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Response of N₂O production rate to ocean acidification in the western North Pacific

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Abstract

Ocean acidification induced by the increase of anthropogenic CO₂ emissions has a profound impact on marine organisms and biogeochemical processes.¹ The response of marine microbial activities to ocean acidification might play a crucial role in the future evolution of air-sea fluxes of biogenic gases such as nitrous oxide (N₂O), a strong greenhouse gas and the dominant stratospheric ozone-depleting substance.² Here, we examine the response of N₂O production from nitrification to acidification in a series of incubation experiments conducted in subtropical and subarctic western North Pacific. The experiments show that, when pH was reduced, the N₂O

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Data Availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information file.

Author contributions

F.B., C.Y., A.M. and S.T. conceived this study, designed and executed the experiments, analyzed the results and wrote the paper. M.W. calculated pH values from DIC and total alkalinity for all cruises. Y.M. measured $\delta^{15}\text{NO}_3^-$ values to estimate nitrification rates. S.K., T.F. and N.H. contributed to the organization of the sampling campaigns. All authors contributed to the interpretation of the results and the preparation of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

production rate during nitrification measured at subarctic stations increased significantly whereas nitrification rates remained stable or decreased. Contrary to what was previously thought, these results suggest that the effect of ocean acidification on N_2O production during nitrification and nitrification rates are likely uncoupled. Collectively these results suggest that, if seawater pH continues to decline at the same rate, ocean acidification could increase the marine N_2O production during nitrification in subarctic North Pacific by 185 to 491% by the end of the century.

The uptake of anthropogenic CO_2 by oceans might lead to a reduction of seawater pH ranging from 0.2 to 0.5 units below the preindustrial level by the year 2100.^{3,4} In fact, surface ocean pH at present is already about 0.1 unit lower than pre-industrial values.⁴ If anthropogenic CO_2 emissions continue at the same rate, the rate of ocean pH change would be faster than changes detected in any geological records.^{5,6} It is thus important to predict future climate changes against the ocean acidification via changes of marine biogeochemical cycles.

N_2O is a strong greenhouse gas, the dominant stratospheric ozone-depleting substance, and formed by microbial processes such as nitrification, denitrification and nitrifier-denitrification.² The net oceanic N_2O production currently represents ~30% of the natural source and ~20% of the total source of atmospheric N_2O and is dominated by nitrification.^{2,7,8} Because the $\text{NH}_3/\text{NH}_4^+$ equilibrium is pH-sensitive and ammonia-oxidizing bacteria (AOB) and archaea (AOA) use preferentially NH_3 rather than NH_4^+ , ocean acidification possibly leads to inhibit marine nitrification and the relevant N_2O production.¹ In fact, Beman et al. (2011) have shown that ocean acidification could reduce nitrification rates in open ocean by 3 to 44% within the next few decades.¹ Based on nitrification rate measurements, Beman et al. (2011) have estimated that the decrease of the nitrification induced by ocean acidification could lead to a reduction of N_2O production ranging from ~0.06 to 0.83 Tg N yr⁻¹, which is comparable to all current N_2O production from industrial processes and fossil fuel burning.^{1,9} Recently, Frame et al. (2017) found increase in N_2O production with decreasing pH in lake water whereas Rees et al. (2016) showed that N_2O production was decreasing with decreasing pH in polar water.^{10,11} The Pacific Ocean constitutes one of the most important marine source of N_2O which is particularly affected by ocean acidification.⁸ Despite the clear influence of the pH on nitrification rate, so far no study has investigated directly the change of N_2O production rates during nitrification in response to acidification in the Pacific Ocean.

To directly assess the response of rates and relations between N_2O production and nitrification to acidification, a series of seawater manipulation experiments were conducted in subtropical and subarctic western North Pacific (WNP) (Fig. 1 and Tab. 1). Seawater samples without the pH manipulation exhibited that both N_2O production and nitrification rates are significantly higher in acidic subarctic waters (stations E1, K2 and KNOT) than those in the subtropical waters (stations S1 and S2) (Fig. 2a and 2b, Fig. S1-2). In subarctic waters N_2O production and nitrification rates respectively range between 1.8 and 37.3 pmol L⁻¹ day⁻¹ and between 17.0 and 50.9 nmol L⁻¹ day⁻¹, whereas in the subtropical waters they range between <0.05 and 0.6 pmol L⁻¹ day⁻¹ and between 3.7 and 11.3 nmol L⁻¹ day⁻¹,

