

Different impacts of an electron shuttle on nitrate- and nitrite-dependent anaerobic oxidation of methane in paddy soil

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Abstract: Quinones, redox-active functional groups in soil organic matter, can act as electron shuttles for microbial anaerobic transformation. Here, we used $^{13}\text{CH}_4$ to trace ^{13}C conversion ($^{13}\text{C}\text{-CO}_2 + ^{13}\text{C}\text{-SOC}$) to investigate the influence of an artificial electron shuttle (anthraquinone-2,6-disulfonate, AQDS) on denitrifying anaerobic methane oxidation (DAMO) in paddy soil. The results showed that AQDS could act as the terminal electron acceptor for the anaerobic oxidation of methane (AOM) in the paddy field. Moreover, AQDS significantly enhanced nitrate-dependent AOM rates and the amount of $^{13}\text{C}\text{-CH}_4$ assimilation to soil organic carbon (SOC), whereas it was remarkably reduced nitrite-dependent AOM rates and ^{13}C assimilation. Ultimately, AQDS notably increased the total DAMO rates and ^{13}C assimilation to SOC. However, the electron shuttle did not change the percentage of $^{13}\text{C}\text{-SOC}$ in total $^{13}\text{C}\text{-CH}_4$ conversion. These results suggest that electron shuttles in the natural organic matter might be able to offset methane emission by facilitating AOM coupled with the denitrification process.

Keywords: global warming; microbial anaerobic metabolism; nitrogen fertilisation; $^{13}\text{CO}_2$ production

The anaerobic oxidation of methane (AOM) is an important methane consumption process, which is of great significance to alleviate global warming. It was first discovered in marine environments and driven by anaerobic methanotrophic (ANME) archaea coupled to sulfate reduction. The sulfate-dependent AOM was previously considered to be the main pathway of methane consumption globally (Knittel and Boetius 2009). However, denitrifying anaerobic methane oxidation (DAMO) processes have been discovered successively in recent decades (Ettwig et al. 2010, Haroon et al. 2013). For example, ANME archaea (ANME-2d) can oxidise CH_4 via reverse methanogenesis pathway to transfer gener-

ated electrons to the outside of cell membranes for the reduction of extracellular NO_3^- to NO_2^- (Haroon et al. 2013). Moreover, a kind of DAMO bacteria (*Methyloirabilis oxyfera*) is found to be able to anaerobically disproportionate NO_2^- into N_2 and O_2 and use the generated O_2 to oxidise CH_4 to CO_2 via an intracellular electron transfer pathway (Ettwig et al. 2010). The DAMO processes have been demonstrated to occur widely in nature, such as in paddy soils (Vaksmas et al. 2017), lake sediments (Shen et al. 2015), freshwater wetlands (Smemo and Yavitt 2011). However, the specific role of organic electron acceptors widely present in terrestrial environments on DAMO is still unclear.

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Quinones, active functional groups in natural humic substances and artificial organic analogs (e.g., anthraquinone-2,6-disulfonate, AQDS), are confirmed to play an important role in microbial anaerobic metabolism (Cervantes et al. 2000). For instance, AQDS has been proved to serve as a terminal electron acceptor for sulfate-dependent AOM (Scheller et al. 2016). Humic acids in natural organic matter can also act as a terminal electron acceptor to facilitate AOM (Valenzuela et al. 2017). More importantly, quinones can also serve as electron shuttles for transferring electrons derived from ANME archaea to distant electron acceptors, such as Fe^{3+} and N_2O (He et al. 2019, Valenzuela et al. 2020).

Flooded paddy fields provide ideal habitats for active methanogenesis, which supplies CH_4 substrate for AOM microbes. Although it is well known that soil organic matter has an important role in the production of CH_4 (Mujiyo et al. 2017), the influence on the dynamics of CH_4 oxidation remains unclear. Furthermore, nitrogen fertilisation in rice production may lead to intense nitrification and denitrification processes that produce a large number of intermediate N compounds such as NO_3^- and NO_2^- acting as electron acceptors for AOM (Ding et al. 2016). Thus, the objective of this study is to investigate how redox-active components of soil organic matter (e.g., quinones) affect nitrite- and nitrate-dependent AOM and $^{13}\text{CH}_4$ assimilation to soil organic carbon (SOC).

