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Viral involvement in microbial anaerobic methane oxidation-mediated arsenic mobilization in paddy soil

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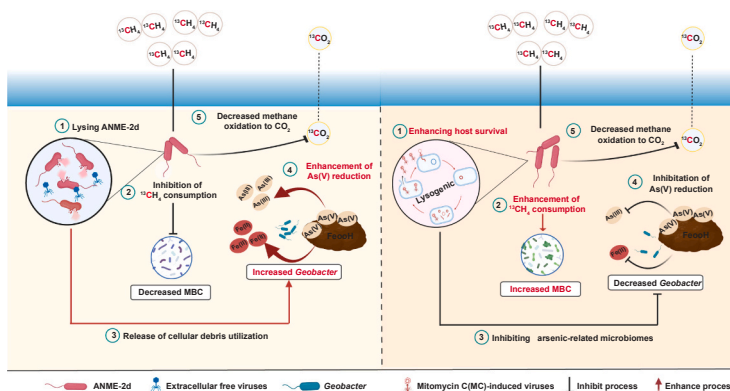
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HIGHLIGHTS

- Extracellular free virus increased $^{13}\text{CH}_4$ -mediated As reduction.
- Extracellular free virus suppressed microbial $^{13}\text{CH}_4$ consumption.
- MC-induced virus had opposite effect on $^{13}\text{CH}_4$ and As behaviors to that of free virus.
- Both types of viruses inhibited $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$.
- Virus-mediated $^{13}\text{CH}_4$ assimilation into MBC influenced microbial populations.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic oxidation of methane (AOM) facilitates arsenic (As) mobilization, posing a significant environmental risk. Soil viruses potentially participate in the microbial AOM process, yet their roles in methane-mediated As mobilization of paddy soil remain elusive. Here, an anaerobic microcosm study was conducted by inoculating microbial suspension with extracellular free virus and mitomycin C (MC)-induced virus, along with $^{13}\text{CH}_4$ injection. The results showed that extracellular free virus enhanced while MC-induced virus suppressed $^{13}\text{CH}_4$ -mediated As mobilization. During the AOM process, both viruses inhibited $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$. However, the extracellular free virus suppressed whereas the MC-induced virus enhanced $^{13}\text{CH}_4$ consumption, likely attributed to the viral influence on the ANME-2d abundance. The methane consumption differences were inferred to influence As reduction, as evidenced by a strong correlation between As(III) and $^{13}\text{CH}_4$ consumption

Abbreviations: AOM, anaerobic oxidation of methane; MC, mitomycin C; TFF, tangential flow filtration; MBC, microbial biomass carbon; AMGs, auxiliary metabolic genes; ANME, anaerobic methanotrophic archaea.

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concentrations. Moreover, virus-mediated methane assimilation into microbial biomass carbon influenced the overall microbial population. An increased abundance of *Geobacter* in the extracellular free virus treatment elevated net As(III) concentrations (up to 260 %) relative to treatment without virus in the presence of $^{13}\text{CH}_4$. In contrast, MC-induced virus led to a net 122 % reduction in As(III) concentration due to decreased *Geobacter* abundance. These findings provide new insights into soil viruses in microbial AOM-driven As mobilization, highlighting their crucial functions in soil ecosystems.

1. Introduction

Arsenic (As) contamination is widespread in paddy soils, leading to a serious food safety issue as rice is consumed daily by half of the global population [1]. Under flooding conditions, As mobilization from soil solids occurs through the reductive dissolution of As-bearing Fe(III) (hydr)oxides and microbial reduction of As(V) to the more mobile and toxic As(III) form [2]. Both cytoplasmic and dissimilatory As(V)-reducing microorganisms are crucial for As(V) reduction [3]. Cytoplasmic As(V)-reducing bacteria can import As(V) into their cytoplasm where it is reduced to As(III) by the soluble arsenate reductase (encoded by the *arsC* gene) before being expelled from the cell [3,4]. Dissimilatory As(V)-respiring bacteria, particularly *Geobacter*, possess the *arrA* gene, which allows them to use As(V) as a terminal electron acceptor, thereby causing its reduction [3,5]. These microbial processes facilitate As accumulation in rice plants, posing significant risks to human health and ecosystems [6]. Therefore, it is crucial to seek methods to mitigate As mobilization through alterations of microbial metabolic activities [4]. In particular, organic matter is a key regulator of the rate and extent of microbially mediated As release from As-contaminated environments [4,7].

Methane (CH_4) is a prominent organic substrate in paddy fields that has been implicated as an electron donor contributing up to 49 % of total As release from soils [7]. Microbial anaerobic oxidation of methane (AOM), serves as an energy source for microbial growth and enables microbial reduction of a diversity of oxidants [8], with CH_4 being oxidized to CO_2 [9]. This process is mainly catalyzed by anaerobic methanotrophic archaea (ANME) or bacteria (NC10 phylum) [9,10]. Recent research revealed that anaerobic methanotrophic archaea (ANME), either alone or synergistically with arsenate-reducing bacteria (*Geobacter*), mediate the coupled pathway through reverse methanogenesis and respiratory arsenate reduction [7]. Moreover, recent studies reveal that up to 60 % of methane carbon can be assimilated into microbial biomass under anoxic/anaerobic conditions, rather than being solely oxidized to CO_2 [11,12]. This suggests that the methane sink capacity in anoxic/anaerobic environments may be appreciably underestimated, as a substantial portion of methane carbon is retained as microbial biomass [12–14]. Specifically, methane-derived C is incorporated into different components of the microbiome, such as phospholipid fatty acids (PLFA) in gram-negative, gram-positive and actinobacterial groups [9]. Therefore, anaerobic methane assimilation, as a crucial part of AOM in paddy soils, has the potential to significantly impact environmental processes. However, most studies have focused on redox coupling between methane and metal oxidants [15], with limited information available concerning the impact of methane assimilation during AOM on microbial As mobilization.

Viruses are the primary biological factor controlling indigenous microbial communities in most anaerobic environments [16]. Due to their high abundance and diversities, viruses garner attention for their importance in carbon biogeochemical cycling, especially regarding their influence on shaping microbial communities and metabolism [17]. Such processes are mediated by viral lysing [18–21] and the reprogramming of microbial metabolic processes through auxiliary metabolic genes (AMGs) [17,22,23]. Genomics provides intriguing insights into the potential interactions between virus-infecting methanotrophs and their impacts on methane oxidation. For instance, some virus-infecting methanotrophs possess large genomes that encode *pmoC*, potentially

accelerating methane oxidation by enhancing the activity of infected methanotrophs to improve their fitness [24]. Viruses also affect mutualistic sulfate-reducing bacteria, including *Deltaproteobacteria* and *Chloroflexi* [16]. These findings broaden our understanding of viruses and offer insights into their survival strategies in anaerobic environments. Recent in-situ soil experiments combining DNA stable-isotope probing with metagenomic analyses have revealed dynamic virus interactions with soil methanotroph or non-methanotrophic methylotroph populations, further confirming their potential roles in methane oxidation [25]. However, viruses in different life cycles (lytic versus lysogenic) play a crucial role in shaping virus-host interactions, significantly influencing microbially-mediated ecological processes [26]. In a lytic infection, viruses commandeer the host's metabolic machinery to produce progeny viruses, culminating in the lysis of the host cell and the subsequent release of both the viruses and cellular debris into the surrounding environment [27]. In contrast, during the lysogenic state, the viral genome integrates into the host genome or exists as a plasmid, facilitating the survival of microbial hosts under adverse environmental conditions [28]. To date, the effects of viruses in different life cycles on microbial AOM, particularly viral involvement in methane-mediated As mobilization, are poorly understood.

To gain a better understanding of virus effects on integrated AOM and As mobilization processes, we conducted an anaerobic incubation by re-inoculating different viral treatments. Extracellular free virus suspension (V1) collected by a tangential flow filtration (TFF) system and mitomycin C (MC)-induced virus suspension (V2) were combined with a microbial suspension (M) and incubated in the presence of argon (Ar) or ^{13}C -labelled methane ($^{13}\text{CH}_4$). We quantified differences in $^{13}\text{CH}_4$ consumption, $^{13}\text{CO}_2$ production and microbial biomass, and further determined how As biotransformations were influenced by the additions of viruses and $^{13}\text{CH}_4$. We further quantified the differences in anaerobic methane oxidation/arsenic-related function genes to gain a mechanistic understanding of the integrated processes. We hypothesize that (i) extracellular free viruses will decrease $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$ and inhibit As reduction by lysing their methanotroph hosts, and conversely, (ii) mitomycin C-induced viruses will enhance microbial $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$ and promote As reduction, as they aid the host bacteria in utilizing methane more efficiently under anaerobic conditions. Our results provide new insights into the roles of viruses in anaerobic methane oxidation and associated As mobilization, thereby enriching our understanding of these intricate biological processes.