respectively (Tab. S2). The high nitrification rate measured in the subarctic region is caused by the high supply of substrate owing to high primary production.¹² A total of fourteen batches of acidification experiments were conducted with seawater samples collected from different depths and locations in WNP (see SI). The experiments revealed that except the stations S1 and S2 where the N_2O production rates are low and near the LOQ, 6 among 10 (60%) N_2O production rates show a significant increase (p -value 0.05) with the decline of pH (Fig. 2b). Moreover, none of the 14 N_2O production rates show a statistically significant decrease with the decline of pH. The N_2O production rates measured in the subarctic sites increased by 19-82% in experiments K2_A, E1_B, KNOT_A, KNOT_C, KNOT_D and KNOT_E in response to a 0.1-unit decrease in ocean pH (Tab. 2). On the other hand, nitrification rates remained constant or decreased in response to decreasing pH (Fig. 2a). The variation of nitrification rates as function of the decline of seawater pH was significant (p -value 0.05) in experiments K2_A, E1_A, KNOT_A, KNOT_C and KNOT_E whereas the other experiments did not show any significant response of the nitrification rate to the reduction of pH (Table 2). The nitrification rates measured in the subarctic sites declined by 3-15% in response to 0.1-unit decrease in pH. These results are in the same range as the reduction of nitrification rates measured by Beman et al. (2011) in response to a 0.1 decrease in pH (3-44%, mean 21%).¹ 60% of the incubation experiments conducted in subarctic WNP show statistically significant increases of the N_2O production rates with the decline of pH (Fig. 2b and Tab. 2). Moreover, the nitrification rates measured in subarctic WNP declined significantly or remained stable after artificial seawater acidification (Fig. 2a and Tab. 2). These findings suggest, contrary to the hypothesis made by Beman et al. (2011), that the effect of ocean acidification on nitrification and associated N_2O production rates are likely uncoupled in some parts of the ocean.

The variability of the influence of pH on nitrification and associated N_2O production rates stems from the multiple factors in addition to pH. In this study, ammonia concentration, light and temperature were controlled but microbial community composition and dissolved oxygen were not. There are some differences in metabolic pathways for N_2O production between AOB and AOA (Fig. 3). The response to pH may vary between AOB and AOA. During nitrification process, ammonia is first oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO) bonded to the cytoplasmic membrane (Fig. 3a).¹ In the case of nitrification by AOB, NH_2OH is subsequently oxidized to nitrite by hydroxylamine oxidoreductase (HAO) located in the periplasmic space.¹³ Several studies suggested that NO intermediate might be produced during the catalytic cycle of HAO.¹⁴ The interaction of NO with the catalytic site leads to the oxidation of HAO and the likely concomitant reduction of NO to N_2O .¹⁷ Similarly, during nitrifier-denitrification process NO_2^- is reduced into NO by nitrite reductase (NIR) which is then reduced to N_2O by the membrane-bound enzyme nitric oxide reductase (NOR) (Fig. 3a).¹⁸⁻²¹ In the case of nitrification by AOA, ammonia is also oxidized to NH_2OH by AMO bonded to the cytoplasmic membrane and then NH_2OH and NO react in an enzymatic reaction leading for NO_2^- formation which is then reduced into NO by NIR (Fig. 3b). During this process NO is first produced from NO_2^- and then consumed during the oxidation of NH_2OH and NO to NO_2^- . In this reaction pathway N_2O might be formed by reaction of NH_2OH and NO from the reduction of NO_2^- . Recently, Frame et al. (2017) have shown that N_2O may be formed by a hybrid mechanism involving

NH_2OH and NO_2^- , or compounds derived from these two molecules¹¹. In this case the enzymatic production of N_2O has not been demonstrated.²² The incubation experiments conducted in WNP show that the hybrid N_2O formation pathway (i.e. $\text{NH}_4^+ + \text{NO}_2^-$) proposed by Frame *et al.* (2017) dominates over the nitrification pathway and increases with decreasing pH (Fig. 2d). Ocean acidification may increase the N_2O production during nitrification by AOA.

