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# Nitrous oxide inhibition of methanogenesis represents an underappreciated greenhouse gas emission feedback

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#### Abstract

Methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) are major greenhouse gases that are predominantly generated by microbial activities in anoxic environments. N<sub>2</sub>O inhibition of methanogenesis has been reported, but comprehensive efforts to obtain kinetic information are lacking. Using the model methanogen *Methanosarcina barkeri* strain Fusaro and digester sludge-derived methanogenic enrichment cultures, we conducted growth yield and kinetic measurements and showed that micromolar concentrations of N<sub>2</sub>O suppress the growth of methanogens and CH<sub>4</sub> production from major methanogenic substrate classes. Acetoclastic methanogenesis, estimated to account for two-thirds of the annual 1 billion metric tons of biogenic CH<sub>4</sub>, was most sensitive to N<sub>2</sub>O, with inhibitory constants (K<sub>1</sub>) in the range of 18–25  $\mu$ M, followed by hydrogenotrophic (K<sub>1</sub>, 60–90  $\mu$ M) and methylotrophic (K<sub>1</sub>, 110–130  $\mu$ M) methanogenesis. Dissolved N<sub>2</sub>O concentrations exceeding these K<sub>1</sub> values are not uncommon in managed (i.e. fertilized soils and wastewater treatment plants) and unmanaged ecosystems. Future greenhouse gas emissions remain uncertain, particularly from critical zone environments (e.g. thawing permafrost) with large amounts of stored nitrogenous and carbonaceous materials that are experiencing unprecedented warming. Incorporating relevant feedback effects, such as the significant N<sub>2</sub>O inhibition on methanogenesis, can refine climate models and improve predictive capabilities.

Keywords: nitrous oxide, methane, greenhouse gas emissions, inhibition, feedback loop, climate change

#### Introduction

Carbon dioxide (CO<sub>2</sub>) receives primary attention as a driver for climate change, but methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) account for  $\sim$ 20% and 7%, respectively, of the net radiative forcing in the atmosphere [1, 2]. The central objective of the Paris Agreement, which is to hold the global average temperature increase to "well below 2°C above preindustrial levels" [3, 4], cannot be met without controlling CH<sub>4</sub> and N<sub>2</sub>O emissions. Global emissions of both CH<sub>4</sub> and  $N_2O$  are ultimately controlled by microbial processes [2, 5]; however, human activities have massively disturbed the natural balance of microbial production and consumption of these greenhouse gases [6]. The current estimated annual net atmospheric emission increases of  $\sim$ 51 Tg of CH<sub>4</sub> [7, 8] and 2.2 Tg of N<sub>2</sub>O [9] are both predicted to accelerate [1, 10]. The key microbial guilds and biogeochemical processes responsible for CH<sub>4</sub> and N<sub>2</sub>O production and consumption are known, and their responses to climate change have been a matter of intense research [11, 12]. One area of considerable uncertainty pertains to positive and negative feedback loops affecting greenhouse gas emissions and climate [13]. An infamous scenario for a positive feedback loop in response to a warming climate is permafrost thawing, which stimulates the microbial activity and the release of  $CO_2$ ,  $CH_4$ , and  $N_2O$  from newly bioavailable carbon and nitrogen pools. The massive release of greenhouse gases will further accelerate radiative forcing and in turn cause more thawing. This example illustrates why a comprehensive understanding of both positive and negative feedback loops is essential. Quantitative information is needed to meaningfully incorporate feedback effects into refined climate models.

Methanogenic archaea (methanogens) drive  $CH_4$  production by utilizing acetate,  $H_2/CO_2$ , and methylated compounds as substrates, generating around 1 billion metric tons of  $CH_4$  annually [14, 15]. Approximately, two-thirds of biogenic  $CH_4$  is produced from acetoclastic methanogenesis (i.e. the conversion of acetate to  $CH_4$  and  $CO_2$ ), with the remaining one-third attributed to hydrogenotrophic  $CO_2$  reduction (hydrogenotrophic pathway) and methylated compound utilization (methylotrophic pathways) [16–20]. While the three major methanogenesis pathways share a core set of enzymes, several mechanistically distinct enzyme

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**Figure 1.** Illustration of the major methanogenic pathways that together account for most of the biogenically produced CH<sub>4</sub> in nature; acetoclastic (left), hydrogenotrophic (top), and methylotrophic (right) conversions channel into the methanogenic pathway; the central circles indicate steps catalyzed by corrinoid-dependent enzyme systems potentially susceptible to N<sub>2</sub>O inhibition; abbreviations: CH<sub>3</sub>-EnzComp, CH<sub>3</sub>-formation enzyme complexes; CH<sub>3</sub>-H<sub>4</sub>MPT, methyl-tetrahydromethanopterin; MTR, N<sup>5</sup>-methyltetrahydromethanopterin: CoM methyltransferase; MTs, substrate-specific methyltransferases; CH<sub>3</sub>-R, methylated compounds (e.g. methanol).

systems with corrinoid prosthetic groups (i.e. vitamin  $B_{12}$  derivatives) are involved in methyl group transfer reactions, energy conservation, and CH<sub>4</sub> production (Fig. 1) [18, 19, 21].

In hydrogenotrophic methanogenesis, CO<sub>2</sub> is sequentially reduced to a methyl group carried by tetrahydromethanopterin (H<sub>4</sub>MPT) with  $H_2$  as electron donor. The corrinoid-dependent  $N^5$ methyltetrahydromethanopterin:CoM methyltransferase (MTR) complex then transfers the methyl group onto another C<sub>1</sub>-carrier, coenzyme M, forming CH<sub>3</sub>–S–CoM, a step associated with energy conservation, followed by CH<sub>4</sub> production catalyzed by methyl coenzyme M reductase (MCR) [22]. Acetoclastic methanogens produce CH<sub>4</sub> by activating acetate to acetyl-CoA, which is then cleaved by the corrinoid-dependent enzyme system carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) to yield an enzyme-bound methyl group and a carbonyl group. The carbonyl moiety is oxidized to  $CO_2$  and the methyl group is transferred to H<sub>4</sub>MPT [23]. The MTR complex catalyzes a methyl group transfer to form CH<sub>3</sub>–S–CoM, a step associated with energy conservation, before MCR mediates the reduction of the methyl group to CH<sub>4</sub> [16, 24]. Methylotrophic methanogens either directly generate CH<sub>3</sub>-S-CoM from methylated compounds (e.g. MeOH) utilizing substrate-specific, corrinoid-dependent MTRs, or cleave the methyl group from methoxylated compounds (e.g. 2methoxybenzoate) and form CH<sub>3</sub>–CoM via H<sub>4</sub>MPT and the MTR complex [25], followed by CH<sub>4</sub> production via MCR. All three methanogenesis pathways utilize corrinoid-dependent enzyme systems for methyl group transfers and have strict requirements for super-reduced Co(I) to generate CH<sub>4</sub> and conserve energy [26, 27]. These features render methanogenesis sensitive to oxidative stress (e.g. fluctuating redox conditions and oxygen intrusion) [19, 28].