2. Material and methods

2.1. Soil sampling and preparation

Soils were collected from a paddy field in Jiaxing, Zhejiang, China ($30^\circ 50' \text{N}$, $120^\circ 43' \text{E}$) in March 2022. This site is utilized for double-rice cultivation in the Yangtze River Delta [29]. Soil samples (0–20 cm) were collected with a stainless steel corer (sterilized with alcohol) after the rice harvest. Five replicate samples were homogenized by passing through a sterilized 2-mm sieve after removing plant residues to create a single composite sample. The soil was transferred on ice to the laboratory. Subsequently, a subsample was air-dried at room temperature for preparation of the experimental As-contaminated soil. Relevant physicochemical properties were previously described as follows: pH, 6.76; organic C, 54.4 g kg^{-1} ; total N, 2.1 g kg^{-1} ; and total As, 16.9 mg kg^{-1} .

[30]. The total As concentration was below the standard established for As-contaminated soils in China (30 mg kg^{-1}) [31].

To prepare the As-contaminated soil, $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich, purity $\geq 98\%$) was added to the air-dried soil to achieve a final total As concentration of 60 mg kg^{-1} soil [32]. After thorough mixing, the As-contaminated soil was pre-incubated in the dark at 25°C for one month to facilitate equilibration. Subsequently, the As-contaminated soil was divided into two subsamples. One subsample was sterilized with 100 kGy γ -radiation for 24 h, and the plate count method was employed to assess the sterilization effectiveness. Briefly, 0.5 g of sterilized soil was plated on TSB and PDA media; no microbial growth was observed on the plates over a 7-day incubation period [33]. The other subsample was used for viral and microbial extraction. The recovery of the indigenous microorganisms and viruses followed a previous report with minor modifications [18]. Specifically, sterile deionized water was added to simulate field flooding (2 cm above soil surface) followed by pre-incubation at 25°C for two weeks.

2.2. Preparation of native soil virus and microbial suspension

Four virus-related treatments were assessed in this study: non-virus (SM), autoclaved virus (DV), extracellular free virus (V1), and mitomycin C (MC)-induced virus (V2). Soil virus extractions were collected according to previous studies [19,34]. The extracellular free virus (V1) sample was collected from soil suspensions undergoing filtration through a TFF system as described previously [19]. Specifically, the pre-incubated soil (1.8 kg) was mixed with 1.8 L of sterile saline magnesium buffer (SM; 5.8 g NaCl , 2 g MgSO_4 , 50 mL Tris-HCl , $\text{pH } 7.5$) and vortexed at maximum speed for 3 min. This mixture was then shaken for 2 h at 4°C and 250 rpm . Subsequently, the soil slurry was centrifuged at 4°C for 20 min ($2500 \times g$), and the supernatant was filtered through a $2\text{-}\mu\text{m}$ cellulose acetate filter. The filtered supernatant was sequentially filtered and concentrated using the TFF system (Cogent μScale , Merck, America) with pore sizes of $0.22 \mu\text{m}$ and 100 kDa . Finally, the obtained retentate ($>0.22 \mu\text{m}$) was taken as the microbial suspension (M), while the concentrated filtrate ($100 \text{ kDa} - 0.22 \mu\text{m}$) was taken as the extracellular free virus suspension (V1). The remaining filtrate ($<100 \text{ kDa}$) was taken as non-virus suspension (SM buffer) [19,35]. In addition, part of the virus filtrate was heat-inactivated to create an “autoclaved virus” treatment (DV).

Another virus sample (V2) was obtained from the microbial suspension induced with mitomycin C (MC) [34]. In details, two equal volumes of microbial suspension (derived from the above-mentioned M suspension) were mixed with and without MC (final MC concentration of $1 \mu\text{g mL}^{-1}$) in sterile 250-mL flasks [36]. All suspensions were incubated for 18 h in the dark (room temperature), and then filtered through a $0.22\text{-}\mu\text{m}$ sterile filter (Thermo Scientific) and further concentrated using Amicon Ultra filters (Millipore) to obtain the MC-induced virus sample (V2) [20].

Overall, 50 mL of autoclaved virus (DV), 50 mL of extracellular free virus suspension (V1), 50 mL of MC-induced virus suspension (V2), 180 mL of microbial suspension (M) and 1400 mL non-virus suspension (SM) were stored at 4°C for use in microcosms within 24 h [35]. Before conducting the microcosms, the presence and purity of each viral and microbial suspension (V1, V2 and M) were verified and enumerated with epifluorescence microscopy (See the [supplementary information](#) for detailed methodology).

2.3. Anaerobic microcosm construction

Anaerobic microcosms were constructed by adding aliquots of dry sterilized soil (18 g) and different virus and microbial mixtures (4 mL) into sterile 100-mL serum vials, with $^{13}\text{CH}_4$ or Ar headspace injection. To simulate the natural abundance of virus and microbial communities in the in-situ soil environment, 1 mL virus suspension (i.e., non-virus SM, DV, V1 and V2) and 3 mL microbial suspension (M) were added

into the sterile vials. The four treatments were as follows: (1) 1 mL SM buffer + 3 mL microbial suspension (SM); (2) 1 mL DV + 3 mL microbial suspension (DV); (3) 1 mL extracellular free virus suspension + 3 mL microbial suspension (V1); and (4) 1 mL MC-induced virus + 3 mL microbial suspension (V2).

After the designated virus and microbial extracts were added to a given soil treatment, anoxic sterile water was added to simulate field flooding (2 cm above the soil surface). The total soil slurry volume in each bottle was 40 mL , thus MC concentration was less than $0.1 \mu\text{g mL}^{-1}$. The vials were sealed with a sterile butyl rubber septum and repeatedly (at least three times) vacuumed and refilled with an equal volume of Ar to produce anoxic soil slurries [37]. Subsequently, the soil slurries were pre-incubated for 24 h to eliminate any residual O_2 . The soil slurries were then treated with two amendments ($10\% \text{ v/v}$ in the headspace): i) 8 mL of $^{13}\text{CH}_4$ ($>99\%$, Sigma-Aldrich) as the CH_4 treatment ($^{13}\text{CH}_4$) or ii) 8 mL of Ar (99.999%) as Ar treatment. Each treatment was performed in triplicate and sampled on day 0 and 90 resulting in a total of 48 individual microcosms ($3 \text{ replicates} \times 2 \text{ gases} \times 4 \text{ treatments} \times 2 \text{ time points}$). The vials were incubated at 25°C in the dark for 90 days without shaking [38].

2.4. Gas and soil physicochemical analyses

Gas, soil and porewater samples were collected at the initial (day 0) and ending (day 90) incubation times. The amount of $^{13}\text{CH}_4$ oxidized was determined from the amount of $^{13}\text{CO}_2$ production [39]. The $^{13}\text{CH}_4$ consumption was calculated as the difference in concentration between days 0 and 90. The $^{13}\text{CH}_4$ and $^{13}\text{CO}_2$ concentrations were quantified using gas chromatography–mass spectrometry (GC-MS, 6890 N–5975 B; Agilent, USA). Briefly, $10 \mu\text{L}$ gas from each vial was sampled with a gas-phase injection needle and analyzed using a GC-MS and HP-PLOT U gas chromatography column (30 m , 0.32 mm , $10.00 \mu\text{m}$, 7-inch column holder) [40]. The total generated $^{13}\text{CO}_2$ included $^{13}\text{CO}_2$ in the headspace and dissolved in the liquid [41]. The generated $^{13}\text{CO}_2$ in the liquid phase was calculated using the method from a previous study [42]. All the net (Δ) values in each group were calculated as:

$$\text{Net } (\Delta) X = (X_{90\text{d}}^{13}\text{CH}_4 - X_{0\text{d}}^{13}\text{CH}_4) - (X_{90\text{d}}\text{Ar} - X_{0\text{d}}\text{Ar})$$

Where X represents the values of different physicochemical factors and functional genes. $X_{90\text{d}}^{13}\text{CH}_4$ and $X_{0\text{d}}^{13}\text{CH}_4$ are the values of X in the $^{13}\text{CH}_4$ treatment on day 90 and day 0, respectively. $X_{90\text{d}}\text{Ar}$ and $X_{0\text{d}}\text{Ar}$ are the values of X in the Ar treatment on day 90 and day 0, respectively.

