Hypoxia-Enhanced N$_2$O Production Under Ocean Acidification in the Bohai Sea

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Nitrous oxide (N$_2$O) is a powerful greenhouse gas that degrades ozone. Hypoxia and ocean acidification are becoming more intense as a result of climate change. The former stimulates N$_2$O emissions, whereas the effects of the latter on N$_2$O production vary by the ocean. Hypoxia and ocean acidification may play a critical role in the evolution of future oceanic N$_2$O production. However, the interactive effects of hypoxia and ocean acidification on N$_2$O production remain unclear. We conducted a research cruise in the Bohai Sea of China to assess the occurrence of ocean acidification in the seasonal oxygen minimum zone of the sea and further conducted laboratory incubation experiments to determine the effects of ocean acidification and hypoxia on N$_2$O production. When pH decreased by 0.25, N$_2$O production decreased by 50.77 and 72.38%, respectively. In contrast, hypoxia had a positive impact; when dissolved oxygen (DO) decreased to 3.7 and 2.4 mg L$^{-1}$, N$_2$O production increased by 49.72 and 278.68%, respectively. The incubation experiments demonstrated that the coupling of ocean acidification and hypoxia significantly increased N$_2$O production, but, individually, there was an antagonistic relationship between the two. Structural equation modeling showed that the total effects of hypoxia treatment on N$_2$O production changes weakened the effects of ocean acidification, with overall positive effects. Generally speaking, our results suggest that N$_2$O production from the coastal waters of the Bohai Sea may increase under future climate change scenarios due to increasingly serious ocean acidification and hypoxia working in combination.

Keywords: ocean acidification, hypoxia, N$_2$O emission, Bohai Sea, oxygen minimum zone

INTRODUCTION

Nitrous oxide (N$_2$O) is an important greenhouse gas with a warming effect 265 times that of carbon dioxide (CO$_2$) (Stocker et al., 2013) and with the ability to destroy atmospheric ozone through photochemical reactions, resulting in significant impacts on global climate change (Crutzen and Ehhalt, 1977; Freing et al., 2012). N$_2$O emissions from oceans are the second-largest natural source of atmospheric N$_2$O and account for $\sim$30% of total natural emissions (Bange, 2006), particularly N$_2$O emissions from the upper ocean (Nevison et al., 1995), making oceans important contributors...
to global climate regulation (Qin, 2014). However, \( \text{N}_2\text{O} \) emissions are not uniformly distributed in the upper ocean due to complicated marine environments. Moreover, the effects of global climate change, including ocean acidification, temperature rise, and oxygen depletion, are likely to disrupt the balance (Codispoti, 2010).

Marine ecosystems play an important role in regulating global climate change through their strong regulatory capacity (Naqvi et al., 1998). Offshore low-oxygen areas are often caused by an increase in the flux of land-based nutrients into the sea and the eutrophication of estuaries and inshore waters. Seasonal stratification of seawater further hinders the transfer of oxygen from the upper layer of seawater to the bottom, producing seasonal variability in offshore hypoxic zones (Zhai et al., 2012; Zhang et al., 2016). Since hypoxic environments can stimulate the production and release of \( \text{N}_2\text{O} \), hypoxic zones are hot spots for global marine \( \text{N}_2\text{O} \) emissions, affecting local and global climate. These areas have become an important subject of research into \( \text{N}_2\text{O} \) emission fluxes and mechanisms in minimum-oxygen zones within open oceans (Naqvi et al., 2000; Kalvelage et al., 2013; Arévalo-Martínez et al., 2015; Babbin et al., 2015; Ji et al., 2015; Kock et al., 2016; Trimmer et al., 2016).

The increase in global temperature due to the enhanced greenhouse effect has been balanced by the exchange of \( \sim \)25% of anthropogenic \( \text{CO}_2 \) into the oceans (Le Quéré et al., 2014). Ocean acidification is the result of the ocean hosting excess \( \text{CO}_2 \), leading to changes in the carbonate system of upper ocean water (Orr et al., 2005). Over the past 20 years, this has resulted in a decrease in seawater pH of \( \sim \)0.0011–0.0024 units per year, with the average marine pH being \( \sim \)0.1 unit lower than before the Industrial Revolution (Stockert et al., 2013). Current research on biogeochemical cycling processes has shown that increasing \( p\text{CO}_2 \) and subsequent pH decline in the oceans are expected to affect microbial nutrient cycling directly and indirectly, for example, by increasing Trichodesmium-fixed \( \text{N}_2 \) and \( \text{CO}_2 \) (Hutchins et al., 2007) and changing ammonia-oxidizing archaea (AOA) abundance that indirectly alters \( \text{N}_2\text{O} \) emissions (Rees et al., 2016). Beman et al. (2011) studied changes in the nitrification rate under ocean acidification, showing that the nitrification rate decreased significantly while the pH dropped to the predicted value (\( \Delta \text{pH} = 0.2 \)) of the future ocean. The \( \text{N}_2\text{O} \) produced by nitrification could be reduced by 0.06–0.83 Tg N year\(^{-1} \) in the next 20–30 years under future ocean acidification. However, predictions of changing \( \text{N}_2\text{O} \) production in ocean acidification scenarios should also consider the effects of ocean acidification on denitrification. Although increasing \( \text{CO}_2 \) could have a negative effect on denitrification (Wan et al., 2016), it remains unclear how ocean acidification affects \( \text{N}_2\text{O} \) production.

