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Mediated electrochemical analysis as emerging tool to unravel links between microbial redox cycling of natural organic matter and anoxic nitrogen cycling

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ABSTRACT

Natural organic matter (NOM) is an important redox-active component in soils and aquifers that comprises numerous functional moieties spanning a wide range of redox potentials. Tracking reversible electron transfer from and to NOM in biogeochemical redox processes has been a challenge for decades. Reasons include side reactions of reactants used to determine the redox state of NOM and slow reaction kinetics of reactants or traditional non-mediated electrochemical methods. Furthermore, partially irreversible reactions/methods employed hamper the experimental determination of redox properties of NOM. Recent advances in mediated electrochemical analysis, however, have greatly improved our ability to characterize the redox properties of NOM. Thus, mediated electrochemical analysis may become an important tool in expanding our understanding of NOM-fueled biogeochemical N cycling in anoxic environments. Nonetheless, this technique has rarely been applied to investigate microbial pathways of reversible NOM redox cycling such as its coupling to anoxic nitrogen (N) cycling.

Here we advocate for employing mediated electrochemical analysis to address such topics in the future and provide recommendations for a successful experimental application of this method in the presence of reactive N-species. To this end, we review recent applications of mediated electrochemical analysis in studying microbial NOM cycling. We exemplify the potential of mediated electrochemical techniques for biogeochemical research by discussing how microbial NOM redox cycling is linked to anaerobic N cycling. We focus on anaerobic ammonium oxidation (anammox) and reduction of N-oxides that are related to N loss and nitrous oxide (N_2O) mitigation. Finally, we present strategies to work around problems arising from electroactive intermediates that hamper the application of mediated electrochemical analysis in microbial experiments.

1. Introduction

Biogeochemical cycling of major elements, including carbon, hydrogen, nitrogen, oxygen, sulfur, and phosphorus, is largely fuelled by microbially mediated electron transfer reactions with a substantial portion thereof taking place in anoxic environments (Falkowski et al., 2008; Pasek, 2008; Figueroa et al., 2018). Tracking electron transfer is central to identify the occurrence and extent of a specific microbial redox process, to elucidate electron flow paths, and to further explore the electron transfer mechanisms involved in element cycling.

Natural organic matter (NOM) dominates the organic carbon stock in terrestrial and aquatic environments (Tratnyek et al., 2011; Schaeffer et al., 2015) and originates from incomplete biomass decay (Stevenson, 1994). Next to the well-established fact that NOM serves as the major electron source for aerobic and anaerobic respiration processes by oxidation to CO_2 (Heitmann et al., 2007; Gao et al., 2019), its redox-active functional moieties can reversibly donate and accept electrons in microbial energy metabolism (Lovley et al., 1996; Lovley et al., 1999; Roden et al., 2010). Microbially mediated NOM redox cycling is known to be linked to greenhouse gas (e.g., CH_4, N_2O, CO_2) emissions and nitrogen loss by coupling C and N cycles (Aranda-Tamaura et al., 2007; Martinez et al., 2013; Scheller et al., 2016; Valenzuela et al., 2017, 2019; Gao et al., 2019; Valenzuela et al., 2020). Nevertheless, significant knowledge gaps regarding the mechanisms and environmental

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niches of such processes exist, primarily due to the lack of appropriate methods for determining the redox properties (i.e., electron exchange capacities and redox state) of NOM. Thus, effective methods for tracking electron transfer flux to and from NOM are crucial for unravelling further processes of NOM driven microbial redox reactions linking C and N turnover.

While electron transfer fluxes in redox reactions involving simple inorganic and/or organic compounds (e.g., acetate) can be calculated from changes in concentrations and valence states of electron donor-acceptor pairs, this approach fails for NOM, which is a vast mixture of organic macromolecules that differ not only in chemical composition but also in the number of redox active groups and their redox state (Stevenson, 1994; Sutton and Sposito, 2005; Lehmann and Kleber, 2015). The ability of NOM to accept and donate electrons can be quantified by determining its electron accepting (EAC) and electron donating capacity (EDC) (Aeschbacher et al., 2010; Tratnyek et al., 2011). Electron transfer fluxes of microbial processes involving NOM as electron donor or acceptor can be obtained by monitoring changes in EAC and EDC of NOM over time. Given its structural complexity and inherent heterogeneity, however, experimental determination of the EAC and EDC of NOM has been a major analytical challenge.

In the past, the EAC and/or EDC of NOM have been assessed by monitoring the consumption of chemical reductants and/or oxidants that react with redox sensitive functional groups of NOM (Lovley et al., 1996; Kappler and Haderlein, 2003; Blodau et al., 2009). However, these methods are tedious and time consuming and results obtained for a specific reactant are difficult to compare with other reactants (Matthiessen, 1995; Bauer et al., 2007). During the last decade, however, Sander and coworkers (Aeschbacher et al., 2010) have evaluated and refined a powerful mediated electrochemical analysis approach to quantify accurately the EAC, EDC, and thus the total electron exchange capacity (EEC) of NOM. This technique is based on chronocoulometry and employs dissolved redox mediators to promote the rate of truly reversible electron transfer and to facilitate redox equilibria between working electrodes and NOM as analyte in the electrochemical cells (Aeschbacher et al., 2010; Sander et al., 2015). Mediated electrochemical analysis can overcome major drawbacks of traditional techniques and has been applied successfully to investigate the redox properties and various biogeochemical redox processes of NOM (Aeschbacher et al., 2011; Aeschbacher et al., 2012; Wenk et al., 2013; Lau et al., 2016; Tan et al., 2017; Wallace et al., 2017), including microbially mediated NOM redox reactions (Klüpfel et al., 2014; Lau et al., 2015; Lau et al., 2017; Gao et al., 2019). This analytical approach thus opens new venues for investigating so far unexplored biogeochemical processes involving NOM.

As nitrogen occurs at various valence states (from +V to -III) and exhibits a pronounced redox-dependent speciation in the biosphere, nitrogen species are prone undergo redox reactions with NOM in environments such as bogs or fens where inorganic electron donors and acceptors are deficient. In particular, the processes governing N loss including N_2O reduction fueled by NOM potentially may contribute to influencing Earth's atmosphere and greenhouse gases budget. Here, we specifically highlight the application of mediated electrochemical analysis in characterizing microbial NOM redox cycling in general and its relevance for N-cycling. First, we summarize applications of chemical methods in studying microbial NOM redox cycling. Second, we review the existing, yet limited, literature wherein mediated electrochemical analysis was employed for quantification of microbial NOM redox cycling and highlight the advantages of this approach compared to chemical methods. Third, we discuss various concepts of the so far less studied NOM-dependent microbial pathways of anoxic N cycling (with emphasis on processes governing N loss including N_2O mitigation), which can be elucidated using mediated electrochemical approaches. Last, we present experimental strategies in the application of the electrochemical approaches to overcome interferences of electroactive substances typically present in microbial experiments or field samples when determining EAC and/or EDC values of NOM.

