

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

3 Chun-Ying Liu<sup>1,2,3</sup>, Wei-Hua Feng<sup>1,4</sup>, Ye Tian<sup>1</sup>, Gui-Peng Yang<sup>1,2,3\*</sup>, Pei-Feng Li<sup>1</sup>, Hermann W.  
4 Bange<sup>5</sup>

5 <sup>1</sup> College of Chemistry and Chemical Engineering, Ocean University of China, Qingdao, 266100, China

6 <sup>2</sup> Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and  
7 Technology, Qingdao, 266071, China

8 <sup>3</sup> Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, Qingdao, 266100, China

9 <sup>4</sup> Key Laboratory of Engineering Oceanography, Second Institute of Oceanography, SOA, Hangzhou, 310012, China

10 <sup>5</sup> GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel, Kiel, 24105, Germany

11

12

13 \* Corresponding author:

14 Prof. Gui-Peng Yang

15 College of Chemistry and Chemical Engineering

16 Ocean University of China

17 238 Songling Road, Qingdao 266100, China.

18

19 Tel.: +86 532 66782657

20 Fax: +86 532 66782540

21 *E-mail address:* [gpyang@ouc.edu.cn](mailto:gpyang@ouc.edu.cn)

22

# 23 **Determination of dissolved nitric oxide in coastal waters of the** 24 **Yellow Sea off Qingdao**

25 Chun-Ying Liu<sup>1,2,3</sup>, Wei-Hua Feng<sup>1,4</sup>, Ye Tian<sup>1</sup>, Gui-Peng Yang<sup>1,2,3\*</sup>, Pei-Feng Li<sup>1</sup>, Hermann W.  
26 Bange<sup>5</sup>

27 <sup>1</sup> College of Chemistry and Chemical Engineering, Ocean University of China, Qingdao, 266100, China

28 <sup>2</sup> Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and  
29 Technology, Qingdao, 266071, China

30 <sup>3</sup> Key Laboratory of Marine Chemistry Theory and Technology, Ministry of Education, Qingdao, 266100, China

31 <sup>4</sup> Key Laboratory of Engineering Oceanography, Second Institute of Oceanography, SOA, Hangzhou, 310012, China

32 <sup>5</sup> GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel, Kiel, 24105, Germany

33 *Correspondence to:* Gui-Peng Yang (gpyang@ouc.edu.cn)

34

35 **Abstract** We developed a new method for the determination of dissolved nitric oxide (NO) in discrete seawater  
36 samples based on a combination of a purge-and-trap set-up and fluorometric detection of NO.  
37 2,3-diaminonaphthalene (DAN) reacts with NO in seawater to form the highly fluorescent 2,3-naphthotriazole  
38 (NAT). The fluorescence intensity was linear for NO concentrations in the range from 0.14 nmol L<sup>-1</sup> to 19 nmol  
39 L<sup>-1</sup>. We determined a detection limit of 0.068 nmol L<sup>-1</sup>, an average recovery coefficient of 83.8% (80.2-90.0%),  
40 and a relative standard deviation of ±7.2%. With our method we determined for the first time the temporal and  
41 spatial distributions of NO surface concentrations in coastal waters of the Yellow Sea off Qingdao and in Jiaozhou  
42 Bay during a cruise in November 2009. The concentrations of NO varied from below the detection limit to 0.50  
43 nmol L<sup>-1</sup> with an average of 0.26 ± 0.14 nmol L<sup>-1</sup>. NO surface concentrations were generally enhanced  
44 significantly during daytime implying that NO formation processes such as NO<sub>2</sub><sup>-</sup> photolysis are much higher

45 during daytime than chemical NO consumption which, in turn, lead to a significant decrease of NO  
46 concentrations during nighttime. In general, NO surface concentrations and measured NO production rates were  
47 higher compared to previously reported measurements. This might be caused by the high NO<sub>2</sub><sup>-</sup> surface  
48 concentrations encountered during the cruise. Moreover, additional measurements of NO production rates  
49 implied that the occurrence of particles and a temperature increase can enhance NO production rates. With the  
50 method introduced here we have a reliable and comparably easy to use method at hand to measure oceanic NO  
51 surface concentrations which can be used to decipher both its temporal and spatial distributions as well as its  
52 biogeochemical pathways in the oceans.

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

55

## 56 1 Introduction

57 As a reactive atmospheric trace gas, nitric oxide (NO) plays important roles in tropospheric  
58 chemistry: It is a key player in the formation of acid rain and ozone (Williams et al., 1992; Lee et al.,  
59 1997; Mazzeo, et al., 2005). NO is an intermediate of both the terrestrial and marine nitrogen cycle  
60 (Ward and Zafiriou, 1988; Williams et al., 1992; Canfield et al., 2010; Chen et al., 2010; Thamdrup,  
61 2012; Voss et al., 2013). It has a variety of sources in seawater, including nitrite photolysis and various  
62 microbial processes such as denitrification, anammox and dissimilatory nitrate reduction to ammonia  
63 (Law, 2001; Schreiber et al., 2012; Martens-Habbena et al., 2015). Because of its chemical reactivity,  
64 NO usually does not accumulate in large amounts in seawater and the ocean as a source of atmospheric  
65 NO is, therefore, negligible in a global context (Zehr and Ward, 2002; Bange, 2008). Moreover, NO  
66 was found to have significant effects on the growth of marine algae (Zhang et al., 2005; Liu et al., 2004;

67 2005; 2006; 2014). To this end, the determination of the spatial and temporal distributions of NO in the  
68 ocean as well as deciphering its oceanic production processes and their major influencing factors are  
69 essential to improve our understanding of the biogeochemical cycling NO in the ocean.

70 Because of its low concentrations in seawater caused by its fast diffusion and high chemical  
71 reactivity, measurements of NO in seawater are very difficult. Therefore, there are only a few methods  
72 available to determine NO (Hetrick and Schoenfish, 2009), see Tab. 1. The electrochemical method  
73 using sensors in seawater medium achieved a detection limit of  $42 \text{ nmol L}^{-1}$  (Xing et al., 2005; Zhang  
74 et al., 2005). Olasehinde et al. (2009) developed a method for the determination of photochemically  
75 generated NO in natural waters adopting 4,5-diaminofluorescein as a probe compound and a  
76 measurement of reversed-phase high performance liquid chromatography (HPLC) with fluorescence  
77 detector. The NO concentrations and signal intensities exhibited a good linearity correlation over the  
78 range of  $0.025\text{-}10 \text{ nmol L}^{-1}$  triazolofluorescein. Zafiriou and McFarland (1980) determined the NO  
79 concentration of seawater by using a flow system to equilibrate the seawater samples with a gas stream  
80 coupled to a chemiluminescence detector. They report an analytical precision of  $\pm 3\%$  and an accuracy  
81 of  $\pm 20\%$ . More recently, Lutterbeck and Bange (2015) developed an improved method of a  
82 chemiluminescence NO analyser connected to a stripping unit, and the limit of detection was  $0.25$   
83  $\text{nmol L}^{-1}$  using a  $20 \text{ mL}$  seawater sample volume. Until now only these two chemiluminescence  
84 methods were applied successfully to determine NO concentrations in seawater samples. The  
85 N-nitrosation of 2,3-diaminonaphthalene (DAN) results in the highly fluorescent 2,3-naphthotriazole  
86 (NAT) which could be used to detect NO concentrations as low as  $10 \text{ nmol L}^{-1}$  (Miles et al., 1995). We  
87 adopted this method for seawater medium instead of NaOH medium and the calibration curve  
88 exhibited linearity over the concentration range of  $1.4 - 1400 \text{ nmol L}^{-1}$  NO (Liu et al., 2009). However,

89 this assay cannot be used to detect trace levels of NO in seawater samples directly.

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

94

## 95 **2 Materials and methods**

### 96 **2.1 Instrumental set-up**

97 The analytical system consists of a degassing column to purge NO from seawater samples, a  
98 reaction chamber where NO reacts to form a fluorescent compound (Fig. 1), and a fluorescence  
99 spectrophotometer (F-4500, Hitachi Co., Japan). The 800 mL degassing column has a sodium silicate  
100 bonded sand core at the bottom to disperse the nitrogen (N<sub>2</sub>) purge gas stream. There are four ports on  
101 the column: (1) a gas port at the bottom of the degassing column where the high purity N<sub>2</sub> purge gas  
102 (99.999%, Qingdao Heli Industry Gas Center, China) or a NO standard gas mixture (5.4 ppmv, NO/N<sub>2</sub>)  
103 (Beijing Sida Standard Substance Co., China) are introduced, (2) a drain port as outlet for water  
104 samples, (3) an inlet port where water samples are pushed into the degassing column with N<sub>2</sub>, and (4) an  
105 outlet port on the top of the degassing column connected with the reaction chamber.