Most known coastal anoxic areas appear in semi-enclosed areas prone to water stratification (Naqvi et al., 2010). The Bohai Sea of China, a shallow semi-enclosed inland sea, is typical of such eutrophic water bodies (Li et al., 2015). Rapid industrial and agricultural development in the region has resulted in high nutrient input into the Bohai Sea, such that the nitrogen and phosphate content exceeds Chinese standards and the eutrophic area has continuously increased (Liu and Yin, 2010). Zhai et al. (2012) and Zhang et al. (2016) found low DO and pH over a wide range of the bottom of Bohai Sea, including a total area with DO < 3.0 mg L\(^{-1} \) of \( \sim \)4.2 \( \times \) 10\(^3 \) km\(^2\). These spatial characteristics are consistent with a double-center cold-water structure (Lin et al., 2006; Zhou et al., 2009).

In this study, we designed a laboratory incubation experiment, investigating the effects of ocean acidification and hypoxia on seawater \( \text{N}_2\text{O} \) production in Bohai Bay, and then conducted aggregated boosted tree (ABT) and structural equation model (SEM) analyses to examine potential factors affecting \( \text{N}_2\text{O} \) production. This study was designed to verify two hypotheses: (1) Ocean acidification alone (Acid) reduces seawater \( \text{N}_2\text{O} \) production, while both hypoxia alone (Hyp) and ocean acidification with hypoxia (Acid + Hyp) conditions increase seawater \( \text{N}_2\text{O} \) production, and (2) hypoxia and ocean acidification change \( \text{N}_2\text{O} \) production by altering seawater properties rather than directly affecting \( \text{N}_2\text{O} \) production. A better understanding of changes in \( \text{N}_2\text{O} \) production and its mechanisms in the Bohai Sea hypoxic zone under future ocean acidification can provide data for improved prediction models.

**MATERIALS AND METHODS**

**Sample Collection and Experimental Manipulation**

A marine survey was conducted in August 2017 (Figure 1). Seawater depth at sampling stations ranged from 6 to 27 m, with the deepest at station A5 (Figure 1). Seawater samples for \( \text{N}_2\text{O} \), pH, and DO were collected, using 5-L Niskin bottles, and vertical profiles of DO and pH were measured simultaneously with the Maestro multiparameter sensor (RBR Maestro 3). Temperature and salinity sensors were calibrated prior to the survey, and DO and pH sensor data were corrected by measured data before use. Samples for the incubation experiment were calibrated on December 21, 2017, at 117°48′11″E, 38°58′41″N (station A: relatively low high-quality exogenous substance input) and 117°43′23″E, 39°55′3″N (station B: relatively high quality of exogenous substance input because of human activity, especially nitrogen). The 5-L polycarbonate culture bottles filled with seawater were airtight and were kept in the dark for 2 h when transporting back to the laboratory. Experiments were performed with the experimental platform already set up and four target \( p\text{CO}_2 \) and oxygen levels, with triplicate samples per treatment (Figure 1, Table 1). All treatments were manipulated by gentle bubbling via plastic diffusers at uniform rates with commercially prepared air (\( \text{CO}_2\text{-O}_2 \) mixture) using acid-washed tubing. The bottle caps were customized for gas inlet/outlet and connected to the air mixture. The experiments were equilibrated for 6 h, distributed in 5-L polycarbonate bottles, sealed for 64 h, and maintained in a laboratory incubator at \( \text{in situ} \) temperature (15°C) in the dark (Table 1). Seawater \( \text{CO}_2 \) parameters were verified by measuring pH and dissolved inorganic carbon (DIC).

