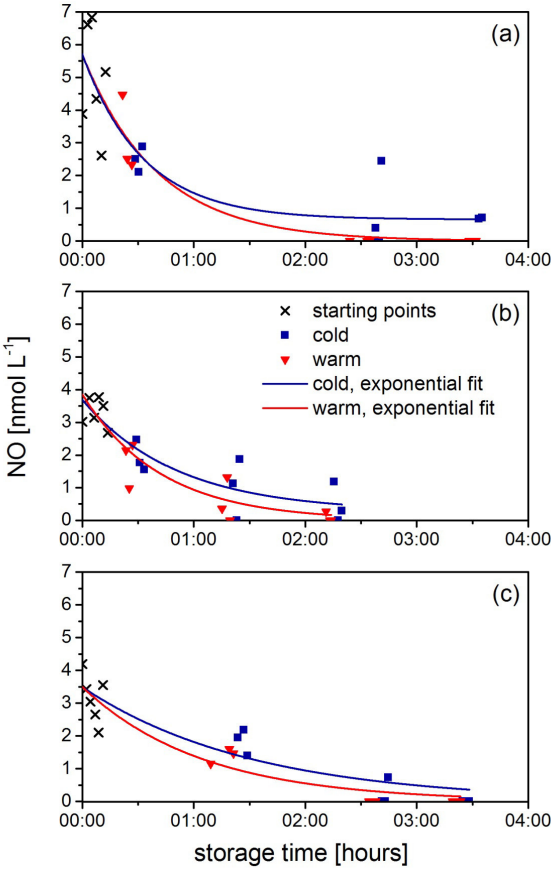

Figure 3. Degradation curves of three sample storage tests. The samples were kept in the dark at room temperature (~ 24 °C, red triangles) and at 6 °C (blue squares). The measurements from the regular sampling (black crosses) were used as starting points for the curve fitting of both temperature settings.

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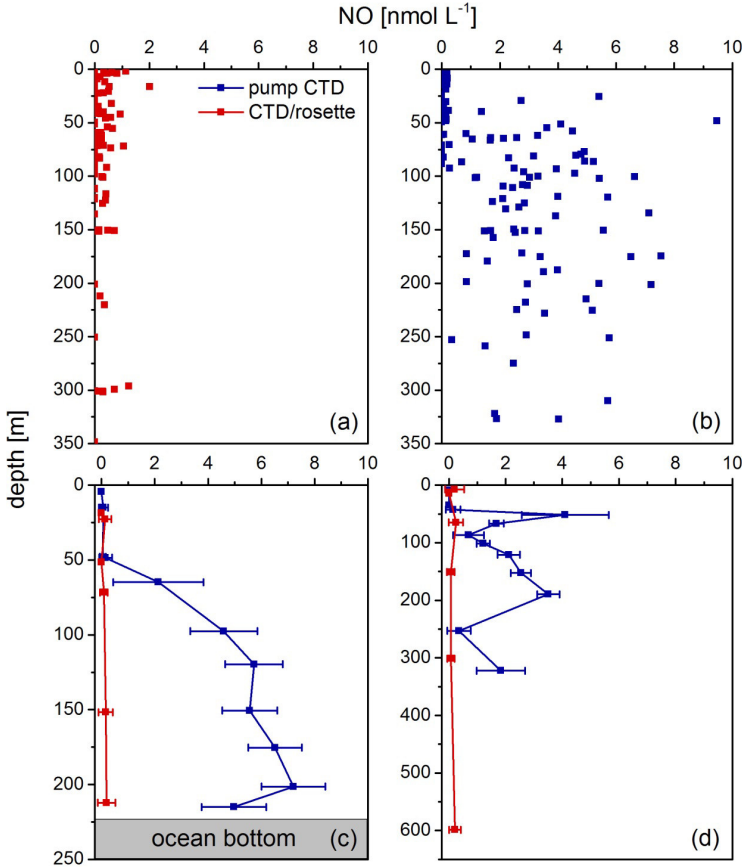

Figure 4. Comparison of the NO measurements from the CTD/rosette (red) and from the pump CTD (blue). **(a, b)** All NO measurements during M93 between 0 and 350 m. **(c)** M93 station 411-6 at 12.377° S, 77.388° W. **(d)** M93 station 391-4 at 12.668° S, 77.821° W, the bottom depth was 1654 m.

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