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Carbon nanotubes accelerate acetoclastic methanogenesis: From pure cultures to anaerobic soils

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ABSTRACT

Direct interspecies electron transfer (DIET) between electricigens and methanogens has been shown to favour CO2 reduction to produce biomethane. Furthermore, DIET is accelerated by conductive materials. However, whether conductive materials can promote other methanogenic pathways is unclear due to a lack of detailed experimental data and the poor mechanistic studies. Here, we hypothesized that conductive carbon nanotubes (CNTs) stimulate acetoclastic methanogenesis independently of electricigens in pure cultures of Methanosarcina spp. and anaerobic wetland soil. We found a significant increase in the methane production rate during the growth phase, e.g. from 0.169 mM to 0.241 mM after addition of CNTs on the 3rd day. CNTs did not increase the abundance of electromicroorganisms or the electron transfer rate in anaerobic soils, using via microbial diversity and electrochemical analysis. 13C-CH3COOH labelling, stable carbon isotope fractionation and the CH3F inhibitor of acetoclastic methanogenesis were used to distinguish methanogenic pathways. CNTs mainly accelerated acetoclastic methanogenesis rather than CO2 reduction in both pure cultures and anaerobic soils. Furthermore, the presence of CNTs slightly alleviate the inhibition of CH3F on acetoclastic methanogenesis during the pure culture of Methanosarcina barkeri and Methanosaricina mazei with the production of more than 0.3 mM methane. CNTs closely attached to the cell surface were observed by transmission electron microscopy. Proteome analysis revealed a stimulation of protein synthesis with about twice the improvement involved in -COOH oxidation and electron transfer. Overall, our findings demonstrate that conducting CNTs favor methane production and that the mechanism involved is acetoclastic methanogenesis via acetate dismutation, at least partly, rather than classical CO2 reduction.

1. Introduction

Methane plays a crucial role globally as a greenhouse gas and a source of renewable fuel. Biomethane is typically produced by the cooperation of various microorganisms such as fermenting microorganisms and methanogenic archaea or methanogens (Liu et al., 2018; Zhu et al., 2018). Fermenting microorganisms are responsible for producing methanogenic substrates, such as acetate and CO2. Methanogens produce methane as the end-product of their anaerobic respiration to obtain energy. The common habitats of methanogens include sediments, anaerobic soils, landfills and so on (Ji et al., 2018; Xiao et al., 2019a). 77.4% of the cultivated methanogens can reduce CO2, and the next most abundant type of metabolism is methylotrophic methanogenesis (Holmes and Smith, 2016). The acetoclastic methanogenic pathway is by far the least metabolism, only 12 species in two genera, namely, Methanosarcina and Methanosaeta (formerly known as Methanobacteri), able to utilize acetate as a carbon and energy source (Holmes and Smith, 2016). Methanosarcina is a metabolically versatile methanogen with the ability to use CO2, acetate and methyl compounds to generate methane (Rother and Metcalf, 2004). For example, research published 40 years ago...
Acetoclastic methanogenesis: \( CH_3COO^{-} + H^+ \rightarrow CH_4 + CO_2 \) (Eq. 1)

Hydrogenotrophic methanogenesis: \( 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \) (Eq. 2)

DIET: \( 8H^+ + 8e^- + CO_2 \rightarrow CH_4 + 2H_2O \) (Eq. 3)

During acetoclastic methanogenesis, methanogenic archaea convert acetate into methane and CO2 by dismutation (Eq. (1)). Here, the acetate carboxyl group is oxidized to CO2 with the release of electrons (Li et al., 2006). Then, electrons are used to reduce CoM-S-S-CoB that transforms into methanofuran-CHO, and then, a series of intermediate metabolites are involved such as methyl-tetrahydrodismethanopterin-HCO- methyl-tetrahydrodismethanopterin-CH3 (H2MPT-CH3). From H2MPT-CH3 to methane, hydrogenotrophic methanogenesis and acetoclastic methanogenesis share the same route. DIET coupling of exoelectrogenic bacteria and methanogens has been further proposed to explain methanogenesis in complex environments (Morita et al., 2011; Song et al., 2019; Xiao et al., 2019a,c). This coupling can be strengthened by the addition of conductive materials (Liu et al., 2012; Lu et al., 2020; Viggli et al., 2014; Xiao et al., 2018; Xiao et al., 2019b).

DIET coupled to CO2 reduction is the main mechanism referred to explain methane production. Nonetheless, this has been unequivocally demonstrated only in cocultures of Geobacter with Methanosaeta or Methanosarcina (Martins et al., 2018; Van Steendam et al., 2019). Interestingly, it is acetotrophic methanogens rather than hydrogenotrophic methanogens that can accept electrons to reduce CO2. In-sights into an alternative mechanism come from enhanced acetate dismutation by conductive materials (Fu et al., 2019; Li et al., 2018). For instance, Fu et al. (2019) disclosed that nano-Fe3O4 acted as an intracellular electron shuttle to accelerate acetoclastic methanogenesis by culturing M. barkeri with acetate as a carbon source. Whereas, redox cycling of Fe (II) and Fe (III) in nano-magnetite in the extracellular space accelerated acetoclastic methanogenesis by cultivating Methanosarcina mazei (Wang et al., 2020). These studies evidence the acceleration of acetate dismutation. However, the mechanism of increased acetoclastic methanogenesis is still poorly understood. Concerning iron ions may be used as nutrients to improve the activity and function of methanogens. Therefore, more work is needed to verify the benefits from conductivity. As human activities increase, more and more carbon nanomaterials end up into soils (Rai et al., 2018; Usman et al., 2020). Nano-magnetite has been found to accelerate methane production by acetate dismutation in pure culture. However, it is still unclear how conductive nanomaterials work to accelerate acetoclastic methanogenesis in multicomunity environments.

To accurately distinguish the source of methane, multiple techniques are necessary. CH3F, an inhibitor of acetoclastic methanogenesis, is widely used to analyze the contribution of CO2 reduction and acetate dismutation (Conrad and Casper, 2010; Ji et al., 2018; Xiao et al., 2018). In brief, methane accumulation comes from both CO2 reduction and acetoclastic methanogenesis without CH3F. Whereas only CO2 reduction can contribute to methane production in the presence of CH3F. The difference between the two treatments thus reveals the contribution of acetoclastic methanogenesis. In addition to the use of CH3F, stable isotope fractionation is a more precise and accurate method (Conrad, 2005). Although these methods are widely used to distinguish traditional methane production pathways, these methods are rarely used to study methane production influenced by electromicroorganisms and conductive materials (Xiao et al., 2020).

Here, we propose an alternative mechanism of acetoclastic methanogenesis involving electron transfer by conductive carbon materials and we explain the mechanism in depth. To test this hypothesis we studied methane production in pure cultures of M. barkeri, M. Mazei and in anaerobic soil containing carbon nanotubes (CNTs). The mechanisms were qualitatively and quantitatively assessed by 13C tracing, CH3F methanogenic inhibition, proteome analysis, thermodynamic analysis, electrochemical analysis, modelling and microscopy.