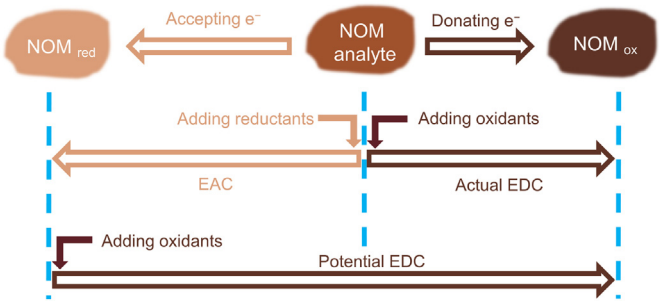

Fig. 1. Schematic illustration of chemical methods to determine the electron donating- (EDC) and electron accepting capacity (EAC) of NOM. The EAC and EDC of NOM were traditionally assed by reacting with chemical reductants and oxidants, respectively.

2. Experimental approaches to determine the redox properties of NOM

2.1. Chemical methods

Chemical methods rely on redox titrations using added reductants and/or oxidants to quantify the redox states of NOM samples (Fig. 1). Initial investigations emphasized on adding oxidants (e.g., ferric citrate ($FeC_6H_5O_7$), potassium ferricyanide ($K_3Fe[CN]_6$) and iodine (I_2)) to measure the EDC of a NOM (Lovley et al., 1996; Matthiessen, 1995; Helburn and MacCarthy, 1994; Benz et al., 1998; Lovley and Blunt-Harris, 1999; Struyk and Sposito, 2001). To determine the potential EDC, the NOM sample is first reduced to its maximum extent either chemically, microbially, or electrochemically (Scott et al., 1998; Benz et al., 1998; Kappler and Haderlein, 2003; Chen et al., 2003; Kappler et al., 2004; Bauer et al., 2007; Ratasuk and Nanny, 2007). Next, an oxidant is added to the fully reduced NOM sample (Fig. 1). From the change in concentration and valence state of the oxidant, the EDC value of the NOM sample can be calculated. The value resulting from this procedure is often referred to as *potential* or *total* electron donating capacity of a given NOM. To obtain the *actual* EDC value, the NOM sample is treated with the oxidant without a previous reduction step. The difference between *potential* EDC and *actual* EDC represents the *actual* electron accepting capacity (EAC) (Fig. 1). Alternatively, the *actual* EAC can be obtained by reacting the native NOM sample with a strong reductant (e.g., hydrogen sulfide (H_2S) or zinc powder (Zn^0)). From the change in concentration and valence state of the reductant the number of electrons taken up by the NOM and thus the *actual* EAC can be calculated (Heitmann and Blodau, 2006; Blodau et al., 2009; Bauer et al., 2007; Heitmann et al., 2007). Note that the electron accepting- or donating capacities as described above are operationally defined parameters that do not necessarily reflect the amount /number of electrons that microbes can transfer in a given environmental setting.

These chemical approaches (Fig. 1) have been commonly used for characterizing the redox properties of NOM in earlier studies. Regarding microbial NOM redox cycling, in particular microbially catalyzed NOM reduction has been studied. NOM on the one hand is known to function as a terminal electron acceptor in anoxic microbial respiration and on the other hand it can serve as an electron transfer mediator (electron shuttle) between Fe(III)-reducing microorganisms and Fe(III) (oxyhydr)oxides (Lovley et al., 1996; Nevin and Lovley, 2000; Kappler et al., 2004; Jiang and Kappler, 2008; Rakshit et al., 2009; Roden et al., 2010; Martinez et al., 2013; Shi et al., 2016). The role of dissolved NOM in electron shutting between microbes and metal oxides was traditionally assessed by comparing the rates and extent of Fe(III) reduction in the presence and absence of NOM. The shutting mechanism was further verified by the abiotic reaction of cell-free

NOM-containing filtrates with Fe(III) minerals (Lovley et al., 1996; Lovley et al., 1998). Indeed, the electron shutting ability of NOM primarily depends on its *potential* EDC (Peretyazhko and Sposito, 2006; Rakshit et al., 2009; Roden et al., 2010; Keller and Takagi, 2013).

Such chemical treatments, however, are tedious (Matthiessen, 1995; Bauer et al., 2007), pH dependent and not versatile regarding the redox potential applied during analysis (Aeschbacher et al., 2010). Furthermore, they may induce side reactions and artifacts that limit accuracy and comparability of such EDC (Peretyazhko and Sposito, 2006) or EAC measurements (Heitmann and Blodau, 2006; Blodau et al., 2009). For example, due to slow reaction kinetics of the two most frequently used Fe(III) oxidants (i.e., $FeC_6H_5O_7$ and $K_3Fe[CN]_6$), the obtained EDC values underestimated the true EDC (Aeschbacher et al., 2010; Rakshit and Sarkar, 2017). Furthermore, the method was not suitable to resolve small differences in EDC values (Rakshit and Sarkar, 2017) or oxidized organic moieties in NOM that are not involved in electron shuttling (Peretyazhko and Sposito, 2006). Also, EDCs determined using $FeC_6H_5O_7$ may differ by one order of magnitude from those obtained using $K_3Fe[CN]_6$ (Peretyazhko and Sposito, 2006; Tratnyek et al., 2011). In addition to artifacts on the oxidative side, there are also issues with reductants used. Using H_2S as reductant, side reactions of H_2S addition to reduced NOM have been reported and could overestimate the EAC and *potential* EDC of NOM (Perlinger et al., 1996; Yu et al., 2016).

2.2. Electrochemical analysis

The electrochemical approaches replaced in the last years more and more the traditional chemical oxidants and reductants by using electrodes as electron acceptors and donors, respectively. Electrochemical methods can potentially overcome the limitations of chemical methods since the number of electrons transferred is measured directly as current rather than indirectly by consumption of a specific reactant. Various electrochemical techniques have been proposed in the past (Buffle and Cominoli, 1981; Mota et al., 1994; Helburn and MacCarthy, 1994; Motheo and Pinhedo, 2000; Nurmi and Tratnyek, 2002), albeit with limited success, primarily because of slow kinetics and thus non-equilibrium of the electron transfer between the electrode and quinone/hydroquinone moieties present in NOM samples. These direct or "non-mediated" electrochemical analysis (Sander et al., 2015) rely on direct physical contact of NOM components with the surface of the working electrode. The need for integrating low current signals over a long period of time hampers the sensitivity and accuracy of this method and prevents its applications to dilute NOM samples or samples with low quinone content (Osterberg and Shirshova, 1997; Tratnyek et al., 2011).

