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 generated 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 (Methylomirabilis 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 (Vaksmaa 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.

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

YAOHONG ZHANG$^1$, FANGYUAN WANG$^{1,2}$

$^1$Collaborative Innovation Center on Forecast and Evaluation of Meteorological Disasters, Jiangsu Key Laboratory of Agricultural Meteorology, School of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing, P.R. China
$^2$State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, P.R. China

*Corresponding author: yhzhang@nuist.edu.cn, 13851760430@163.com


Abstract: Quinones, redox-active functional groups in soil organic matter, can act as electron shuttles for microbial anaerobic transformation. Here, we used $^{13}$CH$_4$ to trace $^{13}$C conversion ($^{13}$C-CO$_2$ + $^{13}$C-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 $^{13}$C-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 $^{13}$C-SOC in total $^{13}$C-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; $^{13}$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 generated 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 (Methylomirabilis 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 (Vaksmaa 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.

Supported by the National Natural Science Foundation of China, Grants No. 41671247 and 41103039; by the Natural Science Foundation of Jiangsu Province, Grant No. BK20171455; by the China Scholarship Council, Project No. 201908320185; and by the Priority Academic Program Development of Jiangsu Higher Education Institutions.
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$_2$O (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$_4$ (Mujio 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 $^{13}$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 according to WRB soil classification. It had a bulk density of 1.28 g/cm$^3$ 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 mixture ratio of 1.5 mmol/L. Immediately after that, 500 μL of Ar-purged NO$_3^-$/NO$_2^-$ 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 $^{13}$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 $^{13}$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 $^{13}$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 $^{13}$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. $^{13}$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 $^{13}$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 with/without AQDS treatments among NO$_3^-$-DAMO, NO$_2^-$-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 $^{13}$CO$_2$ atom percentage increased from its background abundance of 1.08%, up to 1.73% in the treatment 1 amended only with $^{13}$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 CH$_4$ + NO$_2^-$, AQDS addition significantly decreased the $^{13}$CO$_2$ atom percentage in treatment 5 amended with CH$_4$ + NO$_2^-$ + AQDS at Day-60. In contrast, compared with treatment 3 amended with CH$_4$ + NO$_3^-$, AQDS addition remarkably increased the $^{13}$CO$_2$ atom percentage in treatment 6 amended with CH$_4$ + NO$_3^-$ + AQDS at Day-60. Similar results were observed for the $^{13}$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 $^{13}$CO$_2$/g soil/day, respectively (Figure 2). Likewise, the AQDS-mediated AOM rate was 0.86 nmol $^{13}$CO$_2$/g soil/day, much lower than those of NO$_2^-$ and NO$_3^-$-DAMO. AQDS amendment notably decreased the average NO$_3^-$-DAMO rate by 51% but increased the average NO$_2^-$-DAMO rate by 126%. Accordingly, AQDS increased the total DAMO rate by 24%.

The net amounts of $^{13}$C assimilation into SOC via $^{13}$CH$_4$ oxidation over the 60-day incubation were 148 and 118 nmol $^{13}$C/g soil in the processes of NO$_2^-$-DAMO and NO$_3^-$-DAMO, respectively (Figure 2). $^{13}$C assimilation in treatment 5 amended with CH$_4$ + AQDS was 71 nmol $^{13}$C/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 $^{13}$C/g soil in the absence of AQDS to 337 nmol $^{13}$C/g soil in the presence of AQDS. The percentage of $^{13}$C-SOC in the total $^{13}$C-CH$_4$ conversion ($^{13}$C-CO$_2$ + $^{13}$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 $^{13}$CO$_2$ from $^{13}$CH$_4$ oxidation after 30-day and 60-day incubation. $^{13}$C-CO$_2$ atom percentage was calculated from $^{13}$CO$_2$/($^{13}$CO$_2$ + $^{12}$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.](https://doi.org/10.17221/579/2020-PSE)
with AQDS than without AQDS had significant negative correlations with AOM rate, $^{13}$C-SOC and total $^{13}$C-CH$_4$ conversion (Table 2). Additionally, the AOM rate, $^{13}$C-SOC, and total $^{13}$CH$_4$ conversion had a significant positive correlation with each other.