 $N_2O$  is an even stronger oxidant than oxygen (E°'( $N_2O(g)/N_2(g) =$  $+1.355 \text{ V} > E^{\circ'}(O_2(g)/H_2O(l) = +0.818 \text{ V})$ , reacts with Co(I), and has been shown to interfere with Co(I) cobamide-dependent enzyme systems [29, 30]. Demonstrated metabolic consequences include ceased corrinoid-dependent methionine biosynthesis and impaired organohalide respiration [31-33]. Based on this information, elevated N<sub>2</sub>O in anoxic ecosystems would be expected to inhibit other corrinoid-dependent processes such as methanogenesis; however, available studies investigating the impacts of N<sub>2</sub>O on methanogenesis have led to inconsistent N<sub>2</sub>O inhibition patterns for axenic methanogen cultures and CH4 producing microbial communities [34–36]. Laboratory incubations showed that Methanobacterium bryantii strain Bab1 grown with  $H_2/CO_2$  ceased CH<sub>4</sub> production in the presence of 95  $\mu$ M N<sub>2</sub>O, whereas Methanosarcina barkeri strain MS maintained some methanogenic activity at 10-fold higher N2O concentrations under the same growth conditions [35]. Different sensitivities to N<sub>2</sub>O inhibition were also reported for mixed methanogenic cultures maintained with different substrates. For example, inhibition of CH<sub>4</sub> production in a mixed community bioreactor occurred only at N<sub>2</sub>O concentrations exceeding 700  $\mu$ M [36], whereas 20–28  $\mu$ M N<sub>2</sub>O completely inhibited methanogenic activity in salt marsh sediment and Amazon peatland enrichment cultures [34, 37]. These variable sensitivities to N<sub>2</sub>O suggest that inhibition of methanogenesis by N<sub>2</sub>O is organism- and possibly substrate-specific; however, the available data are scarce and do not allow a robust, quantitative assessment of N<sub>2</sub>O inhibition on methanogenesis from relevant methanogenic substrates [34-36].

N<sub>2</sub>O fluxes in soil-water systems have risen sharply due to the intensified use of synthetic N fertilizer in agriculture [6, 9, 38]. As a result, elevated N<sub>2</sub>O concentrations occur more frequently in ecosystems with CH4 production such as rice paddy soils [39], wastewater treatment plants [40], sediments [41], and groundwater aquifers [42]. Also, permafrost thawing accelerates N turnover, releasing large amounts of N<sub>2</sub>O [38, 43]. More detailed knowledge about the interactions between N<sub>2</sub>O concentrations and methanogenesis is needed to advance the predictive capabilities of climate change impacts on future greenhouse gas emission scenarios. To address the existing knowledge gaps, we assessed the inhibitory effect of N<sub>2</sub>O on CH<sub>4</sub> production from major methanogenic substrates in growth experiments with axenic and mixed methanogenic cultures and in wholecell suspension assays. Using cultures performing acetoclastic, methylotrophic, and/or hydrogenotrophic methanogenesis, we determined kinetic parameters that quantitatively describe N<sub>2</sub>O inhibition on methanogenesis.

#### **Materials and methods**

# Methanogenic cultures and growth inhibition experiments

The methanogenic archaeon M. barkeri strain Fusaro metabolizes acetate,  $H_2/CO_2$ , and MeOH while employing different, substrate-specific methanogenic pathways. To determine if M. barkeri

exhibits varied sensitivities to N<sub>2</sub>O, cultures were pregrown with MeOH, H<sub>2</sub>/CO<sub>2</sub>, or acetate for at least three consecutive transfers before the impact of N<sub>2</sub>O on CH<sub>4</sub> production was examined. Also, we analyzed three methanogenic mixed cultures derived from anaerobic digester sludge, which is known to harbor a broad diversity of methanogenic archaea. Three different enrichment cultures from the same source material were obtained with MeOH, H<sub>2</sub>/CO<sub>2</sub>, or acetate as growth substrate. The mixed cultures were transferred at least six times on the respective substrate and were used to examine the impact of  $N_2O$  on CH<sub>4</sub> production from different methanogenic substrates (see Supplemental Information for additional information on the mixed methanogenic cultures). Experiments were performed in triplicate 60-ml glass serum bottles with 30 ml  $N_2/CO_2$  (80/20, v/v) headspace and 30 ml bicarbonate-buffered (50 mM) mineral salt medium (pH 7.2) reduced with 0.2 mM sulfide and 0.2 mM Lcysteine [44]. Acetoclastic, methylotrophic, and hydrogenotrophic cultures received 20 mM acetate, 30 mM MeOH, and 1.24 mmol H<sub>2</sub>, respectively. To avoid overpressure in bottles with H<sub>2</sub> as electron donor, the headspace of culture bottles was replaced with 30 ml of filter-sterilized H<sub>2</sub>. For M. barkeri cultures, 0.1–1.0 ml of N<sub>2</sub>O gas (undiluted or 10-fold diluted in N<sub>2</sub>) was directly added to the incubation vessels to achieve final aqueous N<sub>2</sub>O concentrations of 100 and 200  $\mu$ M in cultures with MeOH, 50 and 100  $\mu$ M in cultures with  $H_2$ , and 20 and 50  $\mu$ M in cultures with acetate. For the methanogenic mixed cultures grown with 30 mM MeOH, 30 ml H<sub>2</sub> (1.24 mmol), and 20 mM acetate, 0.5–1.6 ml of 10-fold diluted N<sub>2</sub>O gas (in N<sub>2</sub>) was introduced to achieve final aqueous phase  $N_2O$  concentrations of 10 and 30  $\mu$ M. More detailed information about N<sub>2</sub>O additions and concentration calculations is provided in the Supplemental Methods. All cultures were incubated without agitation at 37°C in the dark with the stoppers facing up, and replicates without N<sub>2</sub>O and without inoculum served as positive and negative controls, respectively.  $\mbox{CH}_4$  and  $\mbox{N}_2\mbox{O}$  were analyzed throughout the growth experiments by injecting 100  $\mu$ l headspace samples into an Agilent 3000A Micro gas chromatograph equipped with thermal conductivity detectors and a molecular sieve column and a PLOT Q column for  $CH_4$  and  $N_2O$  measurements, respectively.

# Whole-cell suspension assays to determine N<sub>2</sub>O inhibition constants for CH<sub>4</sub> production

The *M. barkeri* and the methanogenic mixed cultures were first grown in 1.6 l medium and harvested via centrifugation when about two-thirds of the initial substrate (i.e. MeOH,  $H_2/CO_2$ , or acetate) had been converted to  $CH_4$ , as calculated based on  $CH_4$  production according to Equations (1)–(3) (see below). The cell pellets collected from 1.6 l of medium were washed and suspended in 1.6 ml of reduced mineral salt medium in sealed 2-ml glass vials, resulting in a 1000-fold concentration of the biomass. A 0.2 ml aliquot of the concentrated cell suspension was sacrificed to measure total protein with the Bradford assay [45].