**\( \text{N}_2\text{O} \) Analysis**

Triplicate samples were collected using acid-cleaned Tygon tubing by siphoning from 5-L incubation bottles into
Acid-washed 60-ml glass vials. Samples were allowed to overflow for two times the volume of the bottle to eliminate air bubbles. Samples were treated with 0.1 ml of saturated mercuric chloride and sealed with butylene rubber stoppers and an aluminum crimp seal. The rubber plug of the sample bottle was first penetrated by an injection syringe; then, 5.0-ml-high purity N₂ (> 99.999%) was injected into the bottle with an airtight syringe. At the same time, 5.0 ml of the sample was discharged from the bottle through the syringe to form headspace in the bottle. The bottle was then shaken for 30 min and balanced for 2 h at room temperature. A subsample of the equilibrated headspace was manually injected into a gas chromatograph (GC) with electron capture detection (SHIMADZU GC-2010 Plus), equipped with an HP-Plot/column (J and WGC Columns, Agilent Technologies, USA). The GC was calibrated daily with three different concentrations of standard gases (291, 617, and 4,980 ppbv N₂O/N₂, Research Institute of China National Standard Materials). The N₂O concentrations in the samples were calculated using the solubility function of Weiss and Price (1980). The detection limit for N₂O analysis was 1.0 nmol L⁻¹, and the precision was ~2%.

Ancillary Measurements

Dissolved oxygen samples were transferred, stored, and analyzed using the Winkler method (Grasshoff et al., 1999). The relative standard deviation of the three samples was 2%. The pH of the collected samples was measured in situ. A Shimadzu TOC-L analyzer was used to repeat the sample three times in the laboratory, and the DIC concentrations of all repeat subsamples were measured. Water samples for nutrient analysis were filtered through 0.45-μm acetate cellulose membrane filters. The filtrates were poisoned with HgCl₂ and stored in the dark at 4°C. Nutrients, including ammonium (NH₄⁺), nitrate (NO₃⁻), and nitrite (NO₂⁻), were determined using a Technicon AA3 Auto-Analyzer (Bran+Luebbe) according to classical colorimetric methods (Wu et al., 2019). NH₄⁺, NO₃⁻, and NO₂⁻ were measured using the indophenol blue method, the
copper–cadmium column reduction method, and the phosphor-molybdate complex method, respectively.

**Statistical Analysis**

Repeated two-way multivariate ANOVA (two-way MANOVA) with IBM SPSS Statistics software (version 23.0) was used to examine the effects of treatments and their interactions on N$_2$O production during incubation. Pearson correlation coefficients were used to evaluate the relationships between N$_2$O production and relevant physical and chemical indicators. This analysis was conducted using the "corrplot" package (Simko, 2017) for R statistical software (version 4.0.3, R Core Team, 2020). ABT modeling was used to explain the relative influence of related parameters on N$_2$O production using the "gbmplus" package in R (De'ath, 2007). SEM was used to assess the relative direct and indirect impacts of ocean acidification and hypoxia on N$_2$O production (Alsterberg et al., 2013; Shi et al., 2016; Zhang et al., 2019; Wang et al., 2020). The SEM fit was evaluated using the chi-square test ($\chi^2$), comparative fit index (CFI), root mean square error of approximation (RMSESEM), and other parameters.

**RESULTS**

**Vertical and Horizontal Profiles of Seawater Chemical Properties**

The sea surface temperature (SST) was horizontally stable at ~28°C across the entire sampling area. Vertical temperature profiles showed an obvious cold water mass in the bottom layer of A3, A4, and A5, implying an obvious thermocline in the middle layer. Vertical density profiles closely mirrored those of temperature, implying that the latter was the dominant factor controlling the former. In contrast, the halocline was much weaker, and the bottom-surface difference in salinity was mostly <1 across the vertical profile. Chlorophyll a showed little vertical variation but increased toward the land, with a maximum concentration of 17.5 µg L$^{-1}$ at A1.

Vertical profiles of DO and pH showed similar stratification patterns, with the surface and bottom layers being uniform or having weak gradients, but the middle layer having rapid changes (Figure 2). DO and pH were higher in the surface layer than in the bottom layer. As for oxygen, A3, A4, and A5 were located in the oxygen minimum zone (O$_2$ < 3.0 mg L$^{-1}$), with the bottom of A3 having the lowest oxygen concentration and highest N$_2$O concentration (37.5 nmol L$^{-1}$). High N$_2$O production was accompanied by ocean acidification and hypoxia. The maximum N$_2$O patterns were consistent with rapid changes in DO and pH.

**Seawater Chemical Properties After Incubation**

Seawater that received the Hyp and Acid treatments differed in pH, DIC, DO, particulate organic carbon and nitrogen [particulate organic carbon (POC) and particulate organic nitrogen (PON)], and various inorganic nitrogen forms (NH$_4^+$, NO$_2^-$, and NO$_3^-$) after 64 h of incubation (Tables 2A,B). For the four treatment groups, the concentrations of seawater NH$_4^+$-N and NO$_3^-$-N at Dongjiang port were four to five times higher than that at the Hongxing wharf. In addition, Hyp produced the highest NH$_4^+$-N and NO$_3^-$-N. For NH$_4^+$-N, all treatment groups were higher than the ambient control (Con), but, for NO$_3^-$-N, all treatment groups performed the same except for Acid (Figure 3). Compared with the Con, DO in Hyp at the Dongjiang port and the Hongxing wharf reached 3.7 and 2.4 mg L$^{-1}$, respectively, while pH for that group at the Dongjiang port and the Hongxing wharf reached 7.69 and 7.71, respectively.