MATERIAL AND METHODS

Soil samples were collected from the paddy field at Agrometeorological Experimental Station (32°12'25"N, 118°43'1"E) in Nanjing in China. The typical rice-wheat rotation is implemented in the field, and rice cultivation is conducted from June to October. Soil samples were collected 0–20 cm depth in October 2018. The paddy soil was originated from river sediment and is classified as a typical Fluvisols based on WRB soil classification. It had a bulk density of 1.28 g/cm³. The total organic carbon and total nitrogen of the soil were 16.36 and 1.76 mg/g, respectively, and the soil pH was 6.72 at a water:soil ratio of 2.5:1.

Potential anaerobic methane oxidation was evaluated using $^{13}\text{CH}_4$ incubation (Fan et al. 2020). All the soil cores were put together to be mixed and homogenised to form one composite sample. Plant remnants and stones were carefully removed before incubation. Then the soil samples (approximately 2 g)

were transferred into 12 mL vials (Labco Limited, Lampeter, UK), and then deionised sterile water (2 mL) was added to the vials. The vials were sealed and vigorously shaken to achieve homogenised soil slurries. To remove the remaining oxygen from the vials, the headspace was evacuated three times and then back-flushed with argon (Ar). The Ar-flushed vials were pre-incubated to eliminate the residual oxygen and background NO_x^- ($\text{NO}_2^- + \text{NO}_3^-$). After pre-incubation, the vials were flushed with Ar again, and then seven different treatments were performed: (1) $^{13}\text{CH}_4$; (2) $^{13}\text{CH}_4 + \text{NO}_2^-$; (3) $^{13}\text{CH}_4 + \text{NO}_3^-$; (4) $^{13}\text{CH}_4 + \text{AQDS}$; (5) $^{13}\text{CH}_4 + \text{NO}_2^- + \text{AQDS}$, and (6) $^{13}\text{CH}_4 + \text{NO}_3^- + \text{AQDS}$. The calculation formulas of AOM rates were: NO_2^- -DAMO rate = treatment 2 – treatment 1, NO_3^- -DAMO rate = treatment 3 – treatment 2, and total DAMO rate = treatment 3 – treatment 1 (Wang et al. 2019). Treatments 4, 5 and 6 were used to detect the effect of AQDS on the AOM rates. Subsequently, 100 μL of Ar-purged $\text{NO}_2^-/\text{NO}_3^-$ the stock solution was injected through the septa into vials, resulting in a final concentration of 1.5 mmol/L. Immediately after that, 500 μL of $^{13}\text{CH}_4$ was injected into each vial, resulting in a final concentration of ~5% (v/v) in the headspace. The vials were shaken for 5 min to reach a liquid-gas equilibrium and then incubated in the biochemical incubator in the dark at 25 °C for 60 days.

Headspace gas samples were collected at 0, 30 and 60 days after $^{13}\text{CH}_4$ injection. 0.5 mL gas samples were collected from the headspace using Ar-flushed syringes, and the equivalent volume of Ar was immediately injected to maintain air pressure balance in the headspace. All gas samples were diluted with Ar (0.5 mL sample into 12 mL Ar). The CH_4 and CO_2 concentrations were measured using a gas chromatograph equipped with a flame ionisation detector (detection limit: 1.4 pg C/s) and an electron capture detector (detection limit: 6 fg/mL) (Agilent 7890, Agilent Technologies, Santa Clara, USA). The ^{13}C -atom percentage of $^{13}\text{CO}_2$ was analysed using an isotope ratio mass spectrometer (isoprime 100, Elementar, Langenselbold, Germany). Rates of nitrite-DAMO, nitrate-DAMO and total DAMO were calculated based on the $^{13}\text{CO}_2$ production. After 60-day incubation, soil slurries in vials were acidified overnight and then freeze-dried to measure SOC concentration. Subsequently, ^{13}C -atom percentage of SOC was analysed using a mass spectrometer, and ^{13}C -SOC accumulation was calculated based on the SOC concentration and ^{13}C -atom percent excess

