# Application of Mediated Electrochemical Analysis to Study Humics Driven Anoxic Nitrogen Cycling

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*三月七日，沙湖道中遇雨。雨具先去，同行皆狼狈，余独不觉，已而遂晴，故作此词。*

莫听穿林打叶声，何妨吟啸且徐行。竹杖芒鞋轻胜马，谁怕？一蓑烟雨任平生。
料峭春风吹酒醒，微冷，山头斜照却相迎。回首向来萧瑟处，归去，也无风雨也无晴。

—— 苏轼 定风波·莫听穿林打叶声
宋代公元 1082 年于黄州（今湖北黄冈，我的家乡）

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# Abstract

Natural organic matter (NOM) is an important electron acceptor and electron donor in soils and aquifers. It comprises redox-active moieties with a wide range of standard reduction potentials. Tracking electron transfer flux from and to NOM in biogeochemical redox processes has been a challenge for decades. However, recent advances in mediated electrochemical analysis has greatly advanced the characterization of redox properties (i.e., electron donating capacity (EDC), electron accepting capacity (EAC), and redox state) of NOM. Nonetheless, this technique has rarely been applied to investigate microbial pathways of NOM redox cycling. The coupling of anoxic nitrogen (N) cycling to NOM redox turnover, however, remains poorly understood in terms of electron transfer mechanisms and environmental niches. The primary goal of my work was to promote and develop the use of mediated electrochemical analysis on studying microbial N cycling driven by humic substances (HS, the largest fraction of NOM) in anoxic environments.

First, in a combination of review and experimental study, I reviewed recent applications of mediated electrochemical analysis in studying microbial NOM redox cycling and highlighted its advantages over the traditional chemical methods and no-mediated electrochemical analysis. Next, I exemplified the prospects of mediated electrochemical techniques for biogeochemical research with linking microbial NOM redox cycling to gaseous C and N emissions (e.g., mitigation of greenhouse gas $CH_4$ and $N_2O$, and N loss) by evaluating the thermodynamics of several enigmatic or yet unraveled pathways. Finally, I presented strategies to overcome problems arising from electroactive intermediates that hamper the application of mediated electrochemical analysis in microbial experiments. For instance, my work revealed that the presence of the N-cycling intermediate nitrite ($NO_2^-$) interferes with the electrochemical determination of the EAC. A sulfamic acid assay was, therefore, established to resolve this obstacle. In addition, my work showed the electro-activity of *Geobacter sulfurreducens* cells in mediated electrochemical analysis as a function of varying cytochrome content. Whereas protocols to eliminate the interference from *Geobacter sulfurreducens* cells in mediated electrochemical analysis of solid NOM samples remain to be developed.

Second, I developed a bioassay to remove nitrite in HS sample using the denitrifying bacterium *Pseudomonas nitroreducens* for an unbiased quantification of the EAC of HS. Cell suspensions of *P. nitroreducens* completely removed $NO_2^-$ at various concentrations (1, 2 and 5 mM) from both humic acid samples (1g HA/L) of different

redox states. As *P. nitroreducens* is not able to exchange electrons with dissolve humic acids, the procedure allows an accurate and precise determination of the EAC of humic acid samples. Further, results of the cell suspension experiments confirmed unequivocally that *Pseudomonas nitroreducens* cannot alter the EDC and EAC of HA samples via redox cycling of HA. The denitrifier assay was proven as robust method at circumneutral pH, thereby avoiding pH-dependent side reactions.

Third, among the NOM-dependent microbial pathways for $CH_4$ and $N_2O$ mitigation and N loss which are outlined in the perspective, my work focused on studying microbial HS oxidation coupled to $N_2O$ reduction (non-denitrifying $N_2O$ reduction and complete denitrification). Mechanistic studies on this process are rare especially for non-denitrifying $N_2O$ reduction. In my study the ability of pure cultures of three $N_2O$ reducing bacteria to couple $N_2O$ reduction with the oxidation of electrochemically reduced hydroquinone $AH_2QDS$ (anthrahydroquinone-2,6-disulfonate, a HS analog) was evaluated. The strains were the putative non-denitrifying $N_2O$ reducer *Desulfitobacterium hafniense* Y51 and the complete denitrifying strains *Paracoccus denitrificans* and *Thiobacillus denitrificans*. Results obtained from cell suspension experiments showed that none of the strains were able to gain electrons from the extracellular hydroquinone $AH_2QDS$ for $N_2O$ respiration. However, the strain *P. denitrificans* oxidized $AH_2QDS$ for denitrification with $N_2O$ as end product, i.e., without further reduction of $N_2O$ to $N_2$. It appeared that neither added (exogenic) $N_2O$ nor biogenic (endogenic) $N_2O$ was usable for hydroquinone oxidation. However, future studies could optimize the incubation strategies to further clarify the process of microbially mediated HS oxidation coupling to $N_2O$ reduction. Also, instead of quinone model compounds, the more complex HS samples can be used as potential electron donors in these studies with mediated electrochemical analysis being employed to quantify the changes in redox properties of HS samples.

Conceptually and methodologically, this study broadens the use of mediated electrochemical analysis on studying humics driven nitrogen cycling (e.g., N-oxides reduction) in anoxic enviroments.

# Zusammenfassung

Natürliches organisches Material (NOM) fungiert als Elektronenakzeptor und Elektronendonor in Böden und Grundwasserleitern. Es besitzt redox-aktive Komponenten, die einen großen Bereich an Standard-Redoxpotentialen überspannen. Den Elektronentransfer von und zu NOM in biogeochemischen Redoxprozessen nachzuweisen ist jedoch eine analytische Herausforderung. Neuere Entwicklungen in der elektrochemischen Analyse haben die Quantifizierung der Redoxeigenschaften (d.h. Elektronen Abgabe Kapazität (EDC), Elektronen Aufnahme Kapazität (EAC)) von NOM jedoch erheblich verbessert. Gleichwohl wird diese Technik bei Untersuchungen von mikrobiellen Prozessen zum NOM Redoxkreislauf nur selten angewendet. Der Zusammenhang zwischen anoxischem Stickstoffkreislauf (N) und NOM Redoxtransformationen ist bislang nur ungenügend untersucht in Bezug auf Elektronentransfermechanismen und ökologische Nischen. Das vorrangige Ziel meiner Arbeit war die elektrochemische Analytik weiter zu optimieren um den durch Huminstoffe (HS, größte Fraktion von NOM) angetriebenen mikrobiellen N Kreislauf in anoxischen Systemen zu untersuchen.

Im ersten Kapitel der Arbeit habe ich den Stand der Forschung bezüglich der Anwendung der elektrochemischen Analyse für die Untersuchung von des mikrobiellen NOM Kreislaufs zusammengetragen. Hierbei wurde der Vorteil der mediierten elektrochemischen Analyse gegenüber traditionellen chemischen Methoden und direkter elektrochemischen Analyse herausgearbeitet. Weiterhin habe ich das Potenzial der mediierten elektrochemischen Analyse für biogeochemische Untersuchungen veranschaulicht. Hierfür wurden mehrere bislang noch nicht beschriebene Zusammenhänge zwischen mikrobellem NOM Redoxkreislauf und C und N Emissionen evaluiert. Abschließend stellte ich Strategien vor um die Problematik, die durch redoxaktive Intermediate entstehen und folglich die Anwendung der vermittelnden elektrochemischen Analyse in mikrobiellen Systemen beeinträchtigen zu überwinden. Meine Arbeit zeigte, dass die Anwesenheit von Nitrit ($NO_2^-$) , ein im N-Kreislauf wichtiges Zwischenprodukt, mit der elektrochemischen Bestimmung der Elektronen Akzeptor Kapazität interferiert. Zur Lösung wurde ein Protokoll mittels Amidosulfonsäure wurde etabliert. Zudemkpnnte nachweisen, dass Zellen von Geobacter sulfurreducens in der mediierten elektrochemischen Analyse elektroaktivität zeigen in Abhängigkeit ihrers Zytochrom Gehalts. Ein Protokoll um die durch *Geobacter sulfurreducens* Zellen hervorgerufen Interferenzen zu eliminieren muss jedoch noch entwickelt werden.

Im zweiten Teil der Dissertation entwickelte ich einen Bioassay für die Eliminierung

von Nitrit aus Huminstoff-Proben mittels des denitrifizierenden Bakteriums Pseudomonas nitroreducens. Mittels Zellsuspensionen von P. nitroreducens konnte $NO_2^-$ (Konzentration von 1, 2, 5 mM) vollständig aus HS Proben (1g HA/L) mit unterschiedlichen Redoxstatus entfernt werden. Da P. nitroreducens nicht in der Lage ist, Elektronen mit gelösten HS auszutauschen, erlaubt das Protokoll eine akkurate und präzise Bestimmung der EAC in Huminsäure Proben auch in Gegenwart von Nitrit. Weitere Ergebnisse des Zellsuspensionsexperiments bestätigten, dass P. nitroreducens weder EDC noch EAC von Huminsäuren verändert. Der Denitrifikationsassay stellt eine robuste Methode dar, die bei circumneutralem pH durchgeführt wird. Daher können pH-induzierte Nebenreaktionen von Nitrit mit Huminstoffen vermieden werden, welche im sauren Milieu auftreten könnten.

Im dritten Teil meiner Arbeit fokussierte ich mich auf die Untersuchung der mikrobiellen HS Oxidation gekoppelt mit der $N_2O$ Reduktion (nicht-denitrifizierende $N_2O$ Reduktion und vollständige Denitrifikation). Mechanistische Untersuchungen der nicht-denitrifizierenden $N_2O$ Reduktion sind rar. In meiner Studie habe ich mit drei $N_2O$ reduzierenden Bakterienkulturen untersucht ob , die $N_2O$ Reduktion mit der Oxidation eines elektrochemisch reduzieren Hydroquinons ($AH_2QDS$; Anthrahydroquinone-2,6-disulfonat) gekoppelt werden kann. $AH_2QDS$ ist eine gut wasserlösliche Modellsubstanz für Huminstoffe. Bei den untersuchten Stämmen handelt es sich um den vermeintlich nicht-denitrifizierende $N_2O$ Reduzierer *Desulfitobacterium hafniense* Y51 und die vollständig denitrifizierenden Stämme *Paracoccus denitrificans* und *Thiobacillus denitrificans*. Die Ergebnisse der Zellsuspensionsexperimente zeigten, dass keiner der Stämme Elektronen für die $N_2O$ Veratmung aus dem extrazellulären Hydroquinon $AH_2QDS$ gewinnen konnte. Der Stamm *P. denitrificans* oxidierte jedoch $AH_2QDS$ im Zuge der Denitrifikation zu $N_2O$. Eine weitere Reduktion von $N_2O$ zu $N_2$ blieb jedoch aus. Es zeigte sich das weder zugebenes $N_2O$ noch biogenes $N_2O$ für die Oxidation des Hydroquinons verwendet wurde. In Folgestudien sollten die Inkubationsbedingungen weiter optimiert werden um weitere Einblicke in die Zusammenhänge zwischen mikrobiellen HS Oxidation und $N_2O$ Reduktion zu erlangen. Neben Model Substanzen sollten komplexere HS Proben als potenzielle Elektonendonoren3 untersucht werden. Zur Quantifizierung der Redoxeigenschaften der HS- Proben steht die vermittelnde elektrochemische Analyse zur Verfügung.

Diese Arbeit erweitert den Anwendungsbereich der mediierten elektrochemischen Analyse für Untersuchungen zum Huminstoff-beeinflussten anoxischen Stickstoff Kreislaufs (z.B. N-Oxid Reduktion) in anoxischen Systemen.

# Chapter 1

## General Introduction

## 1.1 Background

Natural organic matter (NOM) predominates the organic carbon stock in terrestrial and aquatic environments (Tratnyek et al., 2011; Schaeffer et al., 2015), and is often ascribed to its largest fraction, humic substances (HS), which originate from decaying biomass (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 $CO_2$ (Kuzyakov, 2010; Lehmann and Kleber, 2015), 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., $CH_4$, $N_2O$) emissions and nitrogen loss by coupling C and N cycles (Aranda-Tamaura et al., 2007; Martinez et al., 2013; Valenzuela et al., 2017). Nevertheless, significant knowledge gaps regarding the mechanisms and environmental niches of such processes exist, primarily due to the lack of appropriate methods for determining the redox properties (i.e., electron accepting capacity (EAC), electron donating capacity (EDC), and redox state) of NOM.

During the last decade, however, Sander and coworkers (Aeschbacher et al., 2010) have evaluated and refined a powerful mediated electrochemical analysis to quantify accurately the EAC, EDC, and thus the total electron exchange capacity (EEC) of NOM. This technique is based on chronocoulometry and employs soluble redox mediators to promote the rate of electron transfer and to facilitate redox equilibria between working electrodes and NOM analyte in the electrochemical cells (Aeschbacher et al., 2010; Sander et al., 2015). This mediated electrochemical approach can overcome major drawbacks of traditional techniques. Today, mediated electrochemical analysis is applied to investigate the redox properties and 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), and thus opens new venues for investigating so far unexplored biogeochemical processes involving NOM.

## 1.2 Objectives

In general, this PhD thesis aims to evaluate and appraise the use of mediated electrochemical analysis on studying the poorly understood or unraveled avenues of

microbial NOM reduction-oxidation linking to gaseous C and N turnover. I stress on developing the use of this approach on studying humics-driven microbial N cycling from the viewpoints of both the concept and methodology.

The specific objectives of this PhD thesis are:

i) To evaluate the suitability of mediated electrochemical analysis to address microbial pathways of NOM redox cycling in the future.

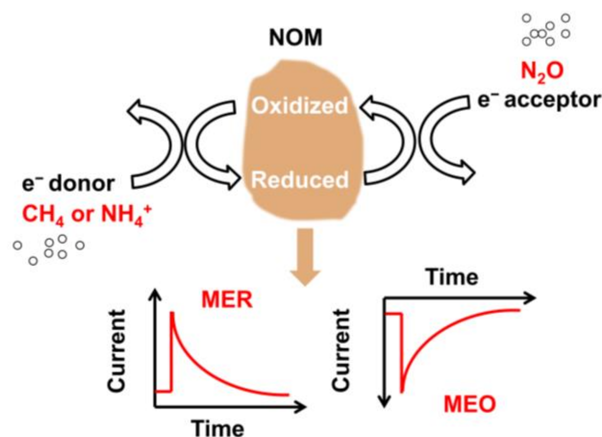
ii) To develop a bioassay for nitrite removal in mediated electrochemical analysis of dissolved humic substances.

ii) To evaluate the ability of a putative non-denitrifying $N_2O$ reducer and two well-defined complete denitrifiers to oxidize hydroquinones with exogenic $N_2O$ reduction.