Cell suspension assays were performed at room temperature in 20-ml glass vials flushed with N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) and were sealed with Teflon-lined butyl rubber stoppers held in place with aluminum crimps. The assay vials received a total of 0.9 ml reduced mineral salt medium, 0.1 ml of cell suspension, and increasing concentrations of substrates (i.e. MeOH, H<sub>2</sub>, or acetate, with N<sub>2</sub>O as indicated in Tables S1–S7). For assay vials receiving H<sub>2</sub> as electron donor, the headspace was replaced with increasing volumes of premixed H<sub>2</sub>/CO<sub>2</sub> (4/1, v/v) to achieve H<sub>2</sub> concentrations ranging from 1.2 to 333  $\mu$ M (Table S3). Small volumes (89–178  $\mu$ l) of undiluted or 10-fold diluted (in N<sub>2</sub>) N<sub>2</sub>O were directly injected into the 20-ml assay vials, what resulted in small pressure changes with negligible impact on the distribution of  $N_2O$  between the aqueous phase and the headspace. All cell suspensions were freshly prepared following identical procedures to ensure consistency between independent experiments. Vials that received 0.1 ml of sterile mineral salt medium or 0.1 ml of heat-killed (i.e. autoclaved) cell suspension served as negative controls.

Following 10 min of equilibration, 0.1 ml of cell suspension was added to initiate the assays. Headspace samples (100  $\mu$ l) were withdrawn with an air-tight syringe with a lock every 30 min over a 3-h incubation period and, for the kinetic experiments, CH<sub>4</sub> was analyzed using an Agilent 7890 GC series gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m length  $\times$  0.32 mm diameter, 1.8  $\mu$ m film thickness). For each treatment at a fixed initial substrate concentration [S], an initial  $CH_4$  production rate v, normalized to the amount of protein per vial in the unit of nmol CH<sub>4</sub> min<sup>-1</sup> mg protein<sup>-1</sup>, was determined. The determined rate data were fit into Michaelis-Menten competitive, noncompetitive, and uncompetitive inhibition models (Table S8) to determine the maximum CH<sub>4</sub> production rate V<sub>max</sub>, the half-velocity constant K<sub>m</sub>, and the inhibitory constant K<sub>1</sub> of N<sub>2</sub>O on CH<sub>4</sub> production from the different substrates. The best-fit inhibition model was chosen based on the highest coefficient of determination (R<sup>2</sup>) and the lowest standard deviation of the residuals (Table S9). Each datum point on the Michaelis–Menten plots (Figure 5) represents a CH<sub>4</sub> production rate generated from at least four time points for one substrate concentration [S].

# Genomic DNA extraction and 16S ribosomal RNA gene amplicon sequencing

DNA extraction and PCR assays followed established procedures [46] and details are provided in the Supplemental Information. The purified DNA samples were processed and barcoded with primers 341F/785R targeting the V3/V4 region of the prokaryotic 16S rRNA gene [47] following established procedures [48]. The resulting sequence data were analyzed using the QIIME 2 v2021.4 environment [49]. The precise programs and settings are described in the Supplemental Information, and the QIIME 2 pipeline script and custom R file employed to parse results are available at https://github.com/rwmurdoch/methanogens\_and\_N2O. The raw amplicon library reads were deposited in the Sequence Read Archive (SRA) under accessions SRR19782291 to SRR19782296.

#### Quantitative real-time PCR and growth yield calculations

Quantitative real-time PCR (qPCR) to enumerate archaeal 16S rRNA genes in *M. barkeri* and methanogenic mixed cultures followed established protocols using primer set Mtgen835F/918R and probe FAM-Mtgen831 (Table S10) [50]. Samples for enumeration of cell numbers were collected at the beginning and at the end of the prolonged growth experiments. Average growth yields of methanogens were calculated from the changes in 16S rRNA gene copy numbers in triplicate culture vessels divided by the total amounts of CH<sub>4</sub> produced over the same time period (Table S11). Reported growth yield (i.e. cells produced per  $\mu$ mol of CH<sub>4</sub> formed) used conversion factors of 3 and 2.5 16S rRNA gene copies per methanogen cell for *M. barkeri* [51] and the enrichment cultures [52], respectively. For comparison with theoretical [53] and reported values from the literature (Table 2), growth yields were also calculated as  $\mu$ g of dry biomass per  $\mu$ mol of CH<sub>4</sub> formed



**Figure 2.** Effect of N<sub>2</sub>O on CH<sub>4</sub> production and growth yields in axenic *M*. Barkeri cultures; the upper panels show time courses of CH<sub>4</sub> production in cultures that received MeOH (A), H<sub>2</sub> (C), or acetate (E); the bottom panels display growth yields after 38-day incubation for *M*. Barkeri growing with MeOH (B), H<sub>2</sub> (D), or acetate (F); error bars represent the standard deviation of replicate samples and are not shown when smaller than the symbol size; n = 3 for (A), (C), and (E); n = 9 (including three technical replicates for triplicate biological samples) for (B), (D), and (F).

making the following assumptions: an average methanogen cell has a volume of 2.5  $\mu$ m<sup>3</sup> [54] with a density equal to water [55]. The dry cell biomass is 30% of the wet cell biomass [56], and 90% of the dry biomass represents organic material [55].

#### **Results**

# N<sub>2</sub>O has distinct impact on CH<sub>4</sub> production from different methanogenic substrates

In the absence of N<sub>2</sub>O, MeOH-grown *M. barkeri* cultures consumed  $895 \pm 10 \ \mu$ mol of MeOH within a 6-day incubation period and produced  $635 \pm 34 \ \mu$ mol of CH<sub>4</sub> (Fig. 2A). This stoichiometry closely matched the expected CH<sub>4</sub> production based on Equation (1):

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad \Delta G^{\circ} = -105 \text{ kJ mol}^{-1}CH_4 \quad (1)$$

Growth yields of  $5.3 \times 10^6 \pm 0.3 \times 10^6$  cells per  $\mu$ mol CH<sub>4</sub> were measured for cultures without N<sub>2</sub>O (Fig. 2B). By contrast, cultures that received 100 or 200  $\mu$ M N<sub>2</sub>O produced negligible amounts of CH<sub>4</sub> during the initial 6-day incubation period without any apparent growth after 6 days. Following an 11-day lag phase, cultures with 100  $\mu$ M N<sub>2</sub>O started consuming MeOH, and 626±20  $\mu$ mol of CH<sub>4</sub> were produced following a 38-day incubation period, indicating that 100  $\mu$ M N<sub>2</sub>O delayed, but

did not prevent, CH<sub>4</sub> production from MeOH by *M. barkeri* (Fig. 2A). Although complete conversion of MeOH to CH<sub>4</sub> according to Equation (1) was achieved in the presence of 100  $\mu$ M N<sub>2</sub>O over a prolonged 38-day incubation period, the growth yield decreased by 63.8% ±7.8% compared to cultures without N<sub>2</sub>O, (i.e. 1.9 × 10<sup>6</sup> ±0.4 × 10<sup>6</sup> vs. 5.3 × 10<sup>6</sup> ±0.3 × 10<sup>6</sup> cells were produced per  $\mu$ mol of CH<sub>4</sub> formed) (Fig. 2B). In the presence of 200  $\mu$ M N<sub>2</sub>O, only 55 ± 11  $\mu$ mol of CH<sub>4</sub> were produced over a 38-day incubation period, and the growth yield decreased by over 80% to 0.9 × 10<sup>6</sup> ±0.4 × 10<sup>6</sup> cells per  $\mu$ mol CH<sub>4</sub>, indicating a pronounced inhibitory effect of N<sub>2</sub>O on CH<sub>4</sub> production and growth of M. *barkeri*.

