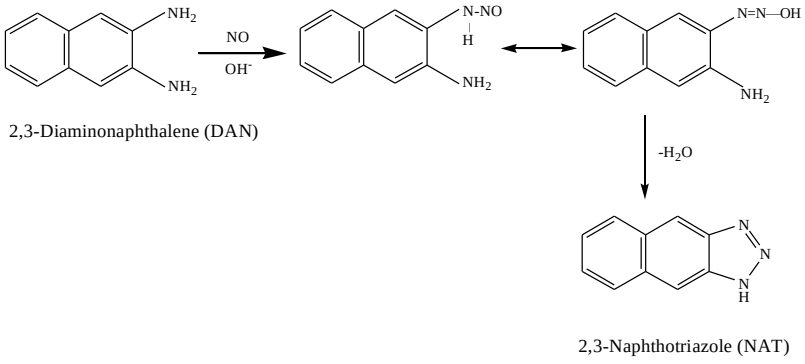
An itemized response (blue words) to the reviewers' comments and suggestions

Anonymous Referee #1

This paper describes a new method for NO in water samples and reports studies of NO in coastal waters. This seems to be the first such report in coastal waters and one of the few in any marine environment. Since NO is undoubtedly a difficult analyte to measure, and since there is little work on it, larger-than-usual uncertainties in results are tolerable; however, at some point very uncertain data lose usefulness.
Although this work appears to be carefully done and well described in many aspects, there are major ambiguities and concerns about this method, including major uncertainties as to how reported [NO] values relate to [NO] in situ. Both aspects need clarification in order to render the MS acceptable

Method – chemistry: (1) The equation (line141-144) is incomplete and disagrees with that reported: It is unbalanced, since NO has an odd number or electrons whereas the products do not.

Line141-144: The reaction diagram is cited from Miles et al. (1995). The relatively nonfluorescent DAN reacts rapidly with the NO-derived N-nitrosating agent to yield its highly fluorescent product NAT. The mistake has been corrected in the revised manuscript, as indicated below.



2,3-Diaminonaphthalene (DAN)

2,3-Naphthotriazole (NAT)

Also, (2) O2 is involved in the DAN->NAT reaction: Biol. Pharm. Bull 21(12) 1247-1250 (1998) states": : : The reaction of NO and O2 with 2,3 diaminonaphthalene (DAN) produced a fluorescent triazole." but in this study the only O2 present is the variable amount in the sample. Less-soluble O2 is stripped out faster than is NO, so as NO reaches the DAN solution, the pO2 varies over time, potentially altering the (NO->NAT) yield.

Yes, the reaction of NO and O_2 with 2,3 diaminonaphthalene (DAN) produced a fluorescent triazole. However, the mechanism of this fluorescence has not yet been established in detail, although the fluorescence increased dose-dependently by NO addition (Nakatsubo et al., 1998). In seawater samples, the concentration of NO (10^{-4} M order of magnitude) was far higher than that of NO (10^{-10} M order of magnitude). Both of them were stripped out and reached the DAN solution finally, thus the NO in samples could almost quantitatively transform into NAT.

(3) The equation (line 232) is incorrect.

Line 232: The equation has been modified, as indicated below.

RC（%）= NO (sw) / NO (DAN) × 100%.

Where NO (DAN) stands for the NO directly injected to the DAN solution and NO (sw) stands for the NO measured from the sample in degassing column according to the method described above.

(4) NO reacts with O2 in both gas phase and solution; rate = k[NO]ˆ2[O2] (ks are known). No evaluation of the roles of these reactions potentially consuming NO is given in the time between sampling and analysis (30 minute stripping period).

The rate law obtained from the oxidation of NO is

$$-d[NO]/dt=4k[NO]^2[O_2]$$

with $k=2*10^6 M^{-2}s^{-1}$. The reaction of NO with O_2 could consume NO in the stripping period indeed. However, we have evaluated the NO recovery coefficient of our purge-and-trap system as described in (3). The resulting NO recovery coefficients ranged from 80.2% to 90.0%, with an average of 83.8%. Furthermore, three replicates of in-situ seawater were measured using our system and method, the aqueous NO solution did not change within one hour, which was also demonstrated by Lutterbeck and Bange (2015).

Method optimization: Table 1's large variations in reaction efficiency clearly establish that near-optimal conditions need to be better defined. At 300 ml/min/45 min (13.5 L gas) the NAT yield is 21%, while at 400 ml/min/30 min (12 L gas), the yield is 69%. Thus, a 12.5% decrease in purge gas volume results in a 328% increase in DAN yield! This huge sensitivity demands better characterization of yield-controlling factors. Also, how can the efficiency also drop at longer times - is the DAN/NAT solution unstable?

This huge sensitivity was related to the status of DAN solution. Under the impact of N_2 gas flow, DAN formed many small bubbles. When the flow reached a certain volume, the trapping liquid was almost bubble - like. The specific surface area was greater when the bubbles were smaller and more; the contact area of NO and DAN was larger; and the reaction was more fully. However, when the flow became even larger, greater bubbles formed, the specific surface area decreased; the reaction yield of the reaction of DAN with NO reduced. The flow rate of 400 mL/min and purging time of 30 min was identified as the optimal experiment condition through the experiments of different flow rate and purging time.
Our experiments showed that the DAN solution was stable in 12 h and the NAT solution did not change within 4 h.

Calculations: Figure 4 lacks critical points at $[NO_2^-]=0$. The bottom 3 curves are roughly the same, _850 units _7%. Is this a "method blank"? Is any blank subtracted? For lab and at-sea measurements, the equation relating fluorescence units to [NO] should be given, along with any blank term(s) used.

According to the reviewer's suggestion, we have added the blank and used [NO] to replace fluorescence units in Figure 4. The bottom 3 curves represent the variations of NO concentrations in different concentrations of nitrite solutions in the dark or under UV-B radiation of 1h. The blank was subtracted.

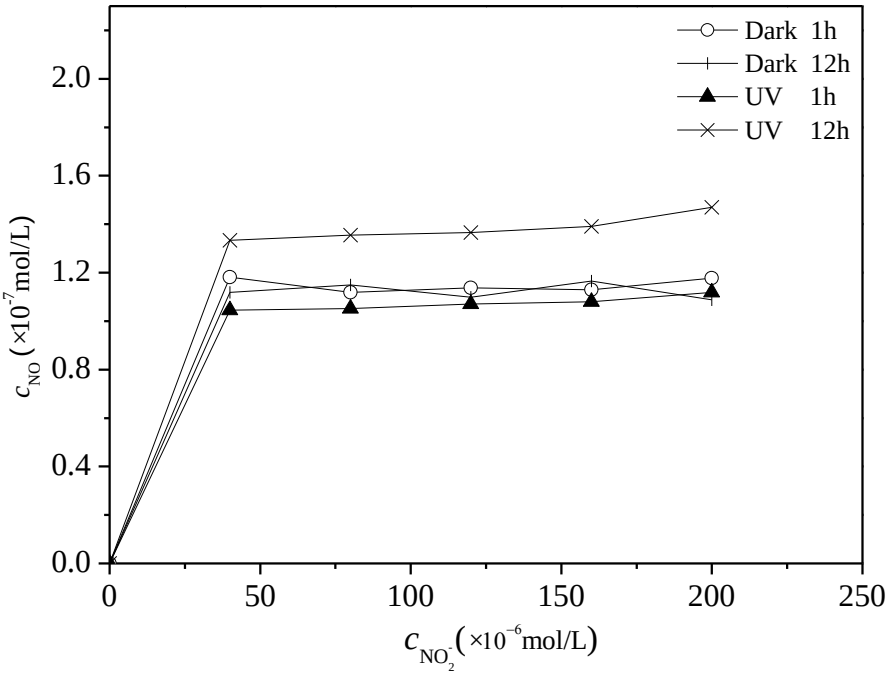


Fig. 4 The variations of NO concentrations in seawater with different concentrations of nitrite in the dark or under UV-B radiation

Here these high NO_2^- concentrations were design to demonstrate no effect caused by NO_2^- on the detection method, thus low concentrations of NO_2^- also has no effect. On the other hand, the fluorescence intensity could not be detected with low concentrations of NO_2^-.

Environmental [NO] data:
(1) In prior marine NO work by others, the NO source has been assigned to microbial processes that were assumed to continue, perhaps at perturbed rates, even during the stripping step, minimizing any losses, or to nitrite photolysis occurring while samples were stripped. Compared to this work, time-dependent losses were previously minimized (most microbial samples were also suboxic or anoxic). Nonetheless, likely all reported data underestimate [NO] to some extent. In contrast, here the minimum time seems to be (?) minutes in the dark, plus 15 minutes on average in the degasser. Thus comparing these data with literature values without qualification/explanation is unjustified.