2. Materials and methods

2.1. Microorganisms and growth conditions for pure culture

Experiment 1: Mineral salt medium, as described elsewhere (Zehnder and Wuhrmann, 1977), was used to culture M. barkeri and M. mazei under strict anaerobic conditions with ~20 mmol L−1 acetate as the methanogenic substrate. In addition to H2, some components with reducibility, such as cysteine and Na2S, may act as electron donors to reduce CO2 to produce methane. In this study, the final concentration of cysteine was about 1 mM, and it was approximately 0.5 mM for Na2S. Therefore, two kinds of methanogenic substrates were provided including acetate and CO2. Anaerobic tubes (25 mL total volume, medium volume of 10 mL) were pressurized with a mixture of N2/CO2 (80%/20%). All incubations were conducted at 30 °C in the dark. In this study, carbon nanotubes were purchased from Macklin (Shanghai, China; CAS: 308068-56-6, Lot#:C10112635, Inner diameter: 5~10 nm, Outer diameter: 10~20 nm, Length: 500~2000 nm). No nanotubes was added in the control group, and the experimental group was added artificially. The final concentration of CNTs was ~0.2 g/L. According to visual observation, most of the CNTs were deposited on the bottom of the vials. The concentration of produced gases was tested using a gas chromatograph (GC; Agilent 7820A, USA) equipped with a flame ionization detector (FID) and a thermal conductivity detector (TCD). A possible dissolution of methane in the medium can be neglected as follows: in the vials with a headspace-to-medium ratio of 15 mL/10 mL, the percentage of methane in the gaseous phase (\( f_g \)) is 97.9% at 30 °C is given by the equation:

\[
f_g = 100\% \times \frac{1}{1 + \frac{R}{V_g} - \frac{1}{V_w}}
\]

where Vg and Vw are the volumes of the gaseous and aqueous phases, respectively. R is the ideal gas constant, and a value of 0.0013 mol L−1 atm−1 is used for Henry’s law constant, K_H. The column is a Haysep Q 80~100 mesh (6 ft × 1.8” × 2.0 mm). High-pressure liquid chromatography (HPLC; Agilent 1260 Infinity) was used to test the acetate concentration.

Experiment 2: CH3F, an inhibitor of acetoclastic methanogenesis (Conrad, 2005), was applied to explore whether CNTs can improve the ability of M. barkeri to endure hostile environments. CH3F was applied at 1.5% v/v to replace the equal gas mixture (N2/CO2, 80%/20%) in the vials, other operations can refer to experiment 1.

2.2. Analysis of potential methane production pathways in pure culture systems with 13C labelling

Experiment 3: To clarify the methanogenic pathways for methane accumulation with and without CH3F, carbon isotopes of CH4 (13CH4/12CH4) were tested. CH4 collected from the headspace was tested to obtain the 813C value using a gas chromatograph combustion isotope ratio mass spectrometer (GC–C–IRMS) system (Thermo Fisher MAT253, Germany). To eliminate the interference of original CO2, separation of CH4/CO2 was performed in a Finnigan Precon. In brief, mixed gas (~1 mL) was injected into a sample container (100 mL),
which was filled with helium gas (99.999% purity) beforehand. Helium loaded the mixture into a chemical trap, which can be applied to scrub CO2 and H2O. Then, CH4 can be oxidized in a combustion reactor at 960 °C and converted to CO2 and water. After that, the combusted CO2 was subsequently purified by two liquid nitrogen cold traps with internal filling of Ni wires and then transferred into the IRMS for determination. The precision of repeated analyses was ±0.2‰ when 1.3 nmol methane was injected. The abundance of 13C in a sample is given relative to a standard using the δ notation:

$$\delta^{13}C = \left( \frac{^{13}C_{\text{sample}}}{^{12}C_{\text{sample}}} \right) / \left( \frac{^{13}C_{\text{PDB}}}{^{12}C_{\text{PDB}}} \right) - 1 \times 1000.$$ 

where PDB refers to the Pee Dee Belemnite carbonate that is used as a standard which has a 13C/12C ratio of 0.0112372.

Experiment 4: To test methane production pathway of M. barkeri and M. mazei in the presence of CH3H2O. Artificial abundance of 13CH3COOH (3% and 5%) was used in pure culture. The test of 13CH4/12CH4 was performed as described in Experiment 3.

2.3. LC-MS/MS based methanogen proteomics

Experiment 5: Proteome analysis was used to determine how CNTs increased the competitiveness of the acetoclastic methanogenic pathway. M. barkeri cells were obtained following the operation of Experiment 1 and proteins of this archaea were analyzed by LC-MS/MS. Three replicates were conducted. Briefly, the total protein collected from each sample, sampled on the 25th day, was digested with trypsin and then the peptides were labelled using a 6-plex TMT reagent Multiplex kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. A Triple TOF 6600 mass spectrometer (SCIEX, Concord, Ontario, Canada) was used to acquire the data. Based on PCA analysis of proteome data (Fig. S1), one of the repeats of the CNT group was not considered in the subsequent analysis. The detailed methods regarding protein extraction and digestion, TMT labelling, and LC-MS/MS analysis can be found in the supporting information.

2.4. Microcosm cultivation and chemical analysis

Experiment 6: After intermittent flooding in October 2017, we sampled soil from the Yellow River Delta, which is a sensitive wetland with serious human intervention (Xiao et al., 2017). To prepare the slurry for the subsequent culture experiment, 150 g soils, 1.5 g dry ground straw of Phragmites australis, and 450 mL sterile water were added into a serum bottle with the total volume of 1000 mL. The detailed operation can be found in the supplementary materials. For the following experiments, vials with a volume of 11 mL were used to test the effect of CNTs on methane production in anaerobic soil. Three cycles of vacuum/charging high-purity N2 were conducted to create anaerobic conditions. Suspensions of 10 g/L CNTs in ultrapure water were N2 flushed and then sterilized. For treatment with CNTs, a CNT suspension (100 µL) was added to each vial to reach a concentration of about 0.2 g/L. An equal amount of water was added to the vial of the control group. A 400 µL acetate solution (0.275 M) was used as the substrate for methanogens with the final acetate concentration of approximately 20 mM. Overall, all treatments contained the same amount of upper space and slurry plus sterile water approximately 5.5 mL. Vials were sacrificed in triplicate to all treatments contained the same amount of upper space and slurry plus with the final acetate concentration of approximately 20 mM. Overall, acetate solution (0.275 M) was used as the substrate for methanogens. The definitions of the parameters are given above, except CacceTa, the acetate concentration, mol·L⁻¹ and NpH, the pH value of the supernatant.

2.5. Calculation of Gibbs free energy for hydrogenotrophic and acetoclastic methanogenesis

Experiment 9: The Gibbs free energy (ΔG) of hydrogenotrophic methanogenesis was calculated based on the concentrations of H2, CO2 and CH4 to analyze the potential contribution of different methanogenic pathways (Xiao et al., 2019b). Briefly, the following equation was used:

$$\Delta G = \Delta G^0 + RT \ln \left( \frac{C_{CH4}}{C_{CO2}^*C_{H2}^*} \right) + \Delta S(T_f - T_0),$$

with ΔG0, ΔG at 273.15 K and 101.325 kPa; R, the ideal gas constant, 8.3145 J·mol⁻¹·K⁻¹; T, the absolute thermodynamic temperature, 303.15 K; CCO2, CCO2, and CCH4 concentrations of methane, CO2, and H2, mol·L⁻¹. For the calculation of ΔG0, ΔG = ΔG0 + ΔS(T_f - T_0), ΔG0 under 298.15 K and 101.325 kPa; ΔS, entropy change at 298.15 K; T_f and T_0, 298.15 K and 273.15 K, respectively. ΔG0 = ΔH-TfΔS, ΔH, enthalpy change at 298.15 K.