## 1.3 Thesis Organization

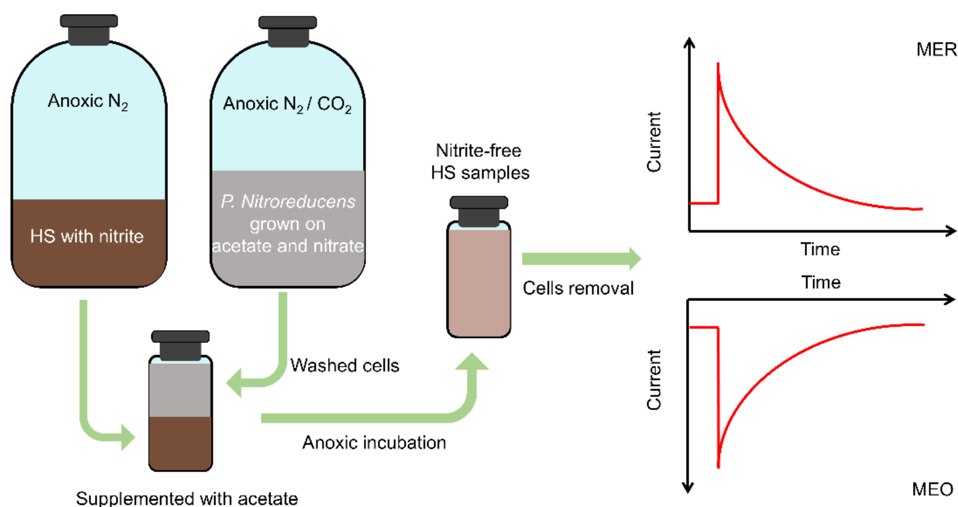
**Chapter 1** is a brief introduction including objectives and structure of this PhD work.

**Chapter 2** provides a critical review and experimental studies on the suitability of mediated electrochemical analysis to address microbial pathways of NOM redox cycling. First, I summarize present-day applications of chemical methods in studying microbial NOM cycling. Second, I review existing, yet limited, literature wherein mediated electrochemical analysis was employed in such topics and stress the advantages of this approach compared to chemical methods. Third, I highlight diverse conceptual or poorly understood mechanisms of microbial NOM redox cycling, with emphasis on processes governing greenhouse gas mitigation and nitrogen loss which can be elucidated using mediated electrochemical approaches. Fourth, I discuss experimental strategies to overcome interferences of electro-active substances typically present in microbial assay or field samples when determining EAC and/or EDC values of NOM.



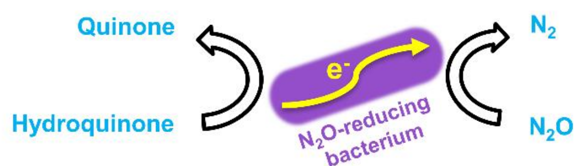

**Scheme 1.1.** Illustration of research topics of the perspective

**Chapter 3** presents a denitrifier method for nitrite removal in mediated electrochemical analysis of the electron accepting capacity dissolved humic substances. The denitrifying (reduce nitrite to nitrogenous gas) bacterium *Pseudomonas nitroreducens* (formerly *denitrificans*), which can neither reduce the humic substance analog anthraquinone-2,6-disulfonate (AQDS) nor oxidize its reduced form anthrahydroquinone-2,6-disulfonate ($AH_2QDS$), was chosen as microbial catalyst for nitrite removal. To validate this bioassay, I treated HA samples containing $NO_2^-$ with a *P. nitroreducens* cell suspension at neutral pH and subsequently quantified the EDC and EAC of HA samples with MEO and MER, respectively.




**Scheme 1.2.** Experimental procedures of the denitrifier assay

**Chapter 4** provides a preliminary evaluation of ability of the potential non-denitrifying $N_2O$ reducer *Desulfitobacterium hafniense* Y51 to oxidize reduced AQDS with $N_2O$ reduction. Two well-defined complete denitrifying strains (*Paracoccus denitrificans* and *Thiobacillus denitrificans*) were also examined. To do this, the HS analog AQDS (anthraquinone-2,6-disulfonate) was electrochemical reduced to $AH_2QDS$ and subsequently used as potential electron donor for a suit of cell suspension experiments. $N_2O$ was supplied as a potential electron acceptor.




**Scheme 1.3.** Hydroquinone as electron donor for microbial $N_2O$ reduction

# Chapter 2

## Microbial redox cycling of natural organic matter linked to greenhouse gases mitigation and nitrogen loss: application of mediated electrochemical analysis

**Abstract**

Natural organic matter (NOM) is an important redox active component in soils and aquifers that comprises numerous moieties spanning a wide range of reduction potentials. Thus, tracking electron transfer from and to NOM in biogeochemical redox processes has been a challenge for decades. However, recent advances in mediated electrochemical analysis have greatly improved the characterization of redox properties of NOM. Nonetheless, this technique has rarely been applied to investigate microbial pathways of NOM redox cycling. Here we aim to provide a guideline for employing mediated electrochemical analysis to address such topics in the future. To this end, we review recent applications of mediated electrochemical analysis in studying microbial NOM cycling and evaluate its prospects. We exemplify the potential of mediated electrochemical techniques for biogeochemical research by addressing how microbial NOM redox cycling is linked to gaseous C and N emissions (e.g., mitigation of greenhouse gas $CH_4$ and $N_2O$, and N loss). Finally, we present strategies to work around problems arising from electroactive intermediates that hamper the application of mediated electrochemical analysis in microbial experiments. Thus, mediated electrochemical analysis is expected to become an important tool in expanding our understanding of microbial sinks of greenhouse gases.

## 2.1 Introduction

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

Natural organic matter (NOM) predominates the organic carbon stock in terrestrial and aquatic environments (Tratnyek et al., 2011; Schaeffer et al., 2015), and is often ascribed to its largest fraction, humic substances (HS), which originate from decaying biomass (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 $CO_2$ (Kuzyakov, 2010; Lehmann and Kleber, 2015), 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., $CH_4$, $N_2O$) emissions and nitrogen loss by coupling C and N cycles (Aranda-Tamaura et al., 2007; Martinez et al., 2013; Valenzuela et al., 2017). Nevertheless, significant knowledge gaps regarding the mechanisms and environmental 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 is 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 straightforward according to changes in valence states and contents of electron donor-acceptor pairs, this approach fails for NOM, which is a mixture of thousands of organic macromolecules that differ not only in chemical composition but also in the number of redox active groups and their redox state. To quantify NOM's ability to accept and donate electrons, the concept of electron accepting (EAC) and electron donating capacity (EDC) is used. In laboratory incubation studies, 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, experimental determination of the EAC and EDC of NOM is a major analytical challenge.

Generally, the EAC and/or EDC of NOM can be assessed by two different

experimental approaches: indirectly by reacting with chemical reductants and/or oxidants, and directly by measuring the electrons exchanged between NOM and an electrode as current using traditional electrochemical techniques. All these methods have multiple limitations, as detailed below. During the last decade, however, Sander and coworkers (Aeschbacher et al., 2010) have evaluated and refined a powerful mediated electrochemical analysis to quantify accurately the EAC, EDC, and thus the total electron exchange capacity (EEC) of NOM. This technique is based on chronocoulometry and employs soluble redox mediators to promote the rate of electron transfer and to facilitate redox equilibria between working electrodes and NOM analyte in the electrochemical cells (Aeschbacher et al., 2010; Sander et al., 2015). This mediated electrochemical approach can overcome major drawbacks of traditional techniques. Today, mediated electrochemical analysis is applied to investigate the redox properties and 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), and thus opens new venues for investigating so far unexplored biogeochemical processes involving NOM.

In this article, we specifically highlight the prospects of mediated electrochemical analysis in characterizing microbial NOM redox cycling. First, we summarize present-day applications of chemical methods in studying microbial NOM cycling. Second, we review existing yet limited literature wherein mediated electrochemical analysis was employed in such topics and stress the advantages of this approach compared to chemical methods. Third, we highlight diverse conceptual or poorly understood mechanisms of microbial NOM redox cycling, with emphasis on processes governing greenhouse gas mitigation and nitrogen loss which can be elucidated using mediated electrochemical approaches. Last, we discuss experimental strategies to overcome interferences of electro-active substances typically present in microbial assay or field samples when determining EAC and/or EDC values of NOM.

## 2.2 Experimental approaches to determine the redox properties of NOM

### 2.2.1 Chemical methods

Chemical methods rely on redox titrations using added reductants and/or oxidants to quantify the redox states of NOM samples (Figure 2.1). Initial investigations emphasized on adding oxidants (e.g., $FeC_6H_5O_7$, $K_3Fe[CN]_6$ and $I_2$) to measure the potential EDC (i.e., total electron exchange capacity, EEC) and *actual* EDC (native state) 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 was either chemically, microbially, or electrochemically reduced to its maximum extent (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) followed by the addition of an oxidant to achieve chemical oxidation of the reduced NOM (Figure 2.1). The total number of electrons accepted by the oxidant with given valence state multiplied by the respective concentration change of the oxidant was considered as the potential EDC. To assess the *actual* EDC, the NOM analyte without previous reduction step was directly treated with an oxidant. Furthermore, the positive difference between potential EDC and *actual* EDC represents the *actual* EAC. Alternatively, the *actual* EAC can be measured by reacting the native NOM sample with a reductant (e.g., $H_2S$, $Zn^0$) and the number of transferred electrons was then set equal to *in situ* EAC (Heitmann and Blodau, 2006; Blodau et al., 2009; Bauer et al., 2007; Heitmann et al., 2007).

These chemical approaches (Figure 2.1) have been commonly used for characterizing the redox properties of NOM in earlier studies. With regard to microbial NOM redox cycling, the widely investigated aspects were the microbially catalyzed NOM reduction and its biogeochemical significance in dual roles: NOM on one hand is known to function as a terminal electron acceptor in anoxic microbial respiration and on the other hand can serve as an essential electron transfer mediator (electron shuttle) between Fe(III)-reducing microorganisms and Fe(III) 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). Specifically, the role of dissolved NOM plays in electron shutting between microbes and metal oxides were traditionally assessed by comparing the rates and extent of Fe(III) reduction in the presence and absence of NOM, and further verified by abiotically reacting the cell-free filtrates from the microbial NOM-reduction step with the terminal acceptor Fe(III) minerals (Lovley et al., 1996; Lovley et al., 1998). Indeed, the electron shutting ability of NOM primarily depends on microbial NOM reduction. As such, previous work addressing the dual roles of NOM mainly focused on the process of microbial NOM reduction, and chemical methods have been applied to monitor EDC of NOM usually only at the beginning and end of an incubation. By and large, the naturally occurring NOM-shuttled microbial Fe(III) reduction proceeds in an analogous manner to artificially assessing the potential EDC of microbially pre-reduced NOM.

Such chemical treatments are experimentally complicated, time consuming (Matthiessen, 1995; Bauer et al., 2007), pH dependent and not versatile regarding the redox potential analyzed (Aeschbacher et al., 2010). They may also induce side reactions and artifacts that limit accuracy and comparability of EDC (Peretyazhko and

Sposito, 2006) or EAC measurements (Heitmann and Blodau, 2006; Blodau et al., 2009). Taking the frequently used two Fe(III) oxidants (i.e., $FeC_6H_5O_7$ and $K_3Fe[CN]_6$) as an example, reaction times often were not sufficient to achieve the true EDC (Aeschbacher et al., 2010; Rakshit and Sarkar, 2017) or the reaction was insensitive (Rakshit and Sarkar, 2017) or oxidized organic moieties in NOM that are not involved in electron shuttling (Peretyazhko and Sposito, 2006). Thus, EDCs determined with $FeC_6H_5O_7$ differed by one order of magnitude from those obtained with $K_3Fe[CN]_6$ (Peretyazhko and Sposito, 2006; Tratnyek et al., 2011).

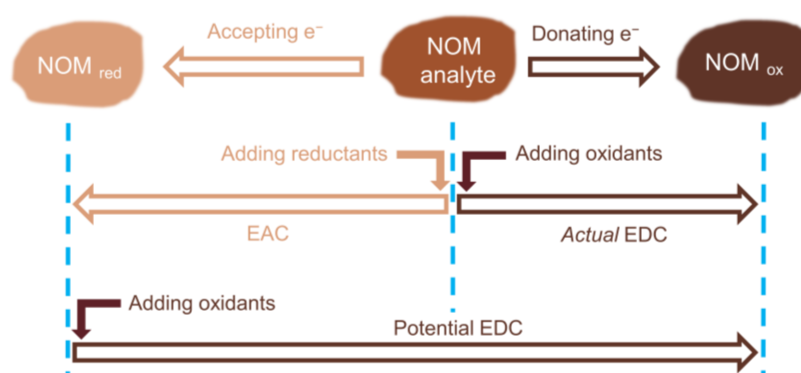



**Figure 2.1.** Schematic illustration of chemical methods to determine the electron donating- (EDC) and electron accepting capacity (EAC) of NOM. The EAC and EDC of NOM were traditionally assed by reacting with chemical reductants and oxidants, respectively.

### 2.2.2 Electrochemical analysis

The electrochemical approaches replaced 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 (Figure 2.2) described by Sander and co-workers (Aeschbacher et al., 2010) allows accurate and fast determination of the EDC and EAC of dissolved electroactive NOM such as humic substances (HS). The use 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 aerobic conditions (Aeschbacher et al., 2012), the abiotic oxidation of dissolved NOM by oxygen (Page et al., 2012; Page et al., 2013), by chlorine dioxide, chlorine, and ozone (Wenk et al., 2013), as well as the photo-oxidation of dissolved NOM (Sharpless et al., 2014). In addition to dissolved NOM, mediated electrochemical analysis has also been validated and applied in assessing redox properties and reactivity of particulate NOM in sediments (Lau et al., 2015; Lau et al., 2016). These are examples of recent advances in examining geochemical redox processes of NOM.

Microbially catalyzed NOM cycling has been demonstrated using mediated electrochemical analysis both in laboratory cultures (Klüpfel et al., 2014) and in complex environments (Lau et al., 2015; Lau et al., 2017). Klupfel et al. (2014) examined the reduction of dissolved HS by the strain *Shewanella oneidensis* MR-1 followed by abiotic re-oxidation of dissolved HS by exposure to oxygen. They quantified changes in EDC and EAC of dissolved HS during repeated anoxic-oxic cycles by the mediated electroanalytical approaches. Lau et al. (2015) successfully utilized this technique to quantify electron transfer to particulate NOM and the contribution of particulate NOM to the overall terminal electron acceptor pool in freshwater sediments during anoxic microbial respiration. The impact of dissolved NOM of different redox states on microbial abundance, activity and community structure in lake waters was also investigated (Lau et al., 2017). These limited but pioneering studies proved the applicability and efficacy of mediated electrochemical analysis to directly track electron fluxes of microbial NOM reduction-oxidation in laboratory incubations.