There may be several reasons for the different trends observed for nitrification and N_2O production rates with the decline of pH, including a shift toward the optimal pH of the N_2O producing enzymes, pH-sensitive regulation of the genes encoding the enzymes involved in N_2O production, a better exchange of $\text{HNO}_2/\text{NO}_2^-$ between the periplasm and the outer environment at lower pH or the displacement of chemical equilibrium leading to N_2O .¹¹ During most of these N_2O formation reactions an HONNOH intermediate is likely formed by dimerization of HNO (nitroxyl) or NH_2OH in aqueous solution (Fig. S3)^{23–25}, although Caranto and Lancaster (2017) recently suggested an ‘ $\text{NH}_2\text{OH}/\text{NO}$ obligate intermediate model’ for bacterial nitrification that does not involve HNO as an intermediate.²⁶ To form the HONNOH intermediate, nitroxyl should be in its non-deprotonated form. Bartberger *et al.* (2001) have shown that HNO has a pKa value of 7.2 ± 1.0 whereas NH_2OH has pKa of 5.94, indicating that about 50% of HNO and 95% of NH_2OH should exist at equilibrium at physiological pH (Fig. S4).²⁸ Diverse mechanisms for pH-homeostasis enable most bacteria and archaea to maintain cytoplasmic pH within a narrower range than external pH. However, Wilks and Slonczewski (2007) have shown that the pH of the periplasm remains equivalent to the extracellular pH.²⁷ Hence, under the current ocean conditions (Fig. S2) the acidification should not significantly affect the dimerization of NH_2OH in the periplasm. However, a reduction of the pH in the periplasmic space may significantly shifts the HNO/NO^- equilibrium toward HNO and therefore promote its dimerization which subsequently undergoes dehydration and form N_2O (Fig. S4). Thus, this latter mechanism might play an important role in the dependence of N_2O production rate to pH.

The response of the oceans to atmospheric CO_2 concentration rise will not be homogenous. The decline of seawater pH shows already different trends depending on the regions, the depths and the microbial activity that may accelerate or attenuate these trends.⁴ Our results demonstrate for the first time that oceanic N_2O production rates during nitrification can increase due to pH changes ($\Delta \text{pH} = 0.02\text{--}0.47$) that mimic anticipated future ocean acidification. Wakita *et al.* (2013) have shown that the seawater pH decreased by $0.0051 \pm 0.0010 \text{ yr}^{-1}$ between 1997 and 2011 at ~200 m depth in subarctic WNP.²⁸ If the decline of seawater pH continues at the same rate, N_2O production rates during nitrification at subarctic stations K2, KNOT and E1 should increase by 185% to 491% by the end of the century ($\Delta \text{pH} = 0.45$). Ishii *et al.* (2011) have shown that the seawater pH also declined by $0.0020 \pm 0.0007 \text{ yr}^{-1}$ between 1994 and 2007 in the subtropical WNP.²⁹ However, the impact of ocean acidification on nitrification and associated N_2O production rates in subtropical WNP remain difficult to predict since no statistically significant change was measured in this study.

If nothing is undertaken to reduce our dependence to fossil fuels in the coming decades, the decline of oceans pH might disturb biogeochemical cycles and therefore affect the climate,

the biodiversity and human activities. Considering a climatic scenario characterized by a very rapid economic growth, a massive use of fossil energies (IPCC scenario A1FI, " $\text{pH}''_{2100}=0.35$), the production rate of N_2O during nitrification might increase by ~166 to 404% by the end of the century in subarctic WNP. For a less severe scenario oriented toward environmental protection (IPCC scenario B2, " $\text{pH}''_{2100}=0.14$), the increase of N_2O production rates during nitrification in subarctic WNP might range between 126 and 222%.^{8,9} If the enhancement of N_2O production is mainly caused during nitrification in polar water, the response of the N_2O production rate during nitrification to ocean acidification in WNP differs from polar water.¹⁰ Thus, there is a possibility of regional differences in the response. Nowadays N_2O emission from WNP represents about 8% of the global oceanic N_2O emissions.⁸ Although it would be inappropriate to extrapolate our data to the global oceans, the results of this study are however relevant to subarctic and subtropical regions of North Pacific. These regions are not only of great global importance for N_2O production but is expected to experience the greatest changes in terms of ocean acidification, warming and stratification. Our findings indicate that, as anthropogenic CO_2 will continue to be trapped in the oceans, pH-driven increases in N_2O production during nitrification rates could drastically raise marine emission of N_2O to the atmosphere.