Soil porewater was sampled by centrifugation at $3100 \times g$ for 8 min and acidified using 6 M HCl to prevent Fe oxidation [40]. Soil pH and Eh were determined using standard probes/meters. Total As, Fe, and Mn concentrations of porewater were determined by ICP-MS (NEX-ION300XX) [43]. The Fe(II) concentration was measured using the phenanthroline-spectrophotometric method [30]. As species were analyzed by HPLC-ICP-MS (PerkinElmer Series 200 HPLC and NEX-ION300XX, ICP-MS) using $20 \text{ mM NH}_4\text{H}_2\text{PO}_4$ ($\text{pH } 6.5$) as the mobile phase [40]. Soil microbial biomass carbon (MBC) was determined by the chloroform fumigation- K_2SO_4 extraction method [44]. Each soil sample was stored at -80°C for subsequent DNA extraction and functional gene analysis.

2.5. DNA extraction and quantification of functional genes

Genomic DNA was extracted from 0.5 g of freeze-dried soil using the Fast DNA SPIN Kit for soil (QBIOM Gene Inc., USA) following the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was used to quantify the absolute abundances of bacteria, archaea, anaerobic methane oxidation and As metabolism related functional genes (Light Cycler 480II; Roche, Switzerland). The PCR mixture contained $1 \mu\text{L}$ of DNA template, $10 \mu\text{L}$ of TB Premix Ex Taq (Takara, Japan), $0.8 \mu\text{L}$ of primer F/R ($10 \mu\text{M}$), and $7.4 \mu\text{L}$ of double-distilled H_2O .

Detailed information concerning primers is provided in [Supplementary Table S1](#). Gene copy numbers were calculated with standard curves generated from six-concentration levels of plasmid DNA. Negative controls were prepared for each assay using sterilized H₂O [18].

2.6. Statistical analyses

All statistical analyses were conducted using R version 3.6.2. Significant differences among treatments were evaluated through one-way ANOVA, performed using the `aov` function in the `stats` package and the `LSD.test` in the `agricolae` package. Linear regression and Pearson correlation were performed using the `lm` function in the `stats` package and `rcorr` in the `Hmisc` package, respectively [18]. Predictor importance for As(III) concentrations was determined using a random forest model [45]. The percentage increase in MSE (mean squared error) was used to assess the importance of predictors, with higher MSE% values indicating greater importance.

3. Results

3.1. Virus inoculation effects on As release and transformation with ¹³CH₄ addition

Fig. 1 displayed the differences in porewater total As, As(III), As(V), and dimethylarsinic acid (DMA) concentrations of different virus groups between day 0 and day 90 in Ar and ¹³CH₄ treatments. In Ar treatments, significant changes in total As concentrations were observed among the four groups: V2 (4115 µg L⁻¹) ≈ SM (3803 µg L⁻¹) > DV (3310 µg L⁻¹) > V1 (2688 µg L⁻¹) ($p < 0.05$, **Fig. 1A** and **S2A**). For the ¹³CH₄ treatment, total As concentrations followed: V1 (4761 µg L⁻¹) ≥ SM (4175 µg L⁻¹) ≥ V2 (3916 µg L⁻¹) ≈ DV (3904 µg L⁻¹) ($p < 0.05$, **Fig. 1A** and **S2A**). Total As concentrations were higher in the ¹³CH₄ versus Ar treatments for the V1, SM and DV treatments, but slightly lower for V2.

As(III) was the dominant As species in porewaters (**Fig. S3**), with its concentration varying in response to both viruses and ¹³CH₄ versus Ar injection (**Fig. 1B** and **S2B**). As(III) concentrations in SM with Ar

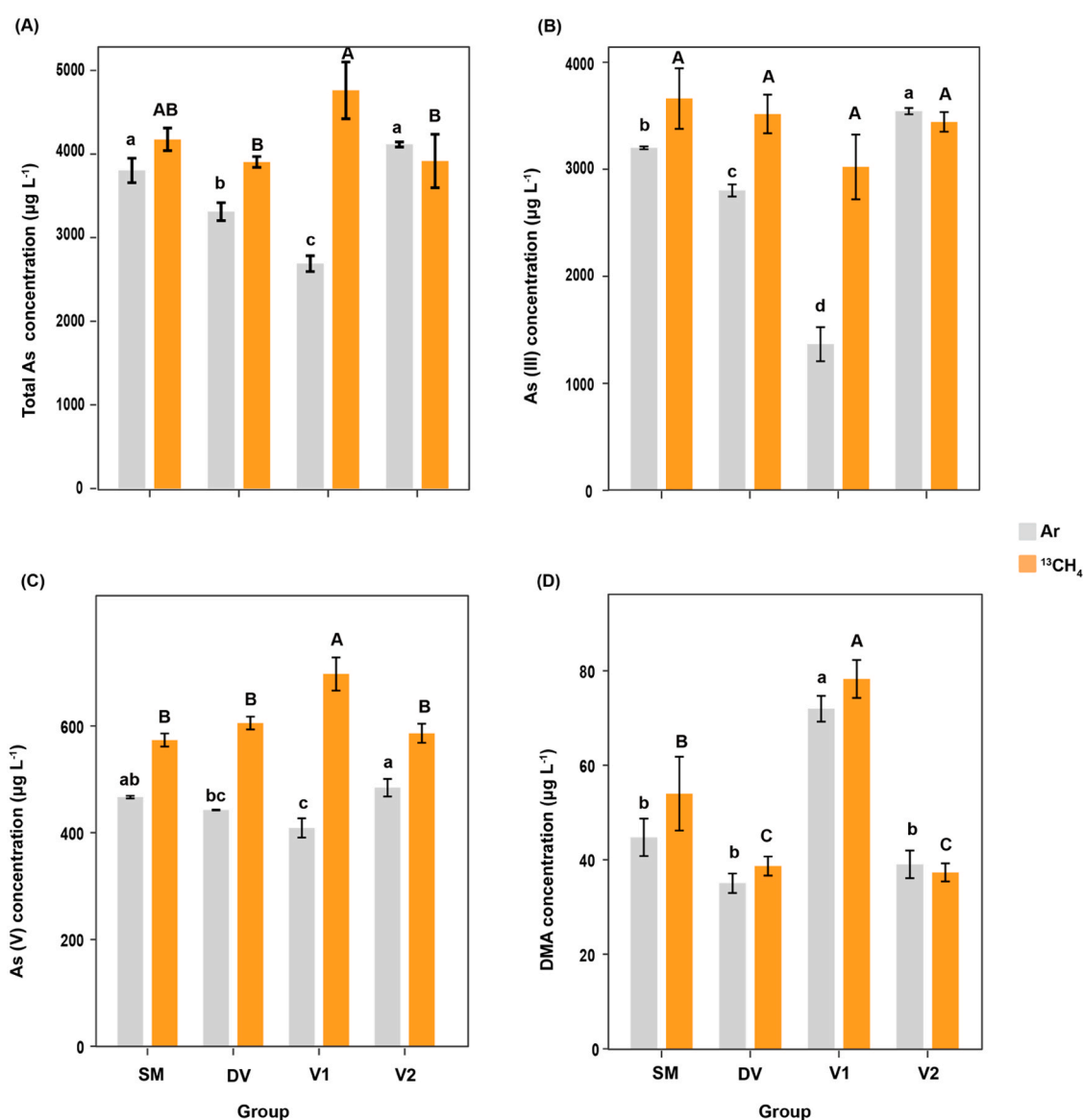


Fig. 1. Differences in porewater As concentration of different virus groups between day 0 and day 90 in Ar and ¹³CH₄ treatments (mean ± SD, n = 3). (A) total As, (B) As(III), (C) As(V) and (D) DMA. SM, non-virus; DV, autoclaved virus; V1, extracellular free virus; V2, mitomycin C (MC)-induced virus; DMA, dimethylarsinic acid. Different lower and upper case letters represent significant differences at $p < 0.05$ in the Ar and ¹³CH₄ treatments, respectively. The differences were calculated by subtracting the values on day 0 from those on day 90 in each group.

