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Elevated ground-level O_3 negatively influences paddy methanogenic archaeal community

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The current knowledge regarding the effect of global climate change on rice-paddy methane (CH_4) emissions is incomplete, partly because information is limited concerning the mechanism of the microbial response to elevated ground-level ozone (O_3). A field experiment was conducted in the China Ozone Free-Air Concentration Enrichment facility in a rice–wheat rotation system to investigate the responses of methanogenic archaeal communities to elevated ground-level O_3 by culture-independent and -reliant approaches. We found that elevated ground-level O_3 inhibited methanogenic activity and influenced the composition of paddy methanogenic communities, reducing the abundance and diversity of paddy methanogens by adversely affecting dominant groups, such as aceticlastic *Methanosaeta*, especially at the rice tillering stage. Our results indicated that continuously elevated ground-level O_3 would negatively influence paddy methanogenic archaeal communities and its critical ecological function. These findings will contribute to a comprehensive understanding of the responses and feedbacks of paddy ecosystems to global climate change.

Methane (CH_4) is a greenhouse gas that has the second largest radiating force after carbon dioxide (CO_2); CH_4 accounts for 78% of total global CO_2-equivalent emissions[1]. As one of the largest anthropogenic sources of CH_4 emissions, paddy fields emit 25–54 Tg of CH_4 annually[2], which is 4–9% of total annual CH_4 emissions (IPCC, 2001)[3]. Therefore, a change in paddy CH_4 emissions is of vital significance to global climate change. It is well known that global climate change affects paddy CH_4 emissions. For example, elevated atmospheric CO_2 and higher soil temperature alone and/or in combination can lead to higher paddy CH_4 emissions[4–7]. Moreover, elevated ground-level ozone (O_3) is a consequence of global climate change[8]. Ground-level O_3 forms from the photochemical oxidation of CH_4, carbon monoxide (CO), and volatile organic components (NMVOCs) in the presence of nitrogen oxides (NO_X = NO + NO_2). However, the impact of elevated ground-level O_3 on potential feedbacks of paddy CH_4 emissions is largely unknown[9], although this information is an indispensable component of a comprehensive understanding of paddy ecosystem responses to global climate change. To the best of our knowledge, only a small number of studies have been conducted, which revealed that elevated ground-level O_3 significantly reduces cumulative CH_4 emissions from paddy soil[9,10] or from peatland[11]. The underlying mechanisms remain elusive.

In terms of the effects on ecosystem production and function, ground-level O_3 is the most important gaseous air pollutant globally[12]; its concentration has been increasing since the industrial revolution and will continue to increase in the coming years[13]. Dentener *et al.*[8] suggested that global annual mean surface O_3 concentrations will increase by between 1.5 ppb (current legislation scenario) and 4.3 ppb (IPCC SRES A2 scenario) over the period of 2000–2030. Ground-level O_3, especially while at an elevated concentration (e.g., over 50 ppb), is phytotoxic with a potential to damage plant photosynthesis, leading to a reduced aboveground biomass and crop yield[12,14]. Negative effects may also be extended to the belowground biomass of plants. Many reports have indicated that O_3 exposure reduces carbon allocation to roots[15], the root/shoot biomass ratio[16] and root exudates[17]. O_3 stress also indirectly affects soil microorganisms through changes in carbon input to soils[15] because soil and vegetation itself can remove most O_3 from the atmosphere[18]. As plants are the main input of carbon and energy to the plant-soil-microbe web, a decrease in the carbon flux from plant to soil due to elevated O_3[19] has been found to adversely influence the diversity of paddy microbes[20,21].

Biologically, CH_4 production is monopolized by methanogenic archaea, which fall into the phylum Euryarchaeota and form six distinct orders: Methanomicrobiales, Methanocellales, Methanosarcinales, Methanobacteriales,

Methanococcales and Methanopyrales. Methanogenic archaeal communities are found to be sensitive to global climate change. Both elevated atmospheric CO_2[22] and higher soil temperatures[7] alter the composition of paddy methanogenic archaeal communities and increase their abundance and activity. However, information regarding the responses of paddy methanogens to elevated ground-level O_3 is limited. Methanogenesis is the final degradation process of organic matter in paddy fields. Organic matter is first anoxically degraded to small molecules, such as acetate, CO_2 and H_2, by diverse bacteria. With the help of methanogenic archaea, some of these molecules are further converted into CH_4[23]. Therefore, the decrease in carbon input from plant to soil due to elevated ground-level O_3 was hypothesized to have a negative effect on methanogenic archaea, especially aceticlastic methanogens, because acetate is quantitatively the most important intermediate in the anaerobic degradation of organic matter and two-thirds of biologically produced methane is derived from the methyl group of acetate[24]. Thus, we conducted a field experiment in the China Ozone Free-Air Concentration Enrichment (FACE-O_3) facility on a rice-wheat rotation system. Changes in paddy methanogenic archaeal composition and abundance in response to elevated ground-level O_3 were investigated using culture-independent methods, including 454 pyrosequencing and real-time quantitative PCR. Methanogenic activity was determined by measuring CH_4 production during microcosm incubation. Our results contribute to a comprehensive understanding of the impact of global climate change on paddy ecosystems as well as feedback between the two.

Results

Soil characteristics. During rice growth, soil dissolved organic C (DOC) concentrations ranged from 80 ± 6.6 to 103 ± 6.1 mg/kg *dry weight soil* (*d.w.s*) (Fig. 1a). According to a pairwise comparison, elevated ground-level O_3 significantly decreased the soil DOC concentration under ambient O_3 ($p < 0.05$), from 100 ± 3.6–103 ± 6.1 to 80 ± 6.6–90 ± 4.0 mg/kg *d.w.s*. The acetate content was measured because it is one of the most abundant low-molecular-weight organic acids in paddy soil and one of the main precursors of CH_4 production. As shown in Fig. 1a, elevated ground-level O_3 significantly decreased the acetate contents under ambient O_3, regardless of the rice growth stage ($p < 0.05$), from 1.80 ± 0.17–2.12 ± 0.20 to 1.34 ± 0.26–1.49 ± 0.13 mM.