According to the reviewer's suggestion, we have tried to discuss the deviation of these detection methods.
NO is a short-lived intermediate of various microbial processes of the nitrogen cycle, which is involved in denitrification (Kampschreur et al., 2007), anammox (Kartal et al., 2011) and archaea ammonia-oxidizing (Martens-Habbena et al., 2015) processes. Zafiriou and McFarland (1981) analyzed NO in seawater samples at the sea surface of the central equatorial Pacific by stripping NO into an air and N_2 stream by passing it through the same chemiluminescence - type detector. Thus, the NO concentrations were underestimated to some extent because seawater samples were suboxic or anoxic. However, time-dependent losses from microbial processes were minimized. Lutterbeck and Bange (2015) improved the method above to determine dissolved NO in discrete seawater samples of the eastern tropical South Pacific Ocean. The contamination by O_2 diffusion into the continue

samples could be further minimized. This work was also designed to detect dissolved NO in discrete seawater samples with a combination of a purge-and-trap set-up and fluorometric NO analyzer. The $HgCl_2$ solution was added to stop biological activities during the stripping. However, the disposal of these Hg-contaminated solutions is a tough proposition.
To improve the method, the purge-and-trap set-up could be modified and the stripping time could be reduced, then the addition of $HgCl_2$ solution may be removed.

(2) In irradiated/sunlit waters, light may also induce NO losses by forming NO-reactive radicals from CDOM. NO loss has been used to estimate rates of CDOM + hv -> radicals {Marine Chem., 30, 45–71 (1990); J. Geophys. Res. 96(C3), 4939–4945 (1991)} and Olasehinde et al. stated, "Thus, our findings indicate that the reaction of NO with photochemically generated free radicals might be a major pathway for NO loss in natural waters." Given high [DOC] in these waters, likely also rich in CDOM (it IS the "Yellow Sea"), it seems likely that NO consumption occurred. Was onlu a small residual [NO] detected?

The production and consumption of NO occur synchronously when sunlight photolyze natural seawater. The photolysis of NO_2^- is to mainly produce NO and OH. On the other hand, the loss of NO happens by forming NO-reactive radicals from CDOM (Zafiriou et al., 1990; Zafiriou and Dister, 1991; Olasehinde et al., 2009).The concentration of NO after exposure to sunlight is a balancing of this production against consumption by radical recombination. The study area has high concentrations of DOC and is rich in CDOM (Liu et al., 2010; Yang et al., 2011), thus, the authentic NO resulted from NO_2^- photolysis was underestimated. The photochemical production rates of NO were only a total value of production and consumption in this study.

(3) What can be reliably said about the reported data's implications for [NO] in-situ, if one ignores the absolute values of [NO] as uncertain and assumes only a roughly constant reaction efficiency? The patterns of Figures 5, 6 seem consistent with a positive light-dependence of the source/sink balance.

NO is a conceptually important intermediate in N-cycle biogeochemistry, product of ocean photochemistry, and putative inter-cellular signal. 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 because a reliable and easy to use method to determine dissolved NO at in-situ concentrations in seawater samples is missing. We try to find a solid method both convenient for many labs and sensitive enough, which seems to have promise (one anonymous reviewer). With our method we determined for the first time the temporal and spatial distributions of NO surface concentrations in coastal waters of the Yellow Sea off Qingdao and in Jiaozhou Bay during a cruise in November 2009. Our results implied the presence of NO formation processes such as NO_2^- photolysis, which was closely related to light intensity and nitrite concentration, and that the occurrence of particles and a temperature increase can enhance NO production rates.

Anonymous Referee #2

Table 2- What are the errors (standard deviations) on these measurements? Were they replicates, triplicates? The very large change in recovery at 400 ml/min compared to other flow rates does not make a lot of sense even with their explanation, and without an idea of the variability at each flow rate it is not possible to determine if the selected optimum is really the best for precision. Why include 200 mL/min if no measurements? Why not run at

350 and 450 mL per minute (or even 410 and 390) to see how sensitive the extraction is to flow rate- that will tell the user just how good their flow control must be to get good results. Perhaps it is possible to obtain even better results with fine tuning? At the very least the recommendation should be that anyone attempting to set this method up on their own will have to run this sort of experiment to determine the optimum flow rates.

The error of these measurements was in the range of 8-25%. They were triplicates. Now the scheme looks a bit rough. We will increase the measurements of the purge flow rates between 350 and 450 mL per minute, especially around 400 mL/min. It is possible to obtain even better results with fine tuning.

Figure 4- This raises more questions than it answers. First, you have a lot of data at unrealistically high nitrite concentrations, why no data at 0.5 or 1 micromole concentrations? Second, the fact that the blank is so high (100 nanomolar if I am reading this correctly) means that the level of contamination is orders of magnitude greater than the signals you report, and the variation in these measurements over both nitrite concentration and time swamps any natural signal. Basically the graph is not going to prove anything except that the nitrite you are using has a huge NO blank associated with it. And if the graph shows anything the UV effect is significant over 1 hour! If you are to prove a lack of a nitrite effect, you need to run samples with some nitrite in it at natural levels (say 0.1 to 1 micromolar) and use nitrite with no appreciable blank, either by purging the solution or using a different nitrite source. I will say that nitrite cannot be causing significant problems with natural samples, or else the signals you see would be dominated by nitrite levels. But this graph doesn't show that.

The fluorescence intensity could not be detected with low concentrations of NO_2^- (0.5-1 mol/L) after UV-B radiation. The high concentrations of NO_2^- (50-200 mol/L) were design to demonstrate the effect caused by the photolysis of NO_2^- on this detection method. Results showed that no obvious effect of high concentrations of NO_2^- on NO detection, thus low concentrations of NO_2^- also has any significant effect.
Figure 4 presents the variations of NO concentrations in seawater added with different concentrations of nitrite in the dark or under UV-B radiation. The blank had no signal when measured with the fluorescence spectrophotometer after UV-B radiation or in the dark. That is, the NO concentrations were zero. Yes, nitrite did not cause significant problems with natural samples during the measurement process.

In terms of the Environmental data, it is unclear if the manuscript has been modified to reflect the Author's clarifications. They need to be in the discussion.

We have carefully considered the reviewers' comments and suggestions and modified the discussion of Environmental [NO] data. We are very grateful to the reviewers for all the constructive comments and helpful suggestions to improve this manuscript.

The following references are added.

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Liu, C. Y., Yang, X. M., Yang, G. P., Zhou, L. M., and Li, P. F.: Composition and characterization of colloidal organic matter in the coastal surface waters of Qingdao, China, Mar. Chem., 121:123-131, 2010.

Determination of dissolved nitric oxide in coastal waters of the Yellow Sea off Qingdao

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Abstract We developed a new method for the determination of dissolved nitric oxide (NO) in discrete seawater samples based on a combination of a purge-and-trap set-up and fluorometric detection of NO. 2,3-diaminonaphthalene (DAN) reacts with NO in seawater to form the highly fluorescent 2,3-naphthotriazole (NAT). The fluorescence intensity was linear for NO concentrations in the range from 0.14 nmol L^{-1} to 19 nmol L^{-1}. We determined a detection limit of 0.068 nmol L^{-1}, an average recovery coefficient of 83.8% (80.2-90.0%), and a relative standard deviation of ±7.2%. With our method we determined for the first time the temporal and spatial distributions of NO surface concentrations in coastal waters of the Yellow Sea off Qingdao and in Jiaozhou Bay during a cruise in November 2009. The concentrations of NO varied from below the detection limit to 0.50 nmol L^{-1} with an average of 0.26 ± 0.14 nmol L^{-1}. NO surface concentrations were generally enhanced significantly during daytime implying that NO formation processes such as NO_2^- photolysis are much higher during daytime than chemical NO consumption which, in turn, lead to a significant decrease of NO concentrations during nighttime. In general, NO surface concentrations and measured NO production rates were higher compared to previously reported measurements. This might be caused by the high NO_2^- surface concentrations encountered during the cruise. Moreover, additional measurements of NO production rates implied that the occurrence of particles and a temperature increase can enhance NO production rates. With the method introduced here we have a reliable and comparably easy to use method at hand to measure oceanic NO

surface concentrations which can be used to decipher both its temporal and spatial distributions as well as its biogeochemical pathways in the oceans.