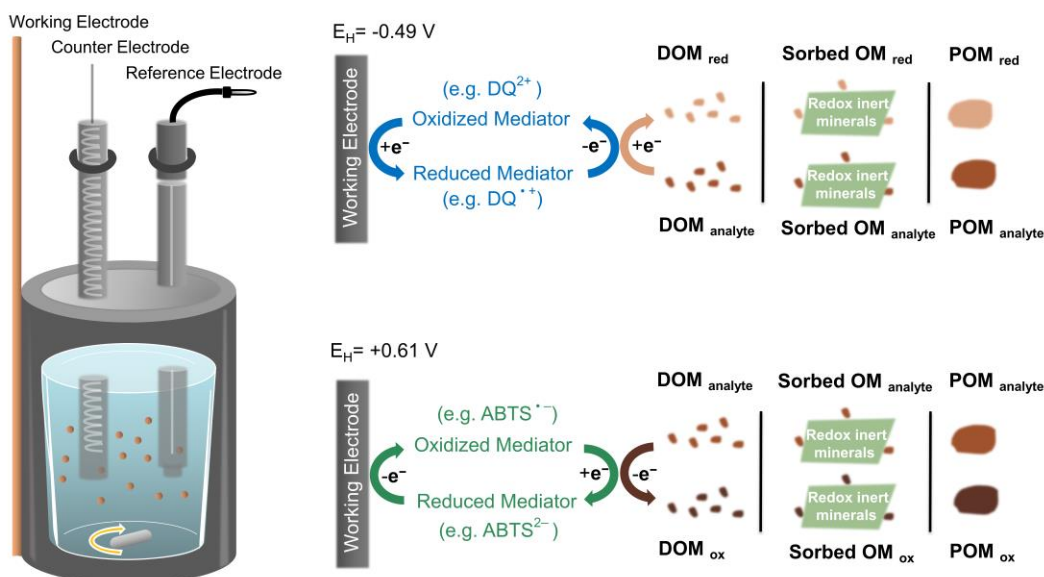



**Figure 2.2.** Set-up and principle of mediated electrochemical (i.e., amperometric) analysis. The electrochemical cell consists of a glassy carbon cylinder as the working electrode and the cell reaction vessel, a Pt wire counter electrode, and an Ag/AgCl reference electrode. The glassy carbon cylinder is often filled with neutral pH-buffered solution containing 0.1 M KCl as the electrolyte and 0.1 M phosphate as the buffering substrate. 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 attainment of redox equilibria, NOM analytes are added and oxidized the reduced mediator, thereby generating reduced NOM and oxidized mediator. The newly formed oxidized mediator is re-reduced at the working electrode, resulting in a reductive and peak-shaped current response until the electrochemical equilibria was re-attained. The number of electrons transferred to the NOM analyte is obtained by integrating the baseline-corrected response current over time and was further mass-normalized to EAC. 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 electron transferring flow wheels in the reversible direction compared to MER. The resultant oxidative and peak-shaped current response is integrated to obtain the number of electrons transferred from the NOM analyte and is mass-normalized to EDC. In general, the speciation of NOM analyte can be dissolved, the form adsorbed at redox inert minerals, particulate.

## 2.3 NOM-dependent microbial pathways for $CH_4$ and $N_2O$ mitigation and N loss

$CH_4$ and $N_2O$ are both highly potent greenhouse gases and $N_2O$ is also a major cause of stratospheric ozone destruction (Ravishankara et al., 2009; IPCC, 2013). Under anoxic conditions, anaerobic microbial $CH_4$ oxidation and microbial $N_2O$ reduction can be sinks of these greenhouse gases. Known anoxic microbial pathways for reactive nitrogen loss include complete denitrification, non-denitrifying $N_2O$ reduction, anaerobic ammonium oxidation, and nitrite-dependent anaerobic methane oxidation with $N_2$ and intra-aerobic $O_2$ production (Ettwig et al., 2010; Stein and Klotz, 2016; Hallin et al., 2017). Given the environmental significances in regulating Earth's climate and balancing global N budget, expanding our understanding of microbial processes eliminating $CH_4$ and $N_2O$ as well as modulating N loss is of great importance. In the following sections, we will discuss how NOM redox cycling contributes to the mitigation of $CH_4$ and $N_2O$ as wells as the loss of reactive N, thus, opening a wider filed of applying electrochemical methods in environmental microbiology.

In anoxic environments, microbially mediated NOM oxidation-reduction can be tightly linked to $CH_4$ and $N_2O$ mitigation and N loss through diverse electron transfer mechanisms. First, NOM can serve as terminal electron acceptor for the oxidation of $CH_4$ to $CO_2$ (Scheller et al., 2016; Valenzuela et al., 2017) and of $NH_4^+$ to $N_2$ (termed NOMammox in this article) (Figure 2.3a). Second, NOM may function as electron donor to reduce nitrogen species including $NO_3^-$, $NO_2^-$ or $N_2O$ to $N_2$ (Lovley et al., 1999; Aranda-Tamaura et al., 2007) (Figure 2.4a). Third, NOM can act as electron shuttle between Fe(III)-reducing microorganisms and Fe(III) oxides, for instance, in the process of Feammox (anaerobic $NH_4^+$ oxidation to $N_2$ coupled to iron reduction) (Zhou et al., 2016), and possibly also in the process of iron dependent anaerobic $CH_4$ oxidation (Figure 2.5a). Thus, microbial redox reactions involving NOM as electron shuttle could regulate either $N_2$ production or $CH_4$ emission by ferrying electrons during microbial iron reduction. Finally, NOM may also play a role 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, we propose that NOM might facilitate MIET between consortia of anaerobic methanotrophic microbes or NOMammox microbes and non-denitrifying $N_2O$ reducers, and therefore constraining $CH_4$ and $N_2O$ emissions to the atmosphere (Figure 2.5b).

**Table 2.1.** Reduction potentials of selected biogeochemical redox couples

| Redox couples | Half-reactions | $Eh^0$ (V) | $Eh^0$ (pH 7) (V) | Eh (pH 7) (V) |
|---|---|---|---|---|
| *Anaerobic $CH_4$ oxidation* | | | | |
| $HCO_3^- / CH_4$ | $HCO_3^- + 9H^+ + 8e^- = CH_4 + 3H_2O$ | 0.227 | -0.238 | -0.218 |
| *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, $Eh^0$, are calculated based on standard Gibbs energies of formation data from Handbook of Chemistry and physics 94th Eds. For quinone species, data is obtained from Clark (1960). Standard reduction potentials at pH 7, $Eh^0$ (pH 7), are all calculated based on $Eh^0$, with activities of 1 for all species except the pH ($10^{-7}$ mol/L). Reduction potentials at pH 7, Eh (pH 7), are all calculated based on $Eh^0$, with activities under realistic environmental conditions. For activities, 1) partial pressures for gases (Bernhardt and Schlesinger, 2013): $N_2$ 0.78084 atm; $CO_2$ $4\times10^{-4}$ atm; $CH_4$ $1.83\times10^{-6}$ atm; $N_2O$ $0.32\times10^{-6}$ atm; $H_2$ $0.51\times10^{-6}$ 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.

### 2.3.1 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 with a range, spanning from +0.15 V to -0.3 V at pH 7 (Aeschbacher et al., 2011). Further, the redox state 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 are well recognized as major reducible moieties in NOM and a representative distribution of standard reduction potentials of such redox active moieties in NOM at pH 7 is shown in Figures 2.3b and 2.4b. For comparison, reduction potentials of canonical biogeochemical redox couples are shown in Table 2.1 and Figures 2.3b and 2.4b 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 methane and ammonium oxidation, respectively.

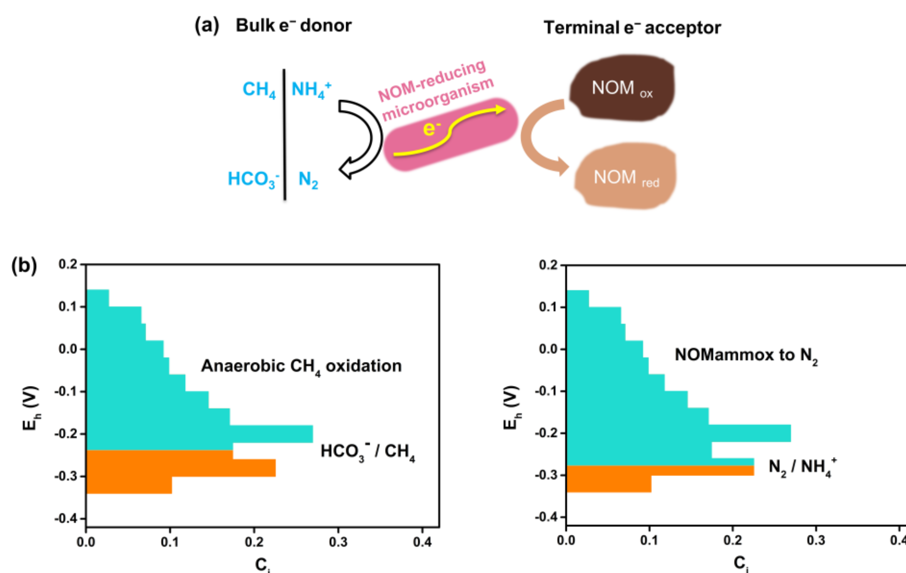



**Figure 2.3.** (a) NOM as terminal electron acceptor in anaerobic microbial methane and ammonium oxidation. (b) Scheme of typical Eh dependent distribution of reducing equivalents in humic acids. Data shown are the average of three different humic acids (Aeschbacher et al., 2011). Ci (mmol $e^-$/g HA) is the molar concentration of redox active moieties at the respective Eh range. The green area indicates the fraction of reducing equivalents that thermodynamically is feasible to drive the processes.

#### 2.3.1.1 Anaerobic $CH_4$ oxidation coupled to NOM reduction

A set of inorganic electron acceptors have been reported for anaerobic oxidation of methane (AOM), including sulfate (Boetius et al., 2000; Holler et al., 2011; Timmers et al., 2016), nitrate (Raghoebarsing et al., 2006; Haroon et al., 2013; Deutzmann et al., 2014), nitrite (Ettwig et al. 2010; Zhu et al. 2018), iron- and manganese oxides (Beal et al., 2009; Ettwig et al., 2016), perchlorate (Luo et al., 2015), bromate (Luo et al., 2017), chromate (Lai et al., 2016) and selenate (Luo et al., 2018). As shown in Figure 2.3b, a large fraction of reducible moieties in HA is also thermodynamically feasible to support anoxic methane oxidation. Indeed, AOM has recently been demonstrated to be coupled to NOM reduction by archaea consortia in marine sediments (Scheller et al., 2016) and in wetland sediments (Valenzuela et al., 2017). This represents an alternative mechanism by which NOM supresses $CH_4$ emissions from humic-rich environments by enhancing anaerobic $CH_4$ oxidation process (Martinez et al., 2013).

#### 2.3.1.2 Anaerobic $NH_4^+$ 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 (Hu et al., 2019) and iron oxides (Feammox) (Yang et al., 2012; Ding et al., 2014). NOM, however, presumably is also linked to anaerobic ammonium oxidation as it is often bound to Fe-(III)-minerals and exists at suitable redox states (Figure 2.3b). Unlike Feammox, NOM reduction coupled to anaerobic ammonium oxidation (NOMammox) to form $N_2$ (Figure 2.3a), however, has not been studied explicitly nor proven clearly heretofore. Zhou et al. (2016) recently demonstrated that the soluble synthetic quinone AQDS functions 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 hydroquinone by Fe(III) minerals (Figure 2.5a). However, the experimental set-ups of this study lacked Fe (III)-free controls. Thus, the formation of $N_2$ from microbial anaerobic ammonium oxidation coupled to AQDS reduction was not studied separately and hence could not be proven directly. Very recently, the occurrence of NOMammox to $N_2$ has been claimed in marine sediments (Rios-Del Toro et al., 2018) but the mechanisms of $N_2$ formation could not be identified. Thus, the evidence of enzymatic NOMammox to $N_2$ is still lacking. As the standard reduction potential of the $N_2/NH_4^+$ couple at pH 7, $Eh^0$(pH7), is -277 mV (Table 2.1), we conclude that compared to synthetic AQDS ($Eh^0$(pH7) =-185 mV) (Table 2.1) naturally occurring quinones (e.g., the plant derived naphthoquinone Lawsone, $Eh^0$(pH7) =-152 mV, (Clark, 1960) and macromolecular dissolved NOM

(Figure 2.3b) are energetically even more favourable for driving NOMammox. As NOM from different sources shows different redox properties, its ability to drive NOMammox may differ with source and redox state.

### 2.3.2 Microbial NOM Oxidation

Phenolic moieties are thought to be the major electron-donating groups in NOM (Aechbacher et al., 2012). NOM in its reduced form has the potential to be microbially oxidized by coupling to N-oxides (e.g., nitrate, nitrite and $N_2O$) reduction, thereby affecting $N_2O$ formation or consumption and also the loss of reactive N.

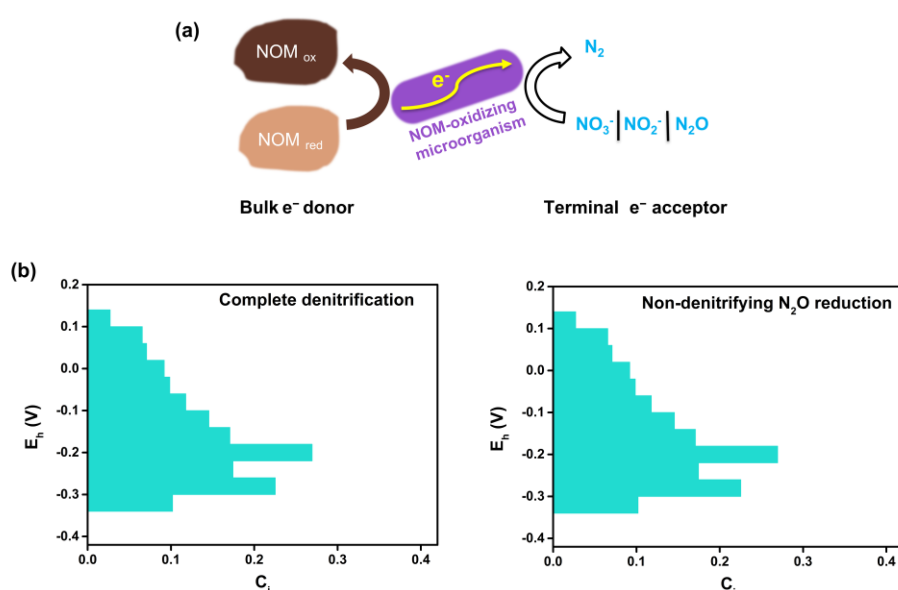



**Figure 2.4.** (a) NOM as electron donor in anaerobic microbial denitrification and non-denitrifying $N_2O$ reduction. (b) Scheme of typical Eh dependent distribution of reducing equivalents in humic acids. Data shown are the average of three different humic acids (Aeschbacher et al., 2011). Ci (mmol $e^-$/g HA) is the molar concentration of redox active moieties at the respective Eh range. The green area indicates the fraction of reducing equivalents that thermodynamically is feasible to drive the process.