Methanogenic archaeal abundance and activity. Copy numbers of 16 s rRNA genes of paddy methanogens and those of *Methanosaeta* were measured using qPCR at both rice growth stages under different ground-level O_3 concentrations (Fig. 1b). Due to its non-specificity, the primer set of 1106F/1378R targeting methanogenic archaeal 16 s rRNA genes can also detect some non-methanogenic archaea[25]. However, the strong relationship between the numbers of methanogenic archaeal 16 s rRNA genes and *mcrA* genes indicates that these primers are suitable for the quantification of methanogenic archaea[26] and implies that those non-specific amplified microbes have the same responses to environmental parameters as paddy methanogens. Therefore, although they could overestimate the abundance of paddy methanogenic archaea, primers 1106F/1378R were still used in this investigation. Before quantification, the interference of inhibitory substances in purified DNA extracts on qPCR was tested by a 10-fold dilution series (up to 1000-fold) of one random DNA extract targeting the 16 s rRNA gene fragment of methanogens. The copy numbers derived from the dilution series were not significantly different with numbers ranging from 2.1×10^9 to 2.3×10^9/g *d.w.s*, which indicated that there were no inhibitory substances in the purified DNA extracts. The copy numbers of the methanogenic archaeal 16 s rRNA gene varied from $1.78 \pm 0.23 \times 10^9$ to $2.44 \pm 0.15 \times 10^9$/g *d.w.s*. There appeared to be a greater abundance of the methanogenic archaeal 16 s rRNA gene at the rice tillering stage than at the rice anthesis stage. A pairwise comparison to methanogenic archaea under ambient O_3 conditions suggested that elevated ground-level O_3 caused a decrease in the copy numbers of the methanogenic archaeal 16 s rRNA gene from $2.44 \pm 0.15 \times 10^9$ to $2.11 \pm 0.23 \times 10^9$/g *d.w.s* at the rice tillering stage and from $2.19 \pm 0.36 \times 10^9$ to $1.78 \pm 0.23 \times 10^9$/g *d.w.s* at the rice anthesis stage, but these differences were not statistically significant. A similar trend for the *Methanosaeta* 16 s rRNA gene was found between the two rice growth stages (Fig. 1b). However, significant decreases were observed for *Methanosaeta* ($p < 0.05$) in response to elevated ground-level O_3 from $7.77 \pm 0.78 \times 10^8$ to $5.09 \pm 0.76 \times 10^8$/g *d.w.s* (decreasing by 34.5%) at the rice tillering stage and from $6.23 \pm 0.70 \times 10^8$ to $3.51 \pm 0.94 \times 10^8$/g *d.w.s* (decreasing by 43.7%) at the rice anthesis stage.

Methanogenic activities were also measured for each soil sample. After a 28-day incubation, CH_4 concentrations in the headspace reached their maximum levels and ranged from 12.94 ± 1.59 to 9.58 ± 1.17 μmol CH_4/g *d.w.s* for the soil samples under ambient O_3 and from 0.62 ± 0.33 to 4.90 ± 1.93 μmol CH_4/g *d.w.s* for the soil samples under elevated ground-level O_3 in the incubated flooded condition (Fig. 1c). Under ambient O_3, the methanogenic activities were significantly higher at the rice tillering stage than those in the rice anthesis stage ($p < 0.05$). By contrast, the methanogenic activities of soil samples under elevated ground-level O_3 were significantly higher at the anthesis stage ($p < 0.05$). At both rice growth stages, however, the methanogenic activities under elevated ground-level O_3 were always significantly lower ($p < 0.05$) than those under ambient O_3, with values ranging from $4.62 \pm 0.57 \times 10^2$ to $3.42 \pm 0.42 \times 10^2$ μmol CH_4/g *d.w.s* per day under ambient O_3 and from $1.75 \pm 0.69 \times 10^2$ to $0.22 \pm 0.12 \times 10^2$ nmol CH_4/g *d.w.s* per day under elevated ground-level O_3.

Taxonomic distribution of methanogenic archaea in flooded paddy soils. A total of 87,688 sequences were obtained (Table S1). Of these sequences, 91.3% were affiliated with methanogenic archaea. Pyrosequencing revealed that the paddy methanogenic archaeal community was dominated by two classes, namely *Methanomicrobia* (81.1%) and *Methanobacteria* (10.2%). With higher resolution, we found that *Methanosarcinales* were most abundant (50.5%), followed by *Methanomicrobiales* (17.6%) and *Methanocellales* (13.0%), at the order level. At the family level, the dominant methanogenic archaea were found to be aceticlastic groups, including *Methanosaetaceae* (32.0%) and *Methanosarcinaceae* (14.1%), followed by *Methanocellaceae* (Rice cluster I) (13.0%) and *Methanobacteriaceae* (10.2%). At the genus level, the dominant methanogenic groups were *Methanosaeta* (32.0%), *Methanosarcina* (14.0%), *Methanobacterium* (10.1%) and *Methanocella* (7.0%) (Fig. 2).

The taxonomic distribution further allowed us to track overall shifts in the structure of the paddy methanogenic archaeal community in response to elevated ground-level O_3 (Fig. 2). Elevated ground-level O_3 significantly decreased the relative abundance of the most dominant genus, *Methanosaeta*, from 39.1% to 29.6% at the rice tillering stage ($p < 0.05$) and from 31.4% to 23.5% at the rice anthesis stage ($p < 0.05$). In addition, examining several other dominant genera revealed that the percentage of *Methanocella* was also significantly decreased ($p < 0.05$), and the percentages of *Methanosarcina* and *Methanoregulaceae* were potentially decreased under elevated ground-level O_3 at the rice tillering stage. By contrast, at the rice anthesis stage, relative abundances of the genus *Methanosarcina* and the order *Methanocellales* were significantly increased in response to elevated ground-level O_3 ($p < 0.05$).

Methanogenic archaeal diversity and richness. The phylogenetic diversity (PD) and Chao1 indices provide estimations of the microbial diversity and richness among different samples. PD and Chao1 consistently indicated that elevated ground-level O_3 significantly ($p < 0.05$) decreased both the diversity (from 37.8 ± 0.9