Keywords: Nitric oxide (NO), determination method, coastal waters of the Yellow Sea, distribution, production rate

1 Introduction

As a reactive atmospheric trace gas, nitric oxide (NO) plays important roles in tropospheric chemistry: It is a key player in the formation of acid rain and ozone (Williams et al., 1992; Lee et al., 1997; Mazzeo, et al., 2005). NO is an intermediate of both the terrestrial and marine nitrogen cycle (Ward and Zafiriou, 1988; Williams et al., 1992; Canfield et al., 2010; Chen et al., 2010; Thamdrup, 2012; Voss et al., 2013). It has a variety of sources in seawater, including nitrite photolysis and various microbial processes such as denitrification, anammox and dissimilatory nitrate reduction to ammonia (Law, 2001; Schreiber et al., 2012; Martens-Habbena et al., 2015). Because of its chemical reactivity, NO usually does not accumulate in large amounts in seawater and the ocean as a source of atmospheric NO is, therefore, negligible in a global context (Zehr and Ward, 2002; Bange, 2008). Moreover, NO was found to have significant effects on the growth of marine algae (Zhang et al., 2005; Liu et al., 2004; 2005; 2006; 2014). To this end, the determination of the spatial and temporal distributions of NO in the ocean as well as deciphering its oceanic production processes and their major influencing factors are essential to improve our understanding of the biogeochemical cycling NO in the ocean.

Because of its low concentrations in seawater caused by its fast diffusion and high chemical reactivity, measurements of NO in seawater are very difficult. Therefore, there are only a few methods available to determine NO (Hetrick and Schoenfisch, 2009), see Tab. 1. The electrochemical method using sensors in seawater medium achieved a detection limit of 42 nmol L^{-1} (Xing et al., 2005; Zhang et al., 2005). Olasehinde et al. (2009) developed a method for the determination of photochemically generated NO in natural waters adopting 4,5-diaminofluorescein as a probe compound and a measurement of reversed-phase high performance liquid chromatography (HPLC) with fluorescence

55 detector. The NO concentrations and signal intensities exhibited a good linearity correlation over the
56 range of 0.025-10 nmol L^{-1} triazolofluorescein. Zafiriou and McFarland (1980) determined the NO
57 concentration of seawater by using a flow system to equilibrate the seawater samples with a gas stream
58 coupled to a chemiluminescence detector. They report an analytical precision of ±3% and an accuracy
59 of ±20%. More recently, Lutterbeck and Bange (2015) developed an improved method of a
60 chemiluminescence NO analyser connected to a stripping unit, and the limit of detection was 0.25
61 nmol L^{-1} using a 20 mL seawater sample volume. Until now only these two chemiluminescence
62 methods were applied successfully to determine NO concentrations in seawater samples. The
63 N-nitrosation of 2,3-diaminonaphthalene (DAN) results in the highly fluorescent 2,3-naphthotriazole
64 (NAT) which could be used to detect NO concentrations as low as 10 nmol L^{-1} (Miles et al., 1995). We
65 adopted this method for seawater medium instead of NaOH medium and the calibration curve
66 exhibited linearity over the concentration range of 1.4 - 1400 nmol L^{-1} NO (Liu et al., 2009). However,
67 this assay cannot be used to detect trace levels of NO in seawater samples directly.

68 In this paper, we describe a modified spectrofluorometric method using a purge-and-trap technique
69 which can be used to quantify NO in seawater samples. This method was applied in a first field study on
70 the distribution and production rates of dissolved NO in coastal waters of the Yellow Sea off Qingdao
71 and Jiaozhou Bay.

72

73 **2** Materials and methods

74 **2.1 Instrumental set-up**

75 The analytical system consists of a degassing column to purge NO from seawater samples, a
76 reaction chamber where NO reacts to form a fluorescent compound (Fig. 1), and a fluorescence
77 spectrophotometer (F-4500, Hitachi Co., Japan). The 800 mL degassing column has a sodium silicate
78 bonded sand core at the bottom to disperse the nitrogen (N_2) purge gas stream. There are four ports on
79 the column: (1) a gas port at the bottom of the degassing column where the high purity N_2 purge gas

(99.999%, Qingdao Heli Industry Gas Center, China) or a NO standard gas mixture (5.4 ppmv, NO/N_2) (Beijing Sida Standard Substance Co., China) are introduced, (2) a drain port as outlet for water samples, (3) an inlet port where water samples are pushed into the degassing column with N_2, and (4) an outlet port on the top of the degassing column connected with the reaction chamber.

The NO standard gas cylinder is linked to the degassing column via a gas-tight syringe (Shanghai Anting Injector Co., China). The N_2 gas cylinder is connected to the degassing column via a deoxygen tube (Agilent Technologies, USA) to remove traces of O_2 and a glass rotameter to monitor the gas flow (0.1-1 L min^{-1}, Jiangyin, China). These two gas streams enter the degassing column via the port at the bottom of the flask, controlled by a three-port valve. The tubing used is made of polytetrafluoretyhylene (PTFE, 1/8-inch tubing outer diameter [o.d.]). Moreover, an Ultraviolet-Visible spectrophotometer (UV-2550, Shimadzu Co., Japan) and an Automatic Analytical Balance (Beijing Sartorius Co., China) were used in this work.

The degassing column, reaction chamber and the syringe were degreased with organic solvents and rinsed several times with methanol and distilled water in order to minimize potential contamination and adsorption effects. The degassing column was cleaned initially with detergent, rinsed with water, acetone, methanol, and distilled water, and then treated for 30 min with 10% (v/v) HCl in an ultrasonic bath, followed by rinsing with distilled water. Subsequently, those parts of the set-up which comes into contact with the sample solutions were rinsed with methanol, water, HCl solution, and dilute NaOH solution. No significant difference was found from the test of the set up loaded with a water sample and without a water sample (dry run).

2.2 Preparation of DAN and NO solutions

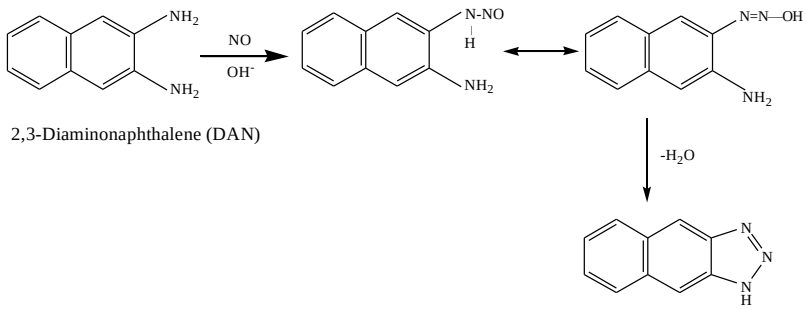
A 2,3-diaminonaphthalene (DAN, ≥ 95%, GC, Sigma-Aldrich Chemical Co., USA) stock solution was prepared fresh with a concentration of 10 mmol L^{-1} in dimethylformamide (Sigma-Aldrich Chemical Co., USA) and kept in the dark at -21 ℃ until used. DAN solutions of 40 μmol L^{-1} were prepared from

the stock solution in Milli-Q water, 10 mmol L^{-1} NaOH aqueous solution and filtered natural seawater, respectively. (Natural seawater was sampled from the coastal waters off Qingdao and was filtered through a 0.45 μm acetate cellulose membrane (Millipore, USA). The DAN solutions were purged with N_2 gas for 30 min to remove oxygen (O_2), then stored on ice and transferred to a refrigerator at 4 ℃ before use.

An aliquot of 10 mL Milli-Q water was bubbled with N_2 gas at a flow of 10 mL min^{-1} for 1h to remove O_2 after 10 min of ultrasonic degassing. The solution was then bubbled with high purity NO gas (99.9%, Dalian Date Gas Ltd, China) for 30 min. The concentration of the saturated NO stock solution was 1.4 mmol L^{-1}, which should be used within 3h (Lantoine et al., 1995). A series of diluted NO solutions were prepared in N_2-purged water from the NO stock solution using a syringe (Xing et al., 2005).

2.3 Fluorometric detection of NO

DAN reacts with NO_x (= NO + NO_2) in alkaline medium and forms the highly fluorescent 2,3-naphthotriazole (NAT):



2,3-Diaminonaphthalene (DAN)

2,3-Naphthotriazole (NAT)

Based on this reaction a fluorometric method was originally developed for the detection of NO in oxygenated media (Misko et al., 1993; Miles et al., 1995) and has been adapted to detect NO in seawater medium instead of aqueous NaOH medium. The wavelength for NAT excitement is 383 nm and the NAT emission is monitored at a wavelength of 410 nm (Liu et al., 2009).