#### 2.3.2.1 Complete denitrification

Denitrification refers to the process of anaerobic respiration of 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 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, the entire reducible moieties in NOM are capable of complete denitrification (Figure 2.4b). The widespread heterotrophic denitrifying bacterium *Paracoccus denitrificans* is well known for its ability to oxidize hydroquinones within humic acids coupled to nitrate reduction (Lovely 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 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 energy source (Coates et al., 2002).

#### 2.3.2.2 Non-denitrifying $N_2O$ reduction

Non-denitrifying $N_2O$ reduction describes the anoxic reduction of $N_2O$ to $N_2$ by non-denitrifying microorganisms, which cannot reduce $NO_2^-$ to nitrogenous gas. 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 (Figure 2.4b). Aranda-Tamaura et al. (2007) described the capability of an anaerobic denitrifying sludge to reduce denitrification intermediates (nitrite and $N_2O$) with the quinone $AH_2QDS$ ($Eh^0(pH7)$ = -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 ($Eh^0(pH7)$ = 1353 mV) (Table 2.1) imply that $N_2O$ may be a highly competitive terminal electron acceptor in enzymatic NOM oxidation. 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 unclear whether *Wolinella succinogenes* can respire $N_2O$ using reduced NOM as electron donor.

### 2.3.3 Electron Shuttling by NOM

In addition to direct coupling to $CH_4$ and 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 $CH_4$ turnover and N loss. By serving as the relay between the microbial cell and a distant extracellular acceptor, such as an iron oxide mineral particle, 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 iron-dependent anaerobic $CH_4$ oxidation and Feammox, and thereby modulating $CH_4$ and N loss, respectively.

#### 2.3.3.1 NOM-shuttled anaerobic $CH_4$ oxidation coupled to iron reduction

NOM-shuttled anaerobic oxidation of $CH_4$ coupled to iron reduction (Figure 2.5a) has been scarcely examined. However, the findings of anaerobic $CH_4$ oxidation coupled to the reduction of environmentally relevant forms of particulate Fe and Mn (Ettwig et al., 2016) by a freshwater enrichment culture, and the discovery of anaerobic $CH_4$ oxidation coupled to NOM reduction in a wetland sediment (Valenzuela et al., 2017) collectively hint at the occurrence of NOM-shuttled iron dependent AOM in freshwater environments with low sulfate concentrations. Until now, NOM-shuttled anaerobic $CH_4$ oxidation coupled to iron reduction has only been demonstrated in coastal wetland sediments (Valenzuela et al., 2019).

#### 2.3.3.2 NOM-shuttled Feammox to $N_2$

As discussed above, NOM presumably exerts a dual role as bulk electron acceptor for NOMammox and as essential electron transfer mediator for Feammox. The feasibility of this process so far has only been supported by the shuttling ability of AQDS, a non-sorbing model quinone (Zhou et al., 2016) while the potential role of sorbing NOM as electron shuttle in the Feammox is still unknown. Sorption of NOM to Fe(III) oxides, however, is expected to alter both the surface chemistry and the redox properties of NOM and Fe(III) oxides (Orsetti et al., 2013; Xue, 2018), and thus may in turn impact the conversion of $NH_4^+$ to $N_2$ by the Feammox process.

Due to its refractory nature NOM can be cycled frequently as electron carrier in

ferrying electrons between Fe(III)-reducing microorganisms and Fe(III) oxides. Moreover, the electron shuttling activities of NOM was shown to be largely dependent on its redox potential (Wolf et al., 2009). 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 and Mn minerals with distinct reduction potentials.

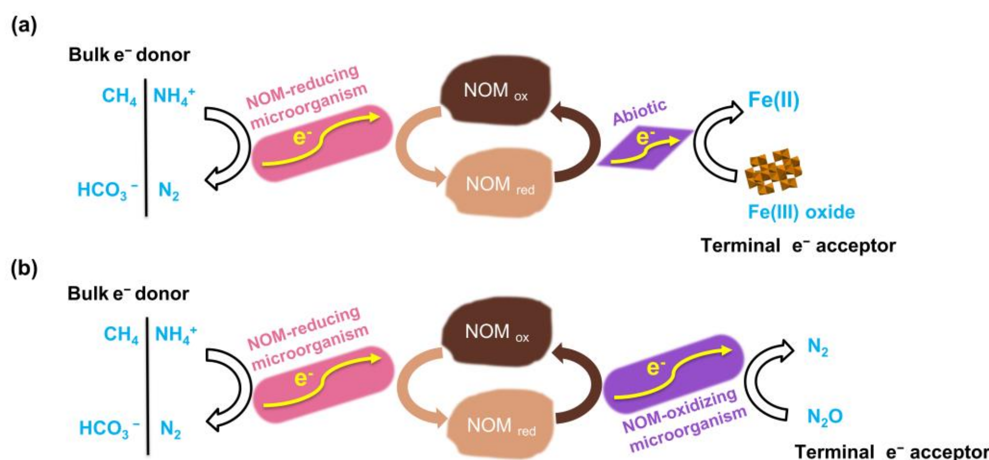



**Figure 2.5.** NOM as electron transfer mediator between microbes and (a) extracellular terminal electron acceptor in anaerobic methane and ammonium oxidation, (b) their syntrophic partners in anaerobic respiration (mediated interspecies electron transfer).

### 2.3.4 NOM-mediated interspecies electron transfer

Interspecies electron transfer (IET) is a microbial syntrophic cooperation enabling different microbial species exchange reducing equivalents under anoxic conditions. 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. Mediated interspecies electron transfer (MIET) can occur via hydrogen (Bryant et al.,1967; Stams and Plugge 2009; Sieber et al., 2014), formate (Boone et al., 1989; Storck et al., 2016), cystine/cysteine (Kaden et al., 2002), $S^0/S^{(-II)}$ (Kaden et al., 2002; Milucka et al., 2012), or quinone/hydroquinone moieties (Lovley et al., 1999; Smith et al., 2015). Known processes for direct interspecies electron transfer (DIET) involve (a) pili which have metallic-like conductivity and function as microbial nanowires (Reguera et al., 2005;

Summers et al., 2010; Rotaru et al., 2014), (b) outer membrane c-type cytochromes (Summers et al., 2010; Lovley, 2012; Shi et al., 2016), and (c) abiotic conductive materials such as granular activated carbon (Liu et al., 2012; Rotaru et al., 2014), carbon cloth (Chen et al., 2014a), biochar (Chen et al., 2014b), or magnetite (Kota et al., 2012a; 2012b; Liu et al., 2015; Kouzuma et al., 2015). 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 modifying *Geobacter sulfurreducens* (Smith et al, 2015).

#### 2.3.4.1 Anaerobic $CH_4$ oxidation coupling to $N_2O$ reduction mediated by NOM?

The discovery of anaerobic $CH_4$ oxidation coupled to sulphate reduction opened an new area in interspecies electron transfer research-It is considered as the main pathway of $CH_4$ mitigation in marine sediments and performed by consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) (Boetius et al., 2000; Holler et al., 2011; Timmers et al., 2016) through direct interspecies electron transfer via conductive filaments as electron transport mechanism (McGlynn et al., 2015; Wegener et al., 2015). Alternative pathways of anaerobic $CH_4$ oxidation, however, have been described involving AQDS, humic acids and Fe(III)-complexes. Such natural or synthetic extracellular dissolved electron-mediators can take over the role of sulfate reducing bacteria in anaerobic $CH_4$ oxidation by ANME (Scheller et al., 2016).

Furthermore, anaerobic $CH_4$ oxidation is also linked to nitrogen cycle. Currently, two microbial pathways are known. First, a denitrifying intra-oxygenic methanotrophic process, where nitrite is metabolized into $N_2$ and $O_2$ via dismutation of NO, carried out by the uncultured NC10 phylum bacterium *Candidatus Methylomirabilis oxyfera* which lacks $N_2O$ reductase (Ettwig et al. 2010; Zhu et al. 2017). Second, ANME-catalysed anaerobic $CH_4$ oxidation coupled to the reduction of nitrate to nitrite (Haroon et al., 2013).

Notably, $N_2O$ as one of the thermodynamically most powerful terminal oxidants in nature, has not been reported to drive anaerobic $CH_4$ oxidation heretofore. Considering the extracellular respiration ability of various ANME lineages (Ettwig et al., 2016; Valenzuela et al., 2017) and the previously discussed process of NOM-coupled non-denitrifying $N_2O$ reduction, an ANME lineage capable of exchanging electrons with a non-denitrifying $N_2O$-reducer via NOM-mediated IET is conceivable (Figure 2.5b). We suggest that screening for microbes capable of anaerobic $CH_4$ oxidation using $N_2O$ as a

terminal oxidant from natural environments will extend our current knowledge on the linkages between $CH_4$ turnover and N-cycling.

#### 2.3.4.2 Anaerobic $NH_4^+$ oxidation coupled to $N_2O$ reduction mediated by NOM?

The pathway of $N_2O$ driven anammox to $N_2$ is thermodynamically feasible but so far has not been discovered in nature (Conthe et al., 2018). Combining the previously discussed NOMammox to $N_2$ and NOM-coupled non-denitrifying $N_2O$ reduction processes, we suggest that NOMammox coupling to non-denitrifying $N_2O$ reduction mediated by NOM is also feasible (Figure 2.5b). Digging into this pathway may deepen our understanding on interconnections of microbial N cycling.

Taken together, based on classifying oxidation-reduction mechanisms, we emphasised the poorly understood or unravelled avenues of microbial NOM reduction-oxidation linking to gaseous C and N turnover. To examine and validate these pathways either in environmental samples or laboratory enrichment/pure cultures, both MER and MEO can be applied in tracking changes in redox states of NOM over the course of laboratory incubations.

## 2.4 Eliminating interference of electro-active substances

Next to the prospects of mediated electrochemical analysis for elucidating and quantifying microbial metabolic pathways involving reversible electron transfer to natural organic matter detailed in section 2, this technique also has its own internal limitations. In certain cases, the presence of electro-active substances in samples could interfere with the determination of the electron accepting (EAC) and/or electron donating capacity (EDC) of NOM. To provide clues for applications of mediated electrochemical analysis in future research on microbial metabolic pathways involving NOM redox cycling, we would like to discuss this issue and propose a suite of strategies to overcome it.

### 2.4.1 Typical medium components

First, ingredients of basic culture medium which might be electro-active in MER and/or MEO cells need to be considered. Take cysteine as an example that is often added into microbial culture medium as a reductant to scavenge oxygen and can cause a current response in MEO analysis (Sections 2.S1 and 2.S2, Figure 2.S1). Therefore, when using mediated electrochemical analysis to examine microbial NOM cycling,

addition of electroactive reducing agents such as cysteine or hydrogen sulfide (Wallace et al. 2017) to the culture bottles has to be avoided due to its interference with EDC measurement of NOM. Furthermore, such compounds may trigger unwanted interspecies electron transfer as has been shown for the cysteine/cystine couple (Nevin and Lovley, 2000; Kaden et al. 2002).

### 2.4.2 Nitrogen cycling intermediate: nitrite

Second, the formation of electroactive intermediates during microbial NOM redox cycling may interfere with mediated electrochemical analysis and must be considered. Regarding anoxic N-cycling, nitrite appears to be the major electrochemically active N-intermediate of concern (Section 2.S1 and Figure 2.6). In some cases, nitrite might transiently accumulate during denitrification, as regulated by nitrate reductase, nitrite reductase and environmental conditions. For example, a transient accumulation of nitrite has been observed in the process of $AH_2QDS$-oxidation coupled to complete denitrification by *Dechloromonas* sp. strain JJ (Coates et al., 2002). Consequently, when studying NOM oxidation coupled to denitrification, the presence of nitrite in NOM analyte may interfere with the assessment of NOM's redox properties. As shown in Figure 2.6, 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 $N_2$. The spike of 0.051 μ mol nitrite caused significant current signals, whereas the spike of 0.021 μ mol nitrite was below the detection limit. To ensure the measured EAC is solely attributed to NOM, strategies to exclude the interference of nitrite must be developed. As such, experiments should be designed to minimize transient nitrite concentrations.

Before discussing protocols of pretreatments to remove nitrite from the sample before electrochemical analysis of NOM, we notice that the NOM may be present in dissolved state, but also associated with particles (Figure 2.2). Recommended methods of nitrite removal differ with the speciation of NOM. For samples containing NOM only in particulate forms (adsorbed to minerals or as particles), nitrite can be removed from the sample by anoxic centrifugation and discarding the 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.

Various reactants, such as azide, hydroxylamine, ascorbate and sulfamic acid, have been described to remove nitrite from aqueous samples in the context of isotope analysis of nitrate (Granger and Sigman, 2009). While azide is highly toxic and dangerous to handle, hydroxylamine is electroactive and prone to react with NOM

(Porter, 1969). Ascorbate is highly electroactive (Walpen et al., 2018) and prone to changes in redox state of NOM. Thus, we evaluated the suitability of the sulfamic acid method (Granger and Sigman, 2009) to quench nitrite interference in the electrochemical determination of EAC of dissolved NOM samples (Sections 2.S1 and 2.S3, Figure 2.S2). The sulfamic acid itself was not electro-active in mediated electrochemical reduction (MER) and had no impact on the redox state of the tested HA samples. The acidification step (to pH≈1.8) that 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 (Figure 2.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) during the acidification step. In this case the sulfamic acid method should only be used with great caution.

Thus, we further recommend the evaluation and application of a bioassay to remove nitrite from anoxic NOM samples containing dissolved NOM. Similar method has been developed and evaluated for N and O isotope analysis of nitrate, and nitrite removal was based on the quantitative conversion of nitrite to $N_2O$ by adding a denitrifying strain which can reduce neither nitrate nor $N_2O$ (Böhlke et al., 2007). With respect to removing nitrite in NOM-containing samples, a requirement is that the denitrifier can neither oxidize nor reduce NOM. Such a metabolism inability has been confirmed for the strain *Pseudomonas nitroreducens* (formerly *Pseudomonas denitrificans*) (Lovely et al., 1998; Lovely et al., 1999). Therefore, *Pseudomonas nitroreducens* is a prime candidate bacterium for nitrite removal in NOM. Other denitrifying strains which are unable to cycle NOM should be further mined in future applications. Among nitrite reducers, in addition to denitrifiers the ammonifiers which perform dissimilatory nitrate reduction to ammonium (DNRA, $NO_3^-/NO_2^- \rightarrow NH_4^+$) potentially can be used for nitrite removal of NOM-containing samples. Hence, ammonifiers cannot cycle NOM warrant screening.