Methods

Sampling and acidification experiments

Seawater samples were collected during the MR13-04, KS-16-8 and YK16-16 cruises at three contrasting times-series stations, the subarctic K2 station (47°00'N – 160°05'E, 100 m and 175 m, July 23rd 2013), the Kyodo North Pacific Ocean Time series (KNOT) station (44°00'N – 155°01'E, 100 m and 175 m, November 19th 2016), and subtropical S1 station (30°00'N – 145°00'E, 175 m and 200 m, July 15th 2013) in the western North Pacific (WNP). Moreover, seawater samples were also collected at an intermediate station E1 (41°15'N – 146°43'E, 100 m and 140 m, July 27th 2013) and in the subtropical S2 station (30°00'N – 142°36'E, 150 and 200 m, July 8th 2016). At each station, seawater samples were collected using 12 L Niskin-X bottles mounted on a CTD rosette sampler. Each sample for N_2O -isotopomer analysis was collected in 230 mL glass vial and the biological activity was stopped by adding 40 μL saturated HgCl_2 solution. All vials were quickly sealed with butyl rubber stoppers and preserved at 4°C in the dark. Measurements of water temperature, salinity, photosynthetically active radiation, oxygen, dissolved inorganic carbon, total alkalinity, chlorophyll-*a* and nutrients concentrations (NH_4^+ , NO_2^- , NO_3^- , PO_4^-) were conducted on board (data available on <http://www.godac.jamstec.go.jp/darwin/e>).

Seawater samples collected at stations K2, S1, and E1 were sub-sampled into two 100 mL glass vials for ammonia oxidation rates measurements and in two 200 mL glass vials for N_2O production rates measurements. The seawater samples used for ammonia oxidation and N_2O production rates determination were quickly sealed with butyl rubber and aluminum caps and were unsealed only for the additions of $^{15}\text{NH}_4^+$ (^{15}N atom fraction: 99.3%) or HCl. The samples were acidified with different amount of 0.5 M HCl solution. Then 1 or 2 μL $^{15}\text{NH}_4\text{Cl}$ solution was added in seawater samples for NH_4^+ oxidation and N_2O production rate analysis using a micro-syringe, so that the final concentration was 233 nmol N L⁻¹. The

samples were incubated in the dark at a temperature close to that at the sampling depth. After incubation, 2 ml of each sample was removed and collected into a 2.5 ml Eppendorf® tube for pH-determination and the biological activity of samples for N₂O production rates was stopped with 40 µl of saturated HgCl₂ solutions and stored in the dark at 4°C until analysis. This aliquot of 2 ml was compensated by adding degased MilliQ water. The samples for ammonia oxidation rates were filtered with DISMIC® filter (0.45 µm) and stored at -20°C until isotopic analysis. The pH of seawater samples measured using a pH electrode (F-72 LAQUA 9615, Horiba, Japan). For the samples collected at stations KNOT and S2 250 ml vials were used and both nitrification and N₂O production rates were measured from the same bottle. After the incubation, 25 ml and 2 ml of each samples were removed for δ¹⁵N-NO₃⁻ and pH measurements. The aliquot of 27 ml was compensated by adding N₂ gas. The other procedures for NO₃⁻ and N₂O samples are same, but pH values were measured on board by using Thermo Fisher, Orion Star A214 pH/ISE benchtop meter.