106 The NO standard gas cylinder is linked to the degassing column via a gas-tight syringe (Shanghai  
107 Anting Injector Co., China). The N<sub>2</sub> gas cylinder is connected to the degassing column via a deoxygen  
108 tube (Agilent Technologies, USA) to remove traces of O<sub>2</sub> and a glass rotameter to monitor the gas flow  
109 (0.1-1 L min<sup>-1</sup>, Jiangyin, China). These two gas streams enter the degassing column via the port at the  
110 bottom of the flask, controlled by a three-port valve. The tubing used is made of polytetrafluorethylyene

111 (PTFE, 1/8-inch tubing outer diameter [o.d.]). Moreover, an Ultraviolet-Visible spectrophotometer  
112 (UV-2550, Shimadzu Co., Japan) and an Automatic Analytical Balance (Beijing Sartorius Co., China)  
113 were used in this work.

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

122

## 123 **2.2 Preparation of DAN and NO solutions**

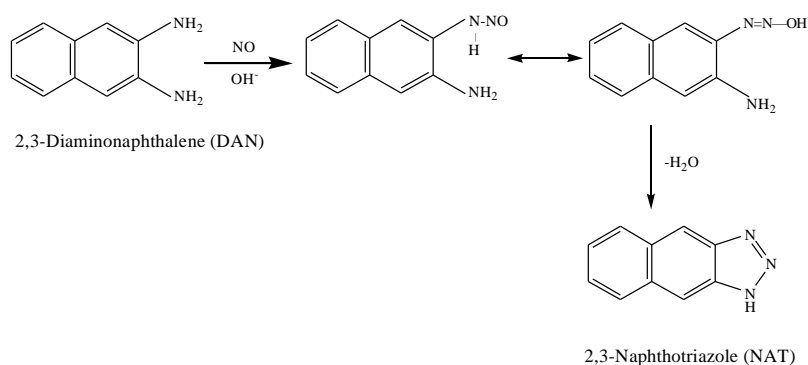
124 A 2,3-diaminonaphthalene (DAN,  $\geq 95\%$ , GC, Sigma-Aldrich Chemical Co., USA) stock solution  
125 was prepared fresh with a concentration of  $10 \text{ mmol L}^{-1}$  in dimethylformamide (Sigma-Aldrich  
126 Chemical Co., USA) and kept in the dark at  $-21 \text{ }^\circ\text{C}$  until used. DAN solutions of  $40 \text{ } \mu\text{mol L}^{-1}$  were  
127 prepared from the stock solution in Milli-Q water,  $10 \text{ mmol L}^{-1}$  NaOH aqueous solution and filtered  
128 natural seawater, respectively. (Natural seawater was sampled from the coastal waters off Qingdao and  
129 was filtered through a  $0.45 \text{ } \mu\text{m}$  acetate cellulose membrane (Millipore, USA). The DAN solutions were  
130 purged with  $\text{N}_2$  gas for 30 min to remove oxygen ( $\text{O}_2$ ), then stored on ice and transferred to a refrigerator  
131 at  $4 \text{ }^\circ\text{C}$  before use.

132 An aliquot of 10 mL Milli-Q water was bubbled with N<sub>2</sub> gas at a flow of 10 mL min<sup>-1</sup> for 1h to  
133 remove O<sub>2</sub> after 10 min of ultrasonic degassing. The solution was then bubbled with high purity NO  
134 gas (99.9%, Dalian Date Gas Ltd, China) for 30 min. The concentration of the saturated NO stock  
135 solution was 1.4 mmol L<sup>-1</sup>, which should be used within 3h (Lantoine et al., 1995). A series of diluted  
136 NO solutions were prepared in N<sub>2</sub>-purged water from the NO stock solution using a syringe (Xing et al.,  
137 2005).

138

### 139 2.3 Fluorometric detection of NO

140 DAN reacts with NO<sub>x</sub> (= NO + NO<sub>2</sub>) in alkaline medium and forms the highly fluorescent  
141 2,3-naphthotriazole (NAT):



142

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

147

### 148 2.4 The influence of nitrite in seawater on the reaction of DAN and NO

149 NO can be formed from nitrite (NO<sub>2</sub><sup>-</sup>) in seawater (Zafiriou and McFarland, 1981). Therefore, we

150 tested a potential interference of dissolved  $\text{NO}_2^-$  by adding different concentrations of  $\text{NO}_2^-$  to  
151 seawater samples. The tests were conducted in the dark or with ultraviolet B (UV-B) radiation (HR  
152 1×18 w, Xinghui Electric Instrument Factory, China). The final concentrations of  $\text{NO}_2^-$  in the seawater  
153 samples were set to 40, 80, 120, 160, and 200  $\mu\text{mol L}^{-1}$ , respectively, and the reaction time was 1 h or 12  
154 h.

155

## 156 2.5 Sampling and analysis

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

162 A 500 mL Wheaton glass serum bottle was rinsed with the seawater three times before it was filled  
163 with seawater quickly through a siphon. When the overflowed sample reached the half volume of the  
164 bottle, the siphon was withdrawn rapidly and 0.5 mL saturated  $\text{HgCl}_2$  solution was added to stop  
165 biological activities and the bottle was sealed quickly. All glass bottles were covered with aluminum  
166 foil to prevent  $\text{NO}_2^-$  photolysis during sampling.

167 Because NO reacts with  $\text{O}_2$  both in the gas phase and in aqueous solution we purged our set-up  
168 for 1h with  $\text{N}_2$  gas and sealed it before the measurements. In a first step, a certain amount of standard  
169 NO gas was transferred to the reaction chamber via the degassing column by injecting it from a gas  
170 tight syringe into the  $\text{N}_2$  carrier gas stream. In the reaction chamber NO reacts with the DAN solution.  
171 After the measurement of the NO gas standard, a 500 mL seawater sample was injected into the



172 degassing column and purged with N<sub>2</sub> gas and immediately transferred into the reaction chamber where  
173 it reacts with 10 mL DAN solution. The gas flow is controlled to ensure that the reaction of NO with  
174 DAN solution was completed. Finally, the fluorescence intensity of the resulting NAT solution was  
175 measured with the F-4500 fluorescence spectrophotometer.

176 In order to prevent NO photochemical generation, the entire glass parts were wrapped with  
177 aluminum foil. The purge-and-trap procedure was conducted at room temperature of 20 °C.

178

## 179 **2.6 O<sub>2</sub>, nutrients, DOC and chlorophyll *a* measurements**

180 Dissolved O<sub>2</sub> (DO) concentrations were determined according to the Winkler method. The  
181 concentrations of dissolved nitrate, nitrite and ammonia were measured by using an AutoAnalyzer 3  
182 (SEAL Analytical, USA). The detection limits of the method were 0.003, 0.015 and 0.040 μmol L<sup>-1</sup> for  
183 nitrate, nitrite and ammonia, with the precision less than 1%. The intensity of sunlight was monitored  
184 by the use of a TES-1322A actionometer (Taishi Co. Taiwan). Dissolved organic carbon (DOC) was  
185 determined by a high-temperature combustion method using a Shimadzu TOC-5000 Analyzer with an  
186 Al-Pt catalyst (Shimadzu Co., Japan). The precision of the DOC measurements was less than 2%.  
187 Concentrations of chlorophyll *a* were measured with a bbe Cuvette Fluorometer (bbe-Moldaenke  
188 GmbH, Kiel, Germany).