More pronounced N<sub>2</sub>O inhibition on CH<sub>4</sub> production and growth was observed in *M. barkeri* cultures that received H<sub>2</sub> as electron donor (i.e. hydrogenotrophic methanogenesis). In the absence of N<sub>2</sub>O, *M. barkeri* cultures, that received H<sub>2</sub> as electron donor, produced  $318 \pm 21 \,\mu$ mol of CH<sub>4</sub> from 1.24 mmol of H<sub>2</sub> over an 11-day incubation period, consistent with Equation (2):

$$4\mathbf{H}_2 + \mathbf{CO}_2 \rightarrow \mathbf{CH}_4 + 2\mathbf{H}_2\mathbf{O} \ \Delta \mathbf{G}^{\circ} = -131 \text{ kJ mol}^{-1}\mathbf{CH}_4 \qquad (2)$$

In the absence of N<sub>2</sub>O, a growth yield of 0.8 ×  $10^6 \pm 0.2 \times 10^6$  cells per  $\mu$ mol of CH<sub>4</sub> formed was measured (Fig. 2C and D). In the presence of 50 or 100  $\mu$ M N<sub>2</sub>O, CH<sub>4</sub> production by M. barkeri

cultures commenced after prolonged lag phases ranging from 13 to 17 days. At the end of the 38-day incubation period, M. *barkeri* cultures with 50  $\mu$ M N<sub>2</sub>O and produced 311±7  $\mu$ mol of CH<sub>4</sub>. The M. *barkeri* cultures that had received 100  $\mu$ M N<sub>2</sub>O only generated 17±3.2  $\mu$ mol of CH<sub>4</sub>, resulting in a 95% decrease in total CH<sub>4</sub> production compared to cultures without N<sub>2</sub>O. The average growth yield in cultures with 50 or 100  $\mu$ M N<sub>2</sub>O decreased by ~70% and 85% to 2.3 × 10<sup>5</sup>±0.3 × 10<sup>5</sup> and 1.2 × 10<sup>5</sup>±0.3 × 10<sup>4</sup> cells per  $\mu$ mol CH<sub>4</sub> formed, respectively, compared to cultures without N<sub>2</sub>O.

The most pronounced N<sub>2</sub>O inhibition was observed in acetatefed *M. barkeri* cultures, and 20  $\mu$ M N<sub>2</sub>O severely diminished CH<sub>4</sub> production (Fig. 2E). In the absence of N<sub>2</sub>O, *M. barkeri* produced 588 ± 24  $\mu$ mol of CH<sub>4</sub> from 605 ± 17  $\mu$ mol of acetate over a 38-day incubation period, consistent with Equation (3):

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \ \Delta G^{\circ} = -35 \text{ kJ mol}^{-1} CH_4 \quad (3)$$

In the presence of 20 or 50  $\mu$ M N<sub>2</sub>O, M. barkeri cultures only produced 21.3  $\pm$  4.1 and 20.4  $\pm$  3.8  $\mu$ mol of CH<sub>4</sub>, respectively, a decline of over 96% in total CH<sub>4</sub> production compared to cultures without N<sub>2</sub>O over the 38-day incubation period. Acetate-grown M. barkeri cultures that had received 20 or 50  $\mu$ M N<sub>2</sub>O generated 1.5  $\pm$  0.2  $\times$  10<sup>6</sup> and 1.0  $\pm$  0.3  $\times$  10<sup>6</sup> cells per  $\mu$ mol of CH<sub>4</sub> produced, decreases of 30.4%  $\pm$  3.3% and 52.5%  $\pm$  12.6%, respectively, compared to the average yield of 2.1  $\times$  10<sup>6</sup>  $\pm$  0.7  $\times$  10<sup>6</sup> cells per  $\mu$ mol CH<sub>4</sub> in cultures without N<sub>2</sub>O (Fig. 2F).

In all culture vessels with observed inhibition of methanogenic activity,  $N_2O$  concentrations remained constant throughout the experiment, consistent with the absence of a "nos" operon on the genome of *M. barkeri*. Taken together, these results demonstrate that micromolar levels of  $N_2O$  inhibit methanogenesis, reduce growth yields of this model methanogen, and further reveal that the inhibition is most pronounced for acetoclastic methanogenesis.

#### N<sub>2</sub>O adversely affects CH<sub>4</sub> production and methanogen growth yields in mixed methanogenic enrichment cultures

To examine the impact of N<sub>2</sub>O on mixed methanogen communities, growth and kinetic assays were performed with enrichment cultures derived from digester sludge. Microbial community analysis revealed the presence of diverse methanogen groups known to utilize acetate, H<sub>2</sub>/CO<sub>2</sub>, and MeOH as substrates, with distinct methanogen taxa prevalent under the different enrichment conditions (Tables S12 and S13). In cultures that received H<sub>2</sub> as electron donor, sequences representing the genus Methanobacterium and other unidentified members of the family Methanobacteriaceae dominated, whereas in acetate-fed cultures, Methanosarcina was the most abundant archaeal taxon. Methanomethylovorans was the most abundant archaeal genus in the MeOH-fed cultures, but sequences representing the genus Methanomassiliicoccus were also prevalent. 16S rRNA gene amplicons representing six families and four of the known eight orders of methanogens were represented in the examined mixed cultures derived from digester sludge (Fig. 3A and B; Table S12).

Without N<sub>2</sub>O addition, the mixed cultures produced 542.6 $\pm$ 23.3, 316.5 $\pm$ 25.4, and 589.2 $\pm$ 33.7  $\mu$ mol of CH<sub>4</sub> from 0.9 mmol of MeOH, 1.24 mmol of H<sub>2</sub>, or 0.6 mmol of acetate, consistent with Equations (1)–(3). When cultures were amended with 10 or 30  $\mu$ M N<sub>2</sub>O, CH<sub>4</sub> production in MeOH-, H<sub>2</sub>-, and acetate-fed methanogenic mixed cultures was substantially or completely

inhibited (Fig. 4A, C, and E). Even over an extended 42-day incubation period, the presence  $10 \,\mu$ M N<sub>2</sub>O still repressed the total CH<sub>4</sub> production in MeOH-, H<sub>2</sub>-, or acetate-grown mixed cultures by ~60%, 80% and 50%, respectively, compared to incubations without N<sub>2</sub>O. With 30  $\mu$ M N<sub>2</sub>O, only negligible amounts of CH<sub>4</sub> were detected in all incubations over a 42-day incubation period. Some N<sub>2</sub>O loss was observed in the mixed culture vessels that received H<sub>2</sub>/CO<sub>2</sub> or MeOH as substrates, with no more than 20% of the initial amount of N<sub>2</sub>O consumed. Taken together, these results demonstrate that N<sub>2</sub>O exerts a stronger inhibitory effect on methanogenesis in the mixed cultures harboring diverse methanogen populations than in axenic *M. barkeri* incubations.