The nitrification is in general more active near the bottom of the euphotic zone. Thus, the depth at which water samples were collected for incubation experiments was determined on the basis of in-situ chlorophyll-*a* fluorescence profiles (Fig. S1). The contribution of the different reaction pathways leading to N₂O production in the water column was evaluated for each sampling sites on the basis of its stable isotope and isotopomer composition of N₂O. Potential N₂O production and ammonia oxidation rates were determined by adding ¹⁵NH₄⁺ to seawater samples. Although the addition of NH₄⁺ might stimulates nitrification, this method enables to measure potential ammonia oxidation rate and N₂O production rate at slightly higher NH₄⁺ concentration than ambient concentration and its dependency of the rates on pH. In this study seawater pH was manipulated by adding HCl solution to avoid N₂O degassing which would result from the acidification by a gentle bubbling of different air/CO₂ mixtures. Moreover, a control experiment with non-acidified sample was carried out for each sampling depth. The addition of a strong acid in a system closed to the atmosphere does not affect the concentration of dissolved inorganic carbon but modifies total alkalinity and *p*CO₂^{30–32}. Although the addition of HCl do not reproduce the increase of dissolved inorganic carbon concentration and the decrease of the total alkalinity induced by natural ocean acidification, this approach enable to precisely control *p*CO₂^{30–32}. The addition of HCl during acidification experiments increase *p*CO₂ and therefore nitrifiers could exhibit a “CO₂-fertilization” effect due to undersaturation of ribulose biphosphate carboxylase oxidase (RuBisCO) at present-day CO₂ concentrations. Several studies have shown that cultivated strains of AOB contain carbon concentrating mechanisms (CCMs) that could reduce their sensitivity to changes in *p*CO₂ and AOA use a different carbon fixation pathway based on 3-hydroxypropionate/4-hydroxybutyrate and utilizes HCO₃⁻ as an inorganic carbon source rather than CO₂.^{33,34} However the effect of increasing *p*CO₂ on marine AOB remains unclear since CCMs are inefficient and energy-consuming processes.

Acidifying seawater samples can potentially lead to production of N₂O when dissolved nitrite is present. Under acidic condition nitrite is prevalent mainly in its protonated form nitrous acid (HNO₂, pK_a 3.398). HNO₂ is not stable in aqueous solution and one of the decomposition pathways leads to N₂O³⁵:

However, in the present study the pH of acidified samples remained above the pK_a value of HNO₂. Under these conditions nitrite anions are not protonated and therefore a contribution of this abiotic pathway to the observed N₂O production is very unlikely.

N₂O isotopomer ratios measured at the stations S1, E1 and K2 range between high site preference N₂O (*SP*≈35‰) produced by marine AOB and AOA and low site preference N₂O (*SP*≈0‰) produced by nitrifier-denitrification^{36,37} (Table S1). These results combined with the oxygen concentrations measured at these stations suggest that nitrification is the main N₂O formation pathway in the lower euphotic and the upper aphotic zones and that the contribution of nitrifier-denitrification pathway ranges between 28% and 51% (Table S1 and Fig. S1). The relatively high δ¹⁵N values of N₂O suggest that AOA might also contribute significantly to the formation of N₂O³⁷. As shallow water masses are well oxygenated, the contribution of denitrifying bacteria to the production of N₂O can be considered as negligible at these stations (Fig. S1).

N₂O stable isotope and isotopomer analysis

Concentrations, isotope and isotopomers ratios of N₂O were measured using a slightly modified version of a gas chromatograph-isotope ratio mass spectrometer (GC-IRMS) system described in an earlier publication³⁸. The butyl rubber septum of the sample vial was pricked with two needles (one for pressurization with He and the other for water transfer) and the water was transferred into a sparging column. Then, the column was purged with ultrapure He (purity 99.9999 %; Japan Air Gases) from the bottom to prevent mixing of laboratory air with the contents of the vial during 18 min at a flow rate of 70 mL min⁻¹ with magnetic stirring. Before and after transfer, the vial was weighed to ascertain the amount of water transferred. The mass transferred to the purging system was used later to calculate the N₂O concentration. The extracted gases were passed through two glass tubes (9 mm o.d.) packed with magnesium perchlorate (8/24 mesh, 8 cm long), Ascarite (20/30 mesh, 5 cm long; Thomas Scientific), and magnesium perchlorate (20/48 mesh, 30 cm long) to remove water vapor and CO₂. After treatment with the chemical trap, the trace components including N₂O were first cryo-trapped in a stainless steel tube (1/8 inch o.d., 40 cm long) filled with glass beads (Flusin GH, 60/80 mesh; GL Sciences, Japan) at -196°C using liquid N₂. The concentrated gases were then purified further by passage through a second chemical trap similar to the first and were focused in a stainless steel tube (1/16 inch o.d., 50 cm long). Finally, the focused gases were injected into a gas chromatograph (HP6890 GC; Agilent Technologies, Santa Clara, CA, USA) equipped with a PoraPLOT Q capillary column (0.32 mm i.d., 25 m long, 10 μm, Chrompack) with He as carrier gas (2 mL min⁻¹). Mass analysis of molecular ion or fragment ion of the purified N₂O was conducted with an IRMS (MAT 252; Thermo Fisher Scientific, Yokohama, Japan). In the fragment ion analysis, separation of interfering components was achieved by use of a GC column (1/8 inch o.d., 150 cm long stainless steel tube packed with silica gel) at 70°C before treatment with the second chemical trap. Calibration methods for concentration and isotopomer ratios have been described elsewhere^{39,40}.