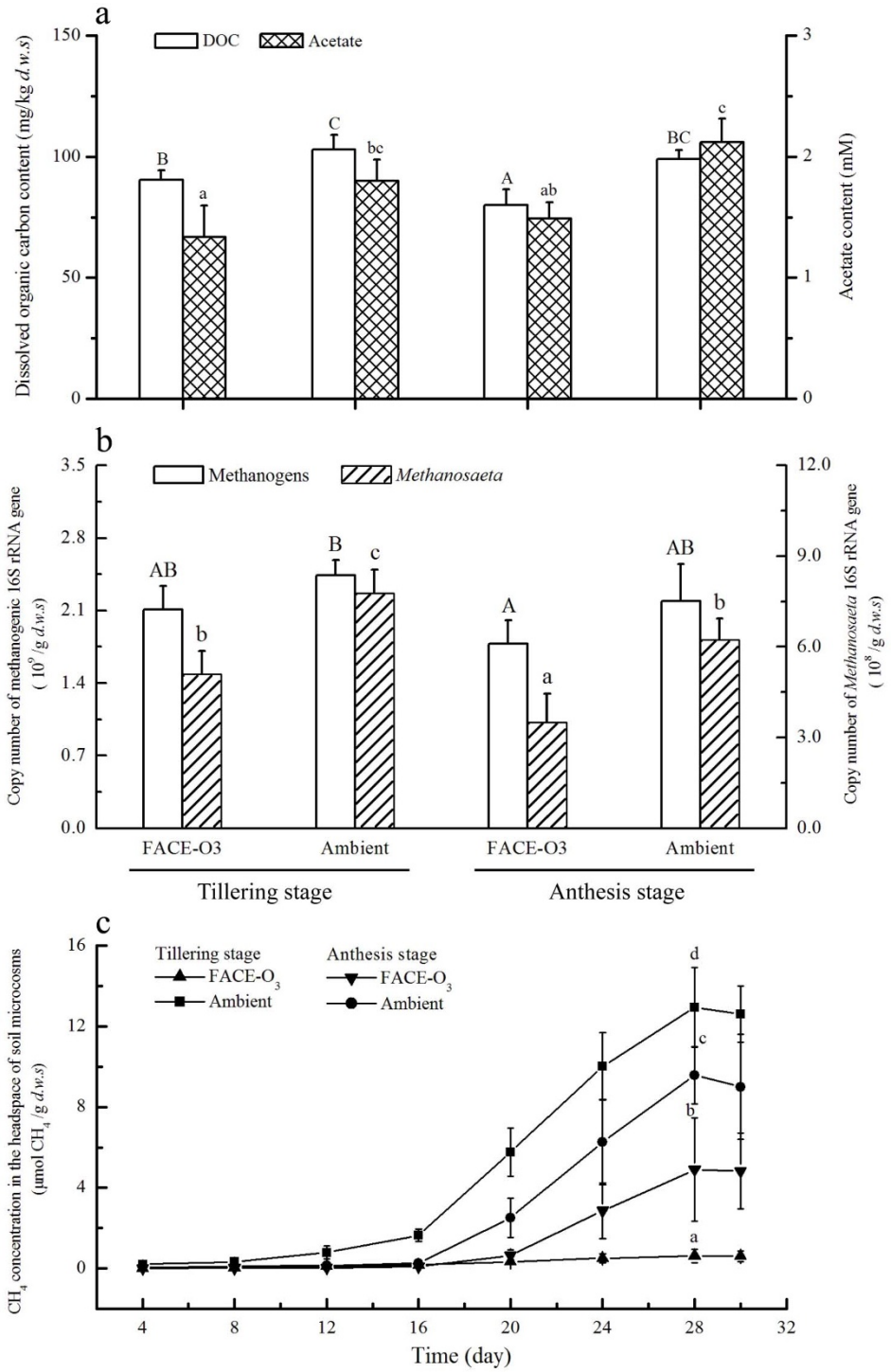

Figure 1 | Concentrations of dissolved organic C (DOC) and acetate in paddy soils (a), copy numbers of methanogenic archaeal and *Methanosaeta* 16 s rRNA genes (b) and the dynamic curves of methane production in incubated flooded paddy soils (c) under elevated ground-level O_3 (FACE-O_3) and ambient O_3 (Ambient) concentrations. Data are expressed as the means with standard deviation (SD). There were 12 soil samples in total. Significant differences are indicated by different letters shown above the error bars ($p < 0.05$), and mean separation was assessed by Tukey's multiple range test. The capital, small and underlined letters indicate different assays.

to 31.3 ± 2.5) and the maximum richness (from 1,891 ± 111 to 1,160 ± 302) of the paddy methanogenic archaeal community at the rice tillering stage (Table 1). Consistently, DGGE fingerprinting analysis found decreases in the intensity of several DGGE bands, including bands 14 and 18, in response to elevated ground-level O_3 (Fig. S1). At the rice anthesis stage, elevated ground-level O_3 had no influence on paddy methanogenic community diversity and richness.

Shifts in methanogenic archaeal assemblages. Variations in the paddy methanogenic archaeal community of different samples were statistically evaluated using a non-metric multidimensional scaling (NMDS) plot of the weighted pairwise UniFrac community distances (Fig. 3). Significant shifts in the assemblage of these functional guilds were observed between elevated ground-level O_3 and ambient O_3, as well as between the two rice growth stages ($p < 0.05$), and were confirmed by the ANOSIM results (Table S2). The

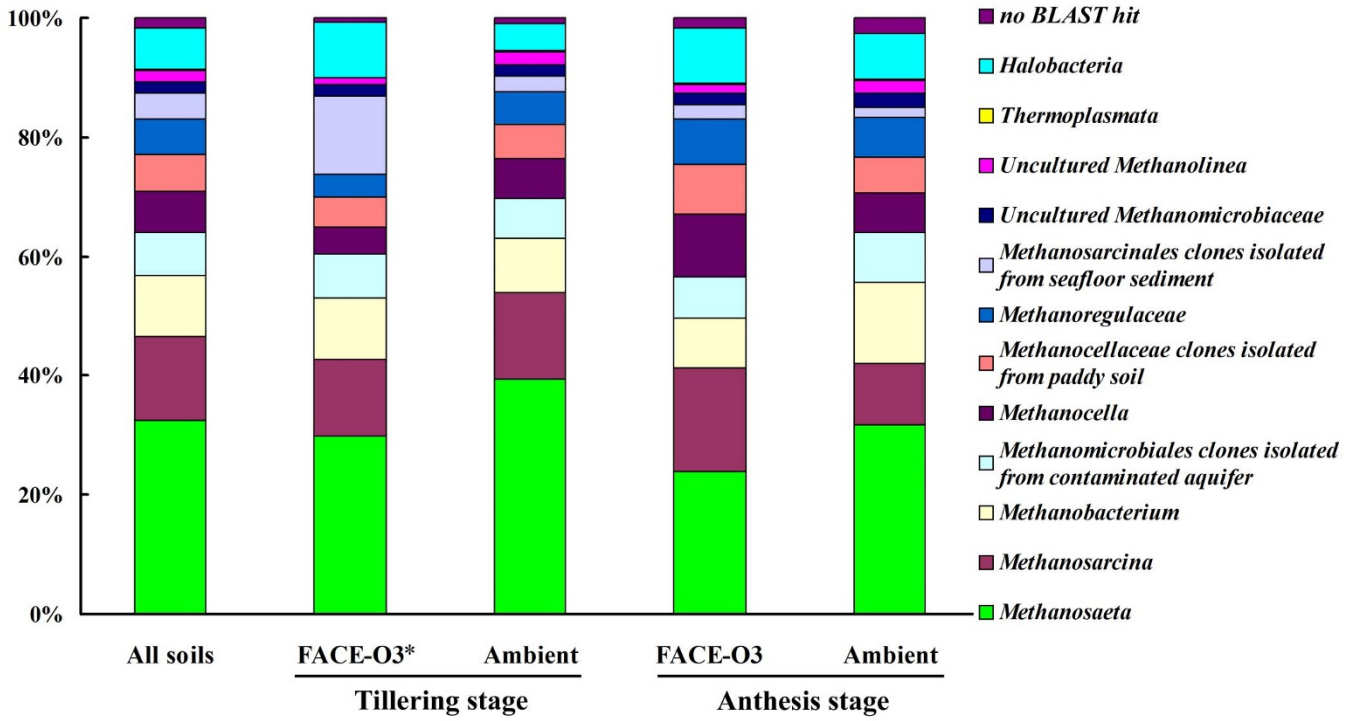

Figure 2 | The 100% stacked column chart of relative abundances of the dominant methanogenic genera derived from 16 s rRNA genes in all paddy soils combined and in each soil sample. *The value of each genus percentage is the mean of triplicates in Table S1.