Instead, the mediated electrochemical (i.e., amperometric) analysis (Fig. 2) described by Sander and co-workers (Aeschbacher et al., 2010) enables truly reversible electron transfer to and from NOM, allowing accurate determination of the EAC and EDC values of dissolved electroactive NOM such as humic substances (HS). The addition of small amounts of dissolved redox mediators enables the rapid electron transfer between the working electrode and the analyte, and prevents the long measurement times due to slow electron exchange kinetics with the working electrode and thus circumvents the limitations of direct (non-mediated) electrochemical measurements. Employing this technique, the coupling between protonation and electron transfer equilibrium in dissolved HS has been studied in detail, as well as the reversibility of NOM redox reactions (Aeschbacher et al., 2011). Subsequent investigations have documented the electron donating properties of dissolved NOM under oxic conditions (Aeschbacher et al., 2012), the chemical oxidation (Page et al., 2012, 2013; Wenk et al., 2013) and photooxidation (Sharpless et al., 2014) of dissolved NOM. Mediated electrochemical analysis has also been applied to quantify the redox properties of particulate NOM (POM) that contribute much higher EAC to total EAC pool of NOM than dissolved NOM in soils and sediments (Lau et al., 2015, 2016; Gao et al., 2019). Mediated electrochemical analysis has also entered the field of environmental microbiology to evaluate microbially catalyzed NOM redox cycling both in laboratory pure cultures (Klüpfel et al., 2014) and in complex environments (Lau et al., 2015, 2017; Gao et al., 2019). These limited but pioneering studies demonstrated the applicability and efficacy of mediated electrochemical analysis to directly track electron fluxes of microbial NOM reduction-oxidation in laboratory incubations and field samples.

3. NOM-dependent microbial pathways for anaerobic ammonium oxidation and N-oxide reduction

Known anoxic microbial pathways for nitrogen loss include anaerobic ammonium oxidation (anammox), complete denitrification, non-denitrifying N_2O reduction, and oxygenic dismutation of NO to N_2 and O_2 (Ettwig et al., 2010; Stein and Klotz, 2016; Hallin et al., 2017; Zhu et al., 2017). The former three are main pathways for nitrogen loss in anoxic environments. Among them, complete denitrification and non-denitrifying N_2O reduction can be sinks of the highly potent greenhouse gas N_2O. N_2O is also a major cause of stratospheric ozone destruction (Ravishankara et al., 2009; IPCC, 2013). Given the environmental significance in balancing global N budgets and regulating Earth's climate, expanding our understanding of microbial processes modulating N loss as well as eliminating N_2O is of great importance. In the following sections, we will discuss how NOM redox cycling contributes to N loss as wells as N_2O mitigation, thus, opening a wider field of applying electrochemical methods in environmental microbiology.

In anoxic environments, microbially mediated NOM oxidation-reduction can be tightly linked to N loss through diverse electron transfer mechanisms. First, NOM can serve as terminal electron acceptor for the oxidation of NH_4^+ to N_2 (Fig. 3a). Second, NOM may function as electron donor to reduce oxidized nitrogen species including NO_3^-, NO_2^- or N_2O to N_2 (Lovley et al., 1999; Aranda-Tamaura et al., 2007) (Fig. 4a). Third, NOM can act as electron shuttle between Fe(III)-/NOM-reducing microorganisms and Fe(III) (oxyhydr)oxides or POM. For example, AQDS (anthraquinone-2,6-disulfonate, a humic substance analog) was found to act as electron shuttle in Feammox (anaerobic NH_4^+ oxidation to N_2 coupled to iron(III) reduction; Fig. 5a) (Zhou et al., 2016). Thus, microbial redox reactions involving dissolved NOM could influence N_2 production by transferring electrons during microbial iron(III) or POM reduction. Finally, NOM may also play a role as electron mediator in mediated interspecies electron transfer (MIET), i.e., acting as an electron carrier between microbes by channelling electrons from an electron-donating cell to an electron-accepting cell (Smith et al., 2015). For instance, NOM might facilitate MIET between consortia of NOM-dependent anammox microbes and complete denitrifiers or non-denitrifying N_2O reducers, and therefore constraining N_2O emissions to the atmosphere (Fig. 5b).

3.1. Microbial NOM reduction

Owing to the inherent heterogeneity of redox-active moieties in NOM, the standard redox potential of a given NOM sample is not a discrete value but typically a range, spanning from +0.15 V to −0.3 V at pH 7 (Aeschbacher et al., 2011). The redox-active functional groups of NOM can oscillate between oxidized and reduced forms as a function of redox milieu, and thus potentially allowing NOM to participate in numerous biogeochemically significant electron-transfer reactions under virtually all environmental redox conditions. Quinone-like functional groups make up the majority of reversibly reducible moieties in NOM (Scott et al., 1998; Struyk and Sposito, 2001; Aeschbacher et al., 2011) and further redox-active moieties of NOM such as phenols or complexed iron may contribute to its redox properties (Ratasuk and Nanny, 2007; Hernández-Montoya et al., 2012). A representative distribution of standard reduction potentials of redox active moieties in