For the calculation of ΔG of acetoclastic methanogenesis, the following equation holds:

$$\Delta G = \Delta G^0 + RT \ln \left( \frac{C_{CH4}*C_{CO2}}{C_{CO2}^*} \right) + 2.303 * RT * NpH$$

The data are presented as the mean ± standard deviation of triplicate cultures. All statistical analyses were performed using Origin 2016 (Origin Lab Corporation, USA) software. A t-test was used to analyze the significance level, and a p-value of less than 0.05 was considered significant.
3. Results

3.1. Effects of carbon nanotubes on methane production in pure cultures

We performed pure cultures of Methanosarcina species to test whether CNTs could promote methane production via acetoclastic methanogenesis. Acetate consumption and methane production were accelerated by CNTs for both species (Fig. 1a and b). However, CNTs showed no significant stimulation of the hydrogenotrophic methanogen M. formicicum (Fig. 1d). The absolute δ\(^{13}\)C values of the produced methane ranged from –40.07 ± 0.90‰ to –36.46 ± 0.66‰ for M. barkeri and from –42.11 ± 0.93‰ to –34.08 ± 0.40‰ for M. mazei (Fig. 1c). These values were in the range of that reported for acetoclastic mechanisms usually have different \(^{13}\)C fractionation. Second, the highest δ\(^{13}\)C values of –34.08‰ and –36.46‰ observed with CNTs implied that CNTs favoured acetate dismutation because CO\(_2\) reduction is expected to fractionate more and yield the more negative values (Conrad, 2005). We further compared the amount of methane produced in the experiments and theoretical calculations for acetate dismutation (Fig. S2). Calculations were consistent with experimental data for CNTs but differed in the control assay. These findings revealed a contribution of acetate dismutation with CNTs. We also performed incubations with the hydrogenotrophic methanogen M. formicicum to check whether the acceleration of methane production was due to CNT chemical reactivity (Fig. 1d). The results showed the absence of acetate consumption and methane production, thus disclosing the CNTs cannot independently accelerate acetate dismutation. Overall, acetoclastic methanogenesis may be the mechanism explaining methane production with CNTs.

To strengthen the acetoclastic methanogenesis hypothesis we performed incubations of M. barkeri with CH\(_3\)F and \(^{13}\)C-labelled acetate (Fig. 2). Without CNTs, CH\(_3\)F inhibited methanogenesis, as expected, as shown by the dotted green and orange bottom lines in Fig. 2a. This inhibition in the control assay was also confirmed by the low \(^{13}\)C–CH\(_4\) abundance below 1.118%, typical of natural abundance, in the presence of highly \(^{13}\)C-labelled acetate (Fig. 2b). The minor methane production observed after 10 days was likely to be due to CO\(_2\) reduction. By sharp contrast, the addition of CNTs induced a rapid increase in methane emission after 6 days in the presence of CH\(_3\)F, as shown by the solid green and orange lines down in Fig. 2a, and the emitted methane was highly \(^{13}\)C-labelled (Fig. 2b). During the incubation of M. barkeri with the addition of CNTs, however, the methane produced in the presence of CH\(_3\)F (Fig. 2a) was only approximately 3% of that in the absence of CH\(_3\)F (Fig. 1b), and the acetate was hardly consumed during the whole incubation with the addition of CH\(_3\)F (Fig. 2b). Thus, CNTs could only slightly relieve the inhibition of acetate dismutation. Since this is a pure culture, interspecies electron transfer can be ruled out. These findings indicated the benefits of CNTs on methane production without the need for other electricigen species by promoting direct acetate dismutation (Eq. (1)).

M. barkeri and M. mazei produced methane through unexclusive acetoclastic methanogenesis even though 20 mM acetate was used as the substrate. Otherwise, the δ\(^{13}\)C–CH\(_4\) should be the same, and the values may be even lower than –30‰ (Penning et al., 2006). Supplemental to a previous study (Salvador et al., 2017; Wang et al., 2020), CO\(_2\) reduction may be involved in methane accumulation even though the substrate of M. barkeri and M. mazei was a high concentration of acetate.

3.2. Proteome analysis

We analyzed the proteome of M. barkeri cultivated with and without CNTs to obtain information on the effect of CNTs on protein expression resulting in an increase in acetoclastic methanogenesis. The abundance of proteins that may be involved in acetoclastic methanogenesis is given in Table S1. The regulation of the expression of proteins and enzymes was modified by the addition of CNTs (Fig. 3). For instance, the enzymes/proteins that control the oxidation of carboxyl groups, such as Ack and Codh-Acs, were highly up-regulated by 1.06 and 1.29 (orange

Fig. 1. Methanogenesis in pure cultures of Methanosarcina species on acetate substrate with carbon nanotubes (CNTs). a) Acetate concentration for M. barkeri and M. mazei. b) Methane production for M. barkeri and M. mazei. c) δ\(^{13}\)C in % of CH\(_4\) produced by M. barkeri and M. mazei. d) Acetate and methane concentrations in pure cultures of M. formicicum; note that the methane control gives similar data as the CNT assay.
arrows), thus showing that CNTs favoured acetoclastic methanogenesis. Moreover, the expression of ferredoxin (Fd) was highly increased by 1.62 and 1.69 for the two subunits of ferredoxin. By contrast, CNTs decreased the expression of proteins involved in methyl reduction, such as mcrB, and energy production, such as atpB. These results indicate the bypass of the classical cycle by CNTs. Overall, CNTs had some impact on the expression of proteins involved in acetoclastic methanogenesis, and suggest that CNTs may strengthen carboxyl oxidation and electron transfer.

### 3.3. Methane production in anaerobic soils amended with carbon nanotubes

We tested whether CNTs enhanced acetoclastic methanogenesis in complex microbial communities of anaerobic soils. The $P_{\text{max}}$ (maximum methane concentration) values of the control and CNT treatments were 0.297 ± 0.016 and 0.304 ± 0.011 mM, respectively. The lag phase of methane production ($\lambda$) was approximately 0.6 days in the vials without CNTs. In the presence of CNTs, $\lambda$ decreased by approximately 28.3%, with a value of ~0.43 days. Furthermore, CNTs promoted methane production, resulting in an increase in $R_{\text{max}}$ with a value of 0.102 ± 0.010 mM D⁻¹ compared to the control (0.087 ± 0.014 mM D⁻¹).

For the kinetics of $\Delta G$ of these two pathways, values were always negative, suggesting that producing methane with acetate or CO₂ as a substrate was feasible (Fig. 4b). The proportion of methane produced by the acetoclastic methanogenic pathway in total methane production remained relatively stable in the range of ~60%–80% for both treatments (Fig. 4c). Direct acetate dismutation contributed more to the net accumulation of methane than did CO₂ reduction (Fig. 4d). The proportion of this pathway reached its peak on the 3rd day. According to the results shown in Fig. 4e, methane yield occurred primarily due to enhancement of acetoclastic methanogenesis in anaerobic soil.