distances between different samples along the NMDS axis 1 further imply that elevated ground-level O_3 concentration has an even greater influence than the rice growth stage. Furthermore, compared to ambient O_3 conditions, it could be inferred that elevated ground-level O_3 had the opposite influences on methanogenic archaeal community composition at the rice tillering and anthesis stages.

Changed methanogenic archaeal OTUs. To demonstrate the effects of elevated ground-level O_3 on paddy methanogenic community composition, a Venn diagram and response ratios were generated to identify the changes in overall and taxa-specific OTUs. Using a subset of 3,500 sequences per sample, a Venn diagram was constructed that calculated the overlap among methanogenic assemblages in different samples. We found that 115 OTUs were shared by all the soils and respectively accounted for 33.2%, 30.7%, 31.3% and 31.8% of OTUs under elevated ground-level O_3 or ambient O_3 at the rice tillering and anthesis stages. Furthermore, unique OTUs for each soil sample were as follows: 114 OTUs (32.9%), 107 OTUs (28.6%), 113 OTUs (30.7%) and 90 OTUs (24.9%) (Fig. 4). At the rice tillering stage, 177 OTUs were shared and accounted for 51.2% and 47.3% of total OTUs under elevated ground-level O_3 and ambient O_3, respectively. At the rice anthesis stage, 196 OTUs were shared by the elevated ground-level O_3 (53.3%) and the ambient O_3 (54.1%). By contrast, the ambient O_3 at the two rice growth stages shared 208 OTUs accounting for 55.6% (tillering) and 57.5% (anthesis) of total OTUs.

Table 1 | Paddy methanogenic archaeal phylogenetic diversity and Chao1 indices under different ground-level O_3 concentrations at the two rice growth stages

Soil samples		Phylogenetic Diversity*	Chao1
Tillering stage	FACE-O_3	31.3(2.5#)A	1160(302)a
	Ambient	37.8(0.9)B	1891(111)b
Anthesis stage	FACE-O_3	37.1(2.0)AB	1548(359)ab
	Ambient	33.5(3.0)AB	1790(105)ab

*Both indices were calculated using the subset of 3,500 sequences per soil sample.
#Numbers in the parenthesis are the standard deviations.

Response ratios were calculated based on the sequence size of each genus (Fig. 5). The 95% confidence interval (CI) at the rice tillering stage ranged from −0.01 to −0.17 and did not overlap with 0, which indicates that elevated ground-level O_3 brings a significant negative influence on the methanogenic archaeal community composition at the rice tillering stage ($p < 0.05$). Compared to ambient O_3, a total of 12 genera were significantly ($p < 0.05$) decreased and 6 genera were significantly ($p < 0.05$) increased under elevated ground-level O_3 at the rice tillering stage. The dominant genera, namely *Methanosaeta*, *Methanosarcina* and *Methanocella*, significantly decreased under elevated ground-level O_3 ($p < 0.05$). At the rice anthesis stage, the 95% CI, ranging from 0.19 to 0.05, revealed a significant positive effect of elevated ground-level O_3 on methanogenic archaeal community composition ($p < 0.05$): under elevated ground-level O_3, 12 genera were significantly increased and 7 genera were significantly decreased ($p < 0.05$). For example, *Methanosarcina*, *Methanocella* and *Methanocellaceae* clones isolated from paddy soil were significantly increased by elevated ground-level O_3 ($p < 0.05$). The results of the Venn diagram and response ratios are consistent with the general trends of the taxonomic distributions of paddy methanogenic archaea (Fig. 2), PD index, Chao1 index (Table 1) and NMDS plot (Fig. 3). In summary, elevated ground-level O_3 influenced the phylogenetic composition of paddy methanogenic archaeal community and significantly decreased their diversity at the rice tillering stage ($p < 0.05$).

Discussion

The community structure of paddy methanogens has been previously studied[25,27,28]. To display a high resolution of the paddy methanogenic archaeal community composition, 454 pyrosequencing technology was utilized in the present investigation. We obtained a

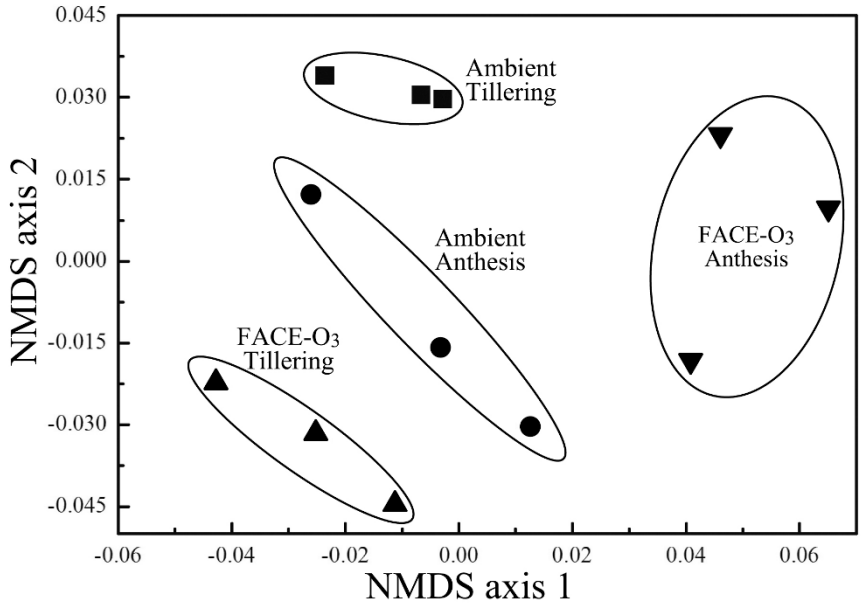

Figure 3 | **Methanogenic archaeal community compositional structure in soils as indicated by a non-metric multi-dimensional scaling plot of the weighted pairwise UniFrac community distances between different soil samples.**

total of 87,688 sequences, of which an overwhelming proportion was highly related to methanogens. These sequences provided detailed information on the methanogenic archaeal community structure in paddy soil and revealed its potential shift in response to elevated ground-level O_3. Such information extends the current knowledge of paddy methanogenic archaeal communities, which is derived from analyses of traditional genetic fingerprinting, clone library and culture-dependent assays.