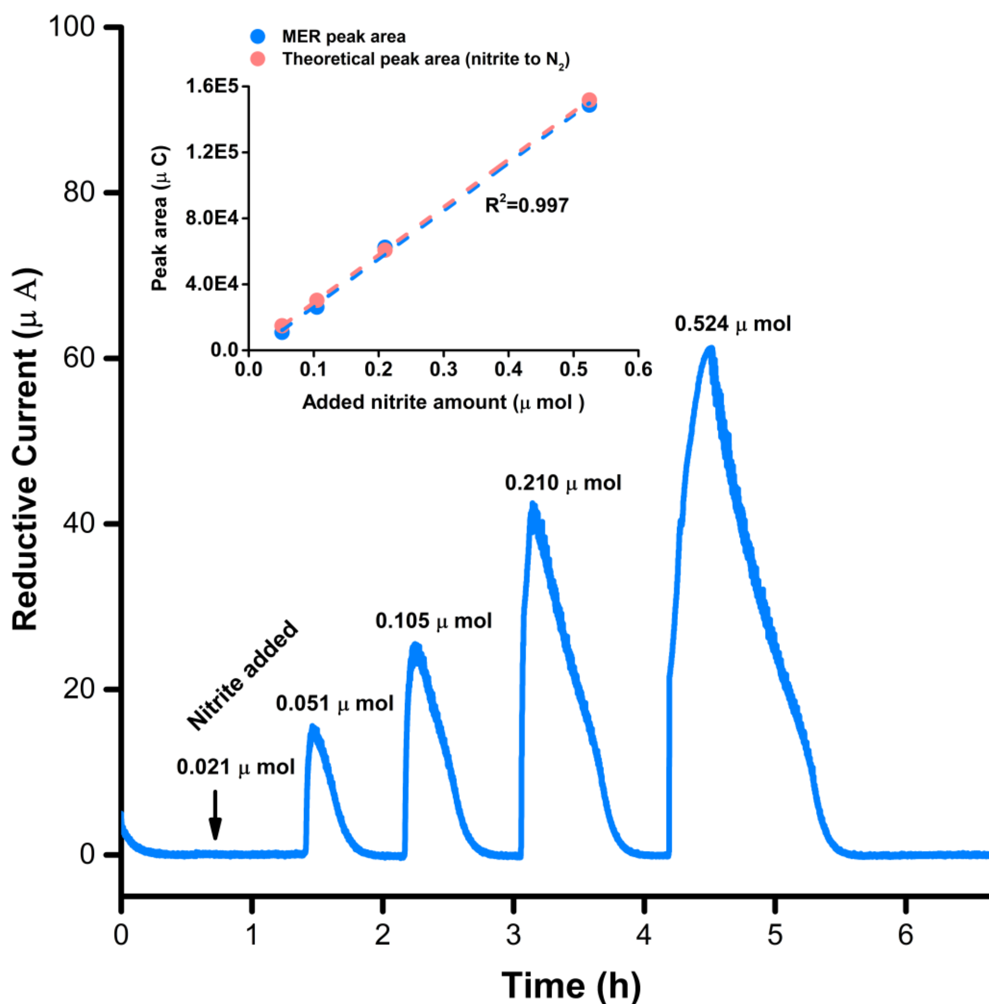



**Figure 2.6.** Reductive current responses (blue trace) to spikes of increasing amounts of nitrite ($NO_2^-$) analyzed by mediated electrochemical reduction (MER, Eh = −0.49 V, pH 7). Numbers above the current peaks indicate the injected amounts of nitrite, ranging from 0.021 to 0.524 μ mol. Nitrite concentration was determined using flow injection analysis (FIA) as described on Section S3. Inset 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.

### 2.4.3 Microbial cells

When microbial cells are associated with particulate NOM, it has to be considered whether the tested strain is electroactive in mediated electrochemical analysis. Inspections with cell-free filtrates, whole cell suspension, and washed cell suspension are thus required in routine cultivation. Our initial trails showed the cells of denitrifying strains such as *Paracoccus denitrificans* (Lovley et al., 1999) and *Thiobacillus denitrificans* (Zheng et al., 2018), potentially being related to topics discussed on

section 3, were not electroactive. However, we observed that acetate-fumarate grown cells of *Geobacter sulfurreducens*, a well-studied humics-respiring bacterium, did cause signals under both MER and MRO modes (Sections 2.S1 and 2.S4, Figure 2.S3), probably due to the presence of periplasmatic and/or membrane bound cytochrome (Esteve-Núñez et al., 2008; Malvankar et al., 2012; Estevez-Canales et al., 2015). We confirmed the role of cytochrome as electroactive component by employing a *Geobacter Sulfurreducens* variant with lower cytochrome content. These cells were grown on iron deficient medium (Estevez-Canales et al., 2015) and showed lower EAC and EDC values compared to cells grown on regular iron containing medium (Figure 2.S3). By contrast, another frequently studied humics-respiring bacterium *Shewanella oneidensis* MR-1 showed negligible or no EAC values (Klüpfel et al., 2014). Nonetheless, protocols to eliminate the interference from *Geobacter sulfurreducens* cells in solid NOM samples remain to be developed.

**Supplementary Information**

Section 2.S1 General Method Description of Mediated Electrochemical Analysis

Section 2.S2 Oxidative Current Response of Cysteine in MEO

Section 2.S3 Experimental Methods and Results of Sulfamic Acid Assay

Section 2.S4 Experimental Methods and Results of *Geobacter sulfurreducens*' Electro-activity

## Section 2.S1 General Method Description of Mediated Electrochemical Analysis

The electron donating capacity (EDC) and electron accepting capacity (EAC) of humic acid were determined by mediated electrochemical oxidation (MEO) and mediated electrochemical reduction (MER), respectively, as described by Aeschbacher et al. (2010) in an anoxic glove box ($N_2$ atmosphere, M. BRAUN UNIlab, Germany). The resulting oxidative and/or reductive current responses of anoxic nitrite, L-cysteine, and washed *Geobacter sulfurreducens* cells in MEO and/or MER were recorded as well. Measurements were conducted in electrochemical cells with each consisted of a glassy carbon cylinder as both the working electrode and reaction vessel (Sigradur G, HTW, Germany), a Pt wire counter electrode (Sigma-Aldrich Co., USA), and an Ag/AgCl reference electrode (Bioanalytical systems Inc., USA). The glassy carbon cylinders were filled with buffer solutions (0.1 M phosphate, 0.1 M KCl, pH 7). The working electrodes were polarized to Eh = +0.61 V (vs. SHE) for MEO and −0.49 V (vs. SHE) for MER, controlled by a potentiostat (1000C Muti-potentiostat, CH Instruments, USA). Dissolved electron transfer mediators, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1,1'-ethylene-2,2'-bipyridyldiylium dibromide (DQ), were used to facilitate electron transfer between the working electrodes and analytes. The resulting oxidative and reductive current responses in MEO and MER of the samples were baseline-corrected, integrated or further mass-normalized to EDC and EAC respectively.

**Section 2.S2 Oxidative Current Response of Cysteine in MEO**

We studied and quantified the electro-activity of L-Cysteine in our MEO setup (Section 2.S1). A 2 mM solution of L-Cysteine was prepared from an anoxic stock solution (0.1 M) by diluting in Millipore water. This concentration is typically used in anoxic microbial culture studies. Clear oxidative current responses of L-Cysteine were obtained in MEO which increased linearly with the spiking volume (Figure 2.S1). The measured numbers of electrons transferred in MEO perfectly matched the values expected for L-Cysteine oxidation to Cystine (one-electron transfer reaction). Take the spiking of 200 μL sample for instance, the detected number of electrons transferred in MEO was 0.399 μ mol, in agreement with the expected value of 0.4 μ mol. As expected, no detectable current response was observed under the MER mode.

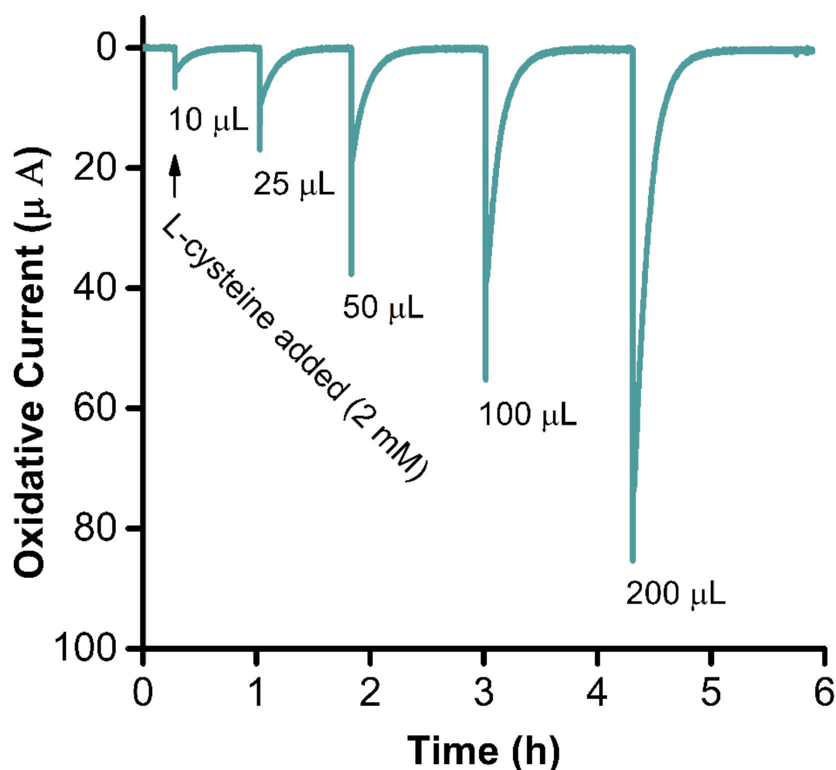



**Figure 2.S1.** Oxidative current responses (green trace) to spikes of increasing volumes of L-cysteine analyzed by mediated electrochemical oxidation (MEO, Eh = + 0.61 V, pH 7). Numbers above the current peaks indicate the injected volumes of 2 mM L-cysteine, ranging from 10 to 200 μL which are typically used in mediated electrochemical analysis.

**Section 2.S3 Experimental Methods and Results of the Sulfamic Acid Assay**

We investigated the suitability of this assay to remove the electroactive nitrite from samples to allow for unbiased MER measurements of humic acids.

Humic acid: Pahokee peat humic acid (PPHA) purchased from the International Humic Substance Society (IHSS) was electrochemically reduced by direct electrochemical reduction (DER) (Aeschbacher et al., 2010) inside the anoxic glovebox. The stock PPHA solution (10g $L^{-1}$) was reduced at a constant negative potential of −0.59V vs standard hydrogen electrode (SHE) and 0.1M KCl solution was used as electrolyte. The unreduced (native) HA solution with a higher redox state (ratio of EAC/(EDC+EAC)) was set as the other treatment that differed in redox state. Both the reduced and native HA solutions were adjusted to pH 7.0 prior to use.
Set-ups: Diluted reduced and native PPHA (1g $L^{-1}$) samples were prepared in 50 mM anoxic phosphate buffer (pH 7, pre-purged with $N_2$), sterile filtered (0.22-μm) and dispensed into brown serum bottles with each containing 15 ml PPHA solution. Sodium nitrite (0.2 and 2 mM) was added from an anoxic sterile stock solution. The treatment without nitrite was set as control. All treatments were performed in independent triplicates. The serum flasks were sealed with butyl rubber stoppers and kept inside the glovebox for equilibration. Samples were taken at 0h, 24h, 48h of incubation, used for EDC, EAC (Section 2.S1) and nitrite measurements.
Nitrite removal: After a 48-hour contact time, five millilitre samples were collected and treated with 40 mM sulfamic acid by adding 200 μL of 1M stock solution. Around 250 μL of 1M HCl solution was added to attain a reaction pH of 1.8. A 10-minute reaction time was sufficient for complete removal of nitrite, confirmed by flow injection analysis (FIA) as described below. Next, the samples were neutralized with 1M KOH solution to pH 7.0. Immediately afterwards, 50 or 100ul samples were spiked into electrochemical cells for EDC and EAC assessments (Section 2.S1).
Analytical methods: The dissolved organic carbon (DOC) content of all samples was determined by a TOC analyzer (Elementar, Hanau, Germany). Nitrite concentration was quantified using a flow injection analysis (FIA) system containing a dialysis membrane for organic ligands removal (Seal Analytical, Norderstedt, Germany). Sampling procedures throughout this experiment were performed inside the anoxic glovebox.

To simulate substrate doses researchers typically apply in laboratory incubations, we used 1) HA of 1g/L, with two different redox states and 2) electron limited (0.2 mM) and excess (2 mM) nitrite. During the 48-hour equilibration, the nitrite concentration and EDC of both native and reduced PPHA samples did not change markedly (Figure

2.S2a-d, One-way ANOVA with Duncan's test or Dunnett's T3 test, $P<0.05$). This implied that in microbial culture studies with DOM and nitrite, hours storage of samples after sampling and before mediated electrochemical analysis could be acceptable as it exerted no significant effect on redox prosperities of DOM samples. For treatments containing no and 0.2 mM nitrite, no significant changes in EDC and EAC of both native and reduced PPHA samples were observed before and after applying sulfamic acid assay (Figure 2.S2e, f, paired-samples T test, $P<0.05$). For treatments with 2 mM nitrite, the observed EAC of PPHA samples before sulfamic acid assay was larger than that after sulfamic acid assay (Figure 2.S2e, f) because the 2 mM nitrite contributed to the reductive current signals in MER (Figure 2.6). For simplicity, the false EAC of PPHA samples was still mass normalized with DOC contents. The loss in EAC of native PPHA samples containing 2 mM nitrite after sulfamic acid assay was 12.55 mmol $e^{-}$/g C, comparable to the expected value (11.62 mmol $e^{-}$/g C) for nitrite reduction to $N_2$ at the electrode. Likewise, the decrease in EAC of reduced PPHA samples was 10.04 mmol $e^{-}$/g C, in line with the expected value (11.52 mmol $e^{-}$/g C) for nitrite reduction to $N_2$. Further on, after nitrite removal by sulfamic acid treatment, the electron exchange capacity (EEC, =EDC+EAC) of PPHA samples between different nitrite addition treatments showed no statistical difference (One-way ANOVA with Duncan's test or Dunnett's T3 test, $P<0.05$), indicating the observed EAC of PPHA in the presence of 2 mM nitrite was equal to its unbiased value.