189

## 190 **2.7 NO production rates**

191 Experiments for NO production by NO<sub>2</sub><sup>-</sup> photolysis were conducted at station 10 as follows:  
192 Aliquots of 10 mL untreated seawater samples from 0.2 m depth or Millipore membrane (0.45 μm)  
193 filtered samples were distributed into three 14 mL glass vials. The initial concentrations of NO<sub>2</sub><sup>-</sup> and

194 DOC in seawater were  $0.75 \mu\text{mol L}^{-1}$  and  $439 \mu\text{mol L}^{-1}$  C, respectively. Then  $200 \mu\text{L}$  of 20%  $\text{NaN}_3$   
195 solutions (instead of saturated  $\text{HgCl}_2$  solution to avoid contamination by the photosensitive Hg) and 20  
196  $\mu\text{L}$  of  $1 \text{ mmol L}^{-1}$  DAN solutions were added. The vials were sealed with rubber septa and aluminum  
197 crimp tops, and were exposed to sunlight on the deck at ambient temperatures ( $17 \text{ }^\circ\text{C}$ ) or at  $13 \pm 2 \text{ }^\circ\text{C}$   
198 in a water bath supplied with the ambient seawater. For “dark” controls vials were wrapped in  
199 aluminum foil. The intensity of sunlight ranged from 67565 lux to 71500 lux (average: 69430 lux).  
200 After irradiation by sunlight for 30 min, the NO concentrations were measured with the method  
201 described above. The NO photolysis production rates were computed as the increase of the NO  
202 concentrations during the incubation time.

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

212

## 213 **3 Results and Discussion**

### 214 **3.1 Method evaluation**

215 Both the purge time and flow of the purge gas ( $\text{N}_2$ ) significantly influence the yield of the NO +

216 DAN reaction and thus, the overall purge efficiency (see Tab. 2). The optimal (i.e. maximum) reaction  
217 yield was 85% after 30 min of purging at a flow of 400 mL min<sup>-1</sup>.

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

224 Seawater samples from coastal waters off Qingdao were analyzed in the lab up to of 7 times and  
225 gave a relative standard deviation of  $\pm 7.2\%$ . The detection limit of our method was determined to be  
226 0.068 nmol L<sup>-1</sup> (S/N = 3), which is lower than most of the reported detection limits for NO  
227 measurements in seawater (see Tab. 1)

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

$$232 \quad \text{RC (\%)} = \text{NO}(\text{sw}) / \text{NO}(\text{DAN}) \times 100\%.$$

233 Where NO (DAN) stands for the NO directly injected to the DAN solution and NO (sw) stands for the  
234 NO measured from the sample in degassing column according to the method described above. The  
235 resulting NO recovery coefficients ranged from 80.2% to 90.0%, with an average of 83.8%.

236 In order to check the linearity of our method, a solution of 10 mL 40  $\mu\text{mol L}^{-1}$  DAN was injected  
237 into the reaction chamber and purged with N<sub>2</sub> gas at a rate of 10 mL min<sup>-1</sup> for 5 min prior to the actual

238 measurements. A series of NO-free seawater samples placed in the degassing column were spiked with  
239 different volumes of the NO standard gas (mixing ratio 5.4 ppmv NO/N<sub>2</sub>) and analyzed according to  
240 the procedure described above. The resulting fluorescence intensity was linear with the NO  
241 concentrations in the range from 0.14 to 19.0 nmol L<sup>-1</sup> ( $y = 7.4286x + 0.6188$ ,  $R = 0.9976$ ,  $P <$   
242  $0.0001$ ) (Fig. 3).

243 The results of the samples spiked with varying concentrations of dissolved NO<sub>2</sub><sup>-</sup> are given in Fig.  
244 4. In general, samples with the same NO<sub>2</sub><sup>-</sup> concentration showed higher fluorescence when  
245 UV-irradiated or kept in dark for 12 h compared to samples under short term (i.e. 1 h) UV irradiation or  
246 kept in dark. This points a significant NO production under UV irradiation ( $n=5$ ,  $F=76.13$ ,  $p=2.32 \times 10^{-5}$ )  
247 and (albeit weaker) NO dark production from NO<sub>2</sub><sup>-</sup>. Higher NO<sub>2</sub><sup>-</sup> concentrations resulted in a slight  
248 increase of fluorescence when irradiated. Therefore we conclude that the measurements of NO should be  
249 done in the dark as soon as possible after sampling when high NO<sub>2</sub><sup>-</sup> concentrations occur.

250 To assess the influence of the interferences of dissolved organic matter, trace metals, nutrients,  
251 and other substances in seawater, the NO/fluorescence intensity relationship should be determined  
252 when the method is applied in different oceanic regions.

253 With our method we are able to detect  $> 0.068$  nmol L<sup>-1</sup> NO in discrete seawater samples with a  
254 volume of 500 mL. With a larger degassing column, even lower concentrations of NO might be  
255 determined.

256 A U-shaped tube and cold bath (i.e. a water trap) was initially placed between the degassing  
257 column and the reaction chamber in order to eliminate small amounts of water carried by the N<sub>2</sub> gas  
258 stream. However, we found that the fluorescence intensities did not show significant differences  
259 when the water trap was removed.

260

### 261 3.2 Distribution of dissolved NO in coastal waters of Qingdao

262 Fig. 5 shows the NO concentrations of surface seawater in coastal waters off Qingdao (stations  
263 S01-S09) and in the Jiaozhou Bay (stations S10 and S11). The concentrations of NO ranged from  
264 below the detection limit (stat. 02 and 03) up to  $0.50 \pm 0.01 \text{ nmol L}^{-1}$  (stat. S08), with an overall mean  
265 of  $0.26 \pm 0.14 \text{ nmol L}^{-1}$ . It is noteworthy that the higher NO concentrations seem to be related to the  
266 time point of sampling (given in local time): Samples of stations 2 and 3 were collected at night time,  
267 22:30h and 00:50h, respectively, while samples for stations 5, 6, 7 and 8 were collected during the  
268 day time (08:58h - 15:38h). (Stations S09 and S10 have been measured in Jiaozhou Bay and, thus,  
269 their NO concentrations are directly not comparable with the stations off Qingdao). Our results are  
270 generally consistent with the findings in the aquatic ecosystem of Daya Bay in China (Zhang et al.,  
271 2006) and the nitrite-rich surface waters of the central equatorial Pacific Ocean (Zafiriou et al., 1980),  
272 indicating that sunlight could be a main factor affecting NO formation in seawater. The  
273 concentrations of NO in coastal surface waters off Qingdao were found to be an order of magnitude  
274 higher than those in surface waters during day time in the central equatorial Pacific Ocean ( $0.05 \text{ nmol}$   
275  $\text{L}^{-1}$ ) (Zafiriou et al., 1980; Zafiriou and McFarland, 1981). This difference is probably related to the  
276 concentrations of  $\text{NO}_2^-$  in seawater. Zafiriou et al. (1980) proposed that sunlight photolyzes  $\text{NO}_2^-$  in  
277 surface water by the following reaction:



279 According to the reaction above, high concentrations of  $\text{NO}_2^-$  together with strong solar irradiation  
280 could cause enhanced concentrations of NO in seawater. The sunlight intensity of the central  
281 equatorial Pacific is generally higher than that of coastal waters of Qingdao (located at  $36^\circ 05' \text{N}$ );

282 however, the coastal waters off Qingdao at the time of our measurements exhibited an average  $\text{NO}_2^-$   
283 concentration of  $0.49 \pm 0.25 \mu\text{mol L}^{-1}$ , which was much higher than that observed concentration in  
284 the central equatorial Pacific Ocean ( $\sim 0.1 \mu\text{mol L}^{-1}$ ).

285 NO is a short-lived intermediate of various microbial processes of the nitrogen cycle, which is  
286 involved in denitrification (Kampschreur et al., 2007), anammox (Kartal et al., 2011) and archaea  
287 ammonia-oxidizing (Martens-Habbena et al., 2015) processes. Zafiriou and McFarland (1981) analyzed  
288 NO in seawater samples at the sea surface of the central equatorial Pacific by stripping NO into an air  
289 and  $\text{N}_2$  stream by passing it through the same chemiluminescence - type detector. Thus, the NO  
290 concentrations were underestimated to some extent because seawater samples were suboxic or anoxic.  
291 However, time-dependent losses from microbial processes were minimized. Lutterbeck and Bange  
292 (2015) improved the method above to determine dissolved NO in discrete seawater samples of the  
293 eastern tropical South Pacific Ocean. The contamination by  $\text{O}_2$  diffusion into the continue samples  
294 could be further minimized. This work was also designed to detect dissolved NO in discrete seawater  
295 samples with a combination of a purge-and-trap set-up and fluorometric NO analyzer. The  $\text{HgCl}_2$   
296 solution was added to stop biological activities during the stripping. However, the disposal of these  
297 Hg-contaminated solutions is a tough proposition.