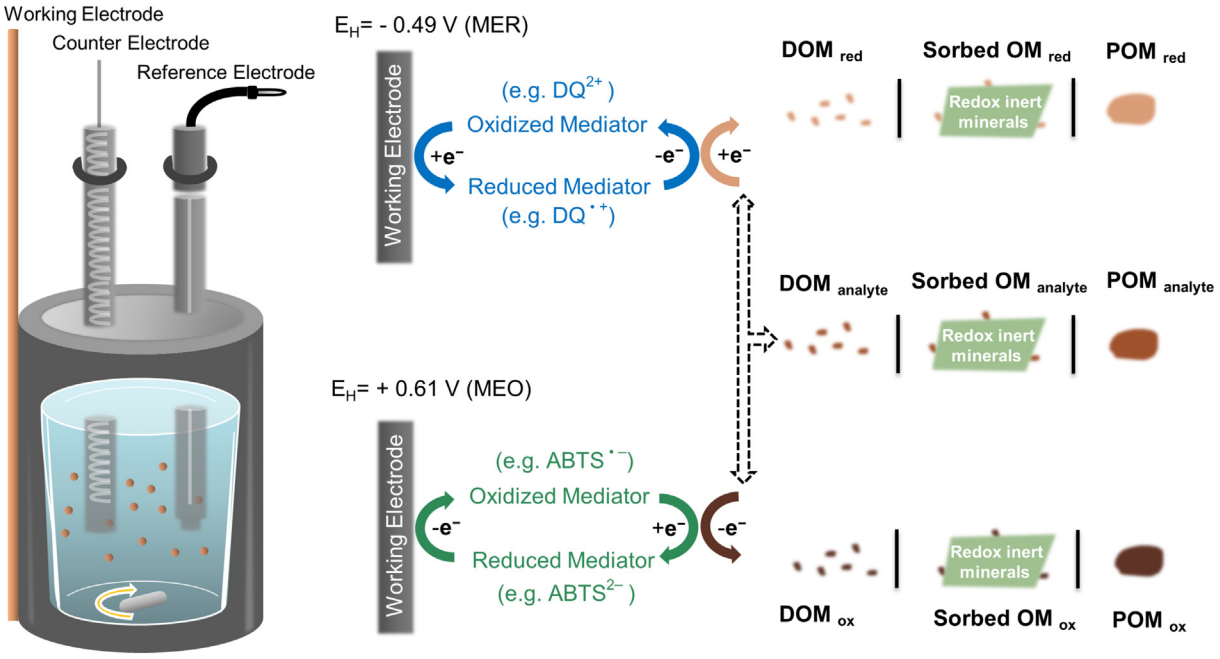

Fig. 2. Setup and principle of mediated electrochemical (i.e., amperometric) analysis. The electrochemical cell consists of a glassy carbon cylinder as the working electrode and the cell reaction vessel, a Pt wire counter electrode, and an Ag/AgCl reference electrode. In MER, a negative potential of −0.49 V vs standard hydrogen electrode (SHE) is applied to the working electrode. Oxidized mediators are firstly added to the pH-buffered solution, being reduced at the working electrode. After reaching redox equilibrium, NOM analytes are added and react with the reduced mediator (the mediator becomes oxidized), thereby generating reduced NOM. The newly formed oxidized mediator is re-reduced at the working electrode, resulting in a reductive and peak-shaped current response until the electrochemical equilibrium was re-attained. The number of electrons transferred to the NOM is obtained by integrating the baseline-corrected response current over time. In MEO, the working electrode is polarized to a positive potential of +0.61 V vs SHE. The reduced mediators and the NOM analytes are added in a similar order as to MER. The electrons flow in the reverse direction compared to MER. The resultant oxidative and peak-shaped current response is integrated to obtain the number of electrons released from NOM. EAC and EDC values are obtained by normalizing the measured number of electrons transferred to the mass of the NOM sample. NOM samples can be analyzed in dissolved, adsorbed, or particulate states.

NOM at pH 7 is shown in Fig. 3b and 4b. For comparison, reduction potentials of canonical biogeochemical redox couples of anoxic N cycling are shown in Table 1 and Figs. 3b and 4b illustrate which fraction of redox-active functional groups in NOM are available to drive a given process. As can be seen, NOM is thermodynamically capable of taking up electrons from microbial anoxic ammonium oxidation.

3.1.1. Anaerobic ammonium oxidation coupled to NOM reduction

Known inorganic electron acceptors for anaerobic NH_4^+ oxidation to produce N_2 include nitrite (anammox) (Dalsgaard and Thamdrup, 2002; Nie et al., 2015), nitric oxide (NO) (Hu et al., 2019), iron(III) oxyhydroxides (Feammox) (Yang et al., 2012; Ding et al., 2014) and sulfate (sulfammox) (Rios-Del Toro et al., 2018a). Since NOM is often associated with Fe(III) minerals and exists at suitable redox states (Fig. 3b) it may also be linked to anaerobic ammonium oxidation. In contrast to Feammox, NOM reduction coupled to anaerobic ammonium oxidation (NOM-dependent anammox) to form N_2 (Fig. 3a) was scarcely studied so far. Zhou et al. (2016) recently demonstrated that the soluble synthetic redox-active quinoid compound AQDS can function as electron shuttle in Feammox by enhancing the N loss. These findings strongly suggest that microbial quinone reduction was coupled to anaerobic ammonium oxidation and abiotic re-oxidation of the hydroquinones by Fe(III) minerals (Fig. 5a), although the experimental setups of this study lacked Fe(III)-free controls to directly prove the formation of N_2 from anammox coupled to AQDS reduction. Recently, the NOM-dependent anammox to N_2 process has been claimed in marine sediments based on ^{15}N isotopic, stoichiometric and spectroscopic evidence (Rios-Del Toro et al., 2018b). A very recent study reported microbial ammonium oxidation to N_2 in an electrochemical setup where electrodes served as electron acceptors (Shaw et al., 2020). However, the mechanistic understanding of enzymatic NOM-dependent anammox to N_2 is still poor. Taking into account the standard reduction potential of the N_2/NH_4^+ couple at pH 7, E_h^0 (pH 7) = −277 mV (Table 1), and of AQDS (E_h^0 (pH 7) = −185 mV) (Table 1), we conclude that naturally occurring quinones (e.g., the plant derived naphthoquinone Lawsone, E_h^0 (pH 7) = −152 mV, (Clark, 1960) and macromolecular dissolved NOM (Fig. 3b) are energetically highly favorable for driving NOM-dependent anammox (Fig. 3b). As NOM from different sources shows different reduction potentials, its ability to drive NOM-dependent anammox may differ with source and redox state.

3.2. Microbial NOM oxidation

Polyphenols and hydroquinone moieties are thought to be the major electron-donating groups in NOM (Struyk and Sposito, 2001; Aeschbacher et al., 2012). NOM in its reduced form has the potential to be microbially oxidized coupled to the reduction of N-oxides (e.g., nitrate, nitrite and N_2O), thereby affecting N_2O formation or consumption and ultimately the loss of reactive N.