Detailed information on the paddy methanogenic community. In general, the paddy methanogenic archaeal community is dominated by two aceticlastic groups of *Methanosaetaceae* (32.0%) and *Methanosarcinaceae* (18.5%) followed by hydrogenotrophic *Methanocellaceae* (Rice cluster I) (13.0%) (Fig. 2 and Table S1). This information supports the findings from denaturing gradient gel electrophoresis (DGGE) fingerprinting based on the biomarker of either the 16 s rRNA gene[25] or the functional gene *mcrA*[28]. Furthermore, the present data provided the respective quantitative percentage in the community. Among low-molecular-weight organic acids, acetate is the most abundant in paddy soil[29], and its concentration can exceed 10 mM in anoxic rice soil[30]. An abundant acetate content can readily stimulate the metabolism of aceticlastic methanogenic archaea, such as *Methanosaeta* and *Methanosarcina*. Consequently, approximately two-thirds of biogenic methane is

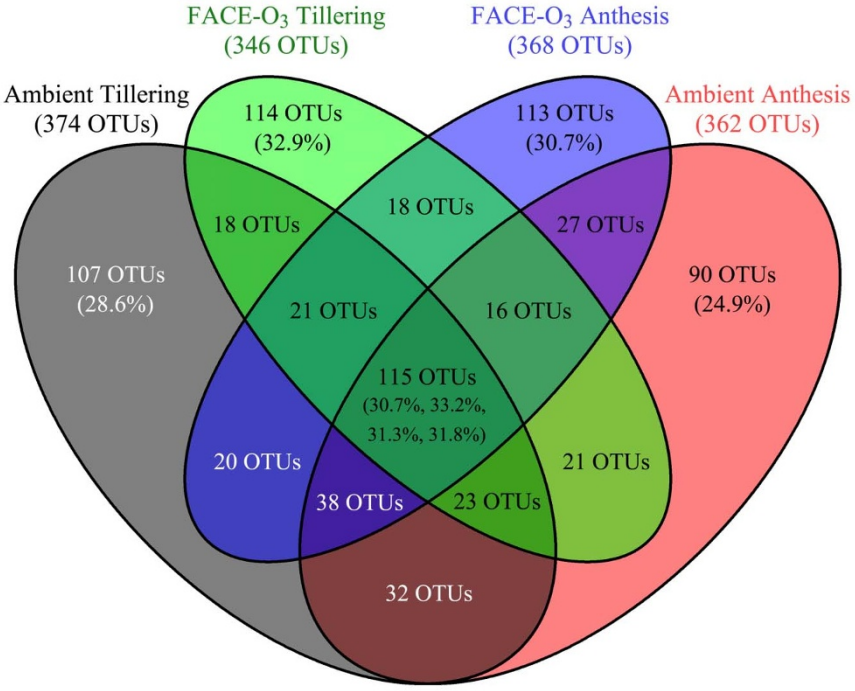

Figure 4 | **Venn diagrams comparing the OTU memberships among different soil samples.** The OTU size in parenthesis under each sample name was calculated from the subset of 3,500 sequences per soil sample. The percentage of unique OTUs was reported in parenthesis.

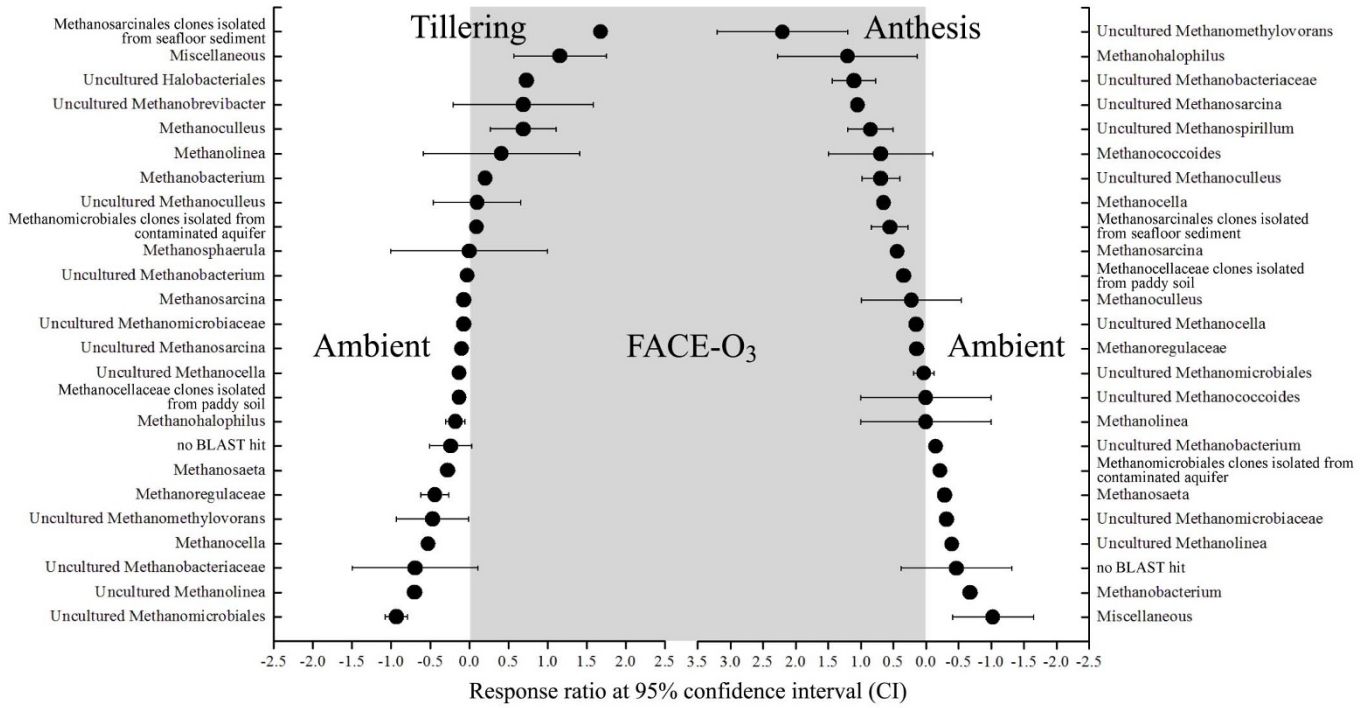

Figure 5 | Significant changes in genera under elevated ground-level O_3 according to the response ratio method at a 95% confidence interval.