### 3.4. Impact of carbon nanotubes on carbon isotopic fractionation

Methane was mainly derived from the reduction of CO₂ in the case of the inhibition of acetoclastic methanogenesis, based on isotope results (Table 1). Combining the equations of hydrogenotrophic methanogenesis (Eq. (2)) and isotope fractionation of this reaction ($\delta^{13}C_{CO_2}/\delta^{13}C_{CH_4} = \delta^{13}C_{CH_4} / \delta^{13}C_{CO_2} = 10^{-1000 \times \Delta_{H}}$), both treatments held the same $\alpha$ value (~1.05). $\delta_{mc}$, which is the $\delta^{13}C$ value of methane from CO₂ reduction, was not influenced by CNTs. In contrast, CNTs changed the value of $\delta_{ma}$ ($p < 0.05$), which is the $\delta^{13}C$ value of methane from acetoclastic methanogenesis.
According to equation $(\delta^{13}CH_4 = f_{\text{ac}}\delta_{\text{ac}} + (1 - f_{\text{ac}})\delta_{\text{mc}})$, the percentages of the contribution of the aceticlastic methanogenic pathway to the total methane under the two experimental conditions were approximately 85 ± 10.18% and 100 ± 9.29% on the 3rd day. The results of the isotope calculations were basically consistent with the data shown in Fig. 4c. That is, carbon isotopic fractionation experiments show that almost all methane produced with acetate as a direct substrate and CNTs strengthened this pathway in anaerobic soil (Table 1).

3.5. Abundance of bacteria and methanogens responding to carbon nanotubes

The known exoelectrogenic bacteria, such as Geobacter and Shewanella, did not benefit from the presence of CNTs with a $p$ value of 0.21 (Fig. S3a). Methanosarcinaceae, which is the most metabolically versatile of the methanogenic archaea, had the highest abundance of methanogens in both treatments (Figs. S3b and S4). The abundance of this kind of methanogen in both treatments exceeded 60%. CNTs significantly increased Methanosarcinaceae abundance ($p = 0.00053$), indicating that methane may derive from the decomposition of acetate by Methanosarcinaceae in anaerobic soil.

4. Discussion

4.1. Carbon nanotubes contributed to an increase of aceticlastic methanogenesis in pure culture

This work highlights that CNTs were favorable to both M. barkeri and M. mazei (Figs. 1 and 2). The culture systems used was pure culture of methanogens. Therefore, there is no DIET process in which electroactive microorganisms generate electrons for methanogens. Very recently, a direct effect of conductive magnetite on pure culture of microorganisms generate electrons for methanogens. The known exoelectrogenic bacteria, such as Geobacter and Shewanella, did not benefit from the presence of CNTs with a $p$ value of 0.21 (Fig. S3a). Methanosarcinaceae, which is the most metabolically versatile of the methanogenic archaea, had the highest abundance of methanogens in both treatments (Figs. S3b and S4). The abundance of this kind of methanogen in both treatments exceeded 60%. CNTs significantly increased Methanosarcinaceae abundance ($p = 0.00053$), indicating that methane may derive from the decomposition of acetate by Methanosarcinaceae in anaerobic soil.

In the presence of CH$_3$F, the slight consumption of acetate caused by the addition of CNTs may be mainly due to the stronger aceticlastic methanogenesis (Fig. 2a). Based on the author’s knowledge of the physiological metabolism of M. barkeri, this kind of methanogenic archaea is basically incapable to oxidizing $^{13}$CH$_3$COOH to produce $^{13}$CO$_2$. Reported that a small percentage (2.3%) of $^{13}$CH$_3$COOH can be converted into $^{13}$CO$_2$ through electron bifurcation when providing a high concentration of ferrihydrite. No ferric iron was added in our research, and additional reducing substances, cysteine and Na$_2$S, were added into the medium. Therefore, it was less likely that methanogenic archaea oxidize acetate through electron bifurcation.

The amount of acetate consumed was approximately 0.01 mmol (Fig. 2a). If the consumed acetate was completely oxidized to CO$_2$ ($^{13}$CH$_3$COOH → $^{13}$CO$_2$ + $^{12}$CO$_2$) without the occurrence of aceticlastic methanogenesis, 3% $^{12}$CO$_2$ and 3% $^{12}$CO$_2$ should be produced with 3% $^{13}$CH$_3$COOH as the substrate. Therefore, the molar amount of produced $^{12}$CO$_2$ was about $3 \times 10^{-7}$ mol. We determined the value of $^{13}$C/$^{12}$C of CO$_2$ in the originally mixed gas, which was approximately 1.088%. Fifteen millilitres of nitrogen and CO$_2$ mixed gas (~20% CO$_2$) was added to the upper space of the anaerobic tube, and the molar amount of $^{13}$CO$_2$ was about $1.32 \times 10^{-4}$ mol. Therefore, the ratio of $^{13}$CO$_2$/$^{12}$CO$_2$ should be approximately 0.227%. Additionally, 1 M CO$_2$ can be converted into 1 M CH$_4$. Correspondingly, the ratio of $^{13}$CH$_4$/$^{12}$CH$_4$ should be about 0.227% as well. The measured value (~3%) was more than one order of magnitude of the theoretically calculated value. Therefore, this assumption seemed to be untenable (0.227% VS ~3%), not to mention that acetate was almost impossible to be completely oxidized without the occurrence of direct acetate dismutation. With 3% $^{13}$CH$_3$COOH as the substrate, 3% $^{13}$CH$_4$ and 3% $^{12}$CO$_2$ should be produced through aceticlastic methanogenesis. The measured value was consistent with the theoretically calculated value. Conclusively, CNTs may promote methane production via direct acetate dismutation in the presence of CH$_3$F.

4.2. Carbon nanotubes stimulated the synthesis of proteins involved in -COOH oxidation and electron transfer

The synthesis of formylmethanofuran dehydrogenase (Fwd), which is a vital enzyme for the generation of methane via CO$_2$ reduction (Wagner et al., 2016), was significantly down-regulated (Supplementary
### Isotopic signatures (δ¹³C values of CH₄ and CO₂ produced in the vials)

| Treatments | δ¹³C values of CH₄ (with CH₃F) (‰) | δ¹³C values of CO₂ (without CH₃F) (‰) |
|------------|----------------------------------|-----------------------------------|
| Control (3rd day) | 67.69 ± 1.13 | 19.66 ± 0.57 |
| CNTs (3rd day) | 68.46 ± 1.35 | 19.16 ± 0.95 |
| Control (5th day) | 68.46 ± 1.47 | 18.48 ± 0.47 |
| CNTs (5th day) | 68.46 ± 1.66 | 17.99 ± 0.30 |

*Note: CNTs = Conductive nano-Fe₃O₄, CNT = Carbon nanotube, CH₃F = Methane trifluoride, CNTs = Conductive nano-Fe₃O₄, CH₃F = Methane trifluoride.*