of SOC. $^{13}\text{CH}_4$ assimilation to SOC was calculated based on the ^{13}C -SOC production during the 60-day incubation (Gupta et al. 2013).

The experiment was conducted by adopting a completely randomised design with seven treatments and three replicates. The experimental data were tested for normality (Shapiro-Wilk) and homogeneity (Levene). The percentage data were arcsine transformed to test normality. Then, the data were subjected to the one-way analysis of variance with Dunnett's test ($P < 0.05$) using the SPSS 19.0 (SPSS Inc., Chicago, USA). Finally, Pearson's estimated correlation coefficients ($P < 0.05$) between the parameters of $^{13}\text{CH}_4$ conversion were calculated.

RESULTS AND DISCUSSION

In the ANOVA ($P < 0.05$), the factor AQDS performed a significant effect on ^{13}C - CO_2 atom percentage. AOM rate showed the same variation tendency as ^{13}C - CH_4 assimilation into SOC under with/without AQDS treatments among NO_2^- -DAMO, NO_3^- -DAMO and total DAMO. Moreover, AQDS exhibited a statistically significant influence on total ^{13}C - CH_4 conversion, and however, it had no effect on the percentage of ^{13}C -SOC.

The $^{13}\text{CO}_2$ atom percentage increased from its background abundance of 1.08%, up to 1.73% in the treatment 1 amended only with $^{13}\text{CH}_4$ at Day-60 (Figure 1). It was further enriched up to 7.53, 11.7, and 4.26% by the addition of NO_2^- , NO_3^- and AQDS at Day-60, respectively. Compared with treatment 2 amended with $\text{CH}_4 + \text{NO}_2^-$, AQDS addition significantly decreased the $^{13}\text{CO}_2$ atom percentage in treatment 5

amended with $\text{CH}_4 + \text{NO}_2^- + \text{AQDS}$ at Day-60. In contrast, compared with treatment 3 amended with $\text{CH}_4 + \text{NO}_3^-$, AQDS addition remarkably increased the $^{13}\text{CO}_2$ atom percentage in treatment 6 amended with $\text{CH}_4 + \text{NO}_3^- + \text{AQDS}$ at Day-60. Similar results were observed for the $^{13}\text{CO}_2$ atom percentage at Day-30 in spite of relatively low values.

The 60 days' average rates of NO_2^- -DAMO, NO_3^- -DAMO and total DAMO were 1.95, 1.43 and 3.39 nmol $^{13}\text{CO}_2/\text{g}$ soil/day, respectively (Figure 2). Likewise, the AQDS-mediated AOM rate was 0.86 nmol $^{13}\text{CO}_2/\text{g}$ soil/day, much lower than those of NO_2^- - and NO_3^- -DAMO. AQDS amendment notably decreased the average NO_2^- -DAMO rate by 51% but increased the average NO_3^- -DAMO rate by 126%. Accordingly, AQDS increased the total DAMO rate by 24%.