3.2.1. Complete denitrification

Denitrification refers to the process of anaerobic respiration of nitrate/nitrite to nitrogenous gases, including complete ($NO_3^-/NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and incomplete ($NO_3^-/NO_2^- \rightarrow NO \rightarrow N_2O$) denitrification. Incomplete denitrification occurs when denitrifying microbes lack the *NosZ* gene that encodes the N_2O reductase with N_2O as the terminal product. Although N_2O is also formed as a side product in complete denitrification, it can be further reduced to N_2. Therefore, mitigation of N_2O emissions can be achieved by suppressing the stepwise reduction to N_2O and/or by selectively promoting the N_2O reduction step. Thermodynamically, all redox-active moieties in NOM (when in their reduced state) can be coupled to complete denitrification

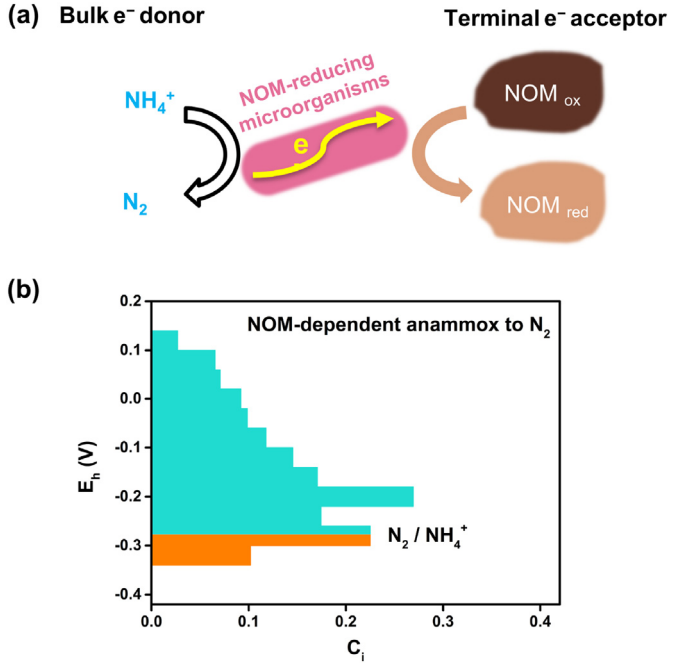

Fig. 3. (a) NOM as terminal electron acceptor in anaerobic microbial ammonium oxidation. (b) Scheme of typical E_h^0 (pH 7)-dependent distribution of reducing equivalents in humic acids. Data shown are the average of three different humic acids (i.e., Leonardite humic acid, Elliot Soil humic acid and Suwanee River humic acid) (Aeschbacher et al., 2011). Ci (mmol e^-/g HA) is the concentration of redox active moieties at the respective E_h^0 (pH 7) range. The green area indicates the fraction of reducing equivalents that thermodynamically is feasible to drive the process.

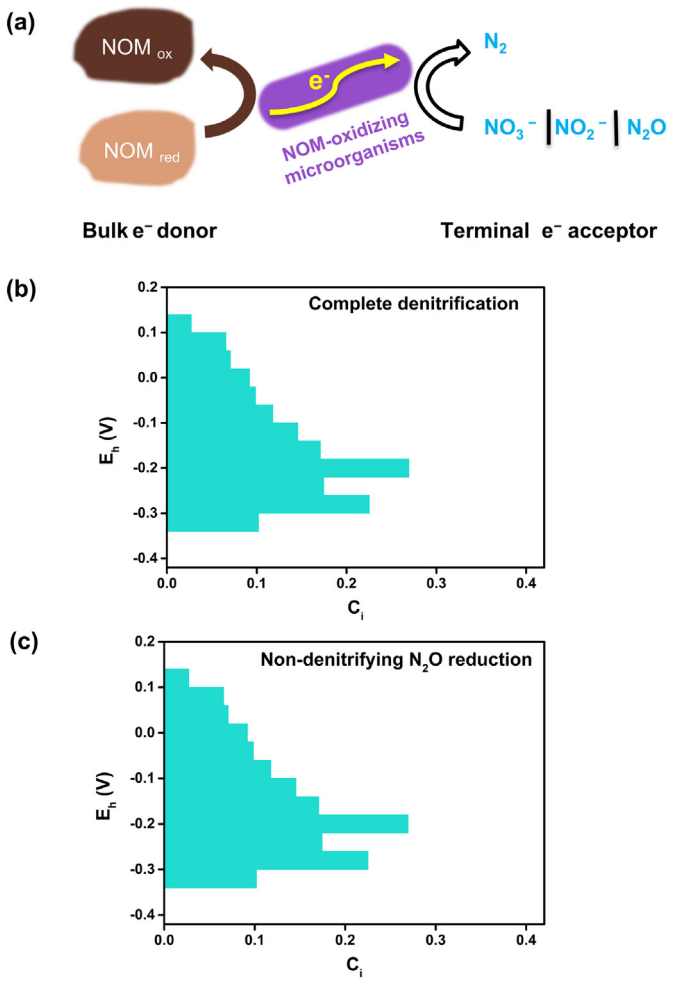

Fig. 4. (a) NOM as electron donor in anaerobic microbial denitrification and non-denitrifying N_2O reduction. (b, c) Scheme of typical E_h^0 (pH 7)-dependent distribution of reducing equivalents in humic acids. Data shown are the average of three different humic acids (i.e., Leonardite humic acid, Elliot Soil humic acid and Suwanee River humic acid) (Aeschbacher et al., 2011). Ci (mmol e^-/g HA) is the concentration of redox active moieties at the respective E_h^0 (pH 7) range. The green area indicates the fraction of reducing equivalents that thermodynamically is feasible to drive the process.

(Fig. 4b). The widespread heterotrophic denitrifying bacterium *Paracoccus denitrificans* is well known for its ability to oxidize hydroquinones within humic acids coupled to nitrate reduction (Lovley et al., 1999). Subsequently, complete denitrifiers oxidizing humic acids have been isolated from many environments such as marine and lacustrine sediments, forest soils, and drainage ditches (Coates et al., 2001; Coates et al., 2002). Nitrate-dependent humic acid-oxidizing bacteria have been isolated from agriculture soils (Van Trump et al., 2011). Apart from dissolved HS, the solid fraction of HS (i.e., humin) has recently been shown to be able to serve as electron donor in denitrification by the heterotrophic denitrifying bacterium *Pseudomonas stutzeri* (Xiao et al., 2016). The strategy of using reduced HS as a recyclable electron source may be significant for environments deficient in readily degradable organic carbon, where heterotrophic microbes could preferentially utilize the limited amount of biodegradable organic compound as a carbon source and the large fraction of refractory reduced organic carbon as an electron donor (Coates et al., 2002).