126 **2.4 The influence of nitrite in seawater on the reaction of DAN and NO**

127 NO can be formed from nitrite (NO_2^-) in seawater (Zafiriou and McFarland, 1981). Therefore, we
128 tested a potential interference of dissolved NO_2^- by adding different concentrations of NO_2^- to seawater
129 samples. The tests were conducted in the dark or with ultraviolet B (UV-B) radiation (HR 1×18 w,
130 Xinghui Electric Instrument Factory, China). The final concentrations of NO_2^- in the seawater samples
131 were set to 40, 80, 120, 160, and 200 μmol L^{-1}, respectively, and the reaction time was 1 h or 12 h.

132

133 **2.5 Sampling and analysis**

134 Sampling was conducted aboard the R/V '*Dong Fang Hong 2*' on a cruise to the coastal waters off
135 Qingdao and Jiaozhou Bay from 4 to 6 November 2009. The locations of sampling stations are shown in
136 Fig. 2. The surface seawater samples were collected from 1 m depth at 11 stations using 8 L Niskin
137 bottles mounted on a Seabird CTD Rosette (Sea-Bird Electronics, Inc., USA). A time-course
138 observation of 24 h was carried out at station 10 near the mouth of Jiaozhou Bay.

139 A 500 mL Wheaton glass serum bottle was rinsed with the seawater three times before it was filled
140 with seawater quickly through a siphon. When the overflowed sample reached the half volume of the
141 bottle, the siphon was withdrawn rapidly and 0.5 mL saturated $HgCl_2$ solution was added to stop
142 biological activities and the bottle was sealed quickly. All glass bottles were covered with aluminum
143 foil to prevent NO_2^- photolysis during sampling.

144 Because NO reacts with O_2 both in the gas phase and in aqueous solution we purged our set-up for
145 1h with N_2 gas and sealed it before the measurements. In a first step, a certain amount of standard NO
146 gas was transferred to the reaction chamber via the degassing column by injecting it from a gas tight
147 syringe into the N_2 carrier gas stream. In the reaction chamber NO reacts with the DAN solution. After
148 the measurement of the NO gas standard, a 500 mL seawater sample was injected into the degassing
149 column and purged with N_2 gas and immediately transferred into the reaction chamber where it reacts
150 with 10 mL DAN solution. The gas flow is controlled to ensure that the reaction of NO with DAN

151 solution was completed. Finally, the fluorescence intensity of the resulting NAT solution was measured
152 with the F-4500 fluorescence spectrophotometer.
153 In order to prevent NO photochemical generation, the entire glass parts were wrapped with
154 aluminum foil. The purge-and-trap procedure was conducted at room temperature of 20 ℃.
155
156 **2.6 O_2, nutrients, DOC and chlorophyll *a* measurements**
157 Dissolved O_2 (DO) concentrations were determined according to the Winkler method. The
158 concentrations of dissolved nitrate, nitrite and ammonia were measured by using an AutoAnalyzer 3
159 (SEAL Analytical, USA). The detection limits of the method were 0.003, 0.015 and 0.040 μmol L^{-1} for
160 nitrate, nitrite and ammonia, with the precision less than 1%. The intensity of sunlight was monitored
161 by the use of a TES-1322A actionometer (Taishi Co. Taiwan). Dissolved organic carbon (DOC) was
162 determined by a high-temperature combustion method using a Shimadzu TOC-5000 Analyzer with an
163 Al–Pt catalyst (Shimadzu Co., Japan). The precision of the DOC measurements was less than 2%.
164 Concentrations of chlorophyll a were measured with a bbe Cuvette Fluorometer (bbe-Moldaenke
165 GmbH, Kiel, Germany).
166
167 **2.7 NO production rates**
168 Experiments for NO production by NO_2^- photolysis were conducted at station 10 as follows:
169 Aliquots of 10 mL untreated seawater samples from 0.2 m depth or Millipore membrane (0.45 μm)
170 filtered samples were distributed into three 14 mL glass vials. The initial concentrations of NO_2^- and
171 DOC in seawater were 0.75 μmol L^{-1} and 439 μmol L^{-1} C, respectively. Then 200 μL of 20% NaN_3
172 solutions (instead of saturated $HgCl_2$ solution to avoid contamination by the photosensitive Hg) and 20
173 μL of 1 mmol L^{-1} DAN solutions were added. The vials were sealed with rubber septa and aluminum
174 crimp tops, and were exposed to sunlight on the deck at ambient temperatures (17 ℃) or at 13 ± 2 ℃
175 in a water bath supplied with the ambient seawater. For "dark" controls vials were wrapped in

aluminum foil. The intensity of sunlight ranged from 67565 lux to 71500 lux (average: 69430 lux). After irradiation by sunlight for 30 min, the NO concentrations were measured with the method described above. The NO photolysis production rates were computed as the increase of the NO concentrations during the incubation time.

We also measured NO production rates in natural seawater at station 10. Three transparent polyethylene buckets (3.5 L) were filled with the surface seawater from 0.2 m depth. The buckets were exposed to sunlight in the water bath on deck. The experiment began at 8:30h (local time) and the NO production rates and chlorophyll *a* concentrations were concurrently measured in 2 h intervals. An aliquot of 10 mL sample was collected from each bucket using a glass syringe, distributed and sealed in a 14 mL glass vial, and then incubated under the same conditions as the bucket samples. Three vials per sample were used in the experiments. After 30 min of incubation, solutions of 20 μL DAN (1 mmol L^{-1}) were injected into the vials, respectively. Concentrations of NO were detected and NO production rates were calculated.

3 Results and Discussion

3.1 Method evaluation

Both the purge time and flow of the purge gas (N_2) significantly influence the yield of the NO + DAN reaction and thus, the overall purge efficiency (see Tab. 2). The optimal (i.e. maximum) reaction yield was 85% after 30 min of purging at a flow of 400 mL min^{-1}.

The set-up was tested for internal NO production or loss by comparing the fluorescence intensity from NO-free gas or NO calibration gas passing through the degassing column with the fluorescence intensity from the same gas bypassing the degassing column. This procedure was repeated with both a dry degassing column and a moistening degassing column (by a minimum amount of filtered seawater). Neither NO production nor NO loss by adsorption was observed in the set-up in all test runs.

Seawater samples from coastal waters off Qingdao were analyzed in the lab up to of 7 times and

gave a relative standard deviation of ±7.2%. The detection limit of our method was determined to be 0.068 nmol L^{-1} (S/N = 3), which is lower than most of the reported detection limits for NO measurements in seawater (see Tab. 1)

The NO recovery coefficient of our purge-and-trap system was estimated by the addition of the same volume of a NO standard solution to (i) 500 mL NO-free seawater in the degassing column and (ii) to 10 mL DAN solution (with a DAN concentration of 40 μmol L^{-1}) in the reaction chamber. The recovery coefficient (RC) of NO was calculated according to:

$$RC（\%）= NO(sw) / NO (DAN) \times 100\%.$$

Where NO (DAN) stands for the NO directly injected to the DAN solution and NO (sw) stands for the NO measured from the sample in degassing column according to the method described above. The resulting NO recovery coefficients ranged from 80.2% to 90.0%, with an average of 83.8%. In order to check the linearity of our method, a solution of 10 mL 40 μmol L^{-1} DAN was injected into the reaction chamber and purged with N_2 gas at a rate of 10 mL min^{-1} for 5 min prior to the actual measurements. A series of NO-free seawater samples placed in the degassing column were spiked with different volumes of the NO standard gas (mixing ratio 5.4 ppmv NO/N_2) and analyzed according to the procedure described above. The resulting fluorescence intensity was linear with the NO concentrations in the range from 0.14 to 19.0 nmol L^{-1} ($y = 7.4286x + 0.6188$, $R = 0.9976$, $P < 0.0001$) (Fig. 3).

The results of the samples spiked with varying concentrations of dissolved NO_2^- are given in Fig. 4. In general, samples with the same NO_2^- concentration showed higher fluorescence when UV-irradiated or kept in dark for 12 h compared to samples under short term (i.e. 1 h) UV irradiation or kept in dark. This points a significant NO production under UV irradiation ($n=5$, $F=76.13$, $p=2.32\times10^{-5}$) and (albeit weaker) NO dark production from NO_2^-. Higher NO_2^- concentrations resulted in a slight increase of fluorescence when irradiated. Therefore we conclude that the measurements of NO should be done in the dark as soon as possible after sampling when high NO_2^- concentrations occur.