Enumeration of total methanogens and total bacteria in the mixed cultures using qPCR revealed that N<sub>2</sub>O diminished methanogen growth yields (Fig. 4B, D, and F). The qPCR analysis further revealed significantly decreased ratios of methanogen-tobacterial 16S rRNA genes in all N<sub>2</sub>O-treated cultures (Fig. 3C–E), illustrating that N<sub>2</sub>O impacted the methanogen populations much more strongly than the bacterial populations. In the absence of N<sub>2</sub>O, the average growth yields of methanogens in the enrichment cultures with MeOH, H<sub>2</sub>, or acetate were 0.6  $\times$  10  $^7\pm0.1$   $\times$  10  $^7,$  0.6  $\times$  10  $^5\pm0.3$   $\times$  10  $^5,$  and 1.0  $\times$  10  $^6\pm0.1$   $\times$  $10^6$  cells per  $\mu$ mol of CH<sub>4</sub> formed, respectively. In the presence of 10  $\mu$ M N<sub>2</sub>O, the growth yields of methanogens declined by ~90%, 60%, and 50% in enrichment cultures that received MeOH, H<sub>2</sub>, and acetate, respectively (Fig. 4B, D, and F). Only negligible CH4 production and methanogen growth were measured in all mixed cultures with 30  $\mu$ M N<sub>2</sub>O (Fig. 4B, D, and F). Taken together, the results of the mixed culture studies corroborate that N2O concentrations in the low micromolar range exhibit pronounced inhibitory effects on hydrogenotrophic, methylotrophic, and acetoclastic methanogenesis in microbial communities.

# Kinetic studies confirm potent N<sub>2</sub>O inhibition of methane formation rates

Whole-cell suspension assays using *M. barkeri* and the methanogenic mixed cultures were performed to quantitatively assess the inhibitory effects of N<sub>2</sub>O on CH<sub>4</sub> production from each methanogenic substrate (Table S1). The Michaelis–Menten single-substrate single-inhibitor model ( $R^2 > 0.95$ ) best explained the trends of CH<sub>4</sub> production rates versus increasing substrate concentrations (Fig. 5). Among all assays with *M. barkeri* and the mixed cultures, maximum CH<sub>4</sub> production rates (i.e.  $V_{max}$  values) of acetate-fed cultures were most strongly affected by increasing N<sub>2</sub>O concentrations, followed by the H<sub>2</sub>- and MeOH-fed cultures (Figs 2 and 3).

In the absence of  $N_2O\!\!,$  the  $V_{max}$  values for  $CH_4$  production in MeOH-, H<sub>2</sub>-, or acetate-amended M. barkeri cell suspension assays were  $440.4 \pm 10.2$ ,  $138.1 \pm 6.9$ , and  $40.8 \pm 4.7$  nmol CH<sub>4</sub> min<sup>-1</sup> mg protein<sup>-1</sup>, respectively (Fig. 5A, Table 1). The addition of  $N_2O$  decreased the  $V_{max}$  of  $CH_4$  production in MeOH-,  $H_2$ -, and acetate-fed M. barkeri cell suspension assays to different extents. Rate data determined in M. barkeri suspensions assays with MeOH fit the Michaelis–Menten inhibition model best. The V<sub>max</sub> values declined by  $\sim$ 45% and 57% to 244.1 ± 38.4 and 188.8 ± 32.5 nmol  $CH_4 min^{-1} mg protein^{-1}$ , respectively, in the presence of 100 and 200  $\mu$ M N<sub>2</sub>O. The determined inhibitory constant, K<sub>I</sub>, of N<sub>2</sub>O on methylotrophic CH<sub>4</sub> production was  $130.9 \pm 4.7 \ \mu$ M in M. barkeri cell suspensions (Table 1), indicating that N<sub>2</sub>O concentrations around 130  $\mu$ M reduced the maximum CH<sub>4</sub> production rate (V<sub>max</sub>) by 50%. More pronounced N<sub>2</sub>O inhibition was observed in M. barkeri whole-cell suspensions assays using H<sub>2</sub> as the electron donor for CO<sub>2</sub> reduction (Fig. 5B). The addition of 50 and 100  $\mu$ M



**Figure 3.** The composition and relative abundances of total sequences representing methanogenic archaea in mixed cultures enriched with acetate,  $H_2/CO_2$ , or MeOH; (A) phylogenetic placements of archaeal 16S rRNA gene amplicons detected in the enrichment cultures; the highlighted lobes indicate major clades of archaea known or suspected to produce  $CH_4$ ; the circles indicate best phylogenetic placements of archaeal taxa identified across all enrichment conditions; the size of the circle is proportional to the number of actual sequence variants (ASVs) detected; large shaded areas indicate archaeal superphyla, including *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanohaloarchaeota*, and *Nanoarchaeota* (DPANN) and *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota* (TACK); see Supplemental Information for details on tree construction and fragment placement methodology; (B) relative abundances of total sequences representing methanogenic archaea in mixed cultures; panels (C) MeOH, (D)  $H_2/CO_2$ , and (E) acetate depict qPCR data showing the proportional changes of total bacterial and total archaeal (methanogen) 16S rRNA genes in the mixed cultures without  $N_2O$  and in the presence of 10 and 30  $\mu$ M  $N_2O$ ; error bars represent the standard deviation of replicate samples).

 $N_2O$  reduced the  $V_{\text{max}}$  values in  $H_2\text{-}\text{fed}$  M. barkeri cell suspension assays by  $\sim$ 40% and 57% to 82.8  $\pm$  29.5 and 59.9  $\pm$  5.9 nmol of CH<sub>4</sub> min<sup>-1</sup> mg protein<sup>-1</sup>, respectively. The model simulation determined a  $K_I$  value of 90.6  $\pm$  10.8  $\mu$ M N<sub>2</sub>O (Fig. 5B, Table 1), indicating a stronger inhibition of N<sub>2</sub>O on CH<sub>4</sub> production in H<sub>2</sub>- versus MeOH-amended M. barkeri cell suspension assays. The M. barkeri assays with acetate as substrate showed the most pronounced inhibition by N<sub>2</sub>O (Fig. 5C, Table 1). In assays amended with 20 and 40  $\mu M$  N\_2O, the  $V_{max}$  values decreased by 21% and 44% to 32.1  $\pm$  4.2 and 22.8  $\pm$  2.5 nmol of CH4 min^1 mg protein^1, respectively. From the best-fit inhibition model, a  $K_I$  value of 24.8±2.6  $\mu$ M was determined for N<sub>2</sub>O inhibition of acetoclastic methanogenesis in M. barkeri cell suspensions. Collectively, the cell suspension assays corroborate strong inhibitory effects of N<sub>2</sub>O on methanogenesis, with the kinetics of acetoclastic methanogenesis being most impacted by N<sub>2</sub>O.