At the Dongjiang port, Hyp had a significant effect on DO ($p < 0.001$) and NH$_4^+$-N ($p < 0.01$) concentrations, while Acid had a significant effect on pH, POC, and PON ($p < 0.05$). At the Hongxing wharf, Hyp had a significant effect on DO ($p < 0.001$) and NO$_3^-$-N ($p < 0.05$) concentrations, while Acid had a significant effect on pH and DIC concentrations ($p < 0.001$). Significantly, interactive effects between Acid and Hyp on pH, POC, and PON ($p < 0.05$) were identified by a two-way MANOVA.

**N$_2$O Concentrations in Incubated Seawater Samples**

After incubation, N$_2$O production showed strong variability between the different treatments (Figure 4). First, both Hyp and Acid treatment had significant effects on N$_2$O potential production from different anthropogenic nitrogen input regions. Hyp had a positive effect on N$_2$O potential production compared with Acid; the higher the anthropogenic nitrogen input, the greater was the effect. Relative to Con, Acid resulted in mean N$_2$O production, dropping from 2.72 to 1.34 nmol L$^{-1}$ at the Dongjiang port and from 23.09 to 6.38 nmol L$^{-1}$ at the Hongxing wharf, while the observed N$_2$O concentrations decreased by 50.77 and 72.38%, respectively. Also, relative to Con, Hyp resulted in mean N$_2$O production, increasing from 2.72 to 4.08 nmol L$^{-1}$ at the Dongjiang port and from 23.09 to 110.78 nmol L$^{-1}$ at the Hongxing wharf, while the observed N$_2$O concentrations increased by 49.72 and 278.68%, respectively (Figure 4, Table 3). Similar to Hyp, Hyp+Acid significantly increased N$_2$O potential production (compared with Con) from 2.72 to 3.17 nmol L$^{-1}$ at the Dongjiang port and from 23.09 to 58.68 nmol L$^{-1}$ at the Hongxing wharf, while the observed N$_2$O concentrations increased by 16.23 and 96.38%, respectively (Figure 4A). As expected, regions with high anthropogenic nitrogen inputs responded more to different treatments, mainly because these inputs increased substrate concentrations for nitrification and denitrification, and nitrogen utilization increased (Figure 4).

**Relationships Between N$_2$O Concentration and Relative Parameters**

Pearson’s correlation analysis showed significant variability between different anthropogenic nitrogen input seawater responses to different treatments (Figure 5). There was a significant positive correlation between N$_2$O concentration and NH$_4^+$-N at the Dongjiang port ($p < 0.001$), indicating that the N$_2$O in this region was mainly produced by nitrification, which was consistent with results from the open ocean (Beman et al., 2011). Seawater pH and DO were positively and negatively correlated with N$_2$O concentration, respectively, consistent
with our first hypothesis. However, at the Hongxing wharf, the results were different; there was a significant positive correlation between N$_2$O concentration and NO$_3$-N ($p < 0.05$), and DO was significantly correlated with N$_2$O concentration ($p < 0.001$) (Figure 5). DO, pH, and DIC accounted for 31.3, 25.1, and 18.2%, respectively, of the relative influencing factors for N$_2$O potential production at the Dongjiang port much larger than for the other parameters. Similarly, DO, pH, and DIC accounted for 47.9, 17.5, and 14.5%, respectively, of the relative influencing factors for N$_2$O potential production at the Hongxing wharf (Figure 6). This result indicated that changes in DO, pH, and DIC might be responsible for the increase in N$_2$O potential production. Overall, hypoxia and acidification are likely to change the future patterns of ocean N$_2$O potential production.

The SEM also showed strong variability between different anthropogenic nitrogen input seawater responses to different treatments (Figure 7). The total effect of ocean acidification and hypoxia on seawater N$_2$O potential production at the Hongxing wharf was greater than at the Dongjiang port, which may be related to the NH$_4^+$-N wastewater discharged by humans. These results showed that the overall effect of hypoxia on seawater N$_2$O potential production was positive, while ocean acidification was negative, in agreement with our incubation experiments. SEM also confirmed the regulatory effect of inorganic nutrients on the effects of hypoxia and ocean acidification on seawater N$_2$O potential production.