To assess the influence of the interferences of dissolved organic matter, trace metals, nutrients, and

other substances in seawater, the NO/fluorescence intensity relationship should be determined when the method is applied in different oceanic regions.

With our method we are able to detect > 0.068 nmol L^{-1} NO in discrete seawater samples with a volume of 500 mL. With a larger degassing column, even lower concentrations of NO might be determined.

A U-shaped tube and cold bath (i.e. a water trap) was initially placed between the degassing column and the recation chamber in order to eliminate small amounts of water carried by the N_2 gas stream. However, we found that the fluorescence intensities did not show significant differences when the water trap was removed.

3.2 Distribution of dissolved NO in coastal waters of Qingdao

Fig. 5 shows the NO concentrations of surface seawater in coastal waters off Qingdao (stations S01-S09) and in the Jiaozhou Bay (stations S10 and S11). The concentrations of NO ranged from below the detection limit (stat. 02 and 03) up to 0.50 ± 0.01 nmol L^{-1} (stat. S08), with an overall mean of 0.26 ± 0.14 nmol L^{-1}. It is noteworthy that the higher NO concentrations seem to be related to the time point of sampling (given in local time): Samples of stations 2 and 3 were collected at night time, 22:30h and 00:50h, respectively, while samples for stations 5, 6, 7 and 8 were collected during the day time (08:58h - 15:38h). (Stations S09 and S10 have been measured in Jiaozhou Bay and, thus, their NO concentrations are directly not comparable with the stations off Qingdao). Our results are generally consistent with the findings in the aquatic ecosystem of Daya Bay in China (Zhang et al., 2006) and the nitrite-rich surface waters of the central equatorial Pacific Ocean (Zafiriou et al., 1980), indicating that sunlight could be a main factor affecting NO formation in seawater. The concentrations of NO in coastal surface waters off Qingdao were found to be an order of magnitude higher than those in surface waters during day time in the central equatorial Pacific Ocean (0.05 nmol L^{-1}) (Zafiriou et al., 1980; Zafiriou and McFarland, 1981). This difference is probably related to the concentrations of

251 NO_2^- in seawater. Zafiriou et al. (1980) proposed that sunlight photolyzes NO_2^- in surface water by the
252 following reaction:

253 $$NO_2^- + H_2O \xrightarrow{hv} NO + \cdot OH + OH^-.$$

254 According to the reaction above, high concentrations of NO_2^- together with strong solar irradiation
255 could cause enhanced concentrations of NO in seawater. The sunlight intensity of the central
256 equatorial Pacific is generally higher than that of coastal waters of Qingdao (located at 36°05'N);
257 however, the coastal waters off Qingdao at the time of our measurements exhibited an average NO_2^-
258 concentration of 0.49±0.25 μmol L^{-1}, which was much higher than that observed concentration in the
259 central equatorial Pacific Ocean (~0.1 μmol L^{-1}).

260 NO is a short-lived intermediate of various microbial processes of the nitrogen cycle, which is
261 involved in denitrification (Kampschreur et al., 2007), anammox (Kartal et al., 2011) and archaea
262 ammonia-oxidizing (Martens-Habbena et al., 2015) processes. Zafiriou and McFarland (1981) analyzed
263 NO in seawater samples at the sea surface of the central equatorial Pacific by stripping NO into an air
264 and N_2 stream by passing it through the same chemiluminescence - type detector. Thus, the NO
265 concentrations were underestimated to some extent because seawater samples were suboxic or anoxic.
266 However, time-dependent losses from microbial processes were minimized. Lutterbeck and Bange
267 (2015) improved the method above to determine dissolved NO in discrete seawater samples of the
268 eastern tropical South Pacific Ocean. The contamination by O_2 diffusion into the continue samples
269 could be further minimized. This work was also designed to detect dissolved NO in discrete seawater
270 samples with a combination of a purge-and-trap set-up and fluorometric NO analyzer. The $HgCl_2$
271 solution was added to stop biological activities during the stripping. However, the disposal of these
272 Hg-contaminated solutions is a tough proposition.

273 The diurnal variation of NO concentrations and other parameters in surface seawater are shown in
274 Fig. 6. Concentrations of NO presented a significant diurnal variation within 24 h. The peak value
275 appeared at 15:00h (local time) with a concentration of 0.81 nmol L^{-1}. After that the concentration of

NO decreased with time gradually until a minimum value occurred at 03:00. Obviously, the concentration of dissolved NO at this station was influenced by the in-situ sunlight intensity. However, the maximum NO concentration appeared not at 12:00h but at 15:00h, which suggesting that there were other influencing factors besides sunlight irradiation.

3.3 NO production rates in coastal waters

The results of the NO irradiation experiments are given in Fig. 7. The production rate of NO through seawater irradiation was 1.52×10^{-12} mol L^{-1} s^{-1} which is slightly higher than that NO production rate of the 0.45 μm Millipore filtered samples (1.46×10^{-12} mol L^{-1} s^{-1}). The difference may indicate that particles in seawater could increase the NO production rate. The non-filtered samples incubated in the water bath had a lower NO production rate (1.44×10^{-12} mol L^{-1} s^{-1}) compared to the other non-filtered treatment, which could be ascribed to the difference of the temperature. The ambient temperature and water bath were 17 ℃ and 13 ℃, respectively, thus the higher temperature may resulted in a higher photolysis rate. The photochemical production rates of NO in Qingdao coastal waters during the daytime were generally higher than that reported from the central equatorial Pacific Ocean (0.4-1.2 $\times10^{-12}$ mol L^{-1} s^{-1}) (Zafiriou and McFarland, 1981).

Previous experiments about NO_2^- photolysis were also carried out in the laboratory (Li et al., 2011): The production of NO was observed after 3 h illumination of 10-100 μmol L^{-1} NO_2^- in Milli-Q water. There was an increasing trend of NO concentrations with the NO_2^- concentrations. For natural seawater, it was observed to have an increasing trend of NO concentration with the illumination time (Li et al., 2011). The process of sunlight photolysis of NO_2^- in surface water was demonstrated, which was consistent with the results of Zafiriou et al. (1980) and Olasehinde et al. (2009).

The production and consumption of NO occur synchronously when sunlight photolyze natural seawater. The photolysis of NO_2^- is to mainly produce NO and OH. On the other hand, the loss of NO happens by forming NO-reactive radicals from CDOM (Zafiriou et al., 1990; Zafiriou and Dister, 1991;

Olasehinde et al., 2009).The concentration of NO after exposure to sunlight is a balancing of this production against consumption by radical recombination. The study area has high concentrations of DOC and is rich in CDOM (Liu et al., 2010; Yang et al., 2011), thus, the authentic NO resulted from NO_2^- photolysis was underestimated. The photochemical production rates of NO were only a total value of production and consumption in this study.

The on-deck incubation experiments for the production rates of NO in Qingdao coastal waters, together with chlorophyll *a* concentrations and sunlight intensities, are shown in Fig. 8. The production rates of NO exhibited a clear variation during the course of the day with a maximum value appearing at 14:30h (local time). The maximum value of 2.52×10^{-12} mol L^{-1} s^{-1} was about seven-fold higher than the minimum value at 08:30h. The production rates of NO kept an increasing trend from 08:30h to 14:30h. The mean production rate in Qingdao coastal waters was 1.51×10^{-12} mol L^{-1} s^{-1} during the day. The variation of the production rates of NO did not follow the trends in chlorophyll *a* concentrations and solar radiation. Therefore, the production pattern of NO in marine environments deserves further research.

4 Summary

For the determination of NO concentrations in discrete seawater samples we developed a new method by combining a purge-and-trap set-up with fluorometric detection of NO. The method showed a linear fluorescence intensity for NO concentrations ranging from 0.14 nmol L^{-1} to 19 nmol L^{-1}. The detection limit is 0.068 nmol L^{-1} (S/N =3), the average recovery coefficient is 83.8% (80.2～90.0%), and the relative standard deviation is ±7.2%. Our method was applied to measure concentrations of NO in surface layer of the coastal waters off Qingdao and Jiaozhou Bay. NO concentrations varied from below the detection limit to 0.50 nmol L^{-1}, with an average of 0.26 ± 0.14 nmol L^{-1}. The concentrations of NO in coastal waters off Qingdao were an order of magnitude higher than those in surface waters of the central equatorial Pacific. NO surface concentrations were generally enhanced

significantly during daytime implying that NO formation processes such as NO_2^- photolysis are much higher during daytime than chemical NO consumption which, in turn leads to the observed significant decrease of the NO concentrations during nighttime. The measurements of NO production rates showed that the occurence of particles and an increase in temperature can enhance NO production.