(3201 $\mu\text{g L}^{-1}$) and $^{13}\text{CH}_4$ (3661 $\mu\text{g L}^{-1}$) were higher than V1 with Ar (1366 $\mu\text{g L}^{-1}$) and $^{13}\text{CH}_4$ (3022 $\mu\text{g L}^{-1}$). However, V2 showed a higher As(III) concentration only in the Ar treatment (3543 $\mu\text{g L}^{-1}$) ($p < 0.05$, Fig. 1B and S2B). In contrast, As(V) concentration was lower in the V1 versus SM with Ar, but was higher in V1 with $^{13}\text{CH}_4$ ($p < 0.05$, Fig. 1C and S2C). The highest DMA concentration in porewater was observed in V1 with both Ar and $^{13}\text{CH}_4$ ($p < 0.05$, Fig. 1D and S2D).

To further quantify the contribution of $^{13}\text{CH}_4$ addition to porewater As dynamics, the concentrations of total As, As(III), As(V) and DMA in the Ar treatment were subtracted from those in the $^{13}\text{CH}_4$ treatment and designated as $\Delta\text{total As}$, $\Delta\text{As(III)}$, $\Delta\text{As(V)}$ and ΔDMA (Fig. 2). For SM, DV and V1, $^{13}\text{CH}_4$ addition significantly increased net porewater total As, As(III), As(V) and DMA concentrations. Conversely, the net total As, As(III) and DMA decreased in V2 (Fig. 2A–B and D), but net As (V) concentrations increased (Fig. 2C). Compared to the Ar treatment, $^{13}\text{CH}_4$ addition increased $\Delta\text{As(III)}$ by 14% (SM), 26% (DV) and 121% (V1), but decreased $\Delta\text{As(III)}$ by 3% in V2.

Compared with SM, V1 virus inoculation increased methane-mediated total As, As(III) and As(V) concentrations by 458%, 260% and 77%, respectively. Conversely, V2 virus inoculation decreased net total As, As(III), As(V), and DMA concentrations by 152%, 122%, 37% and 118%, respectively (Fig. 2). These findings demonstrate that both

viral treatments influenced methane-mediated As release and transformation, but in opposing directions.

3.2. Effects of viruses on $^{13}\text{CH}_4$ consumption and $^{13}\text{CO}_2$ production

Fig. 3 showed the net (Δ) values of $^{13}\text{CH}_4$ consumption and $^{13}\text{CO}_2$ production among the various treatments. After 90 days of anaerobic inoculation, net $^{13}\text{CH}_4$ consumption ranged from 531 to 669 μmol , with average rates varying between 328 and 413 $\mu\text{mol g}^{-1}$ soil d^{-1} . Compared to SM and DV, V2 exhibited higher net $^{13}\text{CH}_4$ consumption and V1 showed lower values ($p < 0.05$, Fig. 3A). In contrast to $^{13}\text{CH}_4$ consumption, the ΔAOM (total $^{13}\text{CO}_2$ production) followed SM (38 μmol) $\geq$ DV (36 μmol) $\geq$ V1 (31 μmol) $\geq$ V2 (28 μmol) ($p < 0.05$, Fig. 3B). Interestingly, no significant difference was observed in total AOM between V1 and V2, and both had lower net $^{13}\text{CO}_2$ production values than SM ($p < 0.05$, Fig. 3B).

A regression analysis between the related methane processes ($^{13}\text{CH}_4$ consumption and $^{13}\text{CO}_2$ production) and As concentrations was conducted to assess possible casual relationships (Fig. 4 and S4A). Net values (Δ) in $^{13}\text{CH}_4$ consumption were negatively correlated with the differences in the concentrations of total As, As(III) and As(V) ($p < 0.05$, Fig. 4A–C), whereas $^{13}\text{CO}_2$ production was positively correlated with

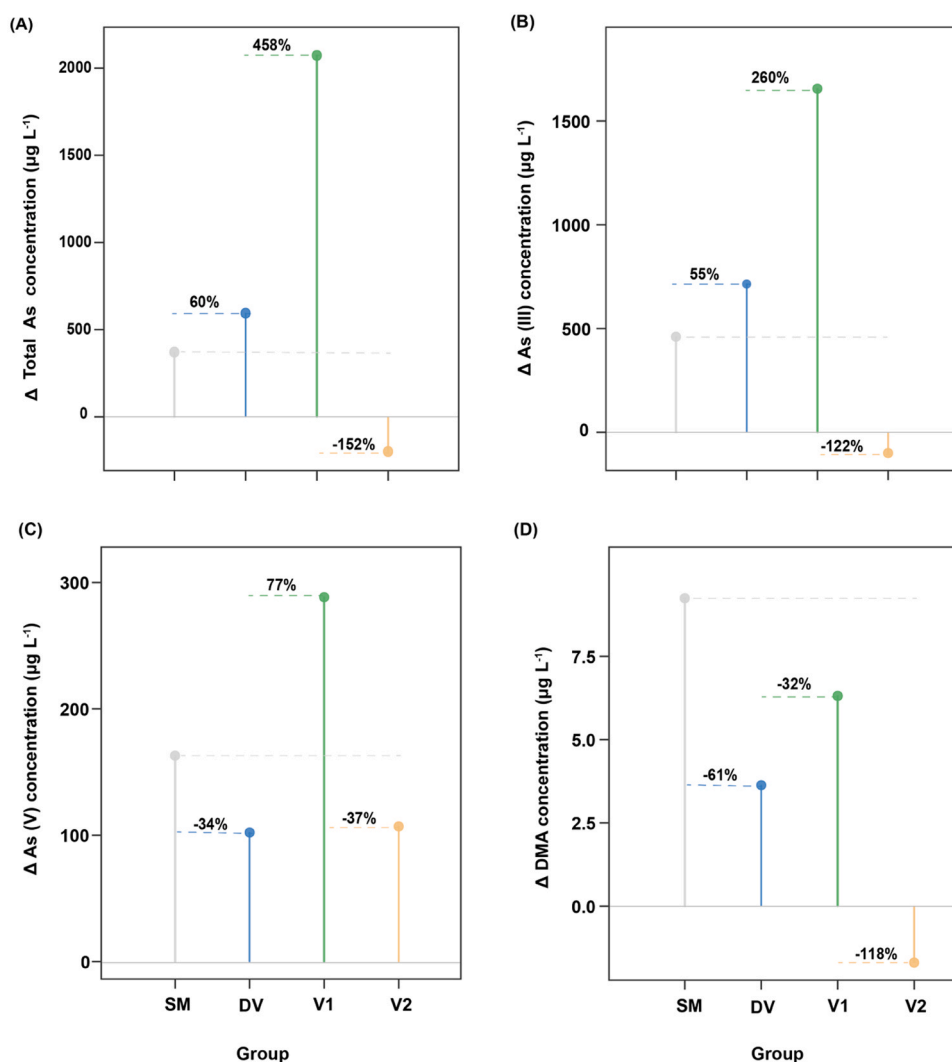


Fig. 2. Net (Δ) values of porewater As concentrations as influenced by $^{13}\text{CH}_4$ addition in different virus groups. $\Delta\text{total As}$ (A), $\Delta\text{As(III)}$ (B), $\Delta\text{As(V)}$ (C) and ΔDMA (D) refer to the difference in the measurements of total As, As(III), DMA and As(V) between Ar and $^{13}\text{CH}_4$ treatment during 90 days. Each percentage value (%) represents the relative contribution of a microbial group compared to the SM group. SM, non-virus; DV, autoclaved virus; V1, extracellular free virus; V2, mitomycin C (MC)-induced virus; DMA, dimethylarsinic acid.