298 The diurnal variation of NO concentrations and other parameters in surface seawater are shown in  
299 Fig. 6. Concentrations of NO presented a significant diurnal variation within 24 h. The peak value  
300 appeared at 15:00h (local time) with a concentration of  $0.81 \text{ nmol L}^{-1}$ . After that the concentration of  
301 NO decreased with time gradually until a minimum value occurred at 03:00. Obviously, the  
302 concentration of dissolved NO at this station was influenced by the in-situ sunlight intensity. However,  
303 the maximum NO concentration appeared not at 12:00h but at 15:00h, which suggesting that there

304 were other influencing factors besides sunlight irradiation.

305

### 306 3.3 NO production rates in coastal waters

307 The results of the NO irradiation experiments are given in Fig. 7. The production rate of NO  
308 through seawater irradiation was  $1.52 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$  which is slightly higher than that NO production  
309 rate of the 0.45  $\mu\text{m}$  Millipore filtered samples ( $1.46 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$ ). The difference may indicate that  
310 particles in seawater could increase the NO production rate. The non-filtered samples incubated in the  
311 water bath had a lower NO production rate ( $1.44 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$ ) compared to the other non-filtered  
312 treatment, which could be ascribed to the difference of the temperature. The ambient temperature and  
313 water bath were 17  $^{\circ}\text{C}$  and 13  $^{\circ}\text{C}$ , respectively, thus the higher temperature may resulted in a higher  
314 photolysis rate. The photochemical production rates of NO in Qingdao coastal waters during the  
315 daytime were generally higher than that reported from the central equatorial Pacific Ocean  
316 ( $0.4\text{-}1.2 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$ ) (Zafiriou and McFarland, 1981).

317 Previous experiments about  $\text{NO}_2^-$  photolysis were also carried out in the laboratory (Li et al.,  
318 2011): The production of NO was observed after 3 h illumination of 10-100  $\mu\text{mol L}^{-1} \text{ NO}_2^-$  in Milli-Q  
319 water. There was an increasing trend of NO concentrations with the  $\text{NO}_2^-$  concentrations. For natural  
320 seawater, it was observed to have an increasing trend of NO concentration with the illumination time  
321 (Li et al., 2011). The process of sunlight photolysis of  $\text{NO}_2^-$  in surface water was demonstrated, which  
322 was consistent with the results of Zafiriou et al. (1980) and Olasehinde et al. (2009).

323 The production and consumption of NO occur synchronously when sunlight photolyze natural  
324 seawater. The photolysis of  $\text{NO}_2^-$  is to mainly produce NO and OH. On the other hand, the loss of NO  
325 happens by forming NO-reactive radicals from CDOM (Zafiriou et al., 1990; Zafiriou and Dister, 1991;

326 Olasehinde et al., 2009).The concentration of NO after exposure to sunlight is a balancing of this  
327 production against consumption by radical recombination. The study area has high concentrations of  
328 DOC and is rich in CDOM (Liu et al., 2010; Yang et al., 2011), thus, the authentic NO resulted from  
329 NO<sub>2</sub><sup>-</sup> photolysis was underestimated. The photochemical production rates of NO were only a total value  
330 of production and consumption in this study.

331 The on-deck incubation experiments for the production rates of NO in Qingdao coastal waters,  
332 together with chlorophyll *a* concentrations and sunlight intensities, are shown in Fig. 8. The production  
333 rates of NO exhibited a clear variation during the course of the day with a maximum value appearing  
334 at 14:30h (local time). The maximum value of  $2.52 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$  was about seven-fold higher than  
335 the minimum value at 08:30h. The production rates of NO kept an increasing trend from 08:30h to  
336 14:30h. The mean production rate in Qingdao coastal waters was  $1.51 \times 10^{-12} \text{ mol L}^{-1} \text{ s}^{-1}$  during the day.  
337 The variation of the production rates of NO did not follow the trends in chlorophyll *a* concentrations  
338 and solar radiation. Therefore, the production pattern of NO in marine environments deserves further  
339 research.

340

#### 341 **4 Summary**

342 For the determination of NO concentrations in discrete seawater samples we developed a new  
343 method by combining a purge-and-trap set-up with fluorometric detection of NO. The method showed  
344 a linear fluorescence intensity for NO concentrations ranging from 0.14 nmol L<sup>-1</sup> to 19 nmol L<sup>-1</sup>. The  
345 detection limit is 0.068 nmol L<sup>-1</sup> (S/N =3), the average recovery coefficient is 83.8% (80.2~90.0%),  
346 and the relative standard deviation is ±7.2%. Our method was applied to measure concentrations of  
347 NO in surface layer of the coastal waters off Qingdao and Jiaozhou Bay. NO concentrations varied



348 from below the detection limit to  $0.50 \text{ nmol L}^{-1}$ , with an average of  $0.26 \pm 0.14 \text{ nmol L}^{-1}$ . The  
349 concentrations of NO in coastal waters off Qingdao were an order of magnitude higher than those in  
350 surface waters of the central equatorial Pacific. NO surface concentrations were generally enhanced  
351 significantly during daytime implying that NO formation processes such as  $\text{NO}_2^-$  photolysis are much  
352 higher during daytime than chemical NO consumption which, in turn leads to the observed significant  
353 decrease of the NO concentrations during nighttime. The measurements of NO production rates  
354 showed that the occurrence of particles and an increase in temperature can enhance NO production.

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

358

### 359 **Acknowledgments**

360 We thank Li Tie and Zhu Chenjian for their assistance during sample collection. This research was  
361 supported by the National Natural Science Foundation of China (Nos. 41176062, 41676065, 40706040),  
362 Natural Science Foundation of Shandong Province (Y2008E04), Scientific Research Promotional Fund  
363 for Middle-age and Young Scientists of Shandong Province (2007BS08015), and Aoshan Talents  
364 Program Supported by Qingdao National Laboratory for Marine Science and Technology (No.  
365 2015ASTP).

366

### 367 **References**

368 Bange, H. W.: Chapter 2 - Gaseous Nitrogen Compounds (NO,  $\text{N}_2\text{O}$ ,  $\text{N}_2$ ,  $\text{NH}_3$ ) in the Ocean in: Nitrogen in the Marine  
369 Environment (Second Edition), Elsevier, Amsterdam, Netherlands, 51-94, 2008.

- 370 Canfield, D. E., Glazer, A. N., and Falkowski, P. G.: The evolution and future of the Earth's nitrogen cycle, *Science*,  
371 330,192-196, 2010.
- 372 Chen, J., Wu, F. H., Xiao, Q., Yang, Z. H., Huang, S. K., Wang, J., Wu, Y. G., Dong, X. J., Pei, Z. M., Zhen, H. L.:  
373 Diurnal variation of nitric oxide emission flux from a mangrove wetland in Zhangjiang River Estuary, China,  
374 *Estuar. Coast. Shelf Sci.*, 90, 212-220, 2010.
- 375 Hetrick, E. M., and Schoenfish, M. H.: Analytical chemistry of nitric oxide, *Annu. Rev. Anal. Chem.*, 2, 409-433,  
376 2009.
- 377 Kampschreur, M. J., Picioreanu, C., Tan, N., Kleerebezem, R., Jetten, M. S. M., and van Loosdrecht, M. C. M.:  
378 Unraveling the source of nitric oxide emission during nitrification, *Water Environ. Res.*, 79, 2499–2509,  
379 2007.
- 380 Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geert, S.W., den Camp, H., Harhangi, H. R.,  
381 Janssen-Megens, E. M., Francoijs, K. J., Stunnenberg, H. G., Keltjens, J. T., Jetten, M. S. M., and Strous, M.:  
382 Molecular mechanism of anaerobic ammonium oxidation, *Nature*, 479, 127–130, 2011.
- 383 Lantoiné, F., Trevin, S., Bedioui, F., and Devynck, J.: Selective and sensitive electrochemical measurement of nitric  
384 oxide in aqueous solution: discussion and new results. *J Electroanal. Chem.*, 392, 85-89, 1995.
- 385 Law, C. S.: Air-Sea Transfer: N<sub>2</sub>O, NO, CH<sub>4</sub>, CO. J.H. Steele, S.A. Thorpe, K.K. Turekian. (eds.) *Encyclopedia of*  
386 *Ocean Sciences*. San Diego, USA, Academic Press, 137-143, 2001.
- 387 Lee, D. S., Köhler, I., Grobler, E., Rohrer, F., Sausen, R., Gallardo-Klenner, L., Olivier, J. G. J., Dentener, F. J., and  
388 Bouwman, A. F.: Estimations of global NO<sub>x</sub> emissions and their uncertainties, *Atmos. Environ.*, 31(12),  
389 1735-1749. 1997.
- 390 Li, P. F., Li, W. S., Liu, C. Y., Zhu, X. C., and Zhang, Q.: The Photodecomposition of Nitrite in Water, *Environ. Chem.*,  
391 30, 1883-1888, 2011. (in Chinese with English abstract).