Our results indicated that apart from NO$_2^-$ and NO$_3^-$, AQDS addition remarkably enhanced $^{13}$CO$_2$ atom percentage compared with the vials amended with the only $^{13}$CH$_4$. 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 $^{13}$C assimilation were much lower in AQDS-mediated AOM than NO$_3^-$-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 NO$_3^-$-dependent AOM rates and $^{13}$C assimilation.

Table 1. Total $^{13}$C-CH$_4$ conversion ($^{13}$C-CO$_2 + ^{13}$C-SOC) and percentage of $^{13}$C-SOC in $^{13}$C-CH$_4$ conversion after 60-day incubation

<table>
<thead>
<tr>
<th></th>
<th>Total $^{13}$C-CH$_4$ conversion (nmol $^{13}$C/g soil)</th>
<th>Percentage of $^{13}$C-SOC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2^-$-DAMO</td>
<td>300.8$^c$</td>
<td>56.6$^a$</td>
</tr>
<tr>
<td>NO$_3^-$-DAMO</td>
<td>203.9$^d$</td>
<td>57.7$^a$</td>
</tr>
<tr>
<td>Total DAMO</td>
<td>504.7$^b$</td>
<td>57.0$^a$</td>
</tr>
<tr>
<td>AQDS</td>
<td>159.1$^e$</td>
<td>58.9$^a$</td>
</tr>
<tr>
<td>NO$_2^-$-DAMO + AQDS</td>
<td>179.4$^e$</td>
<td>60.5$^a$</td>
</tr>
<tr>
<td>NO$_3^-$-DAMO + AQDS</td>
<td>446.3$^b$</td>
<td>56.3$^a$</td>
</tr>
<tr>
<td>Total DAMO + AQDS</td>
<td>625.6$^a$</td>
<td>57.5$^a$</td>
</tr>
</tbody>
</table>

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
AQDS addition increased total DAMO rates and 13C assimilation by 37% and 27%, respectively. This implied that organic fertilisation could potentially facilitate the DAMO process and C-CH4 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 13C-CH4 assimilation into SOC and ultimately increased the total DAMO rate. Thus, humic substances with redox functional groups might play an important role in CH4 consumption under anaerobic conditions in submerged paddy fields.

REFERENCES


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°) of AQDS (−186 mV) is feasible to be reduced by ANME-2d since E° 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 NO3−-dependent AOM. More importantly, AQDS could serve as an electron donor for heterotrophic denitrifying intermediates such as NO2− and N2O (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 N2O (Valenzuela et al. 2020), which potentially contributed to the stimulating effect of AQDS on NO3−-DAMO process in the present study.

AQDS addition remarkably decreased NO2−-dependent AOM rates and 13C assimilation. In fact, NO3− 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 NO3− with M. oxyfera, hence potentially inhibiting the NO2−-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

Table 2. Linear correlation coefficients among anaerobic oxidation of methane (AOM) rate, 13C-SOC, total 13CH4 conversion and the percent of 13C-SOC

<table>
<thead>
<tr>
<th></th>
<th>AOM rate</th>
<th>13C-SOC</th>
<th>Total 13CH4 conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>13C-SOC</td>
<td>0.998*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 13CH4 conversion</td>
<td>0.999*</td>
<td>0.995*</td>
<td></td>
</tr>
<tr>
<td>Percent of 13C-SOC</td>
<td>−0.651*</td>
<td>−0.612*</td>
<td>−0.592*</td>
</tr>
</tbody>
</table>

*P < 0.05; SOC – soil organic carbon


Received: November 4, 2020
Accepted: March 22, 2021
Published online: March 30, 2021