We conclude that our method can be applied to measure (i) NO concentrations in the ocean surface, (ii) NO production and consumption pathways in oceanic waters and (ii) NO production rates in culture experiments.

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Figure Captions

Fig. 1 The purge-and-trap system for the determination of dissolved nitric oxide in seawater

Fig. 2 Loations of the sampling stations in the coastal waters off Qingdao and Jiaozhou Bay

Fig. 3 Relationship between nitric oxide concentrations and fluorescence intensities

Fig. 4 The fluorescence variations of NAT in seawater with different concentrations of nitrite in the dark or under UV-B radiation

Fig. 5 The concentrations of NO in the surface water off Qingdao and Jiaozhou Bay

Fig. 6 The diurnal variations of NO concentrations and related parameters in the surface seawater at station 10

Fig. 7 The production rates of NO by seawater irradiation under natural light after different treatments

Fig. 8 The variations of NO production rates, chlorophyll *a* concentrations and sunlight intensities in the incubation experiments with Qingdao coastal waters

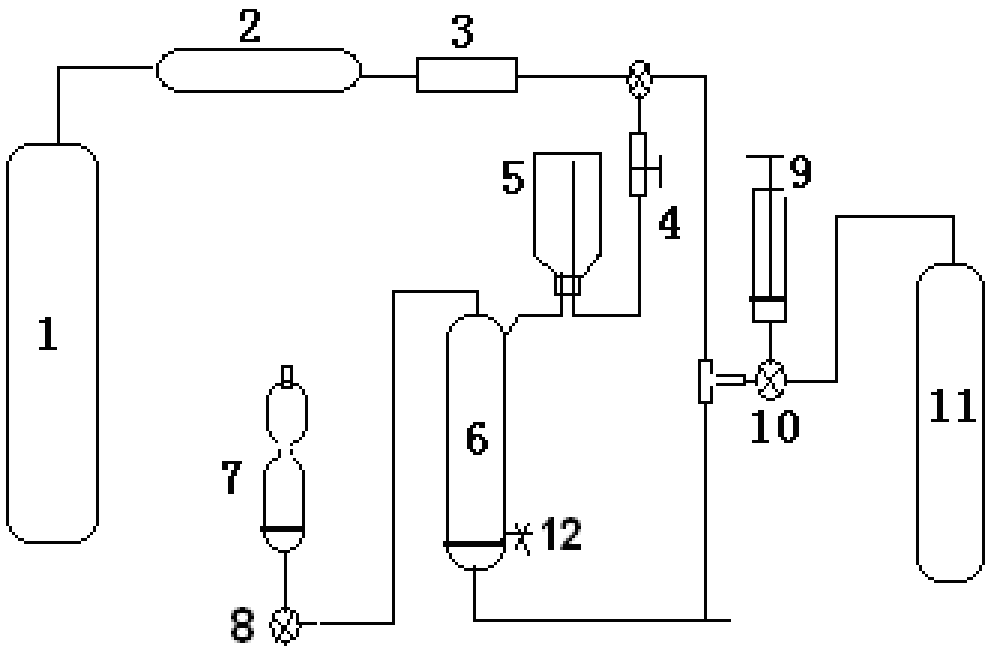


Fig. 1 The purge-and-trap system for the determination of dissolved nitric oxide in seawater (1. N_2 gas; 2. Deoxygenation tube; 3. Glass rotameter; 4. 2-port valve; 5. Sample vial; 6. Degassing column; 7. Reaction chamber; 8 and 10. 3-port valves; 9. Gas-tight syringe; 11. NO standard gas; 12. Drain)

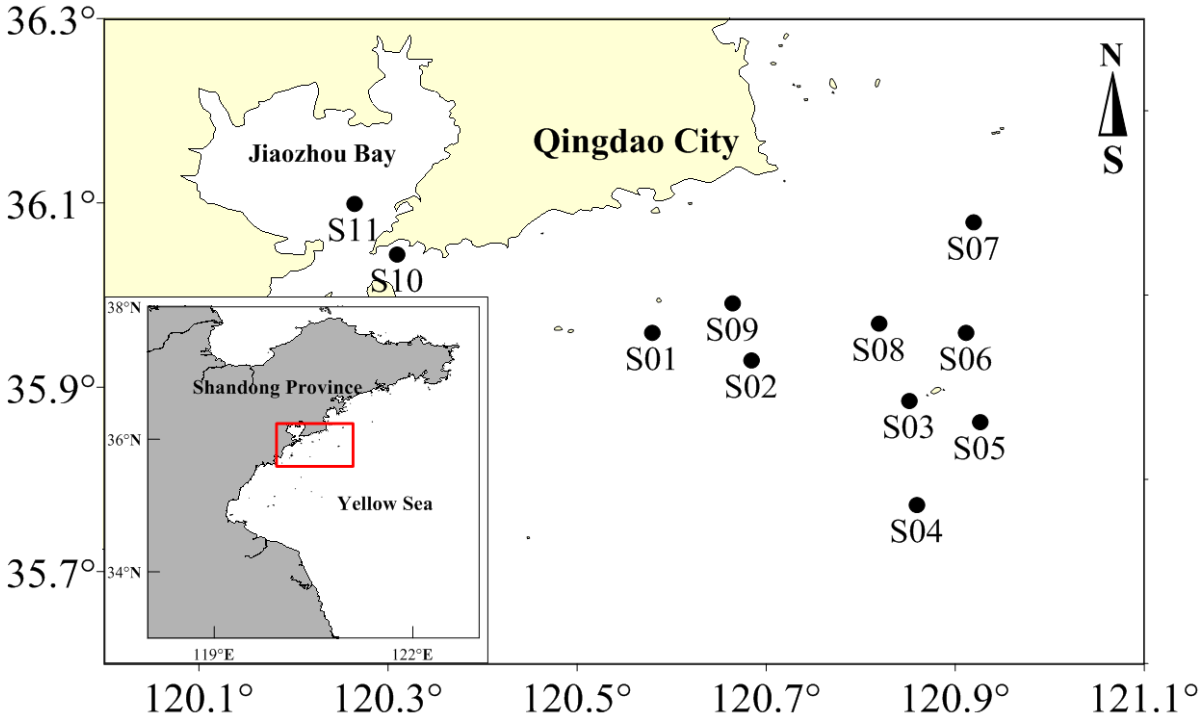
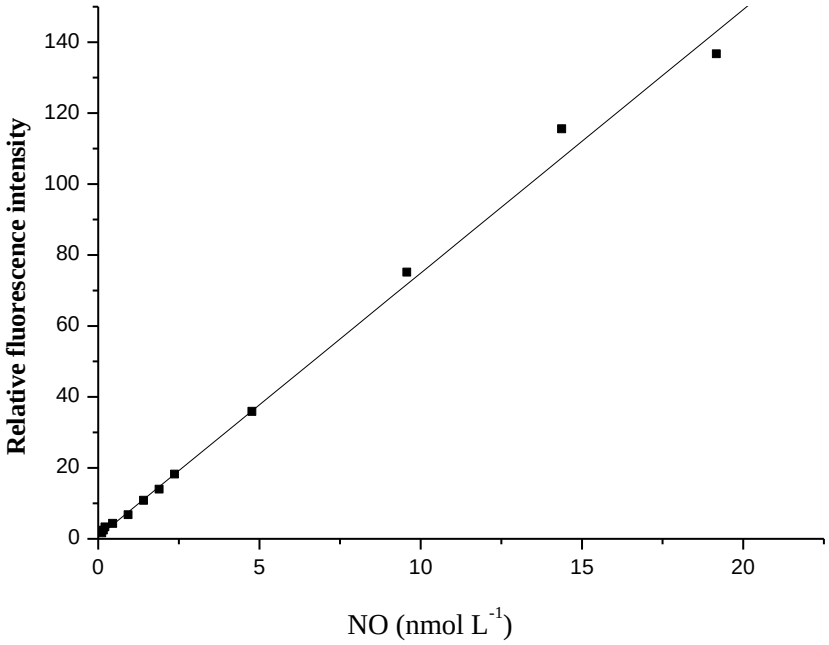