- 392 Liu, C. Y., Kieber, D. J., Yang, G. P., Xue, C., Wang, L. L., and Liu, H. H.: Evidence for the mutual effects of  
393 dimethylsulfoniopropionate and nitric oxide during the growth of marine microalgae, *Nitric Oxide*, 42, 54-61,  
394 2014.
- 395 Liu, C. Y., Yang, X. M., Yang, G. P., Zhou, L. M., and Li, P. F.: Composition and characterization of colloidal organic  
396 matter in the coastal surface waters of Qingdao, China, *Mar. Chem.*, 121:123-131, 2010.
- 397 Liu, C. Y., Zhang, Z. B., and Chen, X. R.: The mutual effects of nitric oxide and iron on the growth of marine algae,  
398 *Acta Oceanol. Sin.*, 24(5), 100-109, 2005.
- 399 Liu, C. Y., Zhang, Z. B., Li, P. F., and Huang, H. W.: Growth effect of exogenous nitric oxide on *Platymonas*  
400 *subcordiformis* and spectrum study, *Chinese J Environ. Sci.*, 27(6), 1062-1067, 2006. (in Chinese with English  
401 abstract).
- 402 Liu, C. Y., Zhang, Z. B., Xing, L., Lin, C., and Wu, Z. Z.: The ocean biogeochemistry of nitric oxide, *Periodical of*  
403 *Ocean University of China*, 34(sup), 16-22, 2004. (in Chinese with English abstract).
- 404 Liu, C. Y., Zhao, M., Ren, C.Y., Yang, G. P., Li, P. F., and Han, Y.: Direct measurement of nitric oxide in seawater  
405 medium by fluorometric method. *Chinese J Anal. Chem.*, 37(10), 1463-1467, 2009.
- 406 Lutterbeck, H. E., and Bange, H. W.: An improved method for the determination of dissolved nitric oxide (NO) in  
407 seawater samples, *Ocean Science*, 12, 959-981, 2015.
- 408 Martens-Habbena, W., Qin, W., Horak, R. E., Urakawa, H., Schauer, A. J., Moffett, J. W., Armbrust, E. V., Ingalls, A.  
409 E., Devol, A. H., Stahl, D. A.: The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition  
410 of archaeal ammonia oxidation by a nitric oxide scavenger, *Environ. Microbiol.* 17(7), 2261-74, 2015. doi:  
411 10.1111/1462-2920.12677.
- 412 Mazzeo, N. A., Venegas, L. E., and Choren, H.: Analysis of NO, NO<sub>2</sub>, O<sub>3</sub> and NO<sub>x</sub> concentrations measured at a  
413 green area of Buenos Aires City during wintertime. *Atmos. Environ.*, 39(17), 3055-3068, 2005.

- 414 Miles, A. M., Chen, Y., Owens, M. W., and Grisham, M. B.: Fluorometric determination of nitric oxide. In *Methods: A Companion to Methods in Enzymology*, 7, 40-47, 1995.
- 415
- 416 Misko, T. P., Schilling, R. J., Salvemini, D., Moore, W. M., and Currie, M. G.: A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.*, 214(1), 11-16, 1993.
- 417
- 418 Olasehinde, E. F., Takeda, K., and Sakugawa, H.: Development of an analytical method for nitric oxide radical determination in natural waters, *Anal. Chem.*, 81(16), 6843-6850, 2009.
- 419
- 420 Schreiber, F., Polerecky, L., and de Beer, D.: Nitric oxide microsensor for high spatial resolution measurements in biofilms and sediments, *Anal. Chem.*, 80, 1152–1158, 2008. doi: 10.1021/ac071563x.
- 421
- 422 Schreiber, F., Wunderlich, P., Udert, K.M. and Wells, G.F.: Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies, *Front. Microbiol.*, 2012. doi: 10.3389/fmicb.2012.0372.
- 423
- 424
- 425 Thamdrup, B.: New pathways and processes in the global nitrogen cycle, *Annual Review of Ecology Evolution and Systematics*, 43, 407-428, 2012.
- 426
- 427 Voss, M., Bange, H. W., Joachim, W. D., Middelburg, J. J., Montoya, J. P., Ward, B.: The marine nitrogen cycle: recent discoveries, uncertainties and the potential relevance of climate change, *Phil. Trans. R. Soc. B*, 368 (1621), 2013. doi: 10.1098/rstb.2013.0121.
- 428
- 429
- 430 Ward, B. B., and Zafiriou, O. C.: Nitrification and nitric oxide in the oxygen minimum of the eastern tropical North Pacific, *Deep-Sea Res.*, 35(7), 1127-1142, 1988.
- 431
- 432 Williams, E. J., Hutchinson, G. L., and Fehsenfeld, F. C.: NO<sub>x</sub> and N<sub>2</sub>O emissions from soil, *Global Biogeochem. Cy.*, 6, 351-388, 1992.
- 433
- 434 Xing, L., Zhang, Z. B., Liu, C. Y., Wu, Z. Z., and Lin, C.: Amperometric detection of nitric oxide with microsensor in the medium of seawater and its applications, *Sensors*, 5(12), 537-545, 2005.
- 435

- 436 Yang, G. P., Ren, C. Y., Lu, X. L., Liu, C. Y., and Ding, H. B.: Distribution, flux and photoproduction of carbon  
437 monoxide in the East China Sea and the Yellow Sea in spring, *J. Geophys. Res.* 116, C02001, 2011,  
438 doi:10.1029/2010JC006300
- 439 Zafiriou, O. C., and McFarland, M.: Determination of trace levels of nitric oxide in aqueous solution, *Anal. Chem.*,  
440 52(11), 1662-1667, 1980.
- 441 Zafiriou, O. C. and Dister, B.: Photochemical free radical production rates: Gulf of Maine and Woods Hole-Miami  
442 transect, *J. Geophys. Res.* 96(C3), 4939–4945, 1991.
- 443 Zafiriou, O. C., and McFarland, M.: Nitric oxide from nitrite photolysis in the central equatorial Pacific, *J Geophys.*  
444 *Res.*, 86(C4), 3173-3182, 1981.
- 445 Zafiriou, O. C., Blough, N. V., Dister , B., Kieber, D., and Moffett, J.: Molecular probe systems for reactive transients  
446 in natural waters, *Mar. Chem.*, 30, 45–71,1990.
- 447 Zafiriou, O. C., McFarland, M., and Bromund, R. H.: Nitric oxide in seawater, *Science*, 207, 637-639, 1980.
- 448 Zehr, J. P., and Ward, B. B.: Nitrogen cycling in the ocean: New perspectives on processes and paradigms, *Appl.*  
449 *Environ. Microb.*, 68, 1015-1024, 2002.
- 450 Zhang, Z. B., Lin, C., Liu, C. Y., Xing, L., Wu, Z. Z., and Sun, F.: Study on patterns and chemical features of NO effect  
451 on marine phytoplankton growth, *Sci. China B: Chem.*, 48(3), 376-384, 2005.
- 452 Zhang, Z. B., Liu, C. Y., Wu, Z. Z., Xing, L., and Li, P. F.: Detection of nitric oxide in culture media and studies on  
453 nitric oxide formation by marine microalgae, *Med. Sci. Monit.*, 12, 75-85, 2006.
- 454 Zhang, Z. B., Xing, L., Wu, Z. Z., Liu, C. Y., Lin, C., and Liu, L.S. Discovery of nitric oxide in marine ecological  
455 system and the chemical characteristics of nitric oxide, *Sci. China B: Chem.*, 49(5), 475-480, 2006.