These results collectively suggested that the sulfamic acid method is validated to eliminate nitrite's interference in mediated electrochemical analysis of DOM (e.g., HA) samples.

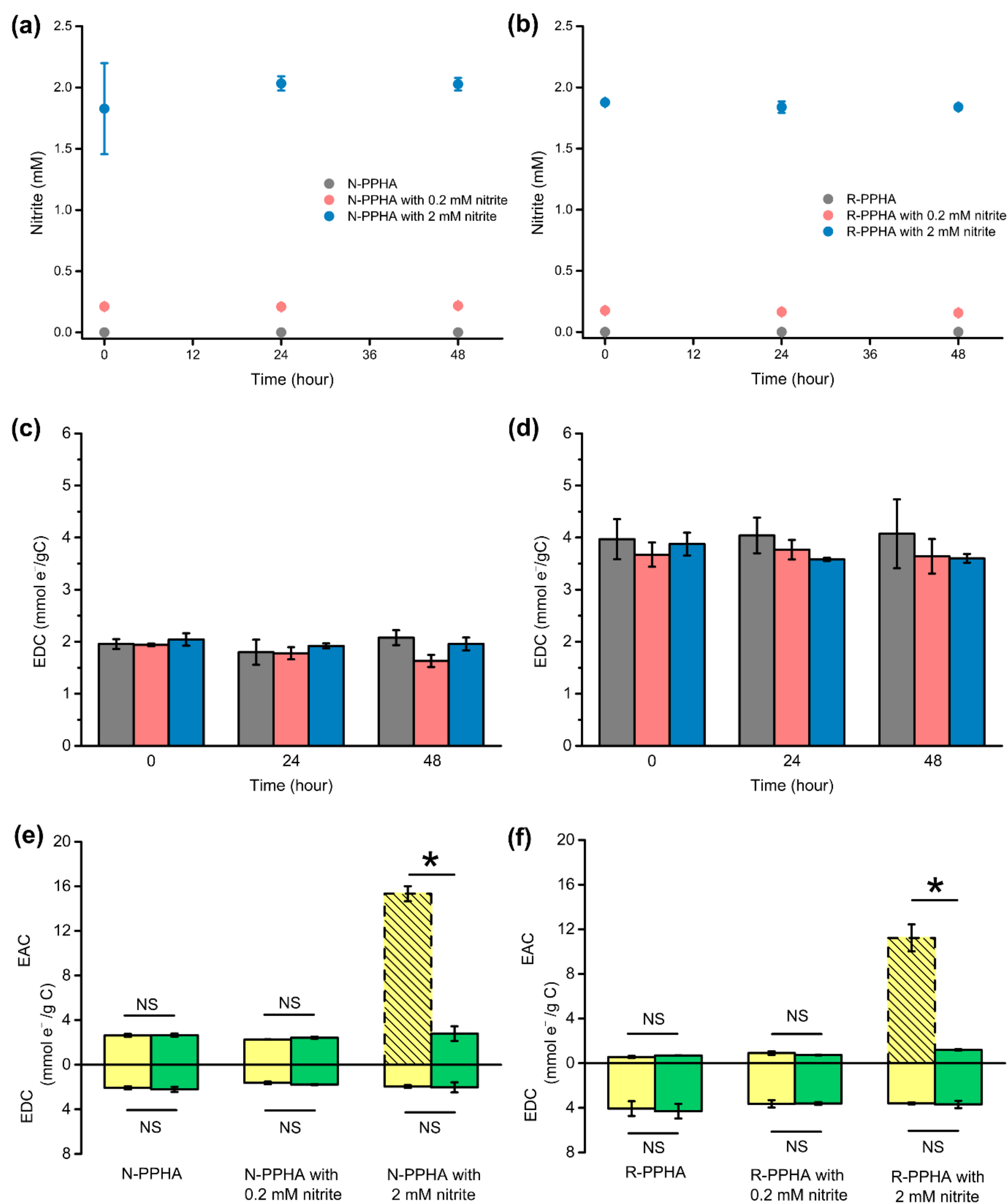



**Figure 2.S2.** Changes in nitrite of (a) native PPHA and (b) reduced PPHA samples with 0.2 mM nitrite (red circles) or 2 mM nitrite (blue circles) over time. The control contained no nitrite (gray circles). Changes in EDC of (c) native PPHA and (d) reduced PPHA samples with 0.2 mM nitrite (red bars) or 2 mM nitrite (blue bars) over time. The control contained no nitrite (gray bars). Changes in EAC and EDC of (e) native PPHA and (f) reduced PPHA samples before (yellow bars) and after (green bars) sulfamic acid assay. NS represents no significant difference and asterisk represents a significant difference before and after the sulfamic acid treatment, paired-samples T test ($P<0.05$). All data shown represent mean values ± 1 SD.

**Section 2.S4 Experimental Methods and Results of *Geobacter sulfurreducens*' Electro-activity**

We evaluated the electro-activity of *Geobacter sulfurreducens* cells in MER and MEO as a function of varying cytochrome content.

Bacteria growth conditions: Strain *Geobacter sulfurreducens* was cultured anaerobically in 30 mM bicarbonate-buffered mineral medium (pH 7.0). The basal medium consisted of (per litre) 0.3 g $KH_2PO_4$, 0.3 g $NH_4Cl$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, and 0.85 g $MgCl_2 \cdot 6H_2O$. After autoclaving and cooling, the medium was supplemented with 1ml (per litre) trace elements solution SL10 (Tschech and Pfennig, 1984), selenate–tungstate (Widdel, 1980) and vitamins (Widdel and Pfennig, 1981) respectively, and distributed into serum bottles. Each serum bottle contained 25ml medium and was sealed with a butyl rubber stopper, the headspace was flushed and filled with $N_2/CO_2$ (80/20%). Strain *Geobacter sulfurreducens* was routinely grown on 10 mM acetate as electron donor and 40 mM fumarate as electron acceptor, incubated at 28 °C in the dark.

Growth on iron deficient medium: *Geobacter sulfurreducens* cells from a preculture (4% v/v inoculum) were grown on iron-free medium as described above by omitting iron from the trace elements solution. After three repeated transfers for eliminating residual iron and cytochromes, the cells (10 % v/v inoculum) were further grown on 20 mM acetate and 40 mM fumarate in three different media: iron-free medium, iron-free medium with the iron chelator of 30 μ M 2,2′-Bipyridyl for ultimately sequestering iron (Estevez-Canales et al., 2015), and regular iron-containing medium as described above (control). All treatments were performed in independent triplicates. Growth was monitored via UV/VIS spectrophotometer (photoLab 6600, WTW, Germany) by measuring the increase in optical density at a wavelength of 600 nm ($OD_{600}$).

Sampling and measurements: Cells were harvested anaerobically in the early stationary phase by centrifugation (10000 rpm; 5 min), washed three times with 30 mM bicarbonate buffer (pH 7, flushed with $N_2/CO_2$), and resuspended to a density with $OD_{600} \approx 0.4$. The washed cell suspension of *Geobacter sulfurreducens* was used for EAC and EDC analysis (Section S1), mass-normalized with the biomass (total cell protein content). The protein of the washed cells was extracted in 0.5 N NaOH solution and determined using the enhanced protocol of bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific, USA) according to the manufacturer's instructions.

We aimed at evaluating the electro-activity of *Geobacter sulfurreducens* in MER and MEO and determining the impacts of cytochromes content on it. To do this, we firstly manipulated the cytochrome levels of *Geobacter sulfurreducens* by cultivating cells in

iron-containing medium (iron sufficient condition) and iron-free mediums with or without iron chelator (iron limitation conditions) (Figure 2.S3a, b). Next, we analysed the EAC and EDC of the washed cell suspensions respectively. As shown in Figure 2.S3c and d, the cells growing under iron rich condition yielded a larger EAC and EDC than that growing under iron deficient conditions. This could largely be explained by a greater content of cytochrome hemes synthesized by *Geobacter sulfurreducens* in regular iron-containing medium, as indicated by the much higher visible pink color (Figure 2.S3b) (Estevez-Canales et al., 2015). The data shown in Figure 2.S3c and d suggest that mediated electrochemical analysis appears to be alternative technique to determine the redox state of cytochromes next to known methods such as fluorescence (Esteve-Núñez et al., 2008), electrochemical impedance spectroscopy (Malvankar et al., 2012) and Confocal Resonance Raman Microscopy (Virdis et al., 2014). We propose to apply mediated electrochemical analysis on studying redox chemistry of cytochromes in future studies.

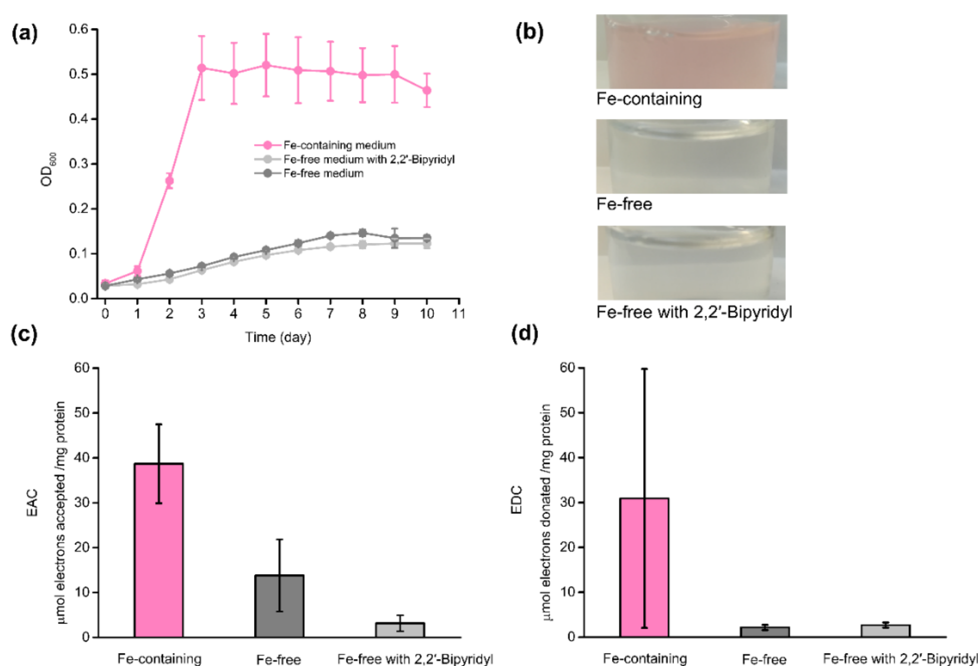



**Figure 2.S3.** (a) Growth of *Geobacter sulfurreducens* in iron-containing medium (pink circles), iron-free medium (gray circles) and iron-free medium containing 30 μ M 2,2'-Bipyridyl (light gray circles) over time. (b) Photographs of culture bottles with 3 different types of medium after 4-day incubation. (c) Electrons accepted by washed *Geobacter sulfurreducens* cells grown on iron-containing medium (sampled at day 4, pink column), iron-free medium (sampled at day 9, gray column) and iron-free medium containing 30 μ M 2,2'-Bipyridyl (sampled at day 10, light gray column) in MER. (d) Electrons donated by washed *Geobacter sulfurreducens* cells grown on iron-containing

medium (pink column), iron-free medium (gray column) and iron-free medium containing 30 μ M 2,2'-Bipyridyl (light gray column) in MEO. All data shown represent mean values ± 1 SD.

# Chapter 3

**A denitrifier method for nitrite removal in electrochemical analysis of the electron accepting capacity of humic substances**

**Abstract**

Humic substances (HS) are important electron acceptors and donors in soils and aquifers. The coupling of anoxic nitrogen (N) cycling to HS redox turnover, however, remains poorly understood. Mediated electrochemical analysis is an emerging tool to determine the redox properties (i.e., electron donating capacity (EDC), electron accepting capacity (EAC), and redox state) of HS in this research field. However, the presence of the N-cycling intermediate nitrite ($NO_2^-$) interferes with the electrochemical determination of the EAC. To eliminate this interference, we developed a bioassay to remove nitrite in HS sample using the denitrifying bacterium *Pseudomonas nitroreducens.* Cell suspensions of *P. nitroreducens* completely removed $NO_2^-$ at various concentrations (1, 2 and 5 mM) from both humic acid samples (1g HA/L) of different redox states. As *P. nitroreducens* is not able to exchange electrons with dissolve humic acids the procedure allows an accurate and precise determination of the EAC of humic acid samples. The proposed method thus opens new perspectives in biogeochemistry to study interactions between HS and N cycling.

## 3.1 Introduction

Humic substances (HS) are redox active and are involved in many redox reactions in anoxic environments, thereby absorbing or discharging electrons depending on their redox state (Lovley et al., 1996; Lovley et al.,1999; Roden et al., 2010). Electron donating capacity (EDC) and electron accepting capacity (EAC) quantify the extent of a HS sample to donate or accept electrons, respectively. Therefore, an exact experimental determination of EDC and EAC of HS is crucial for elucidating electron transfer mechanisms of HS-involved redox processes. However, traditional methods including chemical techniques and non-mediated electrochemical approaches, have multiple drawbacks as described elsewhere (see Chapter 2) (Aeschbacher et al., 2010; Tratnyek et al., 2011). By contrast, the recently developed mediated electrochemical analysis (Aeschbacher et al., 2010), i.e., mediated electrochemical oxidation (MEO) and reduction (MER), was proven for the fast and accurate tracking of changes in EDC and EAC of HS in batch experiments over time (Aeschbacher et al., 2010; Aeschbacher et al., 2011; Aeschbacher et al., 2012; Klüpfel et al., 2014).

Redox processes of humic substances were reported to be coupled to either abiotic or biotic pathways of anoxic nitrogen (N) turnover, such as chemo-denitrification (Stevenson et al., 1970; Zhu-Barker et al., 2015), denitrification (Lovley et al.,1999; Coates et al., 2001; Coates et al., 2002; Xiao et al., 2016) and dissimilatory nitrate reduction to ammonium (DNRA) (Lovley et al.,1999). However, these interactions are largely unexplored in terms of electron transfer mechanisms and their environmental significance due to the lack of sensitive methods to determine the redox state of HS. Although the mediated electrochemical analysis opens many possibilities in this research field, nitrite ($NO_2^-$), a potent reactive intermediate in anoxic N cycling, is electroactive in MER measurements (see Chapter 2) and therefore compromises electrochemical EAC measurements. In anoxic laboratory incubations addressing HS-driven microbial N cycling, nitrite likely accumulates which hampers the application of electrochemical EAC measurements (Coates et al., 2002). While in particulate or sorbed HS samples nitrite can easily be removed via centrifugation the problem will become evident when investigating dissolved HS samples. Using chemical reagents (e.g., hydroxylamine, ascorbate) to remove nitrite in dissolved HS analyte poses the risk to alter the redox state of HS(see Chapter 2) (Porter, 1969). To date, only sulfamic acid assay has been established to resolve this obstacle (see Chapter 2). Nonetheless, if dissolved HS sample harbors chemicals such as thiosulfate, the required reaction acidity may trigger side reactions with HS and cause changes in the redox state of HS(Vairavamurthy et al., 1994; Yu et al., 2015). Hence, an alternative strategy for nitrite

removal at circumneutral pH is warranted.