derived from the methyl group of acetate by aceticlastic methanogens[31]. Moreover, *Methanosaeta* may even be the principal methane producer on Earth[32]. Ranked third, the family *Methanocellaceae* (Rice cluster I), which was previously recognized as the uncultured archaeal group Rice Cluster I, also plays a key role in methane production. RNA-based stable isotope probing analysis strongly suggests that the members of this family are the most active for metabolizing rice root exudates and that they contribute greatly to paddy methane production[33]. The percentage of *Methanocellaceae* (Rice cluster I) observed in the present investigation (13.0%) was substantially lower than the 32.0% reported by Conrad *et al.*[34] but higher than the 7.0% reported in data from a rice field in Fukuoka, Japan[28]. This variation might result from different atmospheric temperatures. Conrad *et al.*[35] found that higher temperatures can increase the percentage of *Methanocellaceae* (Rice cluster I) in paddy methanogenic archaeal communities. The latitude of Hainan Island, China in the report of Conrad *et al.*[34] is lower than that of the location of the present experiment. The mean annual temperature of Hainan Island (26°C) is higher than ours (16°C). Thus, the percentage of *Methanocellaceae* (Rice cluster I) in the present investigation is dramatically lower than the percentage reported in their study. Similarly, the present data are higher than that (7.0%) of data from a rice field in Fukuoka, Japan[28]. Such differences should cause the ecological significance of acetoclastic methanogenic archaea in paddy CH_4 formation to be reconsidered, possibly requiring further examination.

Elevated ground-level O_3 significantly influences the paddy methanogenic community at the rice tillering stage. As initially hypothesized, the paddy methanogenic community was negatively influenced by elevated ground-level O_3, especially at the rice tillering stage. Due to the indirect influence of elevated ground-level O_3 on soil microorganisms via plants, responses of methanogenic archaeal community were studied at the rice tillering and anthesis stages. It is well known that at the rice tillering stage, paddy soil has the highest methanogenic activity, leading to a seasonal peak of CH_4 flux[36] because rice root activity reaches its maximum at this stage. However, we found that at the rice tillering stage, elevated ground-level O_3 significantly decreased paddy methanogenic activity ($p < 0.05$) (Fig. 1c), phylogenetic diversity and richness (Table 1), and potentially the overall abundance of methanogens (Fig. 1b). Correspondingly, the community composition was shifted under elevated ground-level O_3 (Figs. 2, 3 and 5). Samples under elevated ground-level O_3 had the largest number of unique OTUs at the rice tillering stage (Fig. 4). One possible mechanism could be that elevated ground-level O_3 decreases the availability of carbon sources for methanogens. Rai *et al.*[37] have reported high inhibition in root biomass under elevated O_3 due to phytotoxicity. Lower amounts of organic matter partitioning to roots, in turn significantly decreases rice root activity[38]. Correspondingly, elevated ground-level O_3 reduces carbon inputs from plants, such as root exudates[15]. Jones *et al.*[39] reported a reduction of up to 55% in DOC contents under elevated O_3 concentrations. We also consistently found significant decreases in DOC concentrations under elevated ground-level O_3 ($p < 0.05$) (Fig. 1a). A decrease in carbon sources, which are the fuel for microorganism metabolism, changes both the structural and functional aspects of soil biodiversity[40,41]. In a previous investigation, we found that anoxygenic phototrophic purple bacteria negatively respond to elevated ground-level O_3 due to the decrease in carbon bioavailability[21]. Similarly, a negative effect on paddy methanogenic archaeal communities was found in the present investigation.

The negative response of the paddy methanogenic community resulted from the responses of dominant groups; this observation was more obvious for several dominant methanogenic groups. At the rice tillering stage, both the relative and absolute abundances of aceticlastic *Methanosaeta* were significantly decreased ($p < 0.05$) (Figs. 1b and 2), as was the response ratio (Fig. 5). These findings are supported by the DGGE fingerprinting profile (Fig. S1) and the phylogenetic identification (Fig. S2). Acetate is one of the most abundant low-molecular-weight organic acids in paddy soil[29] that is derived both from fermentation[42] and from root exudation[43]. Therefore, inevitably, the acetate content was significantly decreased under elevated ground-level O_3 ($p < 0.05$) (Fig. 1a). In rice fields, acetate levels increase to micromolar levels after sulfate consumption[44]. When acetate reaches micromolar levels, aceticlastic methanogens can use acetate for methanogenesis and dynamically

maintain acetate at the micromolar level. The decrease of the dynamic equilibrium of the acetate content due to elevated ground-level O_3 in this investigation could significantly and negatively influence aceticlastic methanogenesis and aceticlastic *Methanosaeta* ($p < 0.05$), although the exact mechanism remains unknown. *Methanosaeta* might be outcompeted under these negative conditions by other aceticlastic taxa because it is slow growing[45]. Similar phenomena were observed for aceticlastic *Methanosarcina* (Fig. 2, Table S1 and Fig. 5). Interestingly, the extent of *Methanosaeta* responses was greater than that of *Methanosarcina*. A possible explanation is that *Methanosaeta* is a specialist that uses only acetate, unlike *Methanosarcina*, which prefers methylated compounds, such as methanol and methylamines, to acetate[32]. In addition to aceticlastic methanogens, members of the *Methanocellaceae* family, which are the most metabolically active methanogens for rice root exudates[33], were also significantly decreased ($p < 0.05$) under elevated ground-level O_3 at the rice tillering stage (Fig. 2, Table S1 and Fig. 5). As mentioned above, these methanogenic groups play important roles in CH_4 production in paddy soil. Consequently, the overall methanogenic activity at the rice tillering stage was completely inhibited by elevated ground-level O_3 (Fig. 1c). Furthermore, rhizodeposition is regarded as the main origin of CH_4 produced in rice fields[46]. The decrease in DOC could also reduce the methanogenic substrate for methanogens. Collectively, adverse effects on the diversity and functional behavior of methanogens at the tillering stage would lead to a decrease in cumulative CH_4 emissions during the entire rice growing season, which could be the underlying microbial mechanism that explains the observations of Zheng *et al.*[9] and Bhatia *et al.*[10].

Negative influences of elevated ground-level O_3 were still observed at the rice anthesis stage. The negative influence of elevated ground-level O_3 on the paddy methanogenic archaeal community was alleviated at the rice anthesis stage. For example, a positive effect of elevated ground-level O_3 was observed on the community composition at the rice anthesis stage (Fig. 5): the OTU size increased to 368 (Fig. 4), and 12 methanogenic genera were significantly increased ($p < 0.05$) (Fig. 5). Moreover, the methanogenic activity at the rice anthesis stage was significantly higher than that at the rice tillering stage ($p < 0.05$) (Fig. 1c). All of these phenomena may have been due to the increase in the relative abundances of the genus *Methanosarcina* and the order *Methanocellales* (Table S1, Figs. 2 and 5). Both *Methanosarcina* and *Methanocella* can tolerate microoxygen[47], and *Methanocella* is potentially the most oxygen-tolerant methanogen[48]. For similar reasons, the relative abundances of these genera were increased at the rice anthesis stage after mid-season drainage. However, the abundances of overall methanogens and *Methanosaeta* continued to decrease under elevated ground-level O_3 at the rice anthesis stage (Fig. 1b). Moreover, 7 genera were significantly decreased ($p < 0.05$) (Fig. 5) and the overall methanogenic activity was still significantly lower ($p < 0.05$) (Fig. 1c) under elevated ground-level O_3. Therefore, elevated ground-level O_3 still potentially negatively influences the paddy methanogenic archaeal community at the rice anthesis stage.