Similar kinetic responses were observed for whole-cell suspension assays conducted with the methanogenic mixed cultures pregrown with the respective substrates (Fig. 5D–F). In the absence of  $N_2O$ , the determined  $V_{max}$  values for mixed culture cell

suspension assays that received MeOH, H<sub>2</sub>, or acetate were  $209.0 \pm 4.4$ ,  $105.8 \pm 3.7$ , and  $22.8 \pm 0.7$  nmol CH<sub>4</sub> min<sup>-1</sup> mg protein<sup>-1</sup>, respectively (Table 1). Notably, the methanogenic mixed cultures were more sensitive to N2O than axenic M. barkeri cultures irrespective of the type of methanogenic substrate provided. In cell suspension assays that received MeOH, the presence of 50 and 100  $\mu$ M N<sub>2</sub>O reduced the V<sub>max</sub> values by  $37.4 \pm 10.8\%$  and  $75.4 \pm 21.4\%$ , respectively, compared to assays without N2O. The inhibition model determined a K1 value of 109.9  $\pm$  6.8  $\mu M$  for N\_2O inhibition on CH4 production in cell suspension assays amended with MeOH (Fig. 5D, Table 1). In H<sub>2</sub>amended cell suspension assays, the presence of 30 and 60  $\mu$ M  $N_2O$  decreased the  $V_{max}$  values by  $32.3 \pm 9.8$  and  $65.4 \pm 12.9\%$ , respectively, compared to assays without  $N_2O$  (Fig. 5E, Table 1). The best-fit inhibition model determined a  $K_I$  value for  $N_2O$ inhibition of  $62.1\pm6.4~\mu M$  for CH<sub>4</sub> production in assays that received H<sub>2</sub> as electron donor. Consistent with the observations made with M. barkeri, the most pronounced N<sub>2</sub>O inhibition on CH<sub>4</sub> production rates was observed in mixed culture cell suspension assays that received acetate as substrate (Fig. 5F). In



**Figure 4.** Effects of  $N_2O$  on  $CH_4$  production and growth yields in methanogenic mixed cultures enriched with MeOH,  $H_2$ , or acetate; the upper panels depict  $CH_4$  production from MeOH (A),  $H_2$  (C), and acetate (E); the bottom panels demonstrate methanogen growth yield differences in cultures amended with MeOH (B),  $H_2$  (D), or acetate (F); error bars represent standard deviation and are not shown when smaller than the symbol size (n = 3 for upper panels; n = 9 for bottom panels [three technical replicates of triplicate biological samples]).

the presence of 10 and 30  $\mu$ M N<sub>2</sub>O, the V<sub>max</sub> values in suspension assays with acetate decreased by 38±7.8% and 74.9±16.0%, respectively, compared to assays without N<sub>2</sub>O. Using the best-fit Michaelis–Menten inhibition model, a K<sub>I</sub> value of 17.7±1.8  $\mu$ M was determined for N<sub>2</sub>O inhibition of CH<sub>4</sub> production in cell suspension assays with acetate (Table 1).

Taken together, the experimental data demonstrate that dissolved N<sub>2</sub>O concentrations in the low  $\mu$ M range (i.e. 20–100  $\mu$ M) repress CH<sub>4</sub> production and reduce growth yields of *M. barkeri* in axenic culture and of different methanogen guilds in methanogenic mixed cultures. Evaluation of the kinetic data determined in cell suspension assays revealed distinct N<sub>2</sub>O inhibition patterns for CH<sub>4</sub> production from acetate, H<sub>2</sub>, and MeOH, with the rate of acetoclastic CH<sub>4</sub> production being most sensitive to N<sub>2</sub>O inhibition. The K<sub>I</sub> values for N<sub>2</sub>O inhibition of CH<sub>4</sub> production from key methanogenic substrates ranged between 18 and 130  $\mu$ M, suggesting N<sub>2</sub>O can impact CH<sub>4</sub> production and emissions in diverse ecosystems, including critical zone environments (e.g. thawing permafrost).

#### Discussion

Microorganisms drive global C and N cycling and ultimately control  $CH_4$  and  $N_2O$  production, consumption, and thus emissions to the atmosphere [10, 11, 13]. Predictive climate models must consider the responses of microbial  $CH_4$  and  $N_2O$  production

under environmental change scenarios [10, 57]. Quantitative assessment of feedbacks that affect CH<sub>4</sub> production are crucial for refining greenhouse gas emission models. This study investigated the feedback effects between two important greenhouse gases, specifically the inhibitory effect of N<sub>2</sub>O on archaeal CH<sub>4</sub> production, to provide quantitative data that link environmental N<sub>2</sub>O concentrations with methanogenesis. The findings demonstrate that environmentally relevant, micromolar levels of N<sub>2</sub>O suppress CH<sub>4</sub> production and the growth of methanogens. The determined K<sub>1</sub> values reveal a concentration-dependent, progressively negative feedback by N<sub>2</sub>O on archaeal CH<sub>4</sub> formation rates and the total amounts of CH<sub>4</sub> produced and show that the strength of the inhibition is most pronounced for acetoclastic methanogenesis.

Assuming the current day partial pressure of 335 ppb  $N_2O$  in the atmosphere, the theoretical concentration of  $N_2O$  in air-equilibrated water should be around 7 nM; however, substantially elevated levels of dissolved  $N_2O$  have been observed in groundwater and watersheds [33, 42]. In areas impacted by agricultural activities (e.g. fertilizer application), dissolved groundwater  $N_2O$  concentrations can exceed 100  $\mu$ M [42]. Even in some remote, natural aquatic systems, such as ice-covered Antarctic lakes,  $N_2O$  concentrations of up to 86  $\mu$ M have been reported [58, 59].  $N_2O$  is generated during N cycling and major formation processes include microbial denitrification, ammonia oxidation, and abiotic chemodenitrification [44]. Based on the physiology of the microorganisms (e.g. ammonia oxidizers are



**Figure 5.** Kinetics of CH<sub>4</sub> production from MeOH, H<sub>2</sub>, and acetate in whole-cell suspension assays of *M. Barkeri* and the methanogenic mixed cultures in the presence of increasing concentrations of N<sub>2</sub>O; the upper panels show the Michaelis–Menten plots of CH<sub>4</sub> production rates versus the respective substrate concentrations in cell suspensions of *M. Barkeri* without and in the presence of increasing N<sub>2</sub>O concentrations in basal salt medium amended with MeOH (A), H<sub>2</sub> (B), or acetate (C); the bottom panels show Michaelis–Menten plots of CH<sub>4</sub> production rates versus the respective substrate concentrations in concentrated whole-cell suspensions of the methanogenic mixed cultures without N<sub>2</sub>O and in the presence of increasing N<sub>2</sub>O levels in basal salt medium amended with MeOH (D), H<sub>2</sub> (E), or acetate (F); the shaded ribbons represent the standard distances (95% confidence interval) between the measured values and the nonlinear regression lines.

strict aerobes) and the thermodynamics of the processes (e.g. nitrate and nitrite reduction are associated with a greater change in Gibbs free energy than methanogenesis), one might argue that N<sub>2</sub>O formation and methanogenesis are physically separate processes, thus limiting the exposure and inhibitory effects of N<sub>2</sub>O on methanogens. Such redox stratification does occur; however, most environmental matrices, such as soils, are highly heterogenous and characterized by dynamic spatial and temporal gradients resulting in patchy distribution of redox processes. N<sub>2</sub>O is water-soluble and, depending on hydrology, can reach other redox zones [60]. Consequently, impacts of elevated N<sub>2</sub>O on various biogeochemical processes, including those associated with greenhouse gas emissions, are likely. Laboratory studies have reported inhibitory effects of nitrogen oxides (NOx), including N<sub>2</sub>O, on methanogenesis [34–37, 61, 62]; however, the available data are scarce and no uniform pattern has emerged that would support a quantitative relationship between N<sub>2</sub>O and microbial CH<sub>4</sub> production. Consequently, this negative feedback of N<sub>2</sub>O on CH<sub>4</sub> production has not been considered in greenhouse gas emission models.