### Table 1

| Isotopic signatures (δ¹³C values of CH₄ and CO₂ produced in the vials) | Calculated present with CH₃F (%) |
|---------------------------------------------------------------|---------------------------------|
| Isotopic signatures of CH₄ produced in the vials | Calculated present with CH₃F (%) |
| Control (3rd day) | 67.69 ± 1.13 | 19.66 ± 0.57 |
| CNTs (3rd day) | 68.46 ± 1.35 | 19.16 ± 0.95 |
| Control (5th day) | 68.46 ± 1.47 | 18.48 ± 0.47 |
| CNTs (5th day) | 68.46 ± 1.66 | 17.99 ± 0.30 |

*Note: CNTs = Conductive nano-Fe₃O₄, CNT = Carbon nanotube, CH₃F = Methane trifluoride.*

### 4.3. Carbon nanotubes benefited methane production independently of electroactive bacteria

In anaerobic soil, CNTs improved the performance of anaerobic systems, and methane production was comparable (Fig. 4a). The function of CNTs to promote methane production in sediment and sludge has been observed in some studies (Li et al., 2015; Zhang et al., 2018). But the proposed explanations were that CNTs promoted electron transfer between the electrogenic bacteria and the methanogens to reduce CO₂. However, Geobacter spp. are the only bacteria demonstrated to participate in DIET with methanogens (Van Steendam et al., 2019). But CNTs did not affect the abundance of Geobacter or other canonical electrogricans, such as Shewanella, in this study.

We also analyzed the electron transfer by means of an electrochemical method. The addition of CNTs did not significantly change the redox peak (Fig. S5), suggesting that the role of CNTs in promoting electron transfer between electromicroorganisms and methanogenic archaea may also be weak. In view of this, the traditional conclusion that conductive materials facilitate DIET between electron-donating bacteria and methanogens to improve CO₂ reduction may not fully explain the phenomenon well (Fig. 5e). It is worth noting that the abundance of methanogenic archaea was improved by CNTs (Figs. S3b and S4). Therefore, CNTs may only benefit methanogenic archaea just as pure cultures.

### 4.4. Acetoclastic methanogenesis contributed to methane accumulation in anaerobic soils

The inhibitor worked (Fig. 4a), and it was feasible to analyze the contribution of different pathways by inhibiting the acetoclastic methanogenic pathway based on isotopic fractionation (Conrad, 2005). It is widely recognized that the δ¹³C value of methane is more negative when CO₂ is used as a substrate than when methane is obtained using acetate as a precursor (Conrad, 2005). With the application of CH₃F, the δ¹³C-values of methane were very negative and in the range of CO₂ reduction (Conrad and Casper, 2010; Penning et al., 2006). According to Table 1, increased methane production was mainly due to the acetoclastic methanogenesis, which was very consistent with the results shown in Fig. 4e. To the best of our knowledge, this is the first time that
the capability of carbon nanomaterials to improve direct acetate dismutation has been proposed in natural soil. However, this study used only one kind of soil for verification. To make the conclusion more generalizable, it would be better to verify the promotion of carbon nanomaterials on acetoclastic methanogenesis with different characteristics of soils.

Combined with the energy release of methanogenic archaea, acetoclastic methanogenesis actually releases more energy than CO₂ reduction in a nominal environment (Table S3). The energy, ΔGA, is the free energy liberated by the group’s net reaction. According to the data shown in Table S4, for producing 1 mol of methane, 32 or 1 kJ of energy is available by acetoclastic methanogenesis or hydrogenotrophic methanogenesis, respectively, to Methanosarcina (Bethke et al., 2011). Specifically, considering the consumption of ATP during methanogenesis, the useable energy ΔGa is approximately 21 kJ per mole of methane produced for acetoclastic methanogenesis. However, for hydrogenotrophic methanogenesis, this value is approximately –10 kJ. Thus, the production of 1 mol of methane by this pathway requires an additional source of 10 kJ of energy. Under the premise that CNTs promote both methanogenic pathways (Fig. 4b), energy metabolism may also explain why the proportion of methane production from acetate dismutation was much higher than that from CO₂ reduction in both pure culture and anaerobic soil.

5. Conclusion

In addition to the stimulation of the electric syntrophy between exoelectrogenic bacteria and methanogens by conductive C/Fe-bearing materials, the findings of this study revealed that carbon nanotubes directly favoured methane production by promoting a route for acetoclastic methanogenesis independently of electrogenic microorganisms. Moreover, with accurate substrates and methanogens, multiple technologies are still needed to analyze the diverse methanogenic processes. More importantly, we not only found this phenomenon with pure culture, but also systematically verified it in environmental samples. Reliable experiments proposed a new model to expand our knowledge of the role of conductive nanomaterials in methanogenic acetate degradation in natural environments.

CRediT authorship contribution statement

Leilei Xiao: Data curation, Formal analysis, Writing - review & editing. Shiling Zheng: Writing - review & editing. Eric Lichtfouse: Writing - review & editing. Min Luo: Writing - review & editing. Yang Tan: Writing - review & editing. Fanghua Liu: Data curation, Formal analysis.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.107938.

References

Abken, H.J., Tietze, M., Brodersen, J., Bumer, S., Beifuss, U., Deppmeier, U., 1998. Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of Methanosaeta mazei GOL. Journal of Bacteriology 180 (8), 2027–2032.

Bethke, C.M., Sanford, R.A., Kirk, M.F., Jin, Q.S., Flynn, T.M., 2011. The thermodynamic ladder in geomicrobiology. American Journal of Science 311 (3), 183–210.

Conrad, R., 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. Organic Geochemistry 36 (5), 739–752.

Conrad, R., Casper, P., 2010. Stable isotope fractionation during the methanogenic degradation of organic matter in the sediment of an acidic bog lake, Lake Guose Fuchskuhle. Limnology & Oceanography 55 (5), 1932–1942.

Fu, L., Zhou, T., Wang, J., You, L., Li, Y., Yu, L., Zhou, S., 2019. NanoFe3O4 as solid electron shuttle to accelerate acetotrophic methanogenesis by Methanosaetaarkeri. Frontiers in Microbiology 10, 388.

Holmes, D.E., Smith, J.A., 2016. Biologically produced methane as a renewable energy source. Advances in Applied Microbiology 97, 1–61.

Hutten, T.J., Bongaerts, H.C.M., Drift, C., Vogels, G.D., 1980. Acetate, methanol and carbon dioxide as substrates for growth of Methanosaetabarkeri. Antonie Van Leeuwenhoek Journal of Microbiology 46 (6), 601–610.

Ji, Y., Liu, P., Conrad, R., 2018. Response of fermenting bacterial and methanogenic archaeal communities in paddy soil to progressing rice straw degradation. Soil Biology and Biochemistry 124, 70–80.

Kaster, A.K., Moll, J., Parey, K., Thauer, R.K., 2011. Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. Proceedings of the National Academy of Sciences of the United States of America 108 (7), 2981–2986.

Li, J., Xiao, L., Zheng, S., Zhang, Y., Luo, M., Tong, C., Xu, H., Tan, Y., Liu, J., Wang, O., Liu, F., 2018. A new insight into the strategy for methane production affected by conductive carbon cloth in wetland soil: beneficial to acetoclastic methanogenesis instead of CO2 reduction. The Science of the Total Environment 643, 1024–1030.

Li, L.L., Tong, Z.H., Fang, C.Y., Chu, J., Yu, H.Q., 2015. Response of anaerobic granular sludge to single-wall carbon nanotube exposure. Advances in Water Resources 70, 1–8.