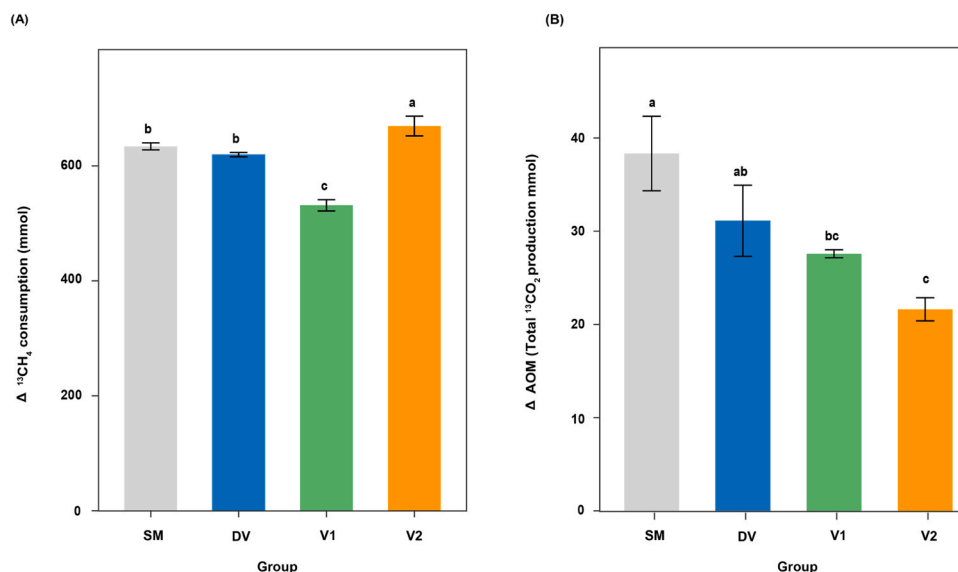


Fig. 3. Net (Δ) values of $^{13}\text{CH}_4$ -related processes in different virus groups (mean $\pm$ SD, $n = 3$). (A) $\Delta^{13}\text{CH}_4$ consumption and (B) ΔAOM (total $^{13}\text{CO}_2$ production). Δ refers to the difference in the measurements between Ar and $^{13}\text{CH}_4$ treatment during 90 days. SM, non-virus; DV, autoclaved virus; V1, extracellular free virus; V2, mitomycin C (MC)-induced virus. Different lowercase letters represent significant differences at $p < 0.05$. AOM refers to $^{13}\text{CO}_2$ production.

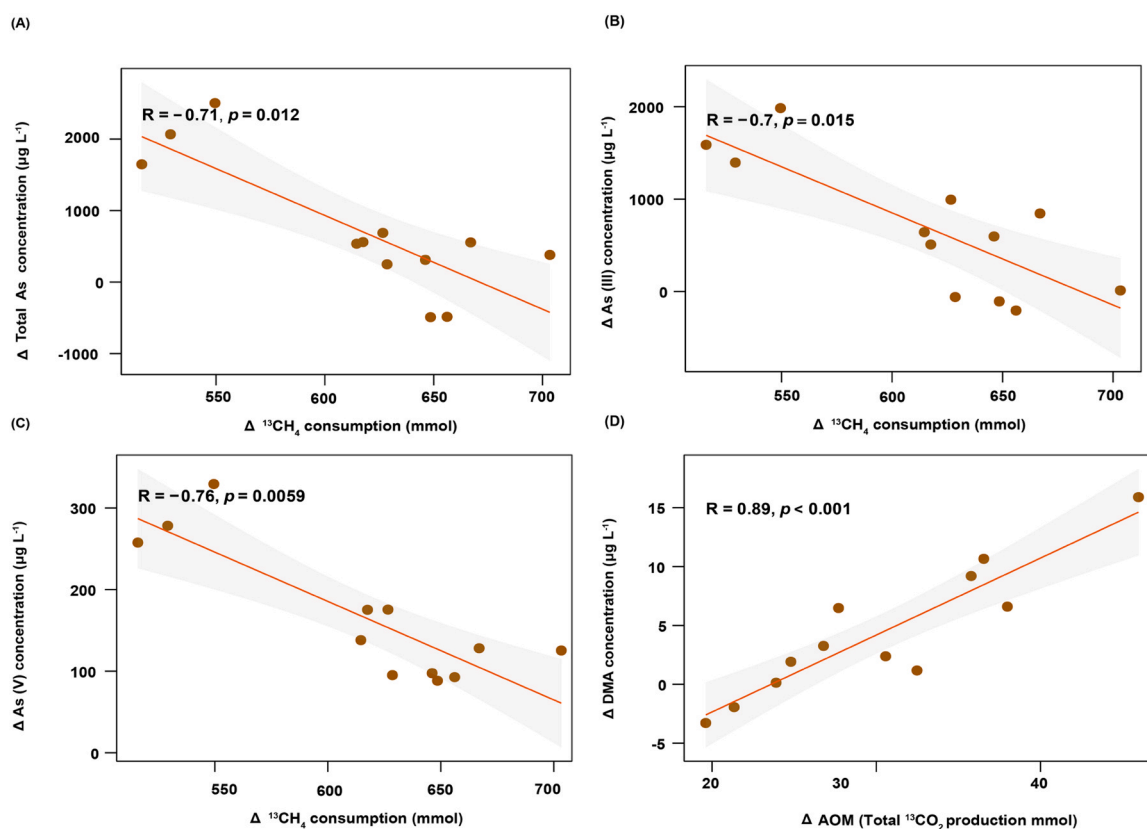


Fig. 4. Relationships between $^{13}\text{CH}_4$ -related processes and As concentrations. Relationships between $\Delta^{13}\text{CH}_4$ consumption and Δ total As (A), As(III) (B) and As(V) (C), and relationships between ΔAOM and ΔDMA (D). Δ refers to the differences in the measurements between Ar and $^{13}\text{CH}_4$ treatment during 90 days. DMA, dimethylarsinic acid. AOM refers to $^{13}\text{CO}_2$ production. The shaded areas represent 95% confidence interval.

ΔDMA concentration ($p < 0.05$, Fig. 4D). Notably, no correlation was found between the changes in $^{13}\text{CO}_2$ production and As(III) ($p > 0.05$, Fig. S4A). These results infer that microbial methane utilization, rather than methane oxidation to CO_2 , influenced As mobilization in the anaerobic paddy soil.

3.3. Methane-mediated changes in microbial abundance with virus inoculation

The qPCR analysis revealed that $^{13}\text{CH}_4$ addition affected the abundances of archaea, bacteria, ANME-2d, NC10, and *Geobacter*, with the effect depending on the type of virus inoculated (Fig. 5). In V2, $^{13}\text{CH}_4$