The evaluation of N<sub>2</sub>O inhibition on CH<sub>4</sub> production from methanogenic substrates (i.e. MeOH, acetate, and H<sub>2</sub>/CO<sub>2</sub>) with both the model methanogen *M. barkeri* and digester sludgederived mixed methanogenic cultures quantitatively links N<sub>2</sub>O concentrations with methanogen activity and growth. The growth experiments illustrate that micromolar N<sub>2</sub>O concentrations affect CH<sub>4</sub> production, and the whole-cell suspension assays and kinetic model simulations provide a plausible explanation for the inconsistent literature reports about N<sub>2</sub>O effects on methanogenesis. Specifically, acetoclastic methanogenesis was most sensitive to N<sub>2</sub>O (K<sub>1</sub> values of 18–25  $\mu$ M), followed by hydrogenotrophic (K<sub>1</sub> 60–90  $\mu$ M) and methylotrophic methanogenesis (K<sub>1</sub> 110– 130  $\mu$ M), indicating that the type of methanogenesis to N<sub>2</sub>O. N<sub>2</sub>O inhibition was significantly more pronounced in methanogenic enrichment cultures than in axenic M. barkeri cultures, and 30  $\mu$ M N<sub>2</sub>O prevented CH<sub>4</sub> production and methanogen growth in the mixed culture experiments regardless of the type of methanogenic substrate utilized. Previous studies support that mixed methanogenic communities are more sensitive to N2O than commonly studied model methanogen isolates [34, 35, 62, 63]. These observations suggest that the axenic methanogen cultures used to elucidate the biochemistry and genetics of methanogenesis may not serve as general models for other features of methanogen biology (e.g. N<sub>2</sub>O inhibition). The reasons for the reduced sensitivity of axenic versus mixed methanogen cultures to N<sub>2</sub>O may be a result of long-term adaptation of the isolates to laboratory cultivation, or not-yet-characterized microbe-microbe interaction networks render mixed cultures more susceptible to  $N_2O$  inhibition. The enrichment cultures used to determine the K<sub>I</sub> values for N<sub>2</sub>O inhibition harbored diverse methanogen groups (Fig. 3A), and kinetic studies (e.g. determination of K<sub>I</sub> values for N<sub>2</sub>O inhibition) with representative isolates of the various lineages are warranted to capture the breadth of methanogen responses to  $N_2O$ .

Taken together, the low  $\mu$ M range K<sub>I</sub> values of N<sub>2</sub>O that impact methanogenesis suggest major consequences of rising N<sub>2</sub>O concentrations for C cycling. Of note, the vast majority of the annual ~1 billion metric tons of biogenic CH<sub>4</sub> is generated from acetateand H<sub>2</sub>-driven CO<sub>2</sub> reduction [16–20, 64], the two processes with the lowest observed K<sub>I</sub> values for N<sub>2</sub>O inhibition. It is therefore reasonable to predict that elevated environmental N<sub>2</sub>O will impact CH<sub>4</sub> production and methanogen growth in ecosystems with high bioavailable C and N loads, such as wetlands, sediments, and permafrost soils.

Key enzyme systems involved in  $CH_4$  production and energy conservation require cobamide prosthetic groups [65] (Fig. 1). The super-reduced Co(I) form of cobamides is susceptible to

Table 1. Kinetic (V <sub>max</sub> , K	(m) and inhibition (K <sub>I</sub> ) para	meters for CH <sub>4</sub> produc	ction from MeOH, H <sub>2</sub>	, and acetate deter	mined in concentrated
whole-cell suspension a	issays of M. barkeri and me	thanogenic mixed cul	ltures in response to	increasing N <sub>2</sub> O co	ncentrations.ª

Culture	Substrate	N <sub>2</sub> Ο (μM)	$V_{max}$ (nmol CH <sub>4</sub> min <sup>-1</sup> mg protein <sup>-1</sup> )	<b>K</b> <sub>m</sub> (μ <b>M</b> )	K <sub>I</sub> (μM)
M. barkeri	MeOH	0	$440.4 \pm 10.2$	$2.1 (\pm 0.1) \times 10^3$	$130.9 \pm 4.7$
		100	$244.1 \pm 38.4$		
		200	$188.8 \pm 32.5$		
M. barkeri	H <sub>2</sub>	0	$138.1 \pm 6.9$	$51.1 \pm 7.2$	$90.6 \pm 10.8$
		50	$82.8 \pm 29.5$		
		100	$59.9 \pm 5.9$		
M. barkeri	Acetate	0	$40.8 \pm 4.7$	$13.4 (\pm 2.7) \times 10^3$	$24.8 \pm 3.1$
		20	$32.1 \pm 4.2$		
		50	$22.8 \pm 2.5$		
Mixed culture	MeOH	0	$209.0 \pm 4.4$	$359.3 \pm 30.8$	$109.9 \pm 6.8$
		50	$130.8 \pm 22.6$		
		100	$51.4 \pm 44.7$		
Mixed culture	H <sub>2</sub>	0	$105.8 \pm 3.7$	$19.0 \pm 2.3$	$62.1 \pm 6.4$
		30	$71.6 \pm 10.4$		
		60	$36.6 \pm 13.6$		
Mixed culture	Acetate	0	$22.8 \pm 0.7$	$302.2 \pm 46.2$	$17.7 \pm 1.8$
		10	$14.1 \pm 1.8$		
		30	5.7 ± 3.6		

<sup>a</sup>Data listed show results from the best fit Michaelis-Menten single-substrate-single-inhibitor models. Error values represent 95% confidence intervals.