**DISCUSSION**

**Potential Factors for Reducing N$_2$O Concentration From Ocean Acidification**

Our results showed that CO$_2$-driven ocean acidification reduced N$_2$O potential production in seawater. In the polar Atlantic Ocean, N$_2$O production was sensitive to pH; when pH decreased by 0.06–0.4, N$_2$O production decreased by 2.4–44% (Rees et al., 2016). The generation of seawater N$_2$O under normal oxygen conditions has always been considered a product of nitrification (Beman et al., 2011). Previous studies suggested that ocean acidification would lead to a decrease in the ammonia oxidation rate, and reducing seawater pH also suppressed seawater N$_2$O emissions (Beman et al., 2011). Sample collection for the incubation experiments was poured from the closed water sampler and immediately kept in the dark, and we assumed that the sampling process and phytoplankton had little influence on

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**TABLE 2A** | Effects of ocean acidification and hypoxia on chemical properties of seawater samples at the Dongjiang port.

| Treatment | pH  | DIC  | DO  | POC  | PON  | TN  | NH$_4^+$ | NO$_2^-$ | NO$_3^-$ | TIN  |
|-----------|-----|------|-----|------|------|-----|----------|----------|----------|------|
| Con       | 7.91 ± 0.10 | 30.62 ± 0.26 | 8.7 ± 0.2 | 0.23 ± 0.04 | 0.04 ± 0.00 | 0.20 ± 0.04 | 2.44 ± 0.49 | 0.79 ± 0.21 | 10.71 ± 6.22 | 13.94 ± 6.72 |
| Hyp       | 7.94 ± 0.01 | 29.51 ± 0.35 | 3.7 ± 0.5 | 0.35 ± 0.10 | 0.07 ± 0.02 | 0.20 ± 0.02 | 3.89 ± 0.46 | 0.78 ± 0.13 | 13.15 ± 3.72 | 17.58 ± 3.55 |
| Acid      | 7.69 ± 0.03 | 31.35 ± 0.73 | 8.9 ± 0.1 | 0.23 ± 0.02 | 0.04 ± 0.00 | 0.20 ± 0.04 | 2.05 ± 0.88 | 0.88 ± 0.14 | 10.04 ± 1.65 | 12.96 ± 0.82 |
| Acid+Hyp  | 7.76 ± 0.05 | 30.87 ± 1.57 | 3.7 ± 0.1 | 0.18 ± 0.00 | 0.03 ± 0.00 | 0.17 ± 0.03 | 2.99 ± 1.37 | 0.76 ± 0.14 | 11.78 ± 0.28 | 15.53 ± 1.28 |

DIC, dissolved inorganic carbon; DO, dissolved oxygen; POC, particulate organic carbon; PON, particulate organic nitrogen; TN, total nitrogen; TIN, total inorganic nitrogen; Con, control treatment; Hyp, hypoxia treatment; Acid, ocean acidification treatment; and Acid+Hyp, the combination of hypoxia and ocean acidification treatment. *; **; and *** indicate significance levels at $p < 0.05, p < 0.01$, and $p < 0.001$, respectively.
TABLE 2B | Effects of ocean acidification and hypoxia on water chemical properties at the Hongxing wharf.

| Treatment | pH     | DIC (mg L⁻¹) | DO (mg L⁻¹) | POC (mg L⁻¹) | PON (mg L⁻¹) | TN (µmol L⁻¹) | NH₄⁺ (µmol L⁻¹) | NO₂⁻ (µmol L⁻¹) | NO₃⁻ (µmol L⁻¹) | TIN (µmol L⁻¹) |
|-----------|--------|--------------|-------------|--------------|--------------|---------------|----------------|----------------|----------------|---------------|
| Con       | 7.90 ± 0.01 | 35.82 ± 0.26 | 6.7 ± 0.3   | 0.23 ± 0.05  | 0.05 ± 0.01  | 0.32 ± 0.16   | 14.20 ± 0.49   | 1.62 ± 0.15   | 42.67 ± 8.24   | 58.49 ± 8.83   |
| Hyp       | 7.95 ± 0.04 | 35.21 ± 0.65 | 2.4 ± 0.2   | 0.40 ± 0.03  | 0.07 ± 0.01  | 0.39 ± 0.03   | 15.90 ± 2.13   | 1.53 ± 0.40   | 54.96 ± 9.74   | 72.38 ± 12.24  |
| Acid      | 7.71 ± 0.02 | 37.74 ± 0.70 | 6.9 ± 0.2   | 0.37 ± 0.09  | 0.06 ± 0.02  | 0.38 ± 0.05   | 14.90 ± 2.46   | 1.83 ± 0.32   | 44.41 ± 3.05   | 60.35 ± 5.80   |
| Acid+Hyp  | 7.69 ± 0.07 | 36.69 ± 0.50 | 2.2 ± 0.4   | 0.43 ± 0.14  | 0.07 ± 0.02  | 0.38 ± 0.03   | 14.93 ± 0.30   | 1.57 ± 0.15   | 47.93 ± 3.95   | 64.43 ± 4.37   |

DIC, dissolved inorganic carbon; DO, dissolved oxygen; POC, particulate organic carbon; PON, particulate organic nitrogen; TN, total nitrogen; TIN, total inorganic nitrogen; Con, control treatment; Hyp, hypoxia treatment; Acid, ocean acidification treatment; and Acid+Hyp, the combination of hypoxia and ocean acidification treatment. *, and *** indicate significance levels at p < 0.05, and p < 0.001, respectively.