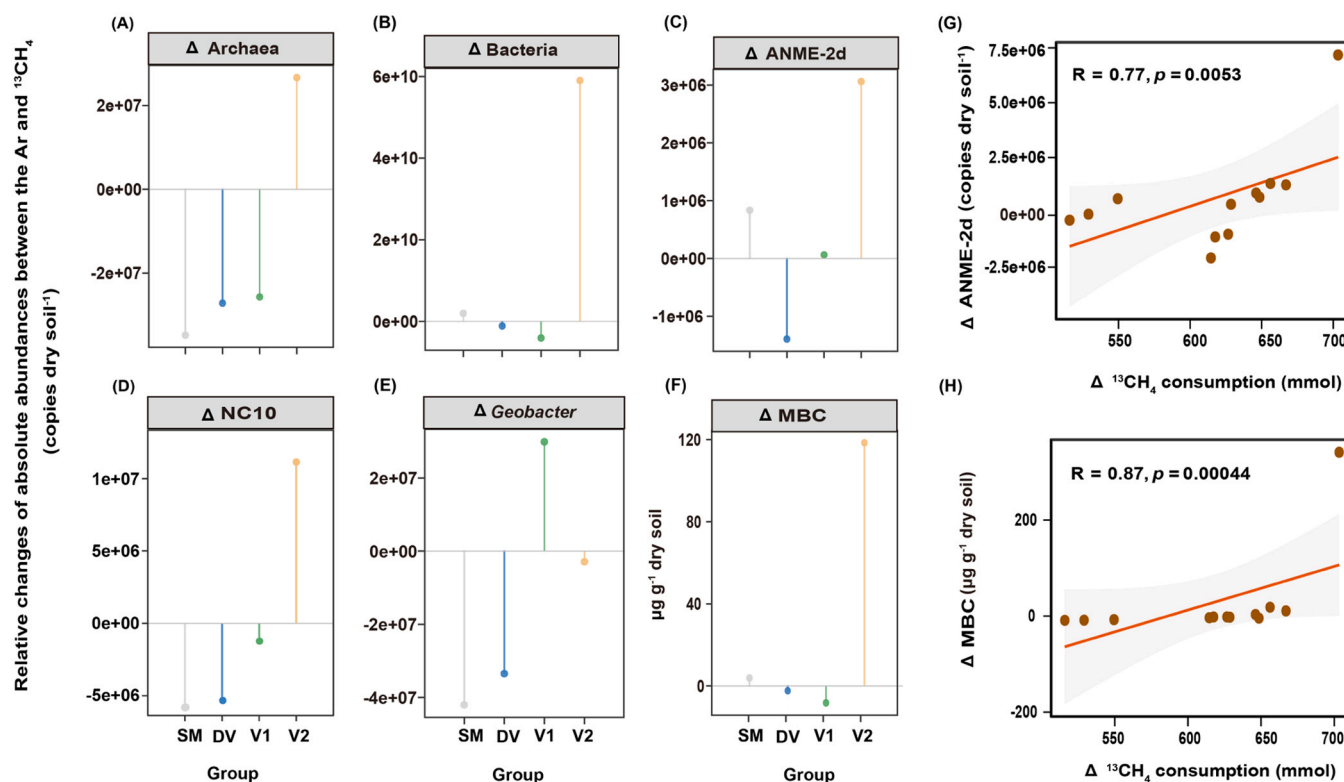


Fig. 5. Effects of $^{13}\text{CH}_4$ addition on changes in the abundance of archaea (A), bacteria (B), ANME-2d (C), NC10 (D), *Geobacter* (E) and microbial biomass carbon (MBC) (F), along with the relationships between changes in $^{13}\text{CH}_4$ consumption and Δ ANME-2d (G) and Δ MBC (H). Δ refers to the differences in the measurements between Ar and $^{13}\text{CH}_4$ treatment during 90 days. SM, non-virus; DV, autoclaved virus; V1, extracellular free virus; V2, mitomycin C (MC)-induced virus. The shaded areas surround the regression line represent the 95% confidence interval.

addition increased the net absolute abundances (Δ values) of archaea, bacteria, ANME-2d, and NC10, and MBC. Conversely, $^{13}\text{CH}_4$ addition decreased these values in V1 (Fig. 5A-D and F). Notably, the $^{13}\text{CH}_4$ addition increased the net abundance of *Geobacter* in V1 by 1.4 times compared to SM (Fig. 5E). Methane utilization was likely associated with changes in ANME-2d abundance and MBC, as evidenced by positive correlations between them (Fig. 5G-H).

3.4. Changes in Fe(II) concentration and its association with As reduction

Virus inoculation modulated porewater Fe(II) concentration as mediated by $^{13}\text{CH}_4$ addition, with changes in the Δ Fe(II) concentration ranging from -1.10 to 4.90 mg L^{-1} (Fig. 6A). The $^{13}\text{CH}_4$ addition increased Δ Fe(II) concentration in V1, but decreased the concentration in V2. Moreover, Δ As(III) concentrations were positively correlated with Δ Fe(II) concentration (Fig. 6B) and the net abundance of *Geobacter* (Fig. 6C). The random forest analysis revealed that the concentrations of Fe(III), MBC and Fe(II) along with total $^{13}\text{CH}_4$ consumption and *Geobacter* were important predictors of the differences in methane-induced As(III) concentrations (Fig. 6D).

4. Discussion

Previous studies have confirmed the presence of methane-related functional genes in virus genomes, suggesting that viruses potentially participate in AOM processes [25,46]. The current study provides experimental evidence for how different viruses impact $^{13}\text{CH}_4$ -mediated As mobilization. Specifically, viruses may dominantly mediate methane utilization by altering the abundance of ANME-2d through lytic and lysogenic infection. This viral involvement affects anaerobic methane assimilation into microbial biomass carbon and microbial population dynamics, ultimately leading to changes in *Geobacter* abundance. These

changes, in turn, affect As reduction and mobilization in anaerobic paddy soil (Fig. 7).

4.1. Opposing effects of viruses on As mobilization in the presence of $^{13}\text{CH}_4$

Our study showed that methane addition significantly promoted As release and reduction (Fig. 2A-B), which was consistent with previous findings [7,40]. This is due to the favorable thermodynamic conditions and chemical feasibility of AOM on the As reduction process (Gibbs free energy change of $\Delta G^0 = -478 \text{ kJ mol}^{-1} \text{ CH}_4$) under anaerobic conditions [7]. This indicates that methane plays a crucial role in driving As biogeochemical cycling in paddy soil with important implications for food safety.

When comparing As concentrations between the Ar and $^{13}\text{CH}_4$ treatments, notable differences emerged in the methane-mediated As release (Δ total As) and reduction (Δ As(III)) between V1 and V2 (Fig. 2). Specifically, the V1 virus inoculation significantly enhanced methane-mediated As release and reduction, whereas the V2 virus showed an inhibitory effect on these processes (Fig. 2A-B). Notably, previous studies have indicated that MC concentrations below $0.1 \mu\text{g L}^{-1}$ had no significant influence on microorganisms compared with the control without MC [47]. Therefore, the observed differences between the viruses obtained through the TFF system and those induced by MC are primarily attributed to the distinct functions of the viruses involved.

The higher methane-mediated Δ total As and Δ As(III) concentrations in the V1 group may be attributed to the presence of more lytic viruses within the viral population [21,35]. Previous studies demonstrated that the addition of virus suspensions collected by TFF and inoculated back into their original host soil significantly decreased the microbial population, suggesting the occurrence of lytic infection within