The net amounts of ^{13}C assimilation into SOC via $^{13}\text{CH}_4$ oxidation over the 60-day incubation were 148 and 118 nmol $^{13}\text{C}/\text{g}$ soil in the processes of NO_2^- -DAMO and NO_3^- -DAMO, respectively (Figure 2). ^{13}C assimilation in treatment 5 amended with $^{13}\text{CH}_4 + \text{AQDS}$ was 71 nmol $^{13}\text{C}/\text{g}$ soil, much lower than those of NO_2^- -DAMO and NO_3^- -DAMO. AQDS addition decreased ^{13}C assimilation derived from NO_2^- -dependent AOM by 42% and increased ^{13}C assimilation derived from NO_3^- -dependent by 114%. Accordingly, ^{13}C assimilation derived from total DAMO increased from 265 nmol $^{13}\text{C}/\text{g}$ soil in the absence of AQDS to 337 nmol $^{13}\text{C}/\text{g}$ soil in the presence of AQDS. The percentage of ^{13}C -SOC in the total ^{13}C - CH_4 conversion (^{13}C - $\text{CO}_2 + ^{13}\text{C}$ -SOC) ranged between 56.3% and 60.5%, which was not affected by AQDS addition (Table 1). However, the percentage of ^{13}C -SOC that was generally higher

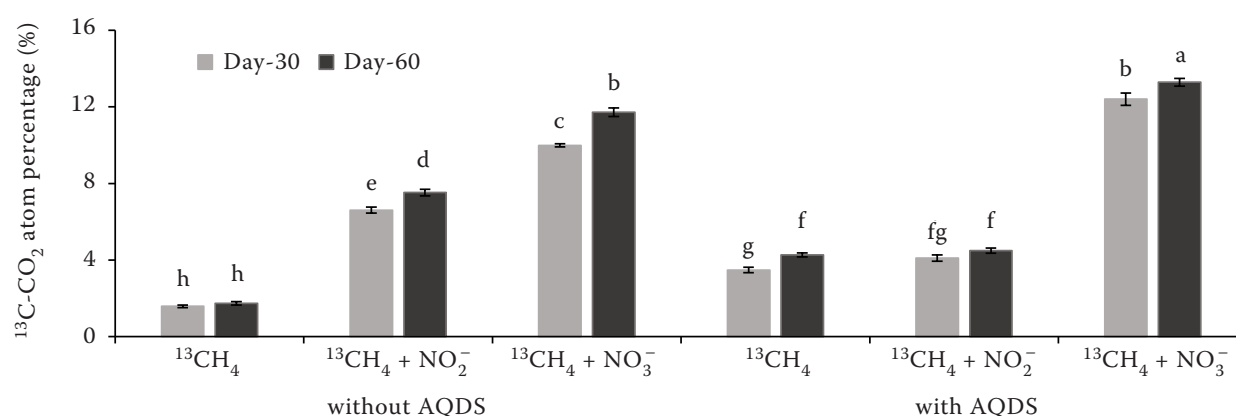


Figure 1. Generation of $^{13}\text{CO}_2$ from $^{13}\text{CH}_4$ oxidation after 30-day and 60-day incubation. ^{13}C - CO_2 atom percentage was calculated from $^{13}\text{CO}_2/(^{13}\text{CO}_2 + ^{12}\text{CO}_2)$. Error bars indicate standard deviation of means ($n = 3$). Different lower-case letters above error bars showed significant differences at $P < 0.05$ level. AQDS – anthraquinone-2,6-disulfonate