Li, Q., Li, L., Reijtar, T., Lesnser, D., Karger, B., Ferry, J., 2006. Electron transport in the pathway of acetate conversion to methane in the marine archaeon Methanosaeta arcivorans. Journal of Bacteriology 188 (2), 702–710.

Liu, F., Rotaru, A.E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., Lovley, D.R., 2012. Magnetic compensation for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. Environmental Microbiology 17 (3), 648–655.

Liu, P.F., Kloese, M., Conrad, R., 2018. Temperature effects on structure and function of the methanogenic microbial communities in two paddy soils and one desert soil. Soil Biology and Biochemistry 124, 236–244.

Lu, J., Chang, J., Lee, D., 2020. Adding carbon-based materials on anaerobic digestion performance: a mini-review. Bioresource Technology 300, 122696.

Martins, G., Salvador, A.F., Pereira, L., Alves, M.M., 2018. Methane production and conductive material: a critical review. Environmental Science and Technology 52 (18), 10241–10253.

Morita, M., Malvankar, N.S., Franks, A.E., Summers, Z.M., Giloteaux, L., Rotaru, A.E., Rotaru, C., Lovley, D.R., 2011. Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. mBio 2, e00159-11.

Penning, H., Claus, P., Casper, P., Conrad, R., 2006. Carbon isotope fractionation during acetoclastic methanogenesis by Methanosaeta concilii in culture and a lake sediment. Applied and Environmental Microbiology 72 (8), 5648–5652.

Rai, P.K., Kumar, V., Lee, S., Raza, N., Kim, K.H., Ok, Y.S., Tsang, D.C.W., 2018. Nanoparticle–plant interaction: implications in energy, environment, and agriculture. Environmental International 119, 1–19.

Rother, M., Metcalf, W., 2004. Anaerobic growth of Methanosarcina acetivorans C2A on carbon monoxide: an unusual way of life for a methanogenic archaeon. Proceedings of the National Academy of Sciences of the United States of America 101 (48), 16929–16934.

Salvador, A.F., Martins, G., Melle-Franco, M., Serpa, R., Stams, A.J.M., Cavaleiro, A.J., Pereira, M.A., Alves, M.M., 2017. Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture. Environmental Microbiology 19 (7), 2727–2739.

Song, X., Liu, J., Jiang, Q., Zhang, P., Shao, Y., He, W., Feng, Y., 2019. Enhanced electron transfer and methane production from low-strength wastewater using a new granular activated carbon modified with nano-Fe3O4. Chemical Engineering Journal 374, 1344–1352.

Stumm, W., Morgan, J.J., 1996. Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters. John Wiley & Sons, Inc., Hoboken.

Usman, M., Farooq, M., Waked, A., Nawaz, A., Cheema, S.A., Behman, H.U., Ashraf, I., Sanaullah, M., 2020. Nanotechnology in agriculture: current status, challenges and future opportunities. The Science of the Total Environment 721, 137778.

Van Steendonk, C., Smets, I., Skerlos, S., Raskin, L., 2019. Improving anaerobic digestion via direct interspecies electron transfer requires development of suitable characterization methods. Current Opinion in Biotechnology 57, 183–190.

Viggi, C.C., Rossetti, S., Fazi, S., Piaano, P., Majone, M., Aulenta, F., 2014. Magnetic particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation. Environmental Science and Technology 48 (13), 7536–7543.

Wagner, T., Ehrler, U., Shima, S., 2016. The methanogenic CO2 reducing–and-fixing enzyme is bifunctional and contains 46 [4Fe–4S] clusters. Science 354 (6308), 114–117.

Wang, H., Byrne, J.M., Liu, P.F., Liu, J., Dong, X.Z., Lu, Y.H., 2020. Redox cycling of Fe (II) and Fe (III) in methanogenic aceticlastic methanogenesis by Methanosaeta arcivorans. Environmental Microbiol Reports 12, 97–109.

Whiticar, M., 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chemical Geology 161, 291–314.

Xiao, L., Liu, F., Lichtfouse, E., Zhang, P., Feng, D., Li, F., 2020. Methane Production by Acetate Dismutation stimulated by Shewanella oneidensis carbon materials: an alternative to classical CO2 reduction. Chemical Engineering Journal 389, 124469.

Xiao, L., Liu, F., Liu, J., Liu, J., Zhang, Y., Yu, J., Wang, O., 2018. Nano-Fe3O4 particles accelerating electromethanogenesis on an hour-long timescale in wetland soil. Environmental Sciences: Nano 5 (2), 436–445.

Xiao, L., Liu, F., Xu, H., Feng, D., Liu, J., Han, G., 2019. Biochar promotes methane production at high acetic acid concentrations in anaerobic soils. Environmental Chemistry Letters 17, 1347–1352.

Xiao, L., Sun, R., Zhang, P., Zheng, S., Tan, Y., Li, J., Zhang, Y., Liu, F., 2019. Simultaneous intensification of direct acetate cleavage and CO2 reduction to generate methane by bioaugmentation and increased electron transfer. Chemical Engineering Journal 378, 122229.

Xiao, L., Wei, W., Luo, M., Xu, H., Feng, D., Yu, J., Huang, J., Liu, F., 2019. A potential contribution of a Fe(III)-rich red clay horizon to methane release: biogenic magnetite-mediated methanogenesis. Catena 181, 104081.

Xiao, L., Xie, B., Liu, J., Zhang, J., Han, G., Wang, O., Liu, F., 2017. Stimulation of long-term ammonium nitrogen deposition on methanogenesis by Methanocellaceae in a coastal wetland. The Science of the Total Environment 595, 337–343.

Yang, Z., Wang, M.Y., Ferry, J.G., 2017. A ferredoxin-and F420H2-dependent, electron-bifurcating, heterodisulfide reductase with homologs in the domains bacteria and archaea. mBio 8 (1), e02285-16.

Zehnder, A.J.B., Wurmhann, K., 1977. Physiology of a methanobacterium strain az. 1. Growth and carbon dioxide as substrates for growth of Methanosaetaarcivorans. Antonie van Leeuwenhoek Journal of Microbiology 46 (6), 1932–1942.

Zhang, X., Wang, J., Li, L., Chen, X., Tian, X., Yang, J., Yuan, M., Pan, H., Shi, J., 2019. Methane production from landfill leachate: the influence of Fe(III)-rich red clay horizon. Bioresour. Technol. 277, 273–280.
Preparation of anaerobic slurry
The bottles were flushed with N$_2$ for approximately 30 min and incubated statically at 30°C in a dark room. After five weeks of incubation, the incubated soil reached a high methane production potential with a methane concentration of about 100,000 ppm. Then, the incubated soil (5 mL) was dispensed into sterile 11-mL serum vials, which were pre-evacuated, flushed with high-purity N$_2$, and incubated statically at 30°C.