NH₃ or NH₄⁺. Since it has been shown that the composition of nitrifying bacterial communities can resist changes in ocean acidification, the observed N₂O concentration changes seem more likely to be related to changes in the NH₄⁺:NH₃ equilibrium (Hutchins et al., 2009; Beman et al., 2011). pH may play an indirect role in controlling N₂O potential production because it changes the chemical speciation of NH₄⁺: NH₃ covaries with other factors. NH₃ acts as a substrate for nitrification (Ward, 2008). According to the equilibrium equation of Bell et al. (2008), a decrease in seawater pH from 8.1 to 7.8 will lead to a 50% decrease in NH₃ concentration under constant temperature (22°C) and salinity (Wyatt et al., 2010). Using that equation, we estimated the NH₃ concentrations based on the calculated pKa values, pH, and NH₄⁺ concentrations measured in situ. A pKa fixed value of 9.25 suggested that NH₃ typically accounted for 63.3% of the total NHx. Beman et al. (2011) found significant correlations between the percentage changes in NH₃, pH, and ammonium oxidation rates (Hutchins et al., 2009) and (Kitidis et al., 2011) between pH and ammonium oxidation. We showed a similar relationship between the absolute values of pH and N₂O and the correlation between N₂O and NH₃ in the incubation experiments (Figure 8) (R² = 0.63, p < 0.05) as a result of the induced shift in the NH₄⁺: NH₃ ratio. Our results indicated that changes in pH of 0.25 in the coastal waters of the Bohai Sea may lead to decreases in N₂O potential production of 50.77 and 72.38% due to a reduced NH₃ regime of 33.8 and 40.2%, respectively.

In contrast, Breider et al. (2019) examined the response of N₂O production from nitrification to acidification in a series of incubation experiments conducted in the subtropical and subarctic western North Pacific, showing that, when the pH decreased, the N₂O production rate significantly increased during the nitrification process, while the nitrification rate remained stable or decreased. We suggest two possible explanations for this difference. First, despite the beginning oxygen of Acid being comparable to the surrounding oxygen concentration, after 64h, the oxygen centralization in the incubation bottles might have slightly diminished, hindering nitrification, on the other hand, according to Ji et al. (2018), when O₂ > 6 µmol L⁻¹ was 98% and showed complete inhibition on N₂O production from NO₂⁻ and NO₃⁻ reduction. The oxygen consumed by our incubation process is limited...
and does not make the oxygen in the incubation bottles reach the threshold value of denitrification, ultimately leading to a reduction in N$_2$O potential production. Second, the methods of acidification treatment were different between the two studies. They used HCl solutions and aerated the system with different concentrations of combined CO$_2$ and O$_2$ to achieve acidification (Breider et al., 2019). We changed the system pH and altered the seawater carbonate system, affecting N$_2$O potential production. In addition, pH reduction had a greater effect on N$_2$O in the anthropogenic nitrogen input samples, which may have been related to the high NH$_4^+$-N in human wastewater discharge.

As ocean acidification intensifies, N$_2$O production decreases, indicating that future ocean acidification is likely to reduce ocean N$_2$O potential production, resulting in negative feedback relative to global warming (Beman et al., 2011). Beman et al. (2011) estimated that an observed reduction in nitrification rates of 3–44% would result in a reduction in global N$_2$O production of 0.06–0.83 Tg N/year over the next 20–30 years. Codispoti (2010) adopted a similar approach and assuming that 50% of the global ocean source of N$_2$O was 3.8 Tg Nyear$^{-1}$. For the less anthropogenic nitrogen input area (pH decreased by 0.25), the estimated reduction of marine N$_2$O sources was $\sim$0.96 year$^{-1}$. For the large anthropogenic nitrogen input area (pH decreased by 2.5), the estimated reduction of marine N$_2$O sources was $\sim$1.38 year$^{-1}$.