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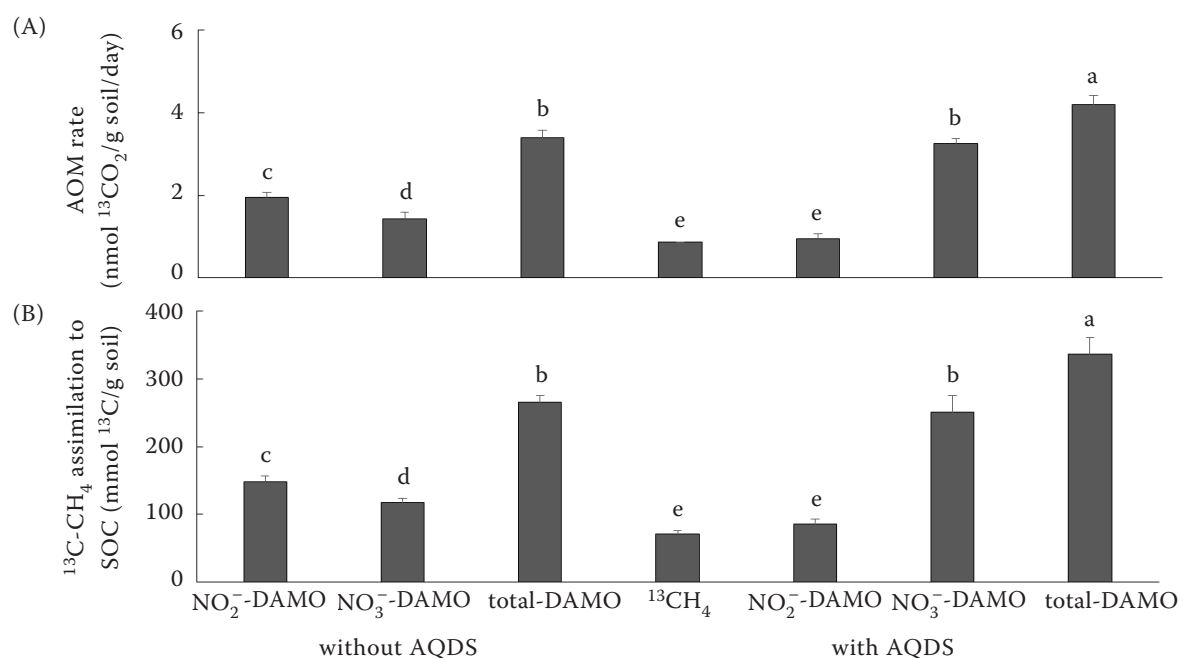


Figure 2. Rates of anaerobic oxidation of (A) methane and (B) ¹³C-CH₄ assimilation to soil organic carbon in the paddy soil microcosms during 60-day incubation. Error bars indicate standard deviation of means ($n = 3$). Different lower-case letters above error bars showed significant differences at $P < 0.05$ level. AOM – anaerobic oxidation of methane; SOC – soil organic carbon; DAMO – denitrifying anaerobic methane oxidation; AQDS – anthraquinone-2,6-disulfonate

with AQDS than without AQDS had significant negative correlations with AOM rate, ¹³C-SOC and total ¹³C-CH₄ conversion (Table 2). Additionally, the AOM rate, ¹³C-SOC, and total ¹³CH₄ conversion had a significant positive correlation with each other.

Our results indicated that apart from NO₂⁻ and NO₃⁻, AQDS addition remarkably enhanced ¹³CO₂ atom percentage compared with the vials amended with the only ¹³CH₄. This was consistent with findings reported by Fan et al. (2020), who argued that quinones present in humic acids could act as effective

electron acceptors to facilitate AOM in paddy soils. Indeed, AQDS has been proved to be able to serve as a terminal electron acceptor for ANME-2d (Bai et al. 2019). However, in this study, both AOM rates and ¹³C assimilation were much lower in AQDS-mediated AOM than NO₃⁻-dependent AOM. This was likely attributed to less thermodynamically favourable AOM coupled to AQDS reduction ($\Delta G^{0'} = -41$ kJ/mol) than nitrate reduction ($\Delta G^{0'} = -765$ kJ/mol).

We found that AQDS addition notably enhanced NO₃⁻-dependent AOM rates and ¹³C assimilation.