Samples Preparation and TMT Labeling
Samples of methanogens were lysed by using lysis buffer (300 µL) supplemented with 1 mM PMSF and sonicated for 3 min. After centrifugation of 15 min (15,000 g), we collected the supernatant. Bradford assay was used to test protein concentration and protein was aliquoted to store at -80°C. For each sample, 100 µg of proteins were mixed with 120 µL reducing buffer (10 mM DTT, 8 M Urea, 100 mM TEAB, pH 8.0) in Amicon® Ultra-0.5 Centrifugal Filter (10 kDa) and incubated at 60°C for 60 min. Then iodoacetamide was added to the solution with the final concentration of 50 mM and incubated for 40 min at room temperature in the dark. After centrifugation at 12000 rpm for 20 min, samples were washed three times with triethylammonium bicarbonate buffer (TEAB) and digested with trypsin (Promega, Madison, WI, USA) (enzyme to protein ratio 1:50) at 37°C overnight. Digested peptides were labeled with TMT reagents (Thermo Fisher Scientific) according to the manufacturer’s instructions. For each 6-plex TMT, control samples were labeled with TMT tags 126, 127, 128 and treatments with CNTs were labeled with TMT tags 129, 130, and 131, respectively. Equal amounts of TMT-labeled peptides were mixed and dried, then resuspended in buffer A (2% acetonitrile, 98% water with ammonia at pH 10) and fractionated by 1100 HPLC System (Agilent).

Proteomic Analysis by LC-MS/MS
Peptides were redissolved with 0.1% formic acid (FA) and analyzed on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a nanospray Flex source (Thermo, USA). Samples were loaded and separated by a C18 column (15 cm × 75 µm) on an EASY-nLC™ 1200 system (Thermo, USA) in Qingdao OeBiotech. Co., Ltd. The flow rate was 300 nL/min and linear gradient was 90 min. The mass spectrometer was operated in the data-dependent mode with positive polarity at electrospray voltage of 2 kV. Full scan MS spectra (m/z 300–1600) were acquired in the orbitrap with the resolution as 70 K, the automatic gain control (AGC) target was 1e6 and the maximum injection time was 80 ms. The top 10 intense ions were isolated for HCD MS/MS fragmentation. In MS2, the resolution was 17500 and the AGC target was 2e5. Fragmentation was performed with normalized collision energy (NCE) of 32% and dynamic exclusion duration of 15 s.

Mass spectrometry data analysis
The mass spectrometry (MS) raw data were analyzed with Proteome Discoverer software (version 2.2) using the Sequest search engine to search against the Methanosarcina barkeri database (UniProtKB, release 2019_03). The following parameters were applied: precursor mass tolerance was 20 ppm; fragment tolerance was 0.5 Da; the dynamic modifications were oxidation (M); the static modification was carbamidomethyl (C) and TMT labeling of amines and lysine; a maximum of two missed cleavages were allowed. Peptides with FDR < 0.01 (based on the target-decoy database algorithm) were used for protein grouping. Protein groups identified ≥ 1 peptides were considered for further analysis and only unique peptides were used for protein quantification. The mass spectrometry raw data were deposited to the iProX (http://iprox.org). URL: https://www.iprox.org/page/PSV023.html?url=1555463853480QxPM, Password: CO2T

By analyzing the clustering results, based on the credible data in the results, the fold change value was obtained by dividing the average value of the CNTs group data by the average value of the control group data, and the p-value of each group was calculated. It was considered that p-value less than or equal to 0.05 was regarded as a differential protein. Cluster Profiler package in R language was used to analyze differential proteins, enrichKEGG function was used to compare differential proteins data in KEGG database, and DOTPLOT was used to map the results of signal pathway comparison. With p-value equal to 0.05 as the standard, we used cnetplot function to map the change relationship between genes and signaling pathways based on the data with significant difference in change, in which the color difference
Electrochemical measurements

A single-chamber three-electrode electrochemical cell was used to conduct CV measurements for characterizing the electron transfer rate. Three electrodes were reference electrode (Ag/AgCl), graphite plate electrode (3.0 cm × 2.5 cm × 0.3 cm, working electrode), and platinum electrode (counter electrode). Incubated soil (~ 70 mL) was poured into the electrochemical cell. CNTs concentration was 0.2g/L as well. CVs were measured using an electrochemical workstation (CHI660e, Chenhua, China). The working electrode had a scan voltage between −1.2 and 1.2 V (versus Ag/AgCl), and the scan rates were 40 –140 mV/s.
| Gena Name  | Shorthand in Figure 3 | Abundance for control | Abundance for treatment with CNTs | p value          |
|----------|----------------------|-----------------------|----------------------------------|-----------------|
| ackA     | ackA                 | 93                    | 96.9                             | 102.7           | 100.8           | 4.69*10^-62 |
| Mbar_A1136 | A1                   | 106.1                 | 117.7                            | 114.5           | 81.6            | 80               | 5.76*10^-63 |
| Mbar_A2520 | A2                   | 69.2                  | 68.3                             | 68.9            | 131.1           | 128.6           | 9.46*10^-66 |
| Mbar_A3525 | Codh-Acs1            | 87                    | 92.9                             | 88.9            | 116.2           | 114.7           | 1.54*10^-63 |
| Mbar_A3717 | Codh-Acs2            | 65.1                  | 66.7                             | 68              | 135.1           | 136              | 9.79*10^-66 |
| Mbar_A0640 | HdrC                 | 88.9                  | 111                              | 95.4            | 101.9           | 103.1           | 6.64*10^-61 |
| Mbar_A0639 | HdrB                 | 91.5                  | 89.4                             | 90              | 107.4           | 107.9           | 2.37*10^-64 |
| Mbar_A1952 | HdrA1                | 116.8                 | 118.3                            | 118.8           | 83.4            | 84.5            | 3.78*10^-65 |
| Mbar_A2589 | HdrA2                | 107.5                 | 106.4                            | 106.9           | 93.5            | 94.3            | 1.29*10^-64 |
| hdrD     | HdrD                 | 112.9                 | 116.9                            | 111.9           | 79.5            | 68.6            | 3.07*10^-63 |
| mtrA     | mtrA                 | 105.1                 | 100.7                            | 102.4           | 96.3            | 96.1            | 2.90*10^-62 |
| mtrB     | mtrB                 | 104.8                 | 101.8                            | 104.1           | 102.5           | 103.5           | 6.76*10^-61 |
| mtrD     | mtrD                 | 117.7                 | 111.2                            | 115.7           | 84.3            | 86.8            | 1.58*10^-63 |
| mtrE     | mtrE                 | 122.5                 | 117.3                            | 120.7           | 79.4            | 79              | 2.45*10^-64 |
| mtrF     | mtrF                 | 93.2                  | 101.7                            | 100.8           | 100.5           | 97.9            | 8.72*10^-61 |
| mtrG     | mtrG                 | 125.9                 | 121.4                            | 122.4           | 74.7            | 74.9            | 1.06*10^-64 |
| mtrH     | mtrH                 | 108.7                 | 103.9                            | 106.7           | 94.4            | 94.4            | 6.80*10^-63 |
| mcrA     | mcrA                 | 100.1                 | 102.5                            | 100.8           | 94.8            | 96.3            | 1.39*10^-62 |
| mcrB     | mcrB                 | 111.5                 | 114.7                            | 112.9           | 84.4            | 83.7            | 1.65*10^-64 |
| mcrC     | mcrC                 | 75.2                  | 75.2                             | 77              | 122.3           | 125.8           | 6.83*10^-66 |
| mcrD     | mcrD                 | 98.7                  | 98.9                             | 100.4           | 97.2            | 102.7           | 7.94*10^-61 |
| mcrG     | mcrG                 | 99.6                  | 102.3                            | 100.8           | 93.3            | 94.1            | 6.37*10^-63 |
| atpA     | atpA                 | 121.3                 | 119.4                            | 121.2           | 77.3            | 79.3            | 3.77*10^-66 |
| atpB     | atpB                 | 137                   | 136.2                            | 137.4           | 61.8            | 63.8            | 3.62*10^-66 |
| atpC     | atpC                 | 114.6                 | 114.6                            | 113.4           | 85.9            | 87.9            | 8.05*10^-66 |
| atpD     | atpD                 | 128.1                 | 125.1                            | 128.3           | 73.3            | 73.5            | 3.39*10^-65 |
| atpE     | atpE                 | 137.3                 | 127                               | 130.7           | 68.3            | 69.8            | 5.37*10^-64 |
| atpF     | atpF                 | 98.5                  | 94.3                             | 94.8            | 112.7           | 109.4           | 5.55*10^-63 |
| atpI     | atpI                 | 127.7                 | 123.4                            | 125.8           | 74.2            | 75.4            | 7.75*10^-66 |
| Mbar_A3421| Fd1                  | 78.4                  | 78                               | 73.2            | 127.2           | 131.4           | 2.81*10^-64 |
| Mbar_A2086| Fd2                  | 76.3                  | 72.6                             | 72.5            | 121              | 117.6           | 2.01*10^-64 |
| Mbar_A0148| EchE                 | 129.9                 | 130.8                            | 130.4           | 68.4            | 68.1            | 4.09*10^-67 |
| Mbar_A0149| EchD                 | 88.7                  | 93                               | 94              | 97.9            | 99.3            | 5.34*10^-62 |
| Mbar_A0150| EchC                 | 113                   | 101.8                            | 107.6           | 104.8           | 106.5           | 6.96*10^-61 |
| Mbar_A0147| EchF                 | 124.4                 | 121.8                            | 122.4           | 77.7            | 79              | 3.55*10^-65 |
| frhA     | frhA                 | 99.2                  | 109.4                            | 102.2           | 92.2            | 92.3            | 6.23*10^-62 |
| frhB     | frhB                 | 106                   | 102.8                            | 107.4           | 95.2            | 98              | 2.31*10^-62 |
| ftr      | ftr                  | 103.8                 | 100.2                            | 102.3           | 100.6           | 101.9           | 5.95*10^-61 |
| fmdC     | fmdC                 | 109.2                 | 105.6                            | 108.1           | 96.3            | 96.7            | 4.00*10^-63 |
| Mbar_A0795| fwdE1                | 94.6                  | 94.6                             | 98              | 107.6           | 113.1           | 1.02*10^-62 |
| Mbar_A1291| fwdD                 | 104.1                 | 109.7                            | 108.7           | 94.7            | 97.1            | 1.69*10^-62 |
| Mbar_A1287| fwdE2                | 121.6                 | 113.9                            | 113.3           | 87.1            | 87.6            | 3.58*10^-63 |
| Mbar_A1763| fwbB2                | 109.8                 | 104.8                            | 106.4           | 94.8            | 96.6            | 1.13*10^-62 |
| Mbar_A1292| fwbB                 | 104.2                 | 96.4                             | 102.3           | 103.5           | 97              | 8.66*10^-61 |
**Table 2** Kinetic parameters of the methane production by fitting the modified Gompertz equation.