oxidants such as N<sub>2</sub>O, a plausible mechanism for the observed inhibition of methanogenesis [29, 34, 63]. The experimental efforts demonstrated that acetoclastic methanogenesis was most sensitive to N<sub>2</sub>O, with  $K_l$  values in the range of 18–25  $\mu$ M, indicating that N<sub>2</sub>O concentrations in the range of 20  $\mu$ M would reduce the  $V_{max}$  of  $CH_4$  production from acetate by 50%. The K<sub>I</sub> values for N<sub>2</sub>O inhibition determined for hydrogenotrophic and methylotrophic methanogenesis ranged between 60-90 and 110–130  $\mu$ M, respectively. The reasons for the apparently substrate-specific K<sub>1</sub> values for N<sub>2</sub>O inhibition likely reflect differences in the pathways leading to CH<sub>4</sub> production from acetate, H<sub>2</sub>/CO<sub>2</sub> and MeOH (Fig. 1). The acetoclastic pathway involves two steps catalyzed by corrinoid-dependent enzyme systems, CODH/ACS and the MTR corrinoid enzyme complex [16, 65]. (Fig. 1), both of which are targets for N<sub>2</sub>O inhibition. Distinct cobamide-dependent enzyme systems catalyze the formation of CH<sub>3</sub>-CoM in the hydrogenotrophic and the methylotrophic pathways [22, 66]. Both the acetoclastic and hydrogenotrophic pathways depend on the MTR corrinoid enzyme complex to generate CH<sub>3</sub>-CoM and to conserve energy [22, 27]. By contrast, methylotrophic methanogens can directly generate CH<sub>3</sub>-CoM without the energy-conserving MTR corrinoid enzyme complex from methylated compounds (e.g. MeOH) via a substrate-specific, corrinoid-dependent methyltransferase complex (i.e. MtaA, MtaB, and MtaC in the case of MeOH) [67]. The experimental efforts consistently demonstrated that CH<sub>4</sub> production from acetate and H<sub>2</sub>/CO<sub>2</sub> exhibited 7- and 3-fold higher sensitivities, respectively, to N<sub>2</sub>O than CH<sub>4</sub> production from MeOH. These findings suggest that N<sub>2</sub>O inhibition of methanogenesis is related to the oxidation of the super-reduced Co(I) cobamide, which is essential for the corrinoid enzyme complexes involved in the different methanogenesis pathways. While the differential susceptibilities of pathwayspecific corrinoid-dependent enzymatic steps can explain the distinct inhibitory effects of N<sub>2</sub>O on CH<sub>4</sub> production via the acetoclastic, hydrogenotrophic, and methylotrophic pathways, detailed enzymatic studies would be needed to assess the responses of individual enzyme systems to N<sub>2</sub>O. Kinetic data for the enzymatic regeneration of the super-reduced Co(I) state are lacking, and the rates of reduction may differ between enzymes and pathways, which could contribute to the observed physiological response of decreased  $CH_4$  production. Recovery from N<sub>2</sub>O inhibition was not a focus, but the experimental data indicate partial recovery of methane formation in N<sub>2</sub>O-treated axenic and mixed cultures; however, the methanogen growth yields remained lower in N<sub>2</sub>O-treated cultures compared to controls without N<sub>2</sub>O even over the extended incubation period. Further studies are needed to generate mechanistic insights into modes of recovery from N<sub>2</sub>O inhibition.

The presence of N<sub>2</sub>O significantly decreased growth yields or completely abolished growth in the examined methanogenic cultures (Table 2). The type of methanogenic substrate utilized determines the fraction of electrons available from electron donor oxidation directed toward cell synthesis and thus governs the growth yields of methanogens [53, 68]. N<sub>2</sub>O affects corrinoiddependent enzymes involved in electron transfer (e.g. the MTR enzyme complex), and it is not surprising that N<sub>2</sub>O interferes with energy conservation in methanogens. Consistently, enumeration of methanogen 16S rRNA genes at the termination of all growth experiments illustrated that N<sub>2</sub>O not only negatively impacted CH4 production but also methanogen growth yields. An alternate explanation for the reduced methanogen growth yields in the mixed cultures exposed to N<sub>2</sub>O could be competition for electron donor (e.g. H<sub>2</sub>); however, the sequencing and the qPCR data do not support this hypothesis, and N<sub>2</sub>O inhibition explains the decline of methanogens and the changes of methanogento-bacteria ratios. The measured methanogen growth yield data in the absence of N<sub>2</sub>O were on par with reported experimental data and closely matched the theoretical values (i.e. yields calculated based on thermodynamics) (Table 2). One exception were the growth yields measured in H<sub>2</sub>/CO<sub>2</sub>-fed M. barkeri cultures, which were  $\sim$ 10-fold lower than data reported in the literature. The most pronounced growth suppression was observed in the mixed methanogenic cultures, where 30  $\mu$ M N<sub>2</sub>O was sufficient to prevent the growth of methanogenic archaea. Collectively, the data show that micromolar concentrations of N<sub>2</sub>O decrease or abolish CH<sub>4</sub> production, reduce methanogen growth yields, and exhibit progressively negative feedback on microbial CH4 production.

**Table 2.** Comparison of growth yields of methanogens utilizing different substrates with values collected from the peer-reviewed literature and calculated values based on thermodynamics and bioenergetic principles.

	Substrates	N2Ο (μΜ)	Growth yield ( $\mu$ g organic matter per $\mu$ mol CH $_4$ formed)		
Cultures			Measured <sup>a</sup>	Predicted from thermodynamics <sup>b</sup>	Range (references)
M. barkeri	MeOH	0	3.57±0.32	5.2	3.3–6.4 ([71, 72])
	MeOH	100	$1.30 \pm 0.28$		
	MeOH	200	$0.62 \pm 0.03$		
	H <sub>2</sub>	0	$0.53 \pm 0.14$	3.6	5.4-8.6 ([73])
	H <sub>2</sub>	50	$0.15 \pm 0.02$		
	H <sub>2</sub>	100	$0.08 \pm 0.02$		
	Acetate	0	$1.45 \pm 0.46$	2.1	1.4-5.7 ([74])
	Acetate	20	$1.02 \pm 0.16$		
	Acetate	50	$0.70 \pm 0.02$		
Mixed methanogenic cultures	MeOH	0	$4.30 \pm 0.56$	5.2	3.3-6.4 ([72])
-	MeOH	10	$0.14 \pm 0.05$		
	MeOH	30	NA		
	H <sub>2</sub>	0	$0.43 \pm 0.05$	3.6	1.3-7.2 ([14])
	H <sub>2</sub>	10	$0.16 \pm 0.01$		(L 27
	H <sub>2</sub>	30	NA		
	Acetate	0	$0.65 \pm 0.05$	2.1	1.4-5.7 ([23, 64, 75])
	Acetate	10	$0.31 \pm 0.02$		(L 2)
	Acetate	30	NA		

<sup>a</sup>Cell numbers were determined with qPCR. Because *M. barkeri* has three copies of the 16S rRNA gene, the qPCR results were divided by a factor of three to obtain cell numbers. Calculation of methanogen cell numbers in the mixed cultures assumed an average 16S rRNA gene content of 2.5. <sup>b</sup>Theoretical values calculated in this study based on thermodynamics and published information [53].

Microbial processes are strongly influenced by environmental factors and their responses to climate change vary both spatially and temporally [11, 69]. To improve the predictive power of climate models and potentially justify the application of biotechnological approaches for managing greenhouse gas emissions, interactions and feedbacks between relevant biotic/abiotic processes must be understood and quantitatively captured [70]. Attempts have been made to include microbial data (i.e. biomass, enzyme, and growth kinetics) to improve climate models [70], but the incorporation of multifactorial, multidirectional, and often nonlinear biotic/abiotic feedbacks underlying the global CH<sub>4</sub> and N<sub>2</sub>O budgets is challenging and requires robust quantitative data. The determined K<sub>I</sub> values for N<sub>2</sub>O inhibition of methanogenesis reveal a relevant negative feedback effect on CH4 emissions, and the new quantitative information generates opportunities to refine CH4 emission models.

### Supplementary material

Supplementary material is available at The ISME Journal online.

### **Conflicts of interest**

The authors declare no competing interests.

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### Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The raw amplicon library reads are available in the SRA repository.

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