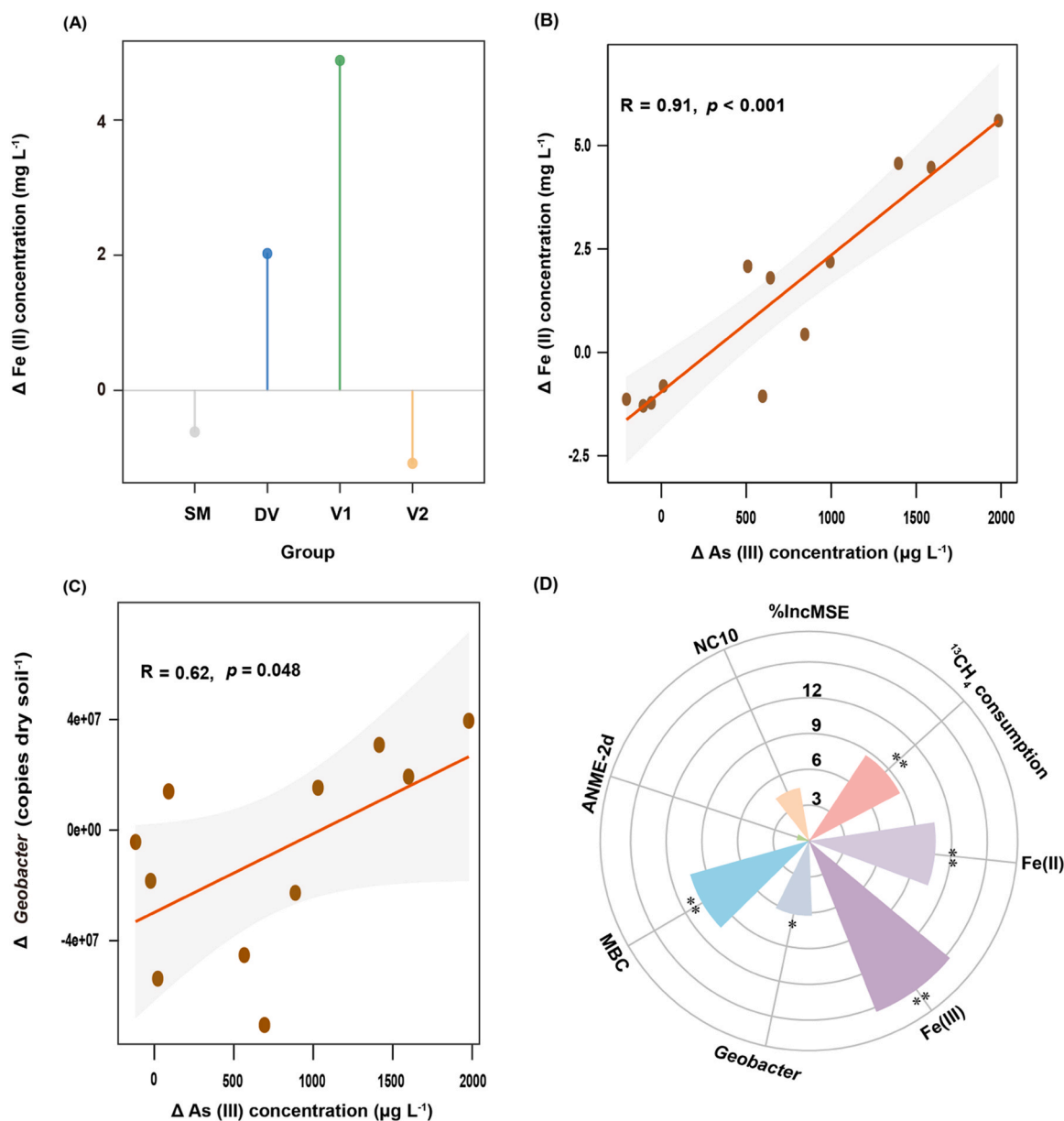


Fig. 6. Differences of Fe(II) influenced by $^{13}\text{CH}_4$ addition in different virus groups and the importance of predictors for $\Delta\text{As(III)}$. (A) $\Delta\text{Fe(II)}$ concentration as influenced by $^{13}\text{CH}_4$ addition in different virus groups during 90 days. (B) and (C) Linear regression (and 95% confidence interval) between $\Delta\text{As(III)}$ and $\Delta\text{Fe(II)}$ concentrations as well as $\Delta\text{Geobacter}$. (D) The predictor importance for the As(III) concentration induced by $^{13}\text{CH}_4$ addition ($\Delta\text{As(III)}$). Different colors refer to the distinct factors. Δ refers to the differences of Fe(II)/As(III) concentrations in the measurements between Ar and $^{13}\text{CH}_4$ treatment during 90 days. SM, non-virus; DV, autoclaved virus; V1, extracellular free virus; V2, mitomycin C (MC)-induced virus. MBC, microbial biomass carbon. Significance levels indicated as *, $p < 0.05$; **, $p < 0.01$.

the viral suspensions [21]. Viruses with a lytic lifestyle have the potential to impose top-down control on microbial composition, obligately infecting and lysing host microbial cells [48]. This process involves lysing the host cell and subsequently releasing cellular debris into the surrounding environment [27,48], thereby reshaping microbial composition and altering metabolic activities by stimulating the growth of microbiomes [26]. In the $^{13}\text{CH}_4$ treatment on day 90, the absolute abundance of archaea (Fig. S8B) and the relative abundance of arsenic functional genes (*arrA*, *arsC*, and *arsM*) in the V1 group were higher than those in the SM group ($p < 0.05$, Fig. S9B). These results indicate that the lytic effects of viruses on their hosts can change the activity of functional microorganisms related to arsenic transformation in soil, including As release, reduction, and methylation [49,50]. This underscores the significance of the lytic activity in the V1 virus and

confirms its important role in methane-mediated arsenic transformation.

The methane-mediated As release and reduction observed in the V2 group contrasted with those in the V1 group, with decreases in $\Delta\text{total As}$ and $\Delta\text{As(III)}$ indicating different viral compositions and functions between V1 and V2 viruses (Fig. 2A-B). These differences may be attributed to the presence of prophages in the V2 group, which may maintain their lysogenic functionality [51]. In the lysogenic state, viral genomes integrate into the host's genome as prophages, potentially influencing host metabolism and/or enhancing the resistance of their hosts to environmental stress [52]. We observed positive effects of $^{13}\text{CH}_4$ addition on the abundances of archaea, bacteria, and ANME-2d and NC10 genes in the V2 group (Fig. 5). This suggests that MC-induced viruses can enhance the methane metabolism capacities of their hosts,

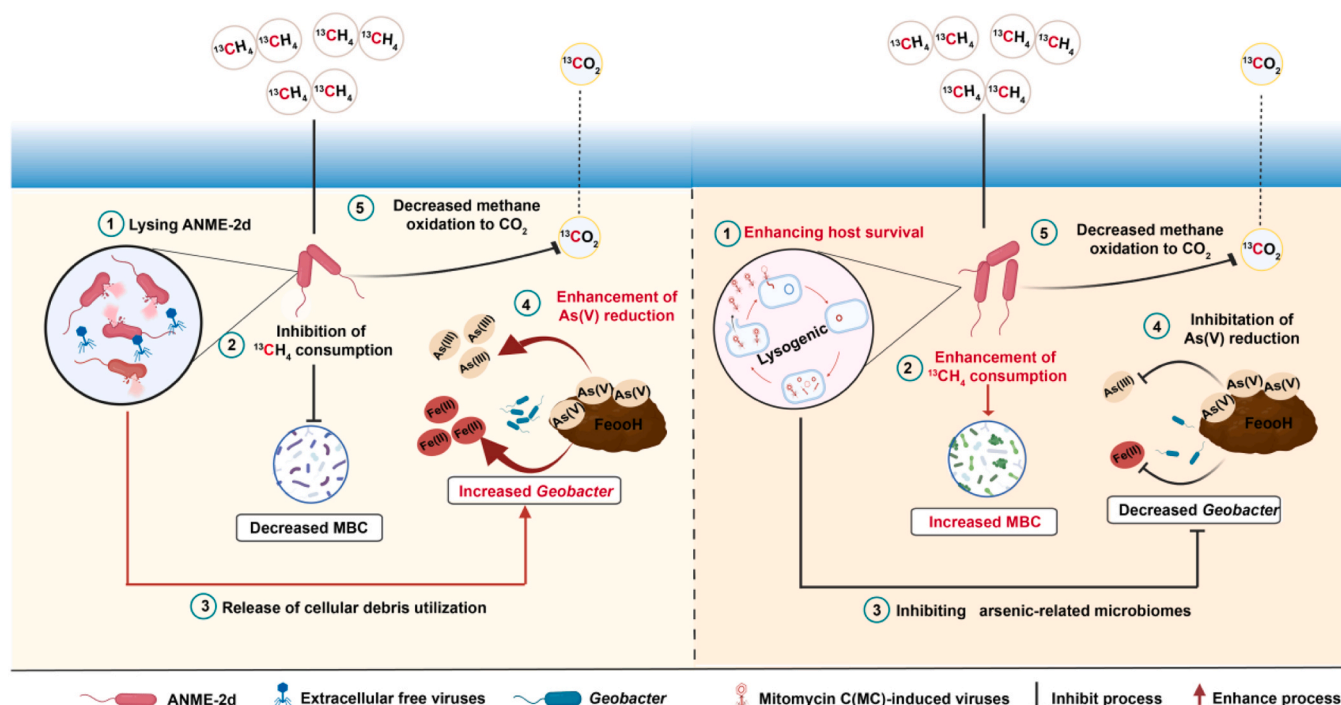


Fig. 7. Conceptual model for the impact of viruses on arsenic reduction during microbial AOM in paddy soil.

providing them with a competitive advantage in resource acquisition. This premise is supported by the observation of the highest net $^{13}\text{CH}_4$ consumption in the V2 group (Fig. 3A). The enhancement of host adaptability (mainly ANME-2d) caused by lysogenic viruses increases their ability to compete for ecological niches with other species (Fig. 5C). This explains the decreased *Geobacter* abundance in our study, which ultimately reduces arsenic reduction functionality (Fig. 5E). Moreover, the increased methane consumption by AOM-related microorganisms in the V2 group resulted in positive effect on methane sequestration (higher MBC) in the soil (Fig. 5F). This could potentially weaken the coupled redox processes of $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$ with As reduction processes. Overall, these results reveal a significant impact of viruses on methane-mediated As mobilization, highlighting the complex interactions between different types of viral activity, methane metabolism, and arsenic reduction.

4.2. Viral influence on AOM through changes in ANME-2d abundance

Under anaerobic conditions, the conversion of ^{13}C from added $^{13}\text{CH}_4$ into $^{13}\text{CO}_2$ confirmed the occurrence of AOM among different virus treatments (Fig. 3B). During the incubation time, there was only a partial conversion of $^{13}\text{CH}_4$ to $^{13}\text{CO}_2$, as the ratio of $^{13}\text{CH}_4$ consumption to $^{13}\text{CO}_2$ production was well over 1. This infers that the remaining methane was sequestered in the soil. During anaerobic CH_4 oxidation, the $^{13}\text{CH}_4$ -derived carbon can be incorporated into microbial biomass and potentially into organic metabolites associated with the AOM process in paddy soils [12,13].