456

Figure Captions

457

458

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

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

461 Fig. 3 Relationship between nitric oxide concentrations and fluorescence intensities

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

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

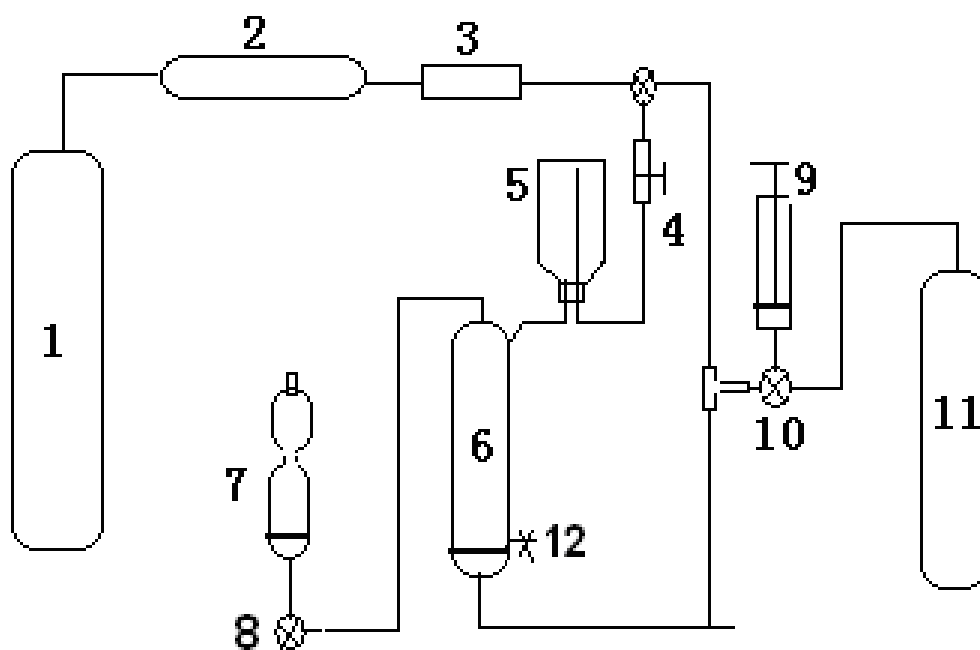
465 Fig. 6 The diurnal variations of NO concentrations and related parameters in the surface seawater at  
466 station 10

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

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

470

471

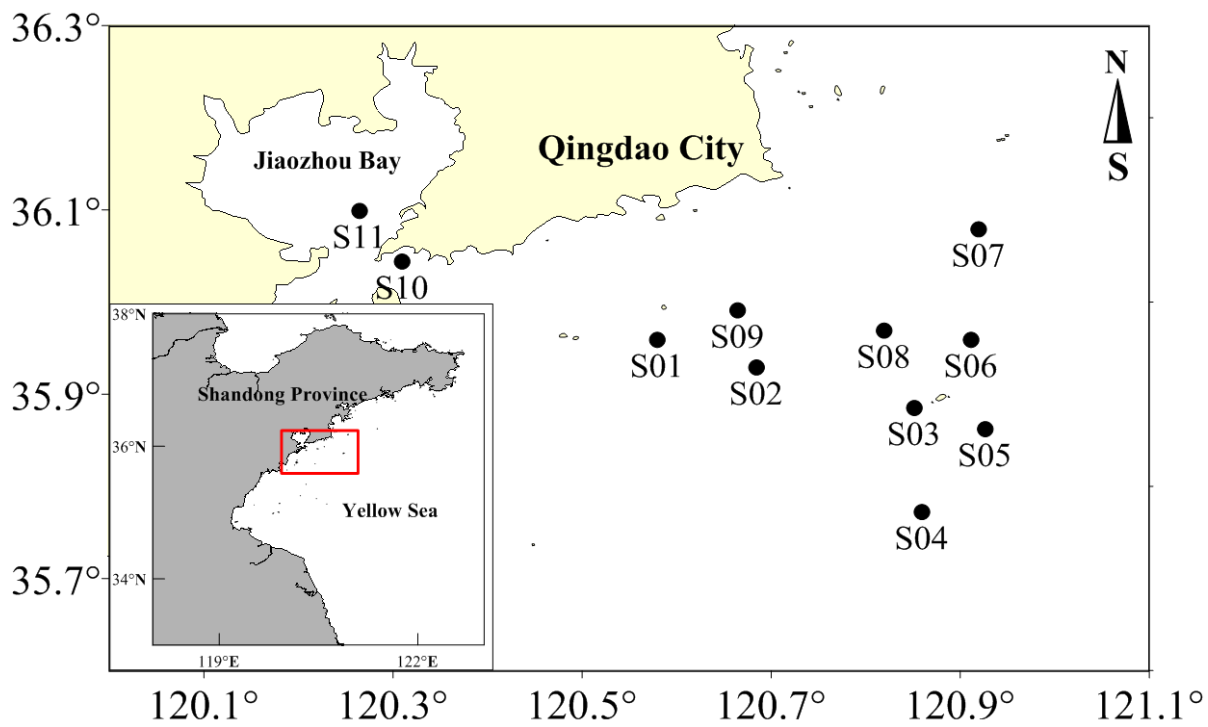


472

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

474 (1. N<sub>2</sub> gas; 2. Deoxygenation tube; 3. Glass rotameter; 4. 2-port valve; 5. Sample vial; 6. Degassing  
475 column; 7. Reaction chamber; 8 and 10. 3-port valves; 9. Gas-tight syringe; 11. NO standard gas; 12.  
476 Drain)