The isotopomer ratios of $^{15}R^{bulk}$, ^{18}R , and $^{15}R^{\alpha}$ were determined and $^{15}R^{\beta}$ was obtained using the following relationships³⁹:

$$^{15}R^{bulk} = \frac{^{15}R^{\alpha} + ^{15}R^{\beta}}{2} \quad [1]$$

Where,

$$^{15}R^{\alpha} = \frac{[^{14}N^{15}N^{16}O]}{[^{14}N^{14}N^{16}O]} \quad [2]$$

$$^{15}R^{\beta} = \frac{[^{15}N^{14}N^{16}O]}{[^{14}N^{14}N^{16}O]} \quad [3]$$

$$^{18}R = \frac{[^{14}N^{14}N^{18}O]}{[^{14}N^{14}N^{16}O]} \quad [4]$$

In this study, the isotopomer δ values were expressed as follows:

$$\delta X = \frac{R - R_{std}}{R_{std}} \quad [5]$$

Where X denotes $^{15}N^{bulk}$, $^{15}N^{\alpha}$, $^{15}N^{\beta}$ or ^{18}O and R denotes the isotope ratio ($^{15}N/^{14}N$ or $^{18}O/^{16}O$) of the central (α), peripheral (β) nitrogen atom or oxygen atom in the N_2O molecule and R_{std} is the isotope ratio of the standard. All δX values were expressed in permil (‰) relative to atmospheric N_2 (Air) or to Vienna Standard Mean Ocean Water (VSMOW). ^{15}N -site preference (SP) value is used to illustrate the intramolecular distribution of ^{15}N in N_2O :

$$SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad [6]$$

The typical analytical precision is 0.6‰, 0.9‰, 1.5‰ and 0.9‰ for $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and $\delta^{18}O$, respectively.

Ammonia oxidation and N_2O production rates

Ammonia oxidation and N_2O production rates were measured by addition of $^{15}NH_4^+$ (^{15}N atom fraction: 99.3%) as a tracer and accumulation of ^{15}N in N_2O , NO_3^- and NO_2^- pool following incubation. The $\delta^{15}N$ values of N_2O was measured using a slightly modified version of a GC-IRMS as described above whereas the $\delta^{15}N$ values of NO_3^- and NO_2^- pool was measured using the “denitrifier method” developed by Sigman et al.⁴¹. Briefly, NO_3^- and NO_2^- were converted into N_2O by denitrifying bacteria that lack N_2O -reductase activity. Then N_2O produced by biodegradation was analyzed using a gas bench pre-concentration system interface and a Delta V plus isotope-ratio mass spectrometer at JAMSTEC. The

calibration standards are USGS-32, USGS-34 and IAEA-NO-3. Ammonia oxidation rates (V_{NOx}) were calculated using the equations following to previous study ⁴²:

$$V_{NOx} = \frac{1}{t} \frac{\Delta [^{15}NO_x]}{^{15}f_{NH_4^+}} \quad [7]$$

$$^{15}f_{NH_4^+} = \frac{[^{15}NH_4^+]}{[^{15}NH_4^+] + [^{14}NH_4^+]} \quad [8]$$

In equation S7, Δ means difference between samples incubated with and without addition of $^{15}NH_4^+$.

The N_2O production rates (V_{N_2O}) were calculated considering the following isotopic species of N_2O :

Mass number	Isotopic species of N_2O considered for calculation of N_2O production rate		
46	$^{15}N^{15}NO$		
45	$^{14}N^{15}N'O$	$^{14}N^{15}N'O$	$^{15}N^{14}N'O$ $^{15}N^{14}N'O$
44	$^{14}N^{14}N'O$	$^{14}N^{14}N'O$	$^{14}N^{14}N'O$