| Treatment | Measured value* | $\lambda$ (Day) | $R_{\text{max}}$ (mM D$^{-1}$) | $P_{\text{max}}$ (mM) | $R^2$ |
|-----------|-----------------|-----------------|-------------------------------|------------------|-------|
| control   | 0.282±0.022     | 0.629±0.223     | 0.087±0.014                   | 0.297±0.016      | 0.999 |
| CNT       | 0.289±0.027     | 0.426±0.137     | 0.102±0.010                   | 0.304±0.011      | 0.999 |

*Measured maximum concentration of methane at the end of the batch experiments (mM)

**Table 3** Electron accepting and donating processes of methane production as defined by free energy change in a nominal anoxic geochemical environment

| Half-reactions                  | Metabolic pathways | Equation | $\Delta G$ (kJ mol$^{-1}$) |
|--------------------------------|--------------------|----------|---------------------------|
| Electron donating half-reactions | acetotrophy        | $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^-$ | -216          |
|                               | hydrogenotrophy    | $4H_2(aq) \rightarrow 8H^+ + 8e^-$                  | -185          |
| Electron accepting half-reaction | methanogenesis     | $HCO_3^- + 9H^+ + 8e^- \rightarrow CH_4 + 3H_2O$     | 184           |

* Related data refer to Bethke et al.$^5$. 25 °C; pH 7; 1 mmol kg$^{-1}$ Ca$^{2+}$, CO$_2(aq)$ + HCO$_3^-$, SO$_4^{2-}$, NO$_3^-$, Fe$^{2+}$, and Mn$^{2+}$; 1 µmol kg$^{-1}$ CH$_3$COO$^-$, CH$_4(aq)$, HS$^-$, and NH$_4^+$; 1 nmol kg$^{-1}$ H$_2(aq)$; N$_2(aq)$ at atmospheric saturation.

**Table 4** Net reactions for two major methanogenesis, the reactions’ available ($\Delta G_A$) and usable ($\Delta G_U$) energies in a nominal geochemical environment

| Metabolic pathways               | Equation                              | $\Delta G_A$ (kJ mol$^{-1}$) | $\Delta G_U$ (kJ mol$^{-1}$) |
|----------------------------------|---------------------------------------|------------------------------|-------------------------------|
| Acetoclastic methanogenesis      | $CH_3COO^- + H_2O \rightarrow CH_4(aq) + HCO_3^-$ | 32                           | 21                            |
| Hydrogenotrophic methanogenesis  | $4H_2(aq) + HCO_3^- + H^+ \rightarrow CH_4(aq) + 3H_2O$ | 1                            | -10                           |

* Environmental conditions are defined the in footnote to Table 3 and refers to Bethke et al.$^5$. 
**Figure S1** Principal component analysis of proteome samples. C1, C2 and C3 were three replicates of the control group. T1, T2 and T3 were three replicates of the carbon nanotubes (CNTs) group. This results showed that the sample 2, T2, was an abnormal sample, which was not in the scope of subsequent analysis.

**Figure S2** Calculated and actual methane concentrations in pure cultures. The calculated methane is based on the equation: \( \text{CH}_3\text{COO}^- + H^+ \rightarrow \text{CH}_4 + \text{CO}_2 \). According to the consumption of acetate, we calculated the methane produced in theory.
Figure S3. Communities of bacteria (a) and methanogenic archaea (b) at the family level in anaerobic soils after 6 days incubation. Relative abundances of less than 0.8%, for bacteria, and 1.9%, for methanogenic archaea, were classified into the group ‘others’.

Figure S4. Community of methanogenic archaea at the genus level in incubated soil.
Figure S5 Cyclic voltammetry at different scan rates of 40, 60, 80, 100, 120, and 140 mV s$^{-1}$ (from the inner to outer) for unamended treatment (a) and treatment with carbon nanotubes (CNTs) (b). (Insets) Peak currents are presented as a function of scan rates.