477

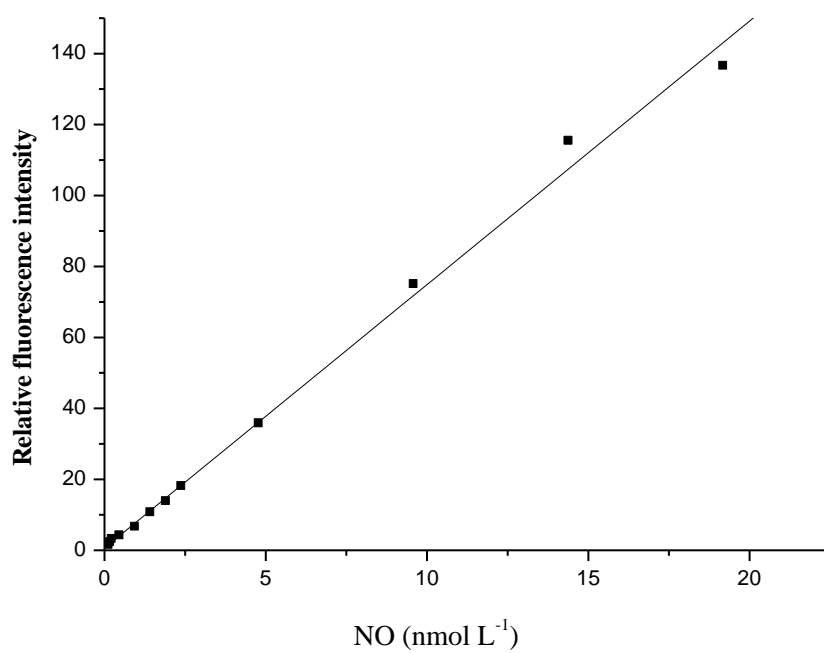


478

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

480

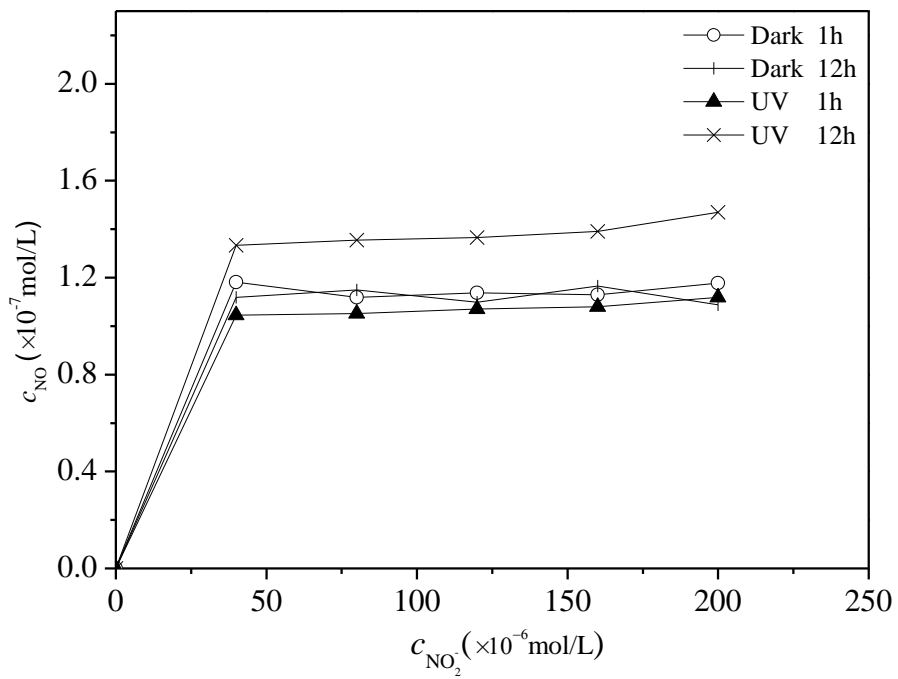




481

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

483

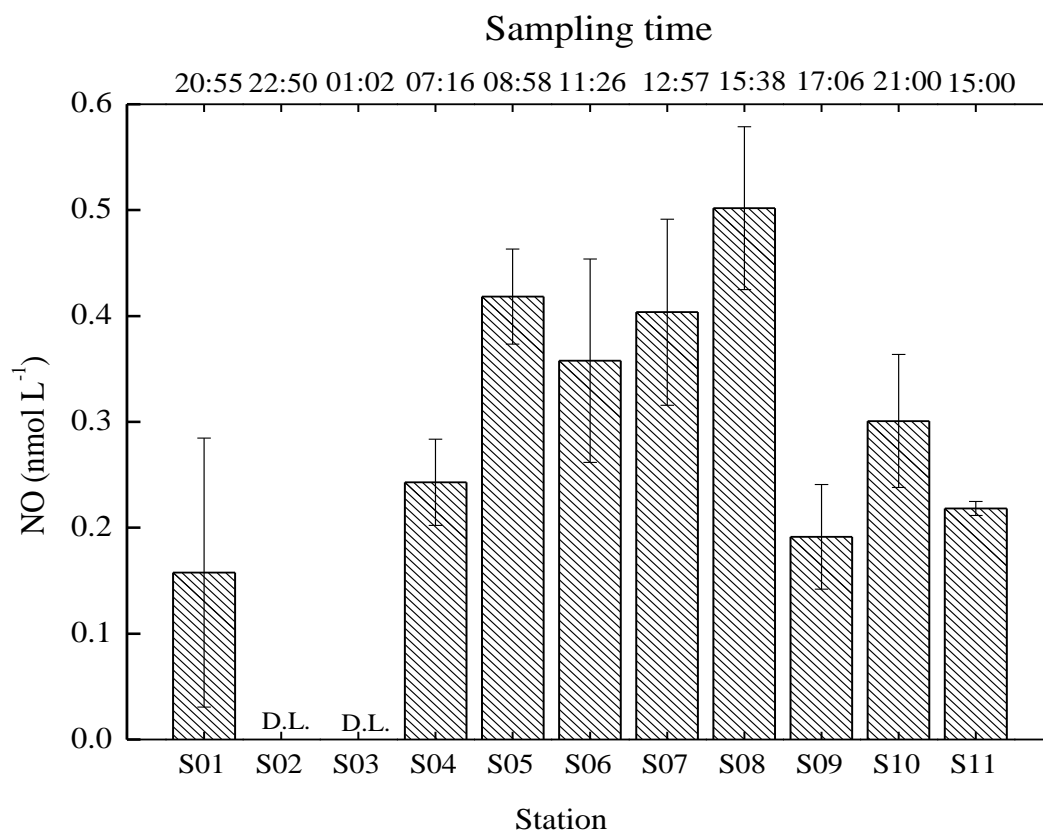


484

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

486 in the dark or under UV-B radiation

487



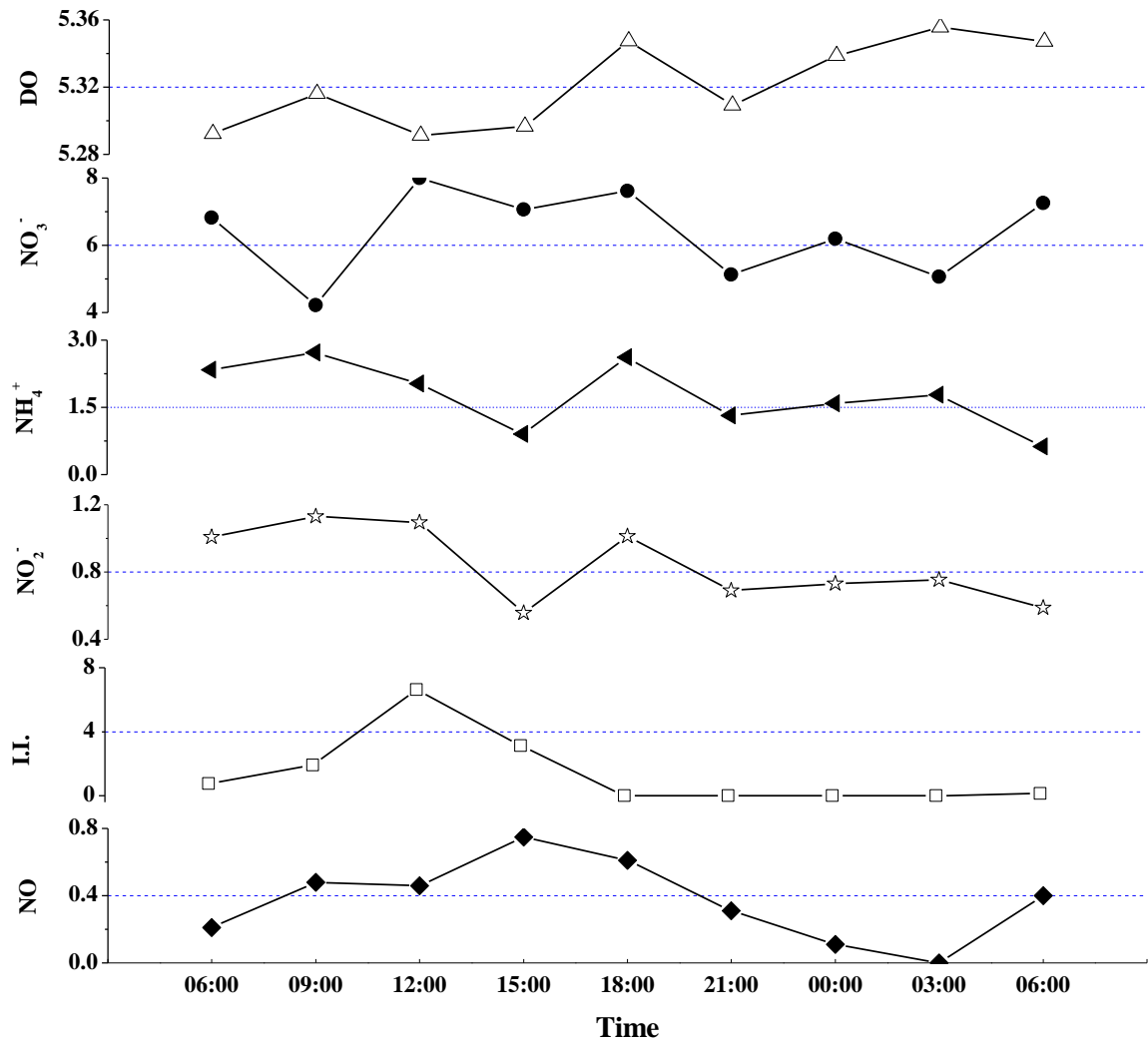
488

489

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

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

493

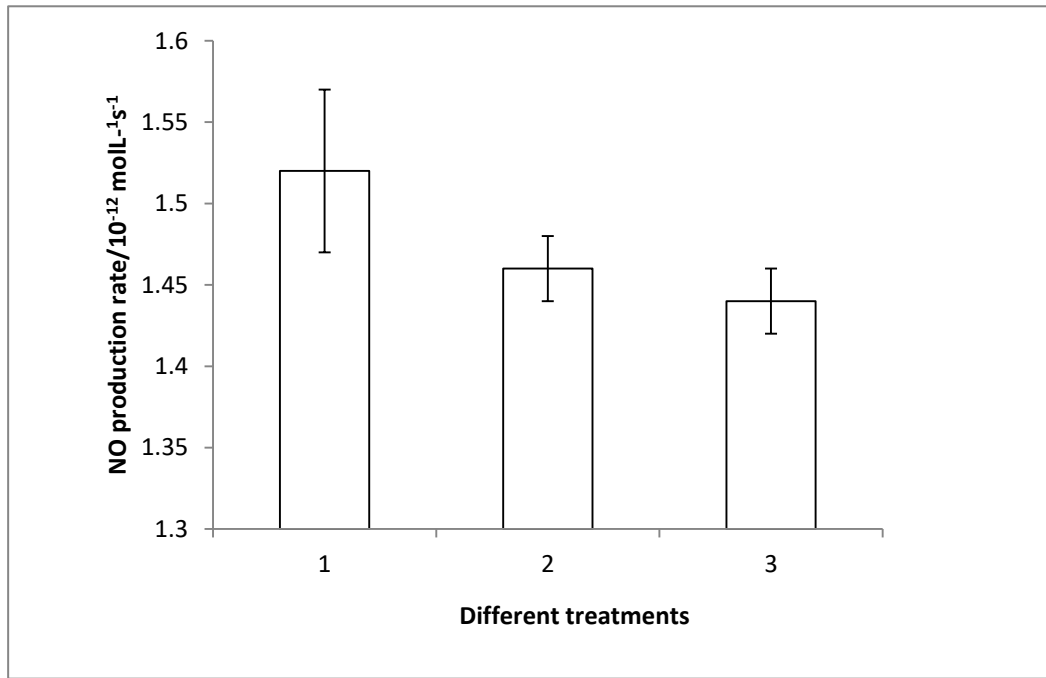


494

495

496 **Fig. 6** The diurnal variations of NO concentrations and related parameters in the surface seawater at  
 497 station 10 (Units: DO (mL L<sup>-1</sup>), NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> (μmol L<sup>-1</sup>), I.I.-illumination intensity (×10<sup>4</sup> lux), NO  
 498 (nmol L<sup>-1</sup>))

499