Where:

xN : Total xN

$^xN'$: xN from $^xNH_4^+$

$^xN''$: xN from $^xNO_2^-$ initially existed

Then formation rates (V_{N_2O}) of N_2O produced with two ammonia molecules and with one ammonia and one nitrite were calculated using the following equations:^{11,42}

$$V_{N_2O} = V_{-N'N'O} + V_{-N'N''O} + V_{N''N'O} \quad [9]$$

$$V_{-N'N'O} = \frac{1}{t} \frac{\Delta [^{15}N'^{15}N'O]}{^{15}f_{NH_4^+} ^{15}f_{NH_4^+}} \quad [10]$$

$$V_{-N'N''O} + V_{N''N'O} = \frac{1}{t} \frac{\Delta [^{14}N''^{15}N'O + ^{15}N'^{14}N''O] - \Delta [^{14}N^{15}NO + ^{15}N^{14}NO]}{^{15}f_{NH_4^+}} \quad [11]$$

$$\Delta \left[{}^{14}\text{N}^{15}\text{NO} + {}^{15}\text{N}^{14}\text{NO} \right] = \Delta \left[{}^{15}\text{N}^{15}\text{NO} \right] \frac{{}^{2^{14}f_{\text{NH}_4^+}} {}^{15}f_{\text{NH}_4^+}}{{}^{15}f_{\text{NH}_4^+} {}^{15}f_{\text{NH}_4^+}} \quad [12]$$

The quantification limits (LOQ) for the rate measurements are based on analytical precision (standard deviation) of the IRMS for $\delta^{45/44}$ and $\delta^{46/44}$ of N_2O , considering that both ammonia oxidation (NO_2^- and NO_3^- production) and N_2O production rates were derived from N_2O measurement. If the difference in measured $\delta^{45/44}$ or $\delta^{46/44}$ between ^{15}N -added and control bottles is three times larger than the precision, ammonia oxidation or N_2O production is considered to be quantifiable. The standard deviation for $\delta^{45/44}$ and $\delta^{46/44}$ were 0.2‰ and 0.5‰, respectively. Using these values, ^{15}f (^{15}N fraction of initial NH_4^+), NO_x^- or N_2O concentration during the incubation, and incubation time, the calculated LOQ for ammonium oxidation or N_2O production rate ranged from 0.02 to 0.30 $\text{nmol L}^{-1} \text{ day}^{-1}$ and from 0.02 to 0.14 $\text{pmol L}^{-1} \text{ day}^{-1}$, respectively. All of the ammonium oxidation rates exceeded the LOQ, but 9 of the N_2O production rates fell below the LOQ. To determine whether the addition of $^{15}\text{NH}_4^+$ stimulate nitrification during the incubation of the samples, experiments with both 51 and 259 nmol L^{-1} of tracer were carried out at KNOT station. No significant difference in ammonium oxidation and N_2O production rates was measured for both concentrations (Table S2). Moreover, no significant difference in ammonium oxidation and N_2O productions rate was measured between experiments incubated during 13-14h and 26-28h (Table S2). Therefore, we can conclude that incubation time and tracer concentration don't affect the rates measured in this study.

Contribution of nitrification and nitrifier-denitrification pathways

In the case where N_2O is only produced by nitrification and nitrifier-denitrification, it is possible to estimate the fraction of N_2O produced by nitrite reduction, ϕ_{ND} , from SP values using the following equation ⁴³:

$$\phi_{\text{ND}} = \frac{SP_{\text{IS}} - SP_{\text{AO}}}{SP_{\text{ND}} - SP_{\text{AO}}} \quad [13]$$

Where SP_{IS} is the SP value of N_2O , SP_{AO} is the SP value attributable to ammonium oxidation (hydroxylamine oxidation pathway) and SP_{ND} is the SP value attributable to nitrite reduction (nitrifier-denitrification pathway). The SP values associated with nitrifier-denitrification and ammonium oxidation pathways were respectively fixed to 0‰ and 36‰ based on the literature discussed above. The contribution of atmospheric N_2O (SP~19‰) was ignored in equation S8. This approximation induce a slight overestimation of ϕ_{ND} .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Experiment and sampling stations.

Fig. 2. Response of nitrification and nitrous oxide production rates to simulated ocean acidification.

(a) Nitrification rates, (b) total N₂O production rates, (c) N₂O production rates from two NH_4^+ and (d) from a hybrid mechanism involving NH_4^+ and NO_2^- . " pH " corresponds to the decrease between seawater pH before and after acidification. The rates in graphs (c) and (d) were calculated using equations (S10) and (S11). Note that results from three independent experiments are plotted as a single symbol for KNOT 100 m and KNOT 150 m (See Table S2).