The present study proposes a bioassay for nitrite removal in mediated electrochemical analysis of dissolved humic substances. The denitrifying (reduce nitrite to nitrogenous gas) bacterium *Pseudomonas nitroreducens* (formerly *denitrificans*), which can neither reduce the humic substance analog anthraquinone-2,6-disulfonate (AQDS) (Lovely et al., 1998) nor oxidize its reduced form anthrahydroquinone-2,6-disulfonate ($AH_2QDS$) (Lovely et al., 1999), was chosen as microbial catalyst for nitrite removal. To validate this bioassay, we treated HA samples containing $NO_2^-$ with a *Pseudomonas nitroreducens* cell suspension at neutral pH and subsequently quantified the EDC and EAC of HA samples with MEO and MER, respectively. We aimed for a selective removal of $NO_2^-$ in HA samples without affecting the EDC and EAC of HA samples.

### 3.2 Materials and Methods

**Humic Acid.** A highly purified soil humic acid, Pahokee peat humic acid (PPHA) purchased from the International Humic Substance Society (IHSS), was used and conditioned with different redox states. Briefly, the PPHA stock solution (10 g HA/L) was prepared in 0.1 M KCl solution (electrolyte) and electrochemically reduced by direct electrochemical reduction (DER) (Aeschbacher et al., 2010) inside an anoxic glovebox ($N_2$ atmosphere, MBRAUN UNIlab, Germany). The applied constant potential was −0.59V vs standard hydrogen electrode (SHE), controlled by a potentiostat (1000C Muti-potentiostat, CH Instruments, USA). Next, both the reduced and the native (non-reduced) HA stock solutions were adjusted to pH 7 and diluted in phosphate buffer (50 mM, pH 7, purged with $N_2$) to give a final concentration of 1g HA/L. Afterwards, they were filtered sterile and anaerobically through 0.22-μm filters for further use.

**Abiotic Set-ups with Humic Acid and Nitrite.** Both the reduced and native PPHA (1g $L^{-1}$) were dispensed into brawn serum bottles inside the anoxic glovebox. Each bottle was filled with 15 ml HA solution. Nitrite concentrations of 1, 2, and 5 mM were prepared using sterile anoxic stock solutions of $NaNO_2$. The treatments without nitrite addition were used as controls. Each treatment was performed in triplicates. All serum bottles were sealed with butyl rubber stoppers and kept in the glovebox for equilibrium. At indicated time interval, samples were collected anoxically for EDC and EAC analyses, as well as nitrite and nitrate measurements.

**Bacterial Strain and Cultivation.** The strain *Pseudomonas nitroreducens* (DSMZ 1650, ATCC 13867) was cultured anaerobically in 30 mM bicarbonate-buffered mineral medium (pH 7.0). The basal medium consisted of (per liter) 0.3 g $KH_2PO_4$, 0.3 g $NH_4Cl$, 0.05 g $MgSO_4•7H_2O$, 0.1g $CaCl_2•2H_2O$, and 0.85 g $MgCl_2$ •$6H_2O$. After autoclaving and cooling, the medium was supplemented with 1 mL (per liter) trace elements solution SL10 (Tschech and Pfennig, 1984), selenate–tungstate (Widdel, 1980) and vitamins (Widdel and Pfennig, 1981). The medium was distributed into 58 mL serum bottles contained 25ml medium and a $N_2/CO_2$ (80/20%, v/v) headspace. All bottles were sealed with butyl rubber stoppers. Strain *Pseudomonas nitroreducens* was grown on 40 mM Na-acetate as the electron donor and carbon source, and 40 mM Na-nitrate as the electron acceptor, incubated at 28 °C in the dark. After 4 days of growth, cells were harvested anaerobically by centrifugation (10000 rpm; 5 min), washed three times with 30 mM $NaHCO_3$ buffer (pH 7, flushed with $N_2/CO_2$, 80/20%, v/v), and resuspended in the buffer to a density with $OD_{600}$≈0.12. The washed cell suspension of *Pseudomonas nitroreducens* was used for denitrifier assay as described later. A complete removal of residual nitrate and nitrite from pre-culture was confirmed with flow injection analysis (FIA) measurements.

**Nitrite Removal.** After a 48-hour exposure, for each abiotic set-up, a five mL HA sample was inoculated with 5 mL washed *Pseudomonas nitroreducens* cells. Na-acetate (10 mM) was added as the electron donor and carbon source to promote the microbial nitrite scavenge. The biological reaction was conducted in a 10-mL crimp vial with only minor headspace, sealed with a butyl rubber stopper. All closed vials were transferred into a wide-mouth bottle and sealed with a big butyl rubber stopper. The bottle was taken out from the anoxic glove box and incubated at 28 °C in the dark for 48 hours. The complete removal of nitrite was confirmed with flow injection analysis (FIA) over time. After 48 hours of incubation, samples were collected and centrifuged anaerobically (10000 rpm; 10 min) to remove cell pellets. The supernatants were used for subsequent EDC, EAC and $NH_4^+$ measurements. Headspace $N_2O$ was quantified by TRACE™ 1310 Gas Chromatograph (Thermo Scientific, USA) equipped with a PDD detector.

**Cells Suspension Experiment.** To further confirm the inability of strain *Pseudomonas nitroreducens* to oxidize and reduce PPHA in the microcosms, we performed a separate cells suspension experiment using nitrate/nitrite as potential electron acceptors and acetate as the potential electron donor. The bacterium was grown, harvested, and washed in the same way as described above, and eventually resuspended to a density with $OD_{600}$≈0.09. Next, the experiment was conducted in an analogous manner as the

nitrite-removal incubation experiments unless otherwise noted. To examine whether *Pseudomonas nitroreducens* can reduce PPHA in the presence of acetate, 5 mL washed cells were added into 5 mL native PPHA solution (1g/L) containing 10mM acetate. To check if this bacterium is able to oxidize PPHA with nitrate or nitrite, 5 mL washed cells were inoculated into 5 mL reduced PPHA (1g/L) containing 2 mM nitrate or 1 mM nitrite, respectively. The three treatments were all performed in triplicates. For cells-free controls, 5 mL washed cells were replaced by 5 mL 30 mM $NaHCO_3$ buffer, running in duplicates. Treatments without PPHA were performed in triplicates and set up as follows: 5 mL washed cells plus 2 mM nitrate or 1 mM nitrite respectively, and 5 mL washed cells plus 2 mM nitrate and 10 mM acetate. Five mL of 50 mM phosphate buffer was added, instead of PPHA solutions. All samples were incubated at 28 °C in the dark. For the treatments containing cells, reduced PPHA and nitrate or nitrite, after 48-h incubation 0.2 mM acetate was added into two of the three replicates as carbon source for a prolonged reaction. Changes in EDC, EAC, nitrate, nitrite and acetate over time were measured as described below.

**Analytical Methods.** The EDC and EAC of HA samples were determined by mediated electrochemical oxidation (MEO) and reduction (MER), respectively (Aeschbacher et al., 2010). In brief, the applied electric potential was controlled with a potentiostat (1000C Muti-potentiostat, CH Instruments, USA) and measurements were conducted using electrochemical cells consisting of a glassy carbon cylinder as both the working electrode and the reaction vessel (Sigradur G, HTW, Germany), a Pt wire counter electrode (Sigma-Aldrich Co., USA), and an Ag/AgCl reference electrode (Bioanalytical systems Inc., USA). The glassy carbon cylinders were filled with buffer solutions (0.1 M phosphate, 0.1 M KCl, pH 7). The working electrodes were polarized to Eh = +0.61 V (vs. SHE) for MEO and −0.49 V (vs. SHE) for MER. Dissolved electron transfer mediators, 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1,1’-ethylene-2,2’-bipyridyldiylium dibromide (DQ), were used to facilitate electron transfer between the working electrodes and the PPHA analyte. The resulting oxidative and reductive current responses in MEO and MER of the samples with time were integrated after baseline correction, and further mass-normalized to EDC and EAC (mmol $e^-$/g C), respectively. The DOC content of HA samples was quantified by a TOC analyzer (Elementar, Hanau, Germany). The concentrations of nitrate and nitrite were quantified using a flow injection analysis (FIA) system containing a dialysis membrane for organic ligands removal (Seal Analytical, Norderstedt, Germany). Acetate concentration was detected by a high-performance liquid chromatography (HPLC) with a Biorad HPX87H Column and a RID 10A and SPD M10A vp Detector (Shimadzu, Japan). The $OD_{600}$ (optical density at a wavelength

of 600 nm) of the cells was determined by a UV/VIS spectrophotometer (photoLab 6600, WTW, Germany). All sampling procedures and electrochemical measurements were performed inside an anoxic glovebox.

## 3.3 Results and discussion

### 3.3.1 Selective Removal of Nitrite by *Pseudomonas nitroreducens*

Nitrite in native and electrochemically reduced PPHA samples was completely removed within 24 hours by washed cells of strain *Pseudomonas nitroreducens* (Figure 3.1). As neither $NH_4^+$ nor $N_2O$ was detected after 48 hours of incubation in the cells suspension for all treatments (not shown) we concluded that nitrite was denitrified to $N_2$ gas as the end product.

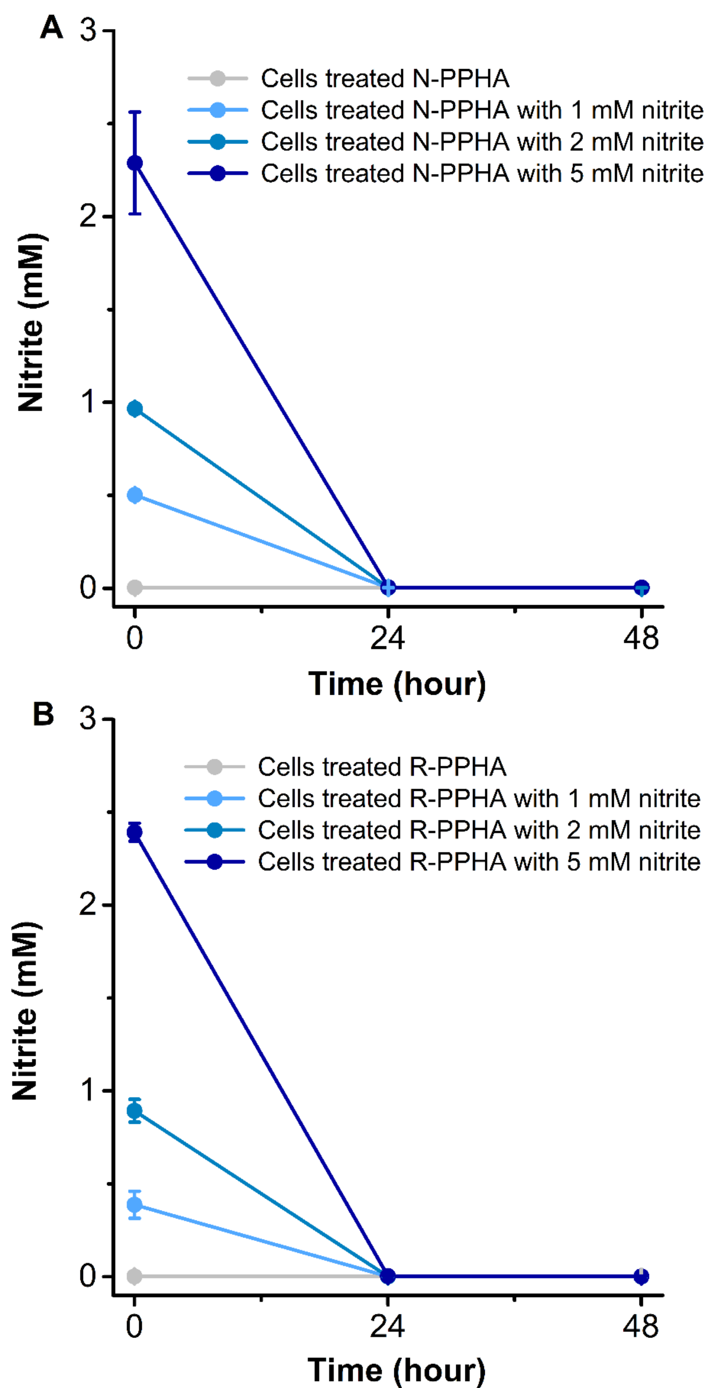



**Figure 3.1.** Removal of nitrite from native (A) and reduced (B) PPHA samples by washed cell suspensions of strain *Pseudomonas nitroreducens*. N-PPHA and R-PPHA represent native PPHA and reduced PPHA, respectively. All data are means ± SD.

### 3.3.2 Redox Capacities of Humic Acid before and after treatment with the Denitrifier Assay

As shown in Figure 3.2, the electron accepting capacities determined for nitrite-free native or reduced PPHA samples were 2.62 and 0.60 mmol $e^-$/g C, respectively. The corresponding EDCs were 2.60 and 4.64 mmol $e^-$/g C, respectively. However, addition of nitrite to native or reduced PPHA samples resulted in an increase of apparent EACs compared to nitrite-free controls (Figure 3.2).