Table 1. Total ¹³C-CH₄ conversion (¹³C-CO₂ + ¹³C-SOC) and percentage of ¹³C-SOC in ¹³C-CH₄ conversion after 60-day incubation

	Total ¹³ C-CH ₄ conversion (nmol ¹³ C/g soil)	Percentage of ¹³ C-SOC (%)
NO ₂ ⁻ -DAMO	300.8 ^c	56.6 ^a
NO ₃ ⁻ -DAMO	203.9 ^d	57.7 ^a
Total DAMO	504.7 ^b	57.0 ^a
AQDS	159.1 ^e	58.9 ^a
NO ₂ ⁻ -DAMO + AQDS	179.4 ^e	60.5 ^a
NO ₃ ⁻ -DAMO + AQDS	446.3 ^b	56.3 ^a
Total DAMO + AQDS	625.6 ^a	57.5 ^a

Different lower-case letters in the same columns showed significant differences at $P < 0.05$ level. SOC – soil organic carbon; DAMO – denitrifying anaerobic methane oxidation; AQDS – anthraquinone-2,6-disulfonate

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Table 2. Linear correlation coefficients among anaerobic oxidation of methane (AOM) rate, ^{13}C -SOC, total $^{13}\text{CH}_4$ conversion and the percent of ^{13}C -SOC

	AOM rate	^{13}C -SOC	Total $^{13}\text{CH}_4$ conversion
^{13}C -SOC	0.998*		
Total $^{13}\text{CH}_4$ conversion	0.999*	0.995*	
Percent of ^{13}C -SOC	−0.651*	−0.612*	−0.592*

* $P < 0.05$; SOC – soil organic carbon

This may be attributed to AQDS acting as an electron shuttle for electron transfer during anaerobic metabolism. AQDS have been reported to act as electron shuttles during AOM coupled to iron (III) reduction (He et al. 2019). ANME-2d may anaerobically oxidise methane to carbon dioxide and transfer electrons generated to outer membrane cytochromes (McGlynn et al. 2015); hence, electron shuttles would facilitate long-distance electron transfer from ANME-2d cell surface to various extracellular electron acceptors. The standard redox potential (E'_0) of AQDS (−186 mV) is feasible to be reduced by ANME-2d since E'_0 values of outer membrane cytochromes of ANME-2d have a large range with −320 mV to −15 mV (Wu et al. 2014). The reduced AQDS carried electrons toward nitrate that was close to ANME-2d cells, hence facilitating NO_3^- -dependent AOM. More importantly, AQDS could serve as an electron donor for heterotrophic denitrifying intermediates such as NO_2^- and N_2O (Aranda-Tamaura et al. 2007), expanding the electron type acceptors for extracellular electron transfer of ANME-2d. In addition, quinones have been recently reported to facilitate extracellular electron transfer from AOM microorganisms (Rice Cluster I) to N_2O (Valenzuela et al. 2020), which potentially contributed to the stimulating effect of AQDS on NO_3^- -DAMO process in the present study.

AQDS addition remarkably decreased NO_2^- -dependent AOM rates and ^{13}C assimilation. In fact, NO_2^- is a substrate for heterotrophic denitrification, and AQDS addition could potentially be beneficial to transfer electrons generated from anaerobic metabolism to denitrifying bacteria (Aranda-Tamaura et al. 2007). These stimulated heterotrophic denitrifiers would predominantly compete for the substrate of NO_2^- with *M. oxyfera*, hence potentially inhibiting the NO_2^- -dependent AOM. Furthermore, *M. oxyfera* was characterised as a slow-growing bacteria compared with denitrifying bacteria (He et al. 2015), resulting in its poor competition for the niche. However, AQDS addition increased total DAMO rates and

^{13}C assimilation by 37% and 27%, respectively. This implied that organic fertilisation could potentially facilitate the DAMO process and C-CH_4 sequestration in paddy soils. Nevertheless, it should be noted that the role of quinone analogs in DAMO might also depend on soil properties; hence, further studies are needed to be conducted in more various paddy soils.

In conclusion, our study showed that AQDS, analogs of natural humic substances, could act as an electron acceptor for AOM in the paddy soil. Moreover, it could act as an electron shuttle to stimulate nitrate-dependent AOM rates and the amount of $^{13}\text{C-CH}_4$ assimilation into SOC and ultimately increased the total DAMO rate. Thus, humic substances with redox functional groups might play an important role in CH_4 consumption under anaerobic conditions in submerged paddy fields.

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