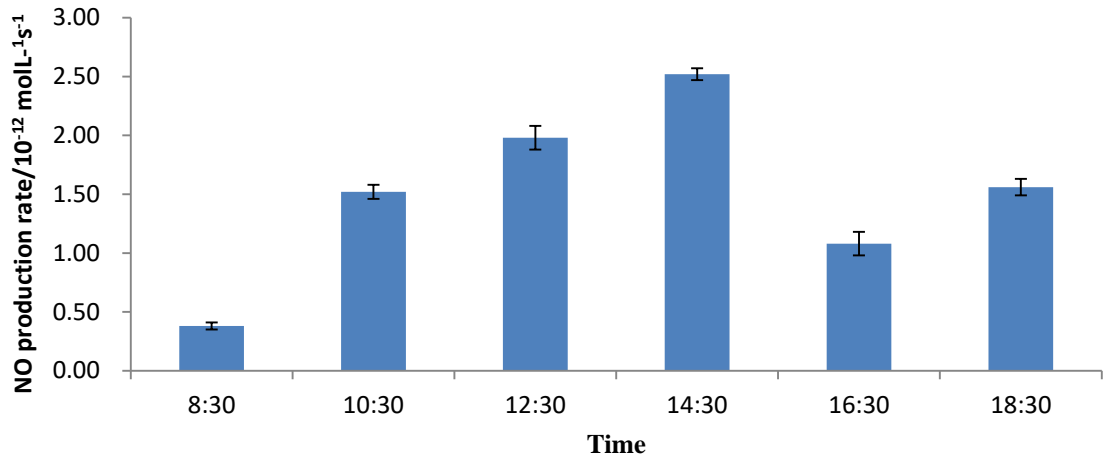
500

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

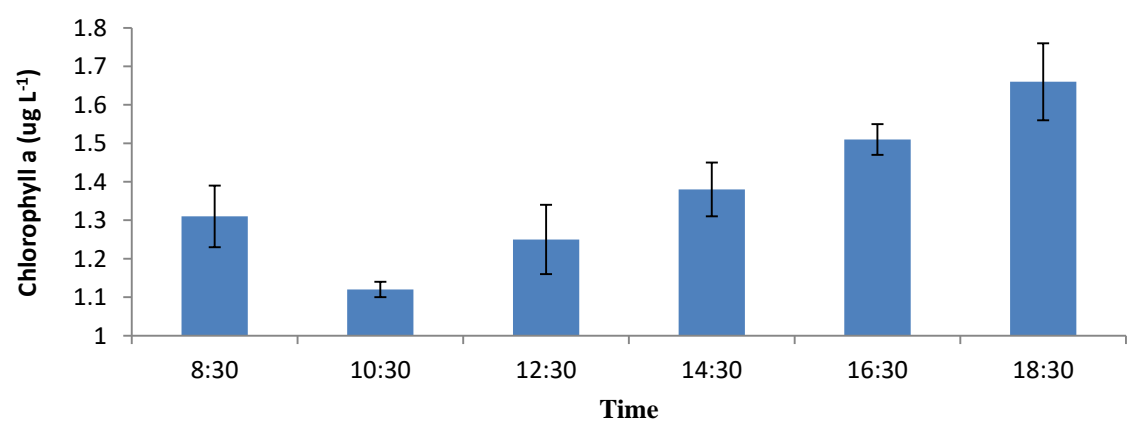
502 (1. Incubated on deck at ambient temperature, 2. 0.45  $\mu\text{m}$  Millipore filtered at ambient temperature, 3.

503 Incubated in water bath supplied with surface seawater)

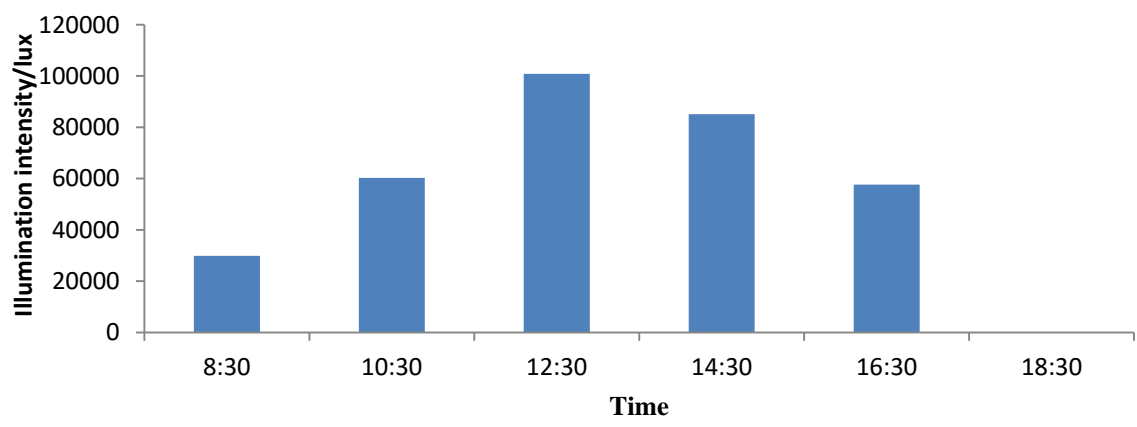
504



505



506



507

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

510

**511** Table 1 The methods for NO detection in seawater

Method	Linearity range (nmol L <sup>-1</sup> )	Detection limit (nmol L <sup>-1</sup> )	Analytical precision	Reference
Microelectrode	140–9900	140	0.24%	Zhang et al. (2003)
Microelectrode	1.4–1400	4.2×10 <sup>-10</sup>	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

**512**

**513**

514

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

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

515

516

517