The observed methanogenic archaeal responses imply that a continuously elevated ground-level O_3 would retard the rate of the increase in methane emissions and further influence global climate change. In view of the cumulative effect of global climate change[49], all of these results indicate that a continuously elevated ground-level O_3 would negatively influence paddy methanogenic archaeal diversity and the ecological behaviors of the community and finally reduce CH_4 production[9,10]. Furthermore, methanotrophs, which utilize CH_4 as their sole source of carbon and energy, would also be influenced. Specifically, with the accumulation of O_3 stress, the decrease in the availability of methane in paddy soil could make high-affinity methanotrophs more active to assimilate atmospheric CH_4 for survival and would increase the sink of atmospheric CH_4. Global climate change encompasses multiple aspects, such as elevated atmospheric CO_2, elevated ground-level O_3 and global warming. Both elevated atmospheric CO_2 and higher soil temperatures can significantly increase paddy CH_4 emission[5]. Therefore, continuously elevated ground-level O_3 would retard the rate of the increase in methane emissions, which are implicated in global climate change. Consequently, decreased paddy CH_4 emission would be expected to mitigate global warming potential (GWP) of greenhouse gases because CH_4 has approximately 25 times higher GWP than CO_2. Moreover, the reduction in CH_4 emission may slow the increase in tropospheric O_3 concentration because CH_4 is one of the major O_3 precursors.

Methods

Site description. As previously described by Feng *et al.*[21], the FACE-O_3 system was established in Jiangdu County, Jiangsu Province, China (119°42′0″E and 32°35′5″N). This site has been in continuous cultivation for over 1,000 years with a rice-wheat rotation. The soil is classified as stagnic anthrosol. The relevant soil properties are as follows: 9.2% sand (1–0.05 mm), 65.7% silt (0.05–0.001 mm), 25.1% clay (<0.001 mm), 1.2 g/cm^3 bulk density, 15.0 g/kg soil organic C (SOC), 1.59 g/kg total N, 1.23 g/kg total P, 10.4 mg/kg available P and pH 6.8. The station sits in the subtropical climatic zone with a mean annual precipitation of 900–1,000 mm, mean annual temperature of 16°C, an average daily integral radiation of 12.3 MJ/m^2, a total annual sunshine time of more than 2,000 h and a frost-free period of more than 230 days.

FACE-O_3 system description. The FACE-O_3 project began on 1 July 2007 and has been carried out for 4 years. The FACE-O_3 system has been described in detail by Tang *et al.*[50]. Briefly, this system has six plots, of which three were under elevated O_3 (hereinafter called FACE-O_3) and three were under ambient O_3 (hereinafter called Ambient). Each plot had an area of approximately 240 m^2. The target O_3 concentration in FACE-O_3 was 50% higher than that in Ambient and was around the average of 60 ppb during the entire rice-growing season. Any one of the FACE-O_3 was separated from the other plots by at least 70 m to avoid cross-contamination. Each FACE-O_3 had a dedicated system for measurement and control of O_3 concentration with an O_3 concentration analyzer (Thermo Electron 49i, Thermo Scientific Co., USA) and a data logger-controller (Campbell CR10X, Campbell Scientific Co., USA). The O_3 concentration analyzers were calibrated against a transfer standard (Thermo Electron 49i-PS, Thermo Scientific Co., USA) on a monthly basis. The O_3 fumigation began at 9:00 a.m. and continued until sunset but was discontinued when the following occurred: (1) leaves were wet, in which case a leaf-wetness sensor was used to shut down the fumigation, or (2) ambient O_3 was lower than 20 ppb. When the target O_3 was higher than 250 ppb, the set-point O_3 was fixed at 250 ppb to prevent the plants from being exposed to extraordinarily high O_3. In the Ambient plots, plants were grown under ambient O_3 without the ring.

Outline of cropping systems. The rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L. cv. Yangmai 14) fields were managed in a rice-wheat rotation system, which is widely distributed in the subtropical area in China. In this system, wheat is sown in early November and harvested in late May or early June of the following year. Rice seeds are sown on a nursery bed in mid-May, and the seedlings are transplanted to fields in mid-June and harvested in mid- to late October. Urea and compound fertilizer are applied as N fertilizer. For wheat cultivation, N fertilizer is applied three times as follows: 50% of nitrogen is basally applied in early November, and the remaining nitrogen is top-dressed in mid-February (10%) and mid-April (40%). For rice cultivation, N fertilizer is applied three times as follows: 36% of nitrogen is basally applied in mid-June, and the remaining nitrogen is top-dressed in mid-June (24%) and mid-July (40%). Detailed information on rice and wheat cultivation has been described previously[51,52].

Sample collection and determination. On 18 July 2010 (rice tillering stage beginning with the appearance of the first tiller and continuing up to the maximum tiller number) and 10 September 2010 (rice anthesis stage beginning with the emergence of the first anthers from the uppermost spikelets on each panicle and continuing for approximately 15 days), soils from three FACE-O_3 plots and three ambient plots were collected. In total, there were 12 soil samples (2 stages × 2 different O_3 concentration × 3 replicates). Each soil sample was collected at a depth of 0 to 5 cm at six points and fully mixed. Aboveground plant materials, roots and stones were removed before homogenizing the soil samples. Samples for molecular studies were maintained at −40°C until further use. Sub-samples for the microcosm incubation assay were pooled and maintained at 4°C without sieving. Soil dissolved organic C (DOC) was extracted by adding 50 ml of 0.5 M K_2SO_4 to 10 g of soil, shaking for 1 h and then vacuum filtering through glass fiber filters (Fisher G4, 1.2 μm pore space). The filtrate was stored at −20°C until further analysis. DOC was determined using a TOC-TN analyzer (Skalar, Netherlands). Acetate in the filtrate was analyzed by high-performance liquid chromatography (Dionex, USA).

Soil DNA extraction. For each soil, genomic DNA was extracted from the same amount of moist soil (0.5 g) on the day after sampling using a FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions.

The extracted soil DNA was dissolved in 50 μl of TE buffer, quantified using a spectrophotometer and stored at −20°C until further use. A total of 12 DNA samples were used for qPCR and bar-coded pyrosequencing analyses.