Fig. 3. Nitrification and nitrifier-denitrification pathways.

Nitrification and nitrifier-denitrification pathways of (a) AOB and (b) AOA. AMO: ammonia monooxygenase, HAO: hydroxylamine oxydoreductase, NIR: nitrite reductase, NOR: nitric oxide reductase, Amt: ammonium transporter system.

Table 1

Overview of experiments, including date, depth of seawater collection, incubation time and temperature, concentration of $^{15}\text{NH}_4^+$ added and variation of seawater pH for the determination of nitrification ("pH"_{nit}) and N_2O production rates ("pH"_{N₂O}).

Station / Experiment	Date	Incub. time h	Depth m	Incub. temperature °C	$^{15}\text{NH}_4^+$ nM	"pH" _{nit}	"pH" _{N₂O}
S1 _A	15/7/2013	12	175	18	185	0.16	0.46
S1 _B	15/7/2013	12	200	18	185	0.20	0.29
S2 _A	8/7/2016	15	150	18	52	0.03	0.02
S2 _B	8/7/2016	15	200	18	52	0.20	0.20
E1 _A	27/7/2013	25	100	7	185	0.27	0.57
E1 _B	27/7/2013	25	140	7	185	0.29	0.16
K2 _A	23/7/2013	24	100	4	185	0.20	0.29
K2 _B	23/7/2013	24	175	4	185	0.20	0.17
KNOT _A	19/11/2016	14	100	5	51	0.22	0.22
KNOT _B	19/11/2016	28	100	5	51	0.30	0.30
KNOT _C	19/11/2016	28	100	5	259	0.25	0.25
KNOT _D	19/11/2016	13	150	5	51	0.14	0.14
KNOT _E	19/11/2016	26	150	5	51	0.18	0.18
KNOT _F	19/11/2016	26	150	5	259	0.20	0.20

Table 2

Slope (m) of the variation of the nitrification (in $\text{nmol L}^{-1} \text{ day}^{-1}$) and N_2O production (in $\text{pmol L}^{-1} \text{ day}^{-1}$) rates as function of the decline of seawater pH, standard error (SE) of slope and p -value (two-sided, n =number of data points). The data highlighted in bold are experiments with a p -value ≤ 0.05 (i.e. slopes significantly different from zero with a confidence level higher or equal to 95%). The estimated limit of quantification (LOQ) for nitrification and N_2O production rates ranged between 0.02 – $0.30 \text{ nmol L}^{-1} \text{ day}^{-1}$ and between 0.02 – $0.14 \text{ pmol L}^{-1} \text{ day}^{-1}$.

Station / Experiment	nitrification rate		N_2O production rate (total)	
	m ($\pm \text{SE}$)	p -value (n)	m ($\pm \text{SE}$)	p -value (n)
S1 _A	21.3 (6.6)	0.19 (3)	N.D.	N.D.
S1 _B	7.7 (6.4)	0.35 (4)	0.2 (1.3)	0.88 (3)
S2 _A	12.28 (18.2)	0.55 (3)	N.D.	N.D.
S2 _B	2.6 (3.1)	0.44 (6)	N.D.	N.D.
E1 _A	-74.7 (21.9)	0.03 (6)	2.6 (1.0)	0.06 (6)
E1 _B	21.1 (18.7)	0.32 (6)	13.9 (3.5)	0.02 (6)
K2 _A	-17.0 (5.3)	0.03 (6)	36.9 (8.0)	0.01 (6)
K2 _B	-11.4 (10.5)	0.39 (4)	-9.5 (25.4)	0.74 (4)
KNOT _A	-12.5 (4.0)	0.0 (6)	23.7 (8.7)	0.05 (6)
KNOT _B	-7.0 (5.9)	0.3 (4)	7.1 (5.6)	0.34 (5)
KNOT _C	-6.4 (2.3)	0.05 (6)	13.0 (2.8)	0.01 (6)
KNOT _D	-17.5 (4.7)	0.06 (4)	38.2 (4.5)	0.01 (4)
KNOT _E	-22.0 (2.8)	0.02 (4)	42.2 (6.3)	0.02 (4)
KNOT _F	-11.2.0 (8.26)	0.12 (5)	65.2 (42.6)	0.22 (5)