When compared to SM, both V1 and V2 viruses suppressed $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$ (Fig. 3B). These results were inconsistent with our hypothesis that V1 virus would inhibit, whereas V2 virus would enhance $^{13}\text{CH}_4$ oxidation to $^{13}\text{CO}_2$. Unlike previous studies [7], this study did not demonstrate a positive correlation between ΔAOM (total $^{13}\text{CO}_2$ production) and $\Delta\text{As(III)}$ concentration (Fig. S4A). This implies that there is not a direct coupling between AOM and As reduction processes in our study. This may result from the occurrence of alternative electron acceptors in paddy soils, such as NO_3^- , NO_2^- , Mn^{4+} , Fe^{3+} , SO_4^{2-} and organic substances (e.g., humic substances), thereby altering AOM pathways

and masking the relationship between AOM and As reduction [9]. This is supported by the differences in the concentrations of Fe(II) and As(III) resulting from $^{13}\text{CH}_4$ addition (Fig. 6 A), suggesting that Fe(III) may serve as another electron acceptor that can be coupled with the AOM process. Furthermore, viral influence on the abundance and activity of AOM-related microorganisms (ANME-2d and *Geobacter*) may potentially disrupt the coupling process between AOM and As reduction (Fig. 5 C and E). However, further research on viral interactions needs to offer deeper insights into these coupling mechanisms.

Under anaerobic conditions, soil microorganisms assimilate methane into microbial biomass providing a potential mechanism to enhance carbon sequestration in paddy soils [9,13,14]. However, our findings revealed that viral influences on $^{13}\text{CH}_4$ consumption patterns were inconsistent with AOM and were in contrast to As(V) reduction (Fig. 2B). V1 virus exhibited the lowest $^{13}\text{CH}_4$ consumption, whereas the V2 group showed the highest $^{13}\text{CH}_4$ consumption (Fig. 3A). The V1 group had a lower MBC content (Fig. 5F), which is consistent with the lower $^{13}\text{CH}_4$ consumption versus the SM treatment (Fig. 3A). Conversely, the V2 group had a higher $^{13}\text{CH}_4$ consumption (Fig. 3A) and a corresponding increase in MBC (Fig. 5F). These observations suggest that distinct virus treatments have the potential to differentially impact methane assimilation processes, subsequently altering the microbial population and As redox functions within paddy soils.

The above inference is supported by the significant changes in the measured abundances of distinct genes between the V1 and V2 group, with net changes noted for archaea, bacteria, ANME-2d, NC10, and *Geobacter* relative to SM (Fig. 5 A-E). Prominently, the arsenic-related microorganisms showed differences between these two treatments upon $^{13}\text{CH}_4$ addition (Fig. S6). Therefore, given that viruses play a pivotal role in regulating soil microbial communities and their functions [17], it is reasonable that viruses may influence microbiomes by mediating microbial methane assimilation into MBC. This in turn indirectly alters As reduction owing to changes in microbial populations and their functions.

Virus-mediated differences in methane consumption were likely related to the altered abundance of ANME-2d rather than NC10, as supported by the positive correlation between ANME-2d and net

methane consumption (Fig. 5G and S4B). The importance of ANME-2d was further confirmed as it was the key taxa responsible for net methane consumption (Fig. S5). Compared to SM, the contrasting trends observed in ANME-2d abundance between the V1 and V2 groups may be attributed to differences in lytic and lysogenic infections (Fig. 5C). This suggests that the distinct virus treatments likely regulate the community composition of their microbial hosts via different infection pathways. Under prolonged methane addition, the AOM-related microbiomes may enrich and become dominant in the soil environment. This is evidenced by the relative abundance of ANME-2d, which is more than two orders of magnitude higher than that of NC10 (Fig. S9B), enhancing their chances of being infected by both lytic and lysogenic viruses [53]. The V1 virus likely follows a “kill-the-winner” pattern [54] by lysing its ANME-2d host, which leads to lower methane utilization and MBC. In contrast, the V2 virus appears to adopt a “piggyback-the-winner” pattern [55], thereby infecting ANME-2d and assisting its host in utilizing methane more efficiently. However, viral genomic information is required to precisely classify differences in virus lifestyles. Further study is also warranted to elucidate the specific mechanisms underlying whether these viruses impact the methane consumption efficiency of ANME-2d and their distinct infection patterns.

Remarkably, the V2 virus increased the abundance of both ANME-2d and NC10, yet maintained the lowest net AOM values (Fig. 3B). Considering that oxidation of some soil organic compounds is more thermodynamically favorable than AOM [9], the shifts in the microbial community induced by the V2 virus may result in preferential microbial oxidation of organic compounds rather than CH₄. Additionally, CO₂ production from non-labeled soil organic matter-derived CH₄ may have diluted ¹³CH₄ concentrations, thereby masking AOM [9].

4.3. Viral influence on methane-driven As mobilization through *Geobacter*

Linkages between soil viruses and microorganisms can trigger a cascade of complex ecological effects, altering the abundance and activity of microorganisms and their influence on soil functions [56]. Our study detected an increase in the abundance of *Geobacter* in the V1 group upon ¹³CH₄ addition (Fig. 5E). Moreover, a simultaneous enhancement of As and Fe mobilization mediated by ¹³CH₄ occurred (Fig. 6B). In soils, As is primarily adsorbed/coprecipitated with Fe(III) (hydr)oxides. Development of anaerobic conditions upon paddy soil flooding induces the reductive dissolution of As-bearing iron (hydr)oxides by *Geobacter*, resulting in As release and reduction [2,57,58]. Thus, the higher As(III) concentrations associated with ¹³CH₄ addition were ascribed to the enrichment of *Geobacter* in the V1 group, which likely resulted from viral lysis of hosts that created new ecological niches for them.

Conversely, the V2 virus suppressed methane-mediated As reduction, possibly due to an inhibitory effect on *Geobacter* (Fig. 5E). This premise is supported by the positive relationship between Δ As(III) and Δ *Geobacter* abundance, with *Geobacter* being the key taxa responsible for As reduction upon ¹³CH₄ addition (Fig. 6 C and D). Overall, our experimental results point to multiple, diverse impacts of viruses on methane-mediated As mobilization in submerged paddy soils, highlighting their complex roles in soil ecosystems.

5. Significances and limitations

The mechanisms of microbial AOM coupled with As reduction have garnered significant interest in paddy soils. As the primary biological factor controlling indigenous microbial communities in soils, viruses exhibit great potential to mediate AOM and As transformation processes through AMGs. This study provides experimental evidence documenting the viral influence on As mobilization by affecting AOM, revealing their potential ecosystem functions and highlighting the previously unrecognized environmental risk of As mobilization in paddy soil. However, limitations remain in precisely classifying the lifestyles of viruses and

elucidating their detailed collaborations with hosts, due to the lack of comprehensive viral genomic information. Thus, future studies of virus-mediated metabolic pathways within the AOM process using omics techniques and DNA/RNA-based stable isotope probing of active microorganisms may be able to enhance our mechanistic understanding of these interrelated processes. Moreover, potential confounding factors, such as soil pH, temperature, MC and organic matter content are not specifically examined and require further investigation. Finally, a viral regulation strategy based on engineering design parameters should be considered for mitigating As risk in paddy soils.

6. Conclusions

To our knowledge, this study marks the first report elucidating the impact of virus-mediated microbial AOM on As mobilization. Our study revealed diverse roles for soil viruses in methane-mediated As mobilization. Extracellular free virus enhanced methane-mediated As mobilization, whereas MC-induced virus inhibited these processes. These contrasting viral effects are attributed to their different infection pathways (lytic versus lysogenic) on ANME-2d, thereby mediating methane assimilation into microbial biomass carbon, which subsequently influences the abundance of *Geobacter*. Consequently, these alterations impact As mobilization by regulating the reductive dissolution of As-bearing iron (hydr)oxides. Our findings not only offer new insights into the intricate relationships between soil viruses, methane oxidation, and As mobilization, but also hold promising implications for using viruses to mitigate As contamination in agricultural ecosystems, thereby protecting food safety.

Environmental Implication

Anaerobic methane oxidation drives the mobilization of arsenic, leading to significant environmental concerns. Soil viruses may be involved in methane oxidation and arsenic reduction.

This study demonstrated that extracellular free virus elevated net As(III) concentrations (up to 260%) relative to treatment without virus in the presence of methane. In contrast, MC-induced virus led to a net 122% reduction in As(III) concentration. Overall, our research underscores a previously unrecognized environmental risk posed by soil viruses in methane-driven arsenic mobilization and its implications for food safety.

CRediT authorship contribution statement

Jianming Xu: Writing – review & editing, Project administration, Funding acquisition. **Randy A. Dahlgren:** Writing – review & editing. **Caixian Tang:** Writing – review & editing. **Youjing Wang:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Yujie Zhou:** Methodology. **Haodan Yu:** Methodology. **Di Tong:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2024.136758](https://doi.org/10.1016/j.jhazmat.2024.136758).

Data availability

Data will be made available on request.

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