### Effect of Hypoxia on N$_2$O Potential Production

The potential production of N$_2$O increased significantly with decreasing DO concentration. Although the regulatory effect of oxygen on N$_2$O production in the ocean is widely accepted in principle (~100 papers cite Goreau et al. (1980) on N$_2$O production in the ocean), to the best of our knowledge, there have been few studies on the effect of oxygen on N$_2$O production in Bohai Bay. Due to eutrophication and environmental degradation, seasonal oxygen shortages in this location may expand in the future. Our results showed a non-linear relationship between N$_2$O concentration and O$_2$ concentration, with the former increasing as the latter decreases (Figure 9A). The basic linear relationship of $\Delta$N$_2$O/AOU is often used to simulate the distribution of N$_2$O in the ocean, in which the slope value of $\Delta$N$_2$O–AOU is regarded as the N$_2$O yield from nitrification, indicating the amount of N$_2$O produced per amount of O$_2$ consumed (Nevison et al., 2003). This theory varies widely (0.08–0.31 nmol N$_2$O per mmolO$_2$), making it difficult to capture the full dynamics of N$_2$O production in hypoxic water (Nevison et al., 2003). Trimmer et al. also proposed a mixed-effects model, which points out that, between 1 and 30 mmol L$^{-1}$ O$_2$ concentration, a decrease in O$_2$ concentration leads to a clear exponential increase in N$_2$O (Yoshida et al., 1984); this is comparable to our results.

Both high and low anthropogenic nitrogen input seawater under Hyp resulted in a decreased NO$_2^-$/NO$_3^-$ ratio after
incubation (Figure 9B). We speculate that nitrification and denitrification may occur together with a decrease in oxygen concentration (even when the oxygen concentration does not reach complete denitrification), and as NO$_2^-$ is the basis for both nitrification and denitrification (Wilson et al., 2014), the consumption of NO$_2^-$ is greater than production. This is similar to Ji et al. (2018), whose $^{15}$N tracer incubation experiments showed that denitrification was the dominant source of N$_2$O production in the anoxic and the peripheral suboxic waters, because rates of N$_2$O production from NO$_2^-$ and NO$_3^-$ reduction were 10- to 100-fold higher than N$_2$O production from NH$_4^+$ oxidation. Our results also support this view, indicating that oxygen concentration in the oxycline supports nitrification and denitrification, resulting in maximum N$_2$O concentration.

**Direct vs. Indirect Effects of Ocean Acidification and Hypoxia on Inorganic Nitrogen and N$_2$O Potential Production**

Our second hypothesis was that hypoxia and ocean acidification changed N$_2$O potential production by altering seawater properties rather than directly affecting N$_2$O production. DO, pH, and DIC were the key factors affecting N$_2$O potential production (Figure 6). SEM also showed strong variability among different anthropogenic nitrogen input seawater responses to different treatments. These results suggested a role for the indirect effects of ocean acidification and hypoxia on inorganic nitrogen and N$_2$O potential production in coastal marine systems. Ocean acidification may directly or indirectly affect N$_2$O potential production through a variety
of mechanisms, including AOA abundance (Rees et al., 2016) and nitrification rates (Beman et al., 2011). In the Dongjiang port, both hypoxia and ocean acidification had direct effects on seawater N$_2$O potential production (Hypoxia $\rightarrow$ N$_2$O, path coefficients $>0.10$, but $P < 0.05$; acidification $\rightarrow$ N$_2$O, path coefficients $>0.10$ and $P > 0.05$) but indirect effects via NH$_4^+$-N, NO$_2^-$-N, and NO$_3^-$-N (Figure 9A). Hypoxia increased NH$_4^+$-N and NO$_3^-$-N, which, in turn, increased N$_2$O potential production, while hypoxia also reduced NO$_2^-$-N and increased N$_2$O potential production. Thus, the total effect of hypoxia on seawater N$_2$O potential production was positive (0.67). Ocean acidification reduced NH$_4^+$-N and NO$_3^-$-N, which, in turn, reduced N$_2$O potential production, and ocean acidification also increased NO$_3^-$-N and reduced N$_2$O potential production. Thus, the total effects of ocean acidification on seawater N$_2$O potential production were negative (0.57). Hypoxia increased, whereas ocean acidification reduced, seawater N$_2$O potential production via direct effects (Figure 9A).