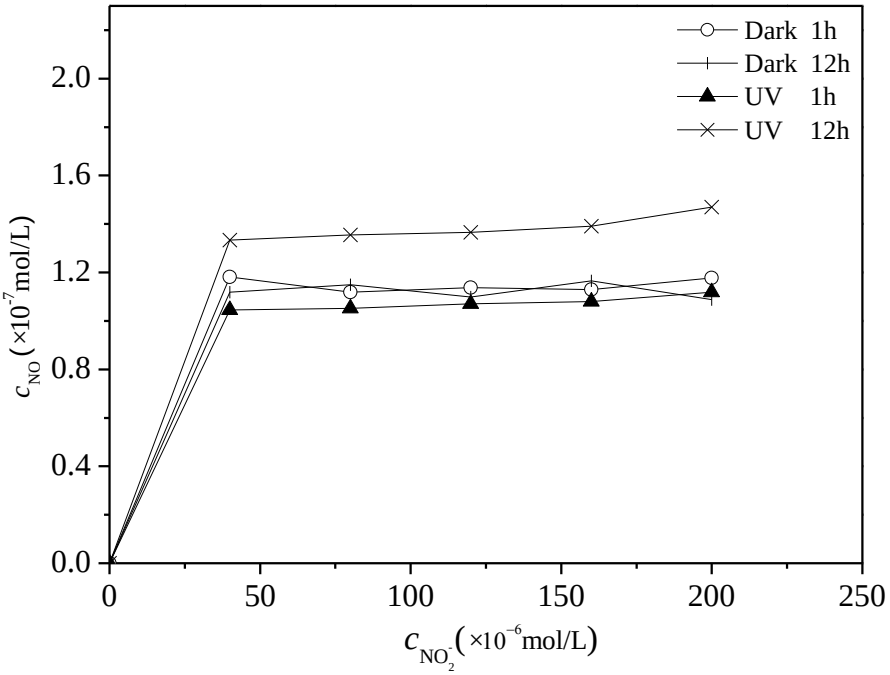
Fig. 2 Location of the sampling stations in the coastal waters off Qingdao and Jiaozhou Bay



456

457 **Fig. 3** Relationship between nitric oxide concentrations and fluorescence intensities

458



459

460 **Fig. 4** The variations of NO concentrations in seawater added with different concentrations of nitrite in

461 the dark or under UV-B radiation

462

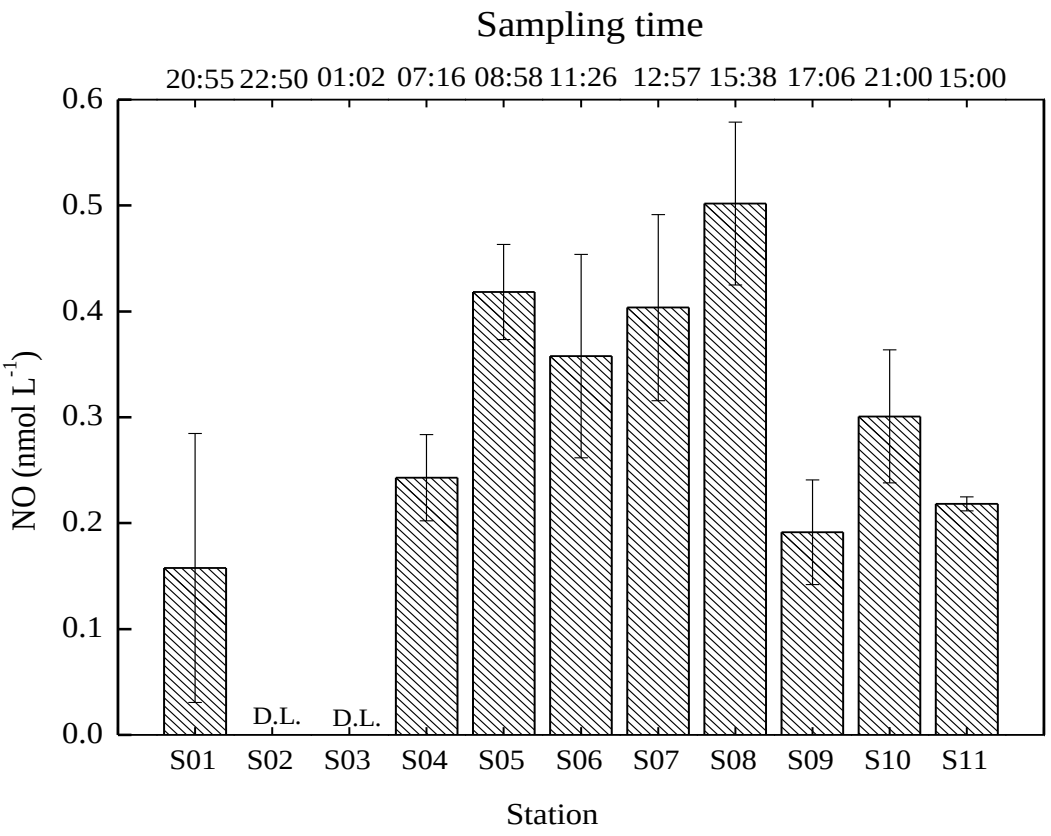


Fig.5 The concentrations of NO in the surface waters off Qingdao (stations S01-S09) and Jiaozhou Bay (stations S10 and S11)

D.L. stands for concentration below the detection limit.

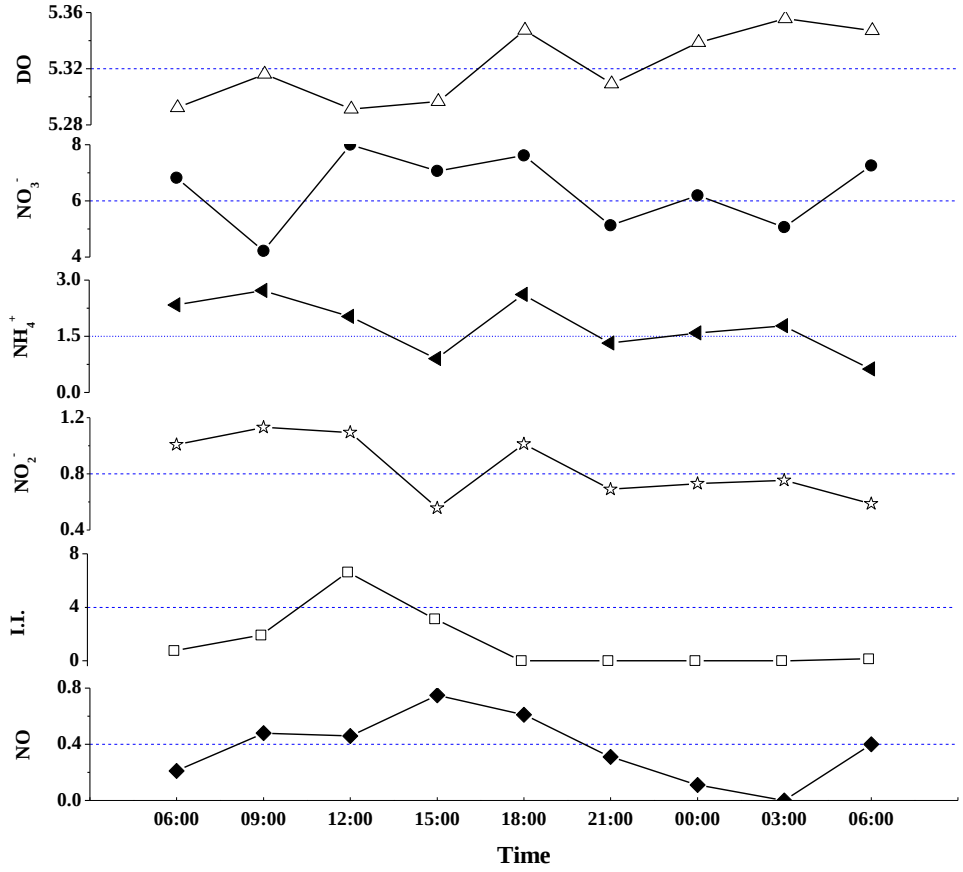
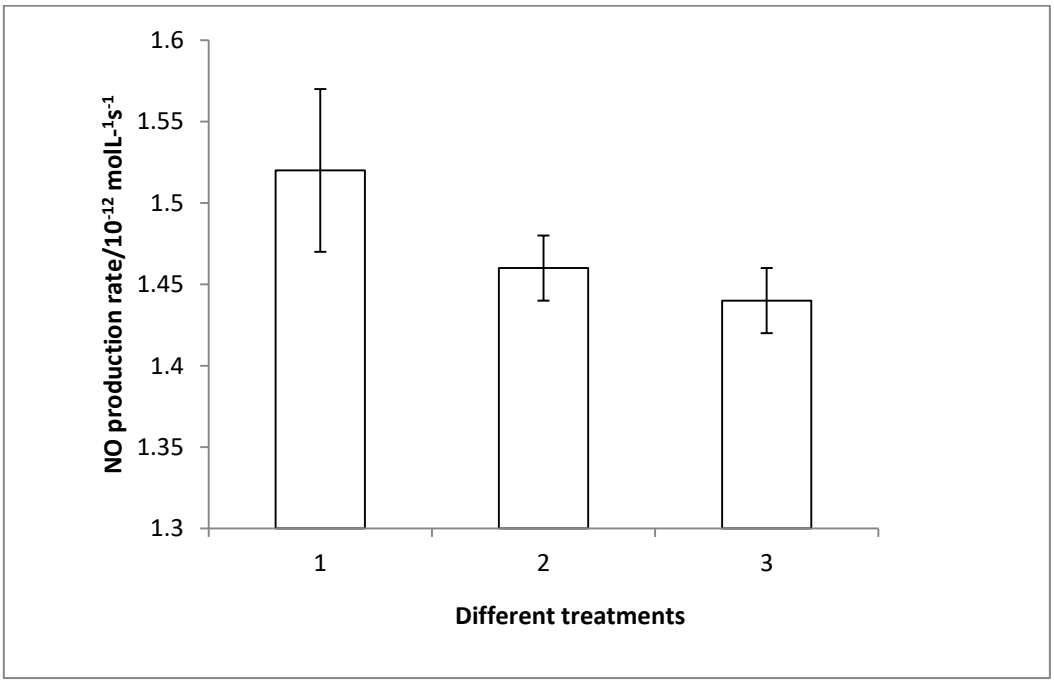