PCR and preparation of the amplicon libraries for 454 pyrosequencing. For each soil sample, the following primer set was used to amplify approximately 280 bp of methanogenic archaeal 16 s rRNA gene fragments for sequencing on the 454 GS-FLX pyrosequencing platform: 1106F (TTWAGTCAGGCAACGAGC) and 1378R (TGTGCAAGGAGCAGGGAC)[23]. The oligonucleotide sequences included the 454 Life Science A or B sequencing adapters (19 bp) fused to the 7-bp bar-coded primer set as follows: Primer B (GCCTTGCCAGCCCGCTCAG) + barcode + forward primer; and Primer A (GCCTCCCTCGCGCCATCAG) + reversed primer. PCR was carried out in 50-μl reaction mixtures with the following components: 4 μl (initial 2.5 mM each) of deoxynucleoside triphosphates, 2 μl (initial 10 μM each) of forward and reverse primers, 2 U of *Taq* DNA polymerase with 0.4 μl (TaKaRa, Japan), and 1 μl of template containing approximately 50 ng of genomic community DNA as a template. Thirty-five cycles (95°C for 45 s, 56°C for 45 s, and 72°C for 60 s) were performed with a final extension at 72°C for 7 min. Triplicate reaction mixtures per sample were pooled, purified using the QIAquick PCR Purification kit (QIAGEN), and quantified using a NanoDrop ND-1000 (Thermo Scientific, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts before pyrosequencing using Genome Sequencer FLX System platform (454 Life Science Branford, CT, USA).

Processing of pyrosequencing data. The methanogenic archaeal 16 s rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) 1.4.0-dev pipeline ([53]; http://www.qiime.org) with the default parameters unless otherwise noted. In brief, sequences were quality trimmed (> 25 quality score and 200 bp in length), and assigned to soil samples based on unique 7-bp barcodes. Sequences were denoised[54] and then binned into OTUs using a 97% identity threshold; the most abundant sequence from each OTU was selected as a representative sequence for that OTU. Taxonomy was assigned to methanogenic archaeal OTUs against a subset of the Silva 104 database (http://www.arb-silva.de/download/archive/qiime/). OTU representative sequences were aligned using PyNAST, and chimera sequences were removed through QIIME. A phylogenetic tree was then constructed using FastTree2[55] to support phylogenetic diversity calculations.

The richness of phylotypes was calculated to compare community-level methanogenic archaeal diversity at a single level of taxonomic resolution. We also estimated phylogenetic diversity using Faith's index[56], which provides an integrated index of the phylogenetic breadth across taxonomic levels. In this diversity analysis, 87,688 methanogenic archaeal sequences that passed QIIME's quality filtering were included. We obtained between 3,551 and 15,387 sequences per sample for all soil samples (mean = 7,307 and median = 6,801) (Table S1). Because an even depth of sampling is required for beta diversity calculations, we reduced the datasets to the lowest number available to correct for differences in survey effort between samples. Namely, we calculated both diversity metrics using a randomly selected subset of 3,500 sequences per soil sample. This approach allows us to compare general diversity patterns among sites even though it is highly unlikely that we surveyed the full extent of diversity in each community[57]. The weighted pairwise UniFrac distances[58] were calculated for community comparisons using QIIME and were visualized using non-metric multidimensional scaling plots as implemented in PRIMER v6[59]. ANOSIM analyses were conducted using PRIMER v6[59].

Real-time quantitative PCR. For each soil, real-time quantitative PCR (qPCR) was used to determine the abundances of the methanogenic archaeal 16 s rRNA gene (1106F and 1378R primer set) and key methanogens, namely the *Methanosaeta* 16 s rRNA gene (1AF (TCYGKTTGATCCYGSCRGAG)[60] and Mst 746r (TCCCTTGCCGTCAGGTC)[61] primer set). Briefly, the target gene copy number was quantified by qPCR analysis with a C1000™ Thermal Cycler equipped with a CFX96™ Real-Time system (Bio-Rad, USA). To generate a qPCR standard curve, a single clone containing the correct insert was grown in Luria-Bertani medium, and then plasmid DNA was extracted, purified and quantified. A 10-fold dilution series of the linear plasmid DNA was made to generate a standard curve covering seven orders of magnitude from 10^2 to 10^8 copies of template per assay. Assays were set up using the SYBR *Premix Ex Taq*™ Kit (TaKaRa) with a 25-μl reaction mixture containing 12.5 μl of SYBR® *Premix Ex Taq*™, 1 μl of the primer set (initial concentration of 10 μM each), 0.5 μl of BSA at 20 mg/ml initial concentration, and 1.0 μl of template containing approximately 2–9 ng of DNA. Blanks were run with water as the template instead of soil DNA extract. Specific amplification of the target gene was confirmed by agarose gel electrophoresis of qPCR amplicons showing the expected band of PCR amplicons sizes and a melting curve analysis resulting in a single peak. qPCR was performed in triplicate, and amplification efficiencies of 97.4–104% were obtained with R^2 values of 0.966–0.977. The final methanogenic archaeal and *Methanosaeta* 16 s rRNA gene quantities were obtained by calibrating against the total DNA concentrations extracted and soil water content.

Methanogenic activity in paddy field soil. Soil samples, which were collected from each FACE-O_3 and ambient plots, were used for the measurement of methanogenic activity in paddy field soils[23]. Briefly, the soils were passed through a 2-mm mesh sieve, and 10 cm^3 of wet soils under flooded conditions were transferred into a 120-ml serum bottle sealed with a butyl rubber septum and an aluminum cap. The headspace in the bottle was flushed with N_2. The preparations with triplicates for one soil sample were incubated statically at 25°C in a dark room, and CH_4 production was determined periodically by a gas chromatograph (Varian 3380) with FID. Methanogenic activity was calculated from the concentration during the period with a linear increase of methane.

Statistical analysis. Statistical procedures were performed with the SPSS 13.0 software package for Windows. Data were expressed as the means with standard deviation (SD), and the letters above the error bar indicate significant differences between the results of the different samples. Mean separation was assessed by Tukey's multiple range test. Differences at $p < 0.05$ were considered statistically significant. Based on the subset of 3,500 sequences per soil sample, a Venn diagram of OTUs among different soil samples was plotted using the R package software (Version 2.12.1), and the response ratios of methanogenic archaeal genera under different ground-level O_3 concentrations were analyzed following the statistical method of Luo *et al.*[62].

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Author contributions

J.G.Z. and X.G.L. designed the study. Y.Z.F. and Y.C.Y. performed the experiments. Y.Z.F., H.Y.Z. and H.Y.C. analyzed the data. Y.Z.F. and X.G.L. wrote the paper. All authors reviewed the manuscript.

Additional information

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