3.2.2. *Non-denitrifying N_2O reduction*

Non-denitrifying N_2O reduction describes the anaerobic reduction of N_2O to N_2 by non-denitrifying microorganisms, which cannot reduce NO_2^- to nitrogenous gases. This process is increasingly being recognized as crucial in alleviating global N_2O emissions (Hallin et al., 2017) as the diversity and abundance of non-denitrifying N_2O-reducers is much greater than previously assumed (Sanford et al., 2012). Reversible NOM oxidation coupled to non-denitrifying N_2O reduction is energetically favorable for all potential NOM redox states (Fig. 4c). Aranda-Tamaura et al. (2007) described the capability of an anaerobic denitrifying sludge to reduce denitrification intermediates (nitrite and N_2O) with the hydroquinone AH_2QDS (E_h^0 (pH 7) = −185 mV) as a sole electron donor, but it remains unclear whether non-denitrifying N_2O-reducers were present in the consortia and contributed to N_2O reduction. However, the finding of N_2O-dependent microbial AH_2QDS oxidation together with the high redox potential of the N_2O/ N_2 couple (E_h^0 (pH 7) = +1353 mV) (Table 1) imply that N_2O may be a highly competitive terminal electron acceptor in enzymatic NOM oxidation. This is further supported by a very recent study that reported the ability of microbial communities in wetland sediments to respire N_2O coupled to the oxidation of reduced humic substances (Valenzuela et al., 2020). Yet to date, NOM oxidation coupled to non-denitrifying N_2O reduction has not been studied and proven in pure cultures. The non-denitrifying N_2O-reducing bacteria *Wolinella succinogenes* (Yoshinari, 1980; Simon et al., 2004) might be a promising candidate, as it oxidizes AH_2QDS with nitrate as electron acceptor (supposedly via dissimilatory nitrate reduction to ammonium) (Lovley et al., 1999). It remains to be investigated whether *Wolinella succinogenes* can use reduced NOM as electron donor for N_2O respiration.

3.3. *Electron shuttling by NOM*

In addition to direct coupling to anoxic nitrogen cycling, reversible microbial NOM oxidation-reduction can be indirectly coupled to the cycling of redox sensitive metals such as Fe and Mn to regulate N loss. By serving as the electron shuttle between the microbial cell and a

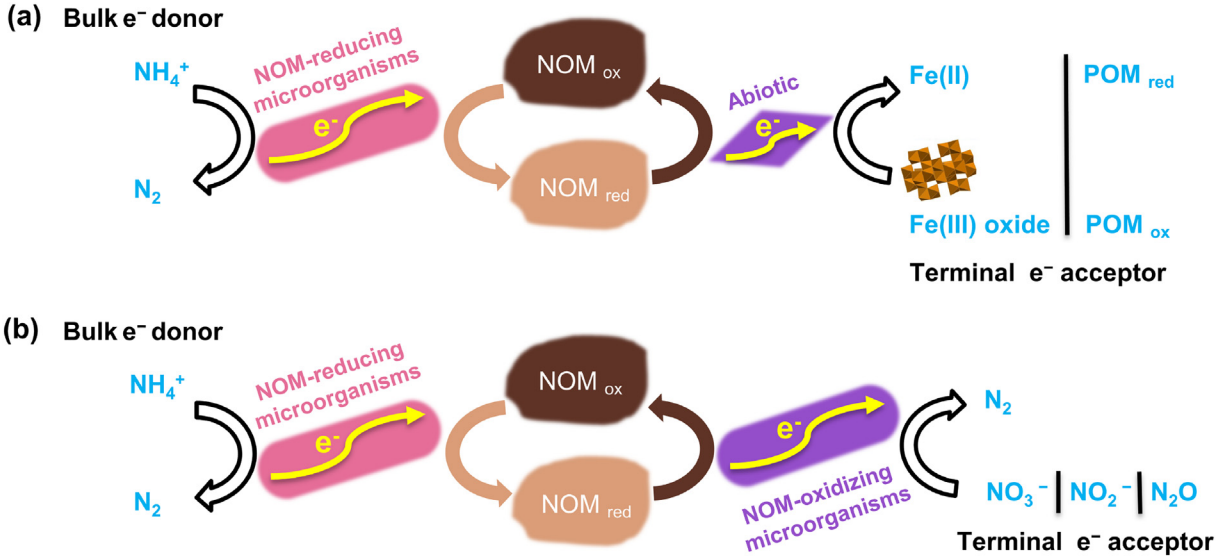

Fig. 5. NOM as electron transfer mediator between microbial cells and (a) extracellular terminal electron acceptor in anaerobic ammonium oxidation, (b) their syntrophic partners in anaerobic respiration (mediated interspecies electron transfer).

Table 1
Reduction potentials of selected biogeochemical redox couples of anoxic N cycling.

Redox couples	Half-reactions	E_h^0 (V)	E_h^0 (pH 7) (V)	E_h (pH 7) (V)
Anaerobic ammonium oxidation				
N_2 / NH_4^+	$N_2 + 8H^+ + 6e^- \rightarrow 2NH_4^+$	0.274	−0.277	−0.199
NO_2^- / NH_4^+	$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$	0.900	0.349	0.349
NO_3^- / NH_4^+	$NO_3^- + 10H^+ + 8e^- \rightarrow NH_4^+ + 3H_2O$	0.880	0.364	0.364
Complete denitrification				
NO_3^- / N_2	$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$	1.244	0.748	0.702
NO_2^- / N_2	$2NO_2^- + 8H^+ + 6e^- \rightarrow N_2 + 4H_2O$	1.527	0.976	0.899
Non-denitrifying N_2O reduction				
N_2O / N_2	$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	1.766	1.353	1.165
Quinone species	$Q^0 + 2H^+ + 2e^- \rightarrow H_2Q$	Eh^0	Eh^0–0.413	Eh^0–0.413
AQDS / AH_2QDS	$AQDS + 2H^+ + 2e^- \rightarrow AH_2QDS$	0.228	−0.185	−0.185

Standard reduction potentials, E_h^0, are calculated based on standard Gibbs energies of formation data from the Handbook of Chemistry and Physics (94th Edn). For quinone species, data is obtained from Clark (1960). Standard reduction potentials at pH 7, E_h^0 (pH 7), are calculated based on E_h^0, with activities of 1 for all species except the pH (H^+ concentration of 10^{-7} mol/L). Reduction potentials at pH 7, Eh (pH 7), are all calculated based on E_h^0, with activities under realistic environmental conditions 1) partial pressures for gases (Bernhardt and Schlesinger, 2013): N_2 0.78084 atm; CO_2 4×10^{-4} atm; CH_4 1.83×10^{-6} atm; N_2O 0.32×10^{-6} atm; H_2 0.51×10^{-6} atm. 2) soluble ions and molecules: H_2O 1; HCO_3^- 10^{-3} mol/L; concentrations of other soluble species are set to 10^{-4} mol/L.

distant extracellular acceptor, such as an iron(III) (oxyhydr)oxide mineral or POM, dissolved NOM with a midpoint reduction potential higher than that of the electron donor and lower than that of the terminal electron acceptor has the potential to enhance Feammox or POM-dependent anammox, and thereby simulating N loss.