Interestingly, the direct and indirect effects of hypoxia treatment on changes in N$_2$O potential production counteracted the effects of ocean acidification. As hypoxia and ocean acidification increased N$_2$O potential production, the negative effects of ocean acidification were canceled, and the total effects were positive (0.10). The situation seems to have changed at the Hongxing wharf, where both hypoxia and ocean acidification had significant direct effects on seawater N$_2$O potential production (Hypoxia $\rightarrow$ N$_2$O, path coefficients $>0.10$ and $P < 0.05$; acidification $\rightarrow$ N$_2$O, path coefficients $>0.10$ and $P > 0.05$), with indirect effects via NO$_3^-$-N (Figure 9B). Hypoxia increased NH$_4^+$-N and NO$_3^-$-N, which, in turn, increased N$_2$O potential production. Thus, the total effect of hypoxia on seawater N$_2$O potential production was positive (0.86). Ocean acidification reduced NH$_3^+$-N and NO$_3^-$-N, which, in turn, reduced N$_2$O potential production. Thus, the total effects of ocean acidification on seawater N$_2$O potential production were negative (0.48). Ocean acidification reduced NH$_3^+$-N and NO$_3^-$-N, which, in turn, reduced N$_2$O potential production. Hypoxia increased, whereas ocean acidification reduced seawater N$_2$O potential production via direct effects (Figure 9B). Interestingly, the direct and indirect effects of hypoxia treatment on changes in N$_2$O potential production counteracted the effects of ocean acidification. As hypoxia and ocean acidification increased N$_2$O potential production, the negative effects of ocean acidification were canceled, and the total effects were positive (0.38).

The causes of hypoxia and acidification at the bottom of the Bohai Sea are very complex, including the coupling of physical, chemical, and biological processes, the most important of which is caused by the oxygen consumption of biological processes.
It is well-known that DO in seawater is significantly related to photosynthesis and the decomposition of organic matter in phytoplankton. The photosynthesis of phytoplankton within the euphotic layer results in high dissolved oxygen. In situ biological production, especially that driven by nitrification, is commonly considered to be the major mechanism of N$_2$O production in the ocean (Naqvi et al., 2010). The formation of a seawater thermocline limits the exchange of DO from the surface to the bottom, a key physical mechanism of the oxycline in the Bohai Sea. Seasonal seawater stratification prevents the bottom N$_2$O from being transported upward, such that nitrification and denitrification occur together, leading to the accumulation of N$_2$O. Generally, phytoplankton blooms occur in spring with increasing temperature and light intensity in the Bohai Sea. The concentration of chlorophyll a reaches its highest level in summer with coefficients sunlight and terrestrial nutrient input (Liu and Yin, 2010). Therefore, due to the propagation of phytoplankton in nutrient-rich layers from spring to summer, the deposition of organic matter and aerobic decomposition provides the basis for hypoxia occurrence at the bottom during summer. This limitation of oxygen supply further increases the pressure of bottom mineralization, exacerbating bottom hypoxia and leading to denitrification. Aerobic decomposition of organic matter increases the pCO$_2$ of the underlying seawater and increases acidification. Our experimental results showed that ocean acidification can attenuate N$_2$O potential production in the OMZ, which may also be an important reason for the lower concentration of N$_2$O at the bottom layer than in the middle layer.

Our experiments also showed that the coupling of ocean acidification and hypoxia increased N$_2$O production in coastal water columns. The decrease in oxygen levels (Gruber, 2011; Gattuso et al., 2015) and the decrease in organic matter output to the deep sea due to ocean acidification (Gehlen et al., 2011, due to reduction in ballast effects) both affect N$_2$O generation and atmospheric release. Hypoxia stimulates seawater N$_2$O potential production with positive feedback effects on global warming, and ocean acidification weakens the increase in N$_2$O caused by hypoxia. Previous studies argued that the occurrence of seasonal hypoxia in the Bohai Sea is often accompanied by ocean acidification (Riebesell and Gattuso, 2015; Wei et al., 2019; Zhai et al., 2019; Song et al., 2020), but the results show that hypoxia and ocean acidification are antagonistic to each other and that artificial nitrogen input can amplify this change. This finding provides a basis for more accurate estimations of offshore greenhouse gas production models.

**CONCLUSIONS**

Ocean acidification and hypoxia decreased and increased N$_2$O potential production, respectively. Incubation experiments showed that the combination of ocean acidification and hypoxia significantly increased N$_2$O potential production, but, individually, there was an antagonistic relationship between the two. Seawater pH, DIC, DO, NH$_4^+$-N, and TIN were the primary predictors of sensitivity to N$_2$O potential production. SEM analysis showed that the direct and indirect effects of hypoxia treatment on N$_2$O potential production changes counteracted the effects of ocean acidification treatment. As hypoxia and ocean acidification together increased N$_2$O potential production, the negative effects of ocean acidification were canceled out, resulting in overall positive effects. Generally speaking, our results showed that N$_2$O potential production from coastal waters of the Bohai Sea may increase under future climate change scenarios by enhancing both ocean acidification and hypoxia. Future research...
should focus on whether these experimental results could be extrapolated to directly assess the response between in situ N$_2$O potential production and climate change. Moreover, further attention should be paid to areas with high anthropogenic nutrient input.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

JS designed the study. TG and DJ performed the experiments. TG, DJ, XM, LP, YW and JS analyzed the data. GZ provided the nutrient data. TG, DJ and JS wrote the manuscript with input from all authors.

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