Fig. 6 The diurnal variations of NO concentrations and related parameters in the surface seawater at station 10 (Units: DO (mL L^{-1}), NO_3^-, NO_2^-, NH_4^+ (μmol L^{-1}), I.I.-illumination intensity ($\times 10^4$ lux), NO (nmol L^{-1}))



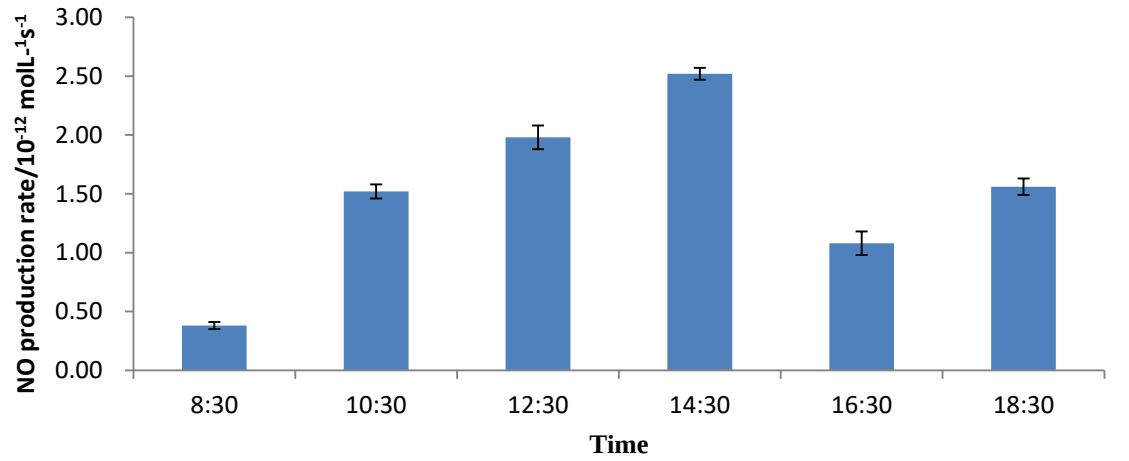
475

476 **Fig. 7** The production rates of NO by seawater irradiation under natural light after different treatments

477 (1. Incubated on deck at ambient temperature, 2. 0.45 μm Millipore filtered at ambient temperature, 3.

478 Incubated in water bath supplied with surface seawater)

479



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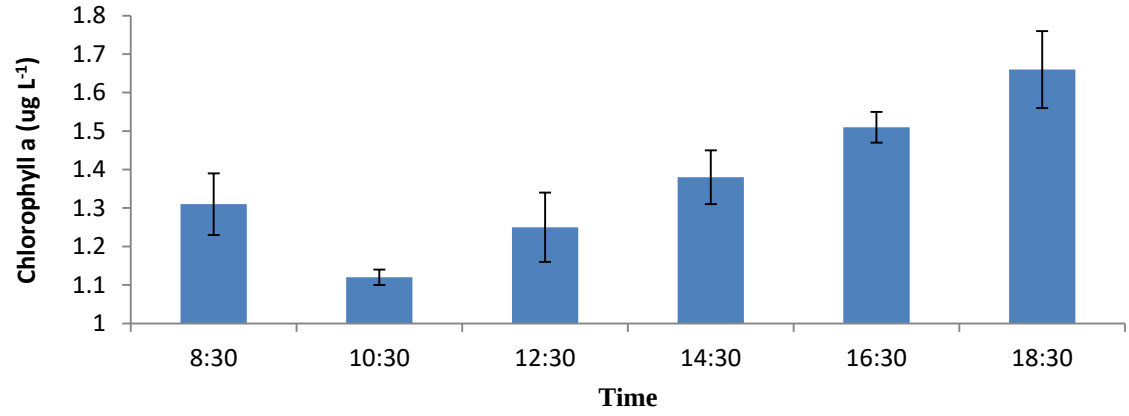

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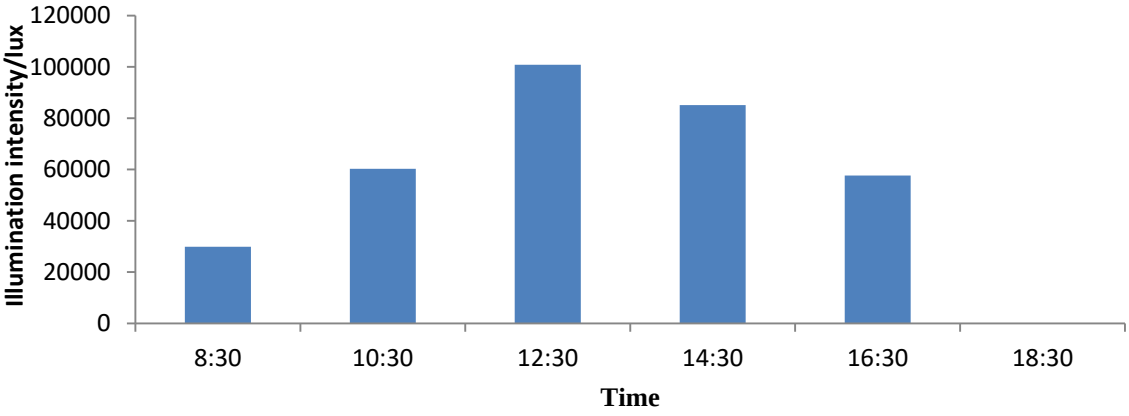

482

483 **Fig. 8** The variations of NO production rates, chlorophyll *a* concentrations and sunlight intensity in the
484 incubation experiments with Qingdao coastal waters

485

Table 1 The methods for NO detection in seawater

Method	Linearity range (nmol L^{-1})	Detection limit (nmol L^{-1})	Analytical precision	Reference
Microelectrode	140–9900	140	0.24%	Zhang et al. (2003)
Microelectrode	1.4–1400	4.2×10^{-10}	6.30‰	Xing et al. (2005)
Microelectrode	0.4 –4000	30	-	Schreiber et al.(2008)
Fluorescence	1.4–1400	1.4	1.63%	Liu et al. (2009)
HPLC with fluorescence	0.025–10	0.025	3-5%	Olasehinde et al. (2009)
Purge-and-trap with chemiluminescence	-	0.0015	3%	Zafirou and McFarland (1980)
Purge-and-trap with chemiluminescence	-	0.25	3-25%	Bange and Lutterbeck (2015)
Purge-and-trap with fluorescence	0.14–19	0.068	7.2%	This study

Table 2 Reaction yields of the reaction of DAN with NO (in %)

Purge flow rate /mL min^{-1}	Purge time /min			
	15	30	45	60
200	—	—	—	—
300	—	—	21	34
400	56	85	69	69
500	—	—	22	26
600	—	—	31	33