3.3.1. *Anaerobic ammonium oxidation to N_2 via NOM electron shuttling*

As discussed above, NOM presumably exerts a dual role as bulk electron acceptor for NOM-dependent anammox and as essential electron transfer mediator for Feammox or even POM dependent anammox (Fig. 5a). The feasibility of NOM-shuttled Feammox to N_2 so far has only been supported by the shuttling ability of AQDS, a non-sorbing model quinone (Zhou et al., 2016). Solid NOM reduction (Roden et al., 2010) might be coupled to anammox (Rios-Del Toro et al., 2018b) with dissolved NOM shuttling electrons between NOM-respiring microbes and the distant electron acceptor POM (Gao et al., 2019).

Due to its refractory nature NOM can be cycled many times as electron carrier in a reversible way by transferring electrons between Fe (III)-/NOM-reducing microbial cells and Fe(III) (oxyhydr)oxides or POM. Moreover, the electron shuttling activities of NOM was shown to be largely dependent on its redox potential (Wolf et al., 2009). Notably, sorption of NOM to oxide minerals, however, is expected to alter the surface chemistry and the redox properties of both the NOM and the oxide minerals (Orsetti et al., 2013; Xiao et al., 2019; Subdiaga et al., 2019), and thus may impact the electron shuttling activities of NOM. In contrast to microbially produced and excreted electron shuttles which show discrete redox potentials, NOM covering a wide range of redox potentials is expected to be universally effective for mediating electron transfer to various Fe(III) and Mn(IV) minerals as well as POM with distinct reduction potentials.

3.4. *NOM-mediated interspecies electron transfer*

Interspecies electron transfer (IET) is a microbial syntrophic cooperation enabling different microbial species exchange reducing equivalents under anoxic conditions (Stams and Plugge, 2009; Lovley, 2012; Kouzuma et al., 2015). The diversity of metabolic strategies for microbial IET is manifold, and despite considerable recent progress, remains partially unexplored (Lovley, 2017; Tremblay et al., 2017). IET proceeds either by direct or by mediated mechanisms (Reguera et al., 2005; Stams and Plugge, 2009; Summers et al., 2010; Lovley, 2017). Humic substances have been investigated extensively for their role as

an electron shuttle in microbial extracellular electron transfer. However, much less studied, and yet important, is the potential role that humic substances play in microbial IET and the mechanisms involved in analogy to the soluble anthraquinone AQDS which enhances IET between *Geobacter metallireducens* and *Wolinella succinogenes* (Lovley et al., 1999) or genetically modified *Geobacter sulfurreducens* (Smith et al., 2015).

3.4.1. Anaerobic ammonium oxidation coupled to N_2O reduction mediated by NOM?

N_2O is one of the thermodynamically most powerful terminal oxidants in nature. The pathway of N_2O driven anammox to N_2 is thermodynamically feasible (Conthe et al., 2018) but in contrast to NO-dependent anammox to N_2 (Hu et al., 2019), so far it has not been experimentally demonstrated. A combination of the previously discussed NOM-dependent anammox to N_2 and NOM-coupled complete denitrification or non-denitrifying N_2O reduction processes, however, is thermodynamically feasible. We hypothesize that an anammox lineage capable of exchanging electrons with a complete denitrifier or a non-denitrifying N_2O-reducer via NOM-mediated IET may be found (Fig. 5b). Digging into such pathways may deepen our understanding on interconnections of microbial N cycling in anoxic environments.

4. Application: Eliminating interference of electro-active substances

Despite the potential of mediated electrochemical analysis for elucidating and quantifying microbial metabolic pathways involving reversible electron transfer to and from NOM in anoxic N cycling, this technique also has certain limitations. For example, the presence of other electro-active substances could interfere with the determination of the EAC and/or EDC of NOM. Nonetheless, in most cases this issue is probably of minor importance compared to the evident drawbacks of chemical and non-mediated electrochemical methods discussed in Section 2. Nevertheless, in the following sections we briefly discuss these limitations based on experimental data and propose possible strategies to overcome these limitations.

4.1. Reducing agents as media components

Ingredients of basic microbial culture media which might be electroactive in MER and/or MEO cells need to be considered. Cysteine or hydrogen sulphide, for example, are added to some culture media as reductants to scavenge oxygen and maintain reducing conditions. However, they can also cause a current response in MEO analysis (Sections S1 and S2, Fig. S1) (Wallace et al., 2017; Gao et al., 2019) and therefore, when using mediated electrochemical analysis to examine microbial NOM cycling, the addition of cysteine or hydrogen sulfide should be minimized or even avoided. Furthermore, such compounds can function as electron shuttles and may trigger interspecies electron transfer and electron shuttling as has been shown for the cysteine/cystine couple and for redox-active sulfur species (Nevin and Lovley, 2000; Kaden et al., 2002; Lohmayer et al., 2014).

4.2. Nitrite as reactive nitrogen cycling intermediate

The formation of electroactive intermediates during microbial NOM-N redox cycling may interfere with mediated electrochemical analysis (Li et al., 2020). In contrast to most inorganic N species such as ammonium, N_2, nitrate and N_2O (Lau et al., 2016; Gao et al., 2019; Kwon et al., 2019) nitrite is electroactive in MER and thus of potential concern (Section S1 and Fig. 6). Nitrite might transiently accumulate during denitrification, regulated by the activity of nitrate and nitrite reductases as well as influenced by environmental conditions. A transient accumulation of nitrite has been observed for example in the process of AH_2QDS-oxidation coupled to denitrification by

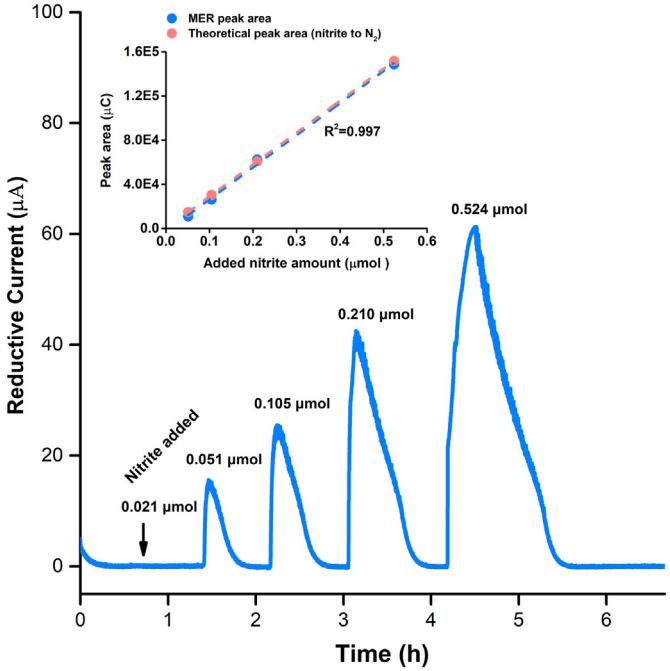

Fig. 6. Responses of reductive current to spikes of increasing amounts of nitrite (NO_2^-) analyzed by mediated electrochemical reduction (MER, Eh = −0.49 V, pH 7). Numbers above the current peaks indicate the injected amounts of nitrite, ranging from 0.021 to 0.524 μmol. Nitrite concentration was determined using flow injection analysis (FIA) as described on Section S3. Insert panel presents the linear increase in area of current peaks with increasing spiked amounts of nitrite, theoretical peak areas (assuming nitrite is reduced to N_2 at the electrode) are set as references.

Dechloromonas sp. strain JJ (Coates et al., 2002). When studying NOM oxidation coupled to denitrification, the presence of nitrite in the analyte may interfere with the assessment of NOM's redox properties using mediated electrochemical analysis. As shown in Fig. 6, the area of the reductive current peaks increased linearly with increasing spiked amounts of nitrite in accordance with the value expected for reduction of nitrite to N_2. To ensure the measured EAC is solely attributed to NOM, strategies to exclude the interference of nitrite must be developed. Recommended methods of nitrite removal depend on the type of NOM present. For samples containing NOM in particulate form (adsorbed to minerals or as particles), nitrite can be removed from the sample by anoxic centrifugation and discarding the nitrite-containing supernatant, followed by re-suspension of the solid NOM in fresh basic medium or buffer solution. For samples containing dissolved NOM, we suggest pretreatment steps to selectively remove nitrite from culture solution without affecting the redox properties of dissolved NOM.

To achieve this, we evaluated the suitability of sulfamic acid as a reagent to remove nitrite (Granger and Sigman, 2009; Klueglein and Kappler, 2013) and avoid its interference in the electrochemical determination of EAC of dissolved NOM samples (Sections S1 and S3, Fig. S2). Sulfamic acid itself was shown not to be electroactive in MER and had no impact on the redox state of the tested HA. The acidification step (to pH ≈ 1.8) that is required to initiate the reaction between sulfamic acid and nitrite as well as the subsequent neutralization step (to pH ≈ 7.0) did recover the redox state of HA samples without significant changes (Fig. S2). We, thus, suggest that in future studies this method could be an option for electrochemical analysis of dissolved NOM in the presence of nitrite. If other electroactive compounds such as thiosulfate, however, are present in DOM-nitrite samples, they might alter the redox state of dissolved NOM (Vairavamurthy et al., 1994; Yu et al., 2015) during the acidification step. In this case the sulfamic acid method should only be used with great caution.

Therefore, we recently developed a bio-pretreatment to remove nitrite from dissolved NOM samples under pH-neutral conditions (Li et al., 2020). The denitrifying strain *Pseudomonas nitroreducens* (formerly *Pseudomonas denitrificans*) that can neither oxidize nor reduce humic acids (Lovley et al., 1998, 1999; Li et al., 2020) completely removed nitrite from humic acid-containing samples and retained their original EAC values (Li et al., 2020). Other denitrifying strains which are unable to cycle NOM could be applied in future studies. Among nitrite-reducers, in addition to denitrifiers, the ammonifiers which perform dissimilatory nitrate reduction to ammonium (DNRA, NO_3^-/NO_2^- → NH_4^+), potentially can be used for nitrite removal of NOM-containing samples.

4.3. Cells of NOM-respiring microbes

When microbial cells are associated with POM, the possibility needs to be took into account that the present cells are electroactive in mediated electrochemical analysis. Controls using cell-free filtrates, whole cell suspensions, and washed cell suspensions are thus required. Experiments with cells of the denitrifying strains *Paracoccus denitrificans* (Lovley et al., 1999) and *Thiobacillus denitrificans* (Zheng et al., 2019) revealed that they are not electroactive (not shown). However, we observed that acetate-fumarate grown cells of *Geobacter sulfurreducens*, a well-studied humics-respiring bacterium that was reported to be able to exchange electrons with *Thiobacillus denitrificans* mediated by humic acids (Zheng et al., 2019), did produce signals under both MER and MRO modes (Sections S1 and S4, Fig. S3). This is probably due to the presence of periplasmatic and/or membrane-bound cytochromes (Esteve-Núñez et al., 2008; Malvankar et al., 2012; Estevez-Canales et al., 2015), as confirmed by analyzing a *G. sulfurreducens* variant with lower cytochrome *c*ontent grown on Fe-deficient medium (Estevez-Canales et al., 2015) which showed lower EAC/EDC values compared to cells grown on regular iron-containing medium (Fig. S3). By contrast, another frequently studied humics-respiring bacterium, *Shewanella oneidensis* MR-1, showed negligible or no EAC values (Klüpfel et al., 2014).

5. Outlook

By mimicking electron shuttle-based electron transfer processes occurring in nature, the application of mediated electrochemical analysis allows tracing the reversible electron flux to and from NOM during microbial and abiotic redox processes. Nevertheless, mediated electrochemical analysis is still an underemployed tool in exploring the poorly understood linkages between NOM redox cycling and N turnover driven by microorganisms in anoxic environments. We anticipate that future studies will increasingly employ the potential of mediated electrochemical analysis and the still existing (minor) limitations of this technique can be overcome. In summary, mediated electrochemical analysis is expected to be utilized as a crucial and essential tool in biogeochemistry to study redox couplings between NOM and N cycling, but also other NOM redox processes.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

The supporting information contains a general method description of mediated electrochemical analysis performed in this study, it shows oxidative current response of cysteine in MEO, it provides experimental methods and results of the sulfamic acid assay, as well as experimental methods and results of the *Geobacter sulfurreducens*' electro-activity experiments. Supplementary data to this article can be found online at https://doi.org/10.1016/j.earscirev.2020.103281.

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