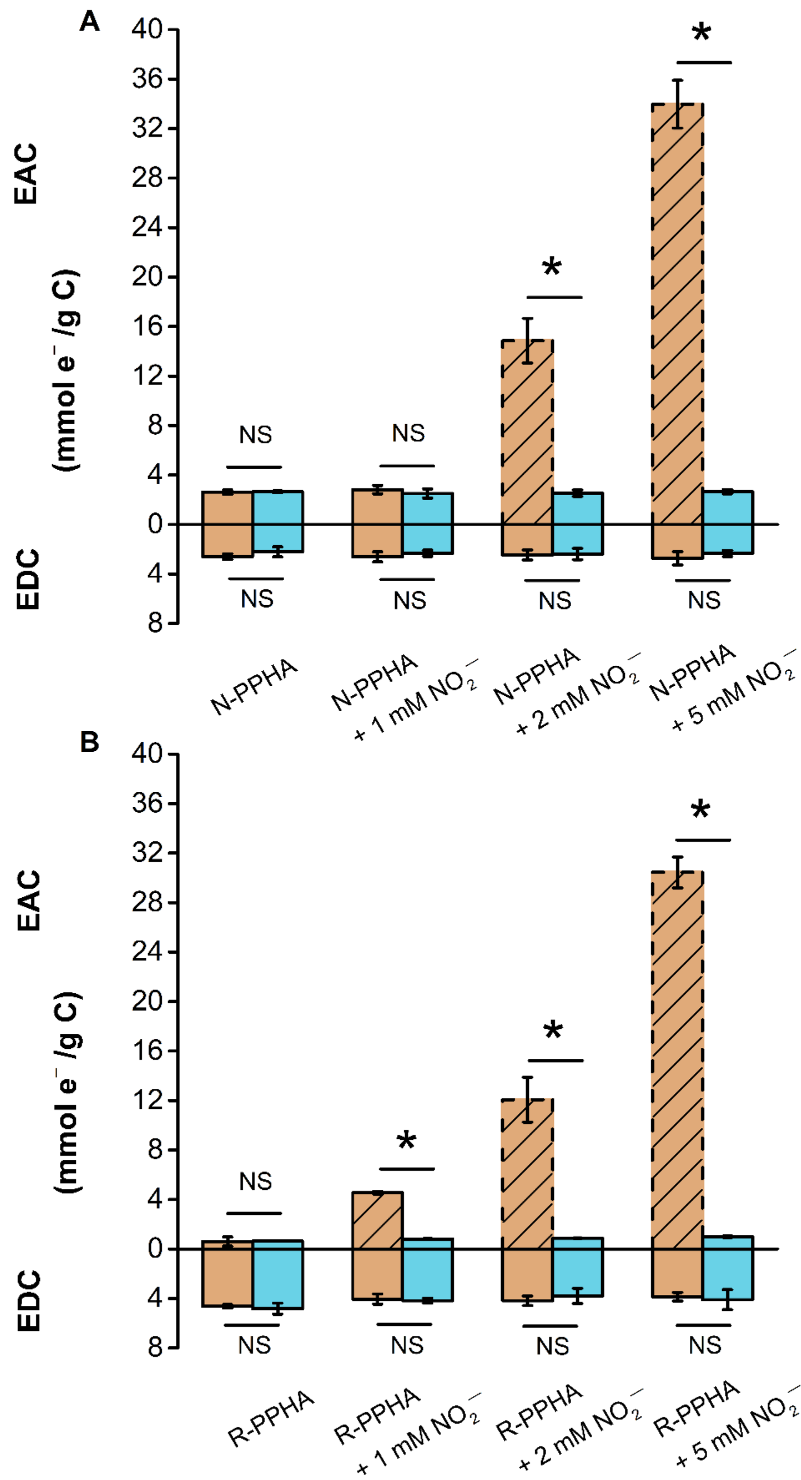



**Figure 3.2.** The EAC and EDC of native (A) and reduced (B) PPHA samples before (brown bars) and after (blue bars) treatment with the denitrifier assay. NS represents difference is not significant and asterisk denotes a significant difference before and after the denitrifier assay (paired-samples T test, $P<0.05$). N-PPHA and R-PPHA represent native PPHA and reduced PPHA, respectively. All data are means ± SD.

After complete nitrite removal by the denitrifier assay, however, the EACs of all PPHA samples were recovered (Figure 3.2). For both native and reduced PPHA samples amended with, after treatment with the denitrifier assay, the decrease in apparent EACs agreed well with the values for stoichiometric nitrite reduction to $N_2$ at least for the higher nitrite setups. The recovery ranged from 92.6% to 100.2% (Table 3.1). At lower nitrite concentrations, the response of the electrochemical setup was understoichiometric with regard to nitrite. In all treatments, however, the measured EDCs of both native and reduced PPHA samples were comparable before and after application of the nitrite removal procedure (Figure 3.2, paired-samples T test, $P<0.05$). Furthermore, after nitrite removal, also the electron exchange capacity of the humic acids (EEC, =EDC+EAC) were similar compared to the nitrite-free control (Figure 3.2, One-way ANOVA with Duncan's test or Dunnett's T3 test, $P<0.05$) both in native and reduced PPHA samples. Overall, these results proved that the denitrifier bioassay did eliminate the interference of nitrite in mediated electrochemical analysis and recovered the original EAC of PPHA. Consequently, the developed denitrifier bioassay offers a fast and robust method to selectively remove nitrite from PPHA samples without changing their redox states.

**Table 3.1.** Electron Budgets of Nitrite-Interfered PPHA Samples in MER before and after Nitrite Removal by Denitrifier Assay.

| Treatment | Loss in observed EACs after nitrite removal (mmol $e^-$/g C) | Expected amount of electron transferred for nitrite reduction to $N_2$ [a] (mmol $e^-$/g C) | Recovery rate [b] |
|---|---|---|---|
| N-PPHA + 2 mM nitrite | 12.33 ± 1.75 | 12.32 ± 0.16 | 100.21 ± 15.41 % |
| N-PPHA + 5 mM nitrite | 31.32 ± 2.08 | 31.68 ± 1.25 | 98.85 ± 5.23 % |
| R-PPHA + 1 mM nitrite | 3.74 ± 0.12 | 5.97 ± 0.16 | 62.68 ± 1.68 % |
| R-PPHA + 2 mM nitrite | 11.19 ± 1.82 | 12.07 ± 0.29 | 92.63 ± 13.71 % |
| R-PPHA + 5 mM nitrite | 29.45 ± 1.23 | 31.43 ± 0.54 | 93.69 ± 2.30 % |

[a] Calculated based on measured nitrite concentration. [b] Ratio of measured difference in EACs to theoretical value assuming nitrite reduction to $N_2$. N-PPHA and R-PPHA represent native PPHA and reduced PPHA, respectively.

### 3.3.3 Inability of *Pseudomonas nitroreducens* to Reduce or Oxidize Humic Acid

The denitrifier assay experiment showed that the presence of *Pseudomonas nitroreducens* cells did not alter the EDC and EAC of PPHA samples. In further cell experiments we specifically investigated the potential of *Pseudomonas nitroreducens* to oxidize reduced PPHA with nitrate/nitrite as terminal electron acceptors or to reduce native PPHA in the presence of acetate as electron donor.

As shown in Figure 3.3A, within 48-hour incubation no significant decrease in EDC of reduced PPHA was observed in the presence of cells and 2 mM nitrate (excess in electron equivalents). Even the addition of a carbon source (0.2 M acetate) after a 48-h reaction time did not cause changes in the EDC of reduced PPHA over the prolonged 96-hours incubation. In parallel, a consumption of nitrate was observed after addition of 0.2 mM acetate (Figure 3.3B). This indicated the activity and viability of cells after 48-hours of incubation. Similar results were also observed for treatments with 1mM nitrite (excess in electron equivalents) as potential electron acceptor (Figure 3.3C and D). Additionally, we observed no changes in EAC of reduced PPHA in nitrate reduction experiment (not shown). In the nitrite reduction experiment, EAC was not used as proxy of redox properties of reduced PPHA because nitrite interfered with the electrochemical analysis. Therefore, we conclude that *Pseudomonas nitroreducens* is unable to oxidize PPHA with nitrate/nitrite as electron acceptors. Figure 3.3B and D show that the presence of reduced PPHA slightly enhanced nitrate/nitrite reduction by cells, likely due to consumption of an internally stored C source as electron donor. This might be explained by a lower redox potential of the system due to the presence reduced PPHA.

As shown in Figure 3.3E and F, we found no significant decrease in EAC of native PPHA in the presence of cells and 10 mM acetate (excess in electron equivalents) within 48-h incubation and a prolonged 96-hours incubation (not shown). In addition, the rapid consumption of nitrate in the treatment containing nitrate and acetate plus cells (Figure 3.3B) within 24 hours suggested the cells viability at the beginning of the cell suspension experiment. In addition, no changes in EDC of native PPHA was observed in acetate oxidation experiment (not shown). Hence, *Pseudomonas nitroreducens* is not capable of reducing PPHA with acetate as electron donor.

The results obtained from the cell suspension experiment confirmed unequivocally that *Pseudomonas nitroreducens* cannot alter the EDC and EAC of PPHA samples via redox cycling of HA.

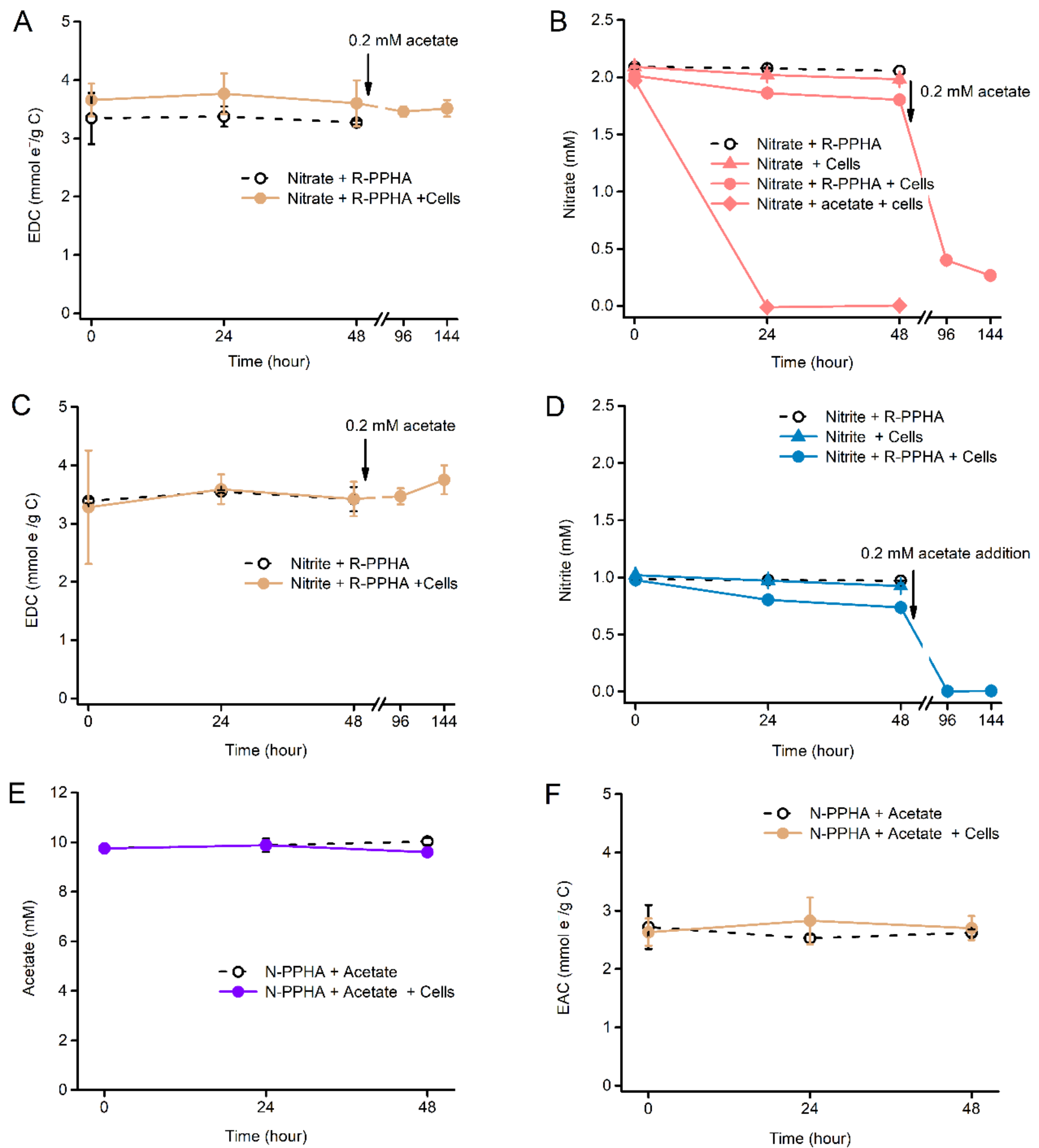



**Figure 3.3.** Changes in EDC of reduced PPHA samples (A) and nitrate (B) across treatments over time. Changes in EDC of reduced PPHA samples (C) and nitrite (D) across treatments over time. Changes in acetate (E) and EAC of native PPHA samples (F) across treatments over time. N-PPHA and R-PPHA represent native PPHA and reduced PPHA, respectively. All data are means ± SD or means ± ranges of duplicates.

### 3.4 Implications for Experiments Studying HS-Driven N Cycling in Anoxic Environments.

The experimental conditions of our study regarding the concentrations of nitrite, humic acids and substrate concentrations are typical for many microbial batch studies (Lovley et al.,1999; Van Trump et al., 2011). Furthermore, we studied the PPHA at two different and defined redox states to cover the majority of redox states may present in batch studies. The nitrite concentrations used (2 and 5 mM) were chosen to match reported toxicity threshold values for N-cycling microbes, e.g., denitrifiers (Mahne and Tiedje, 1995) and nitrate-respiring ammonium producers (Cruz-García et al., 2007), and to demonstrate the efficiency of denitrifier method in the presence of excess nitrite.

It is important to note that HA samples should not be stored longer than two days before performing the bioassay. As shown in Supplementary Figure 3.S1, we monitored a prolonged equilibration of the abiotic set-ups with PPHA and nitrite up to 68 days and found that the redox state of PPHA was conserved for 2 days whereas longer storage time (e.g., 6 days) caused significant decrease in EDC of reduced PPHA. A plausible cause might be nitration of phenolic moieties in reduced PPHA taking place with slow kinetics (Stevenson et al.,1970).

Control experiments with reduced HA only are required when studying HS redox cycling. For example, we performed the cell suspension experiment weeks later than the denitrifier assay but using the same stock reduced PPHA sealed in a serum bottle kept inside the glove box. However, a slight loss in EDC was found (Figure 3.2B, Figure 3.3A and C). The observation was in line with Supplementary Figure 3.S1B showing even in the nitrite-negative control the EDC of reduced PPHA had a slight decrease over time scale. Thus, we suggest that in batch studies, the control with reduced HA only should be set up to follow changes in EDC (if any).

HS-coupled denitrification and DNRA processes so far received little attention and are only partially understood (Lovley et al.,1999; Coates et al., 2001; Coates et al., 2002; Xiao et al., 2016) as well as HS-coupled anaerobic ammonium oxidation (Zhou et al., 2016; Rios-Del Toro et al., 2018). Currently, however, their contributions to anoxic N cycling including N loss is unclear. Future batch studies addressing these important coupled pathways using the powerful tool of mediated electrochemical analysis may shed further light on the accumulation of biogenic nitrite. The denitrifier bioassay developed in this study provides a robust method to completely remove nitrite without altering the redox states of HS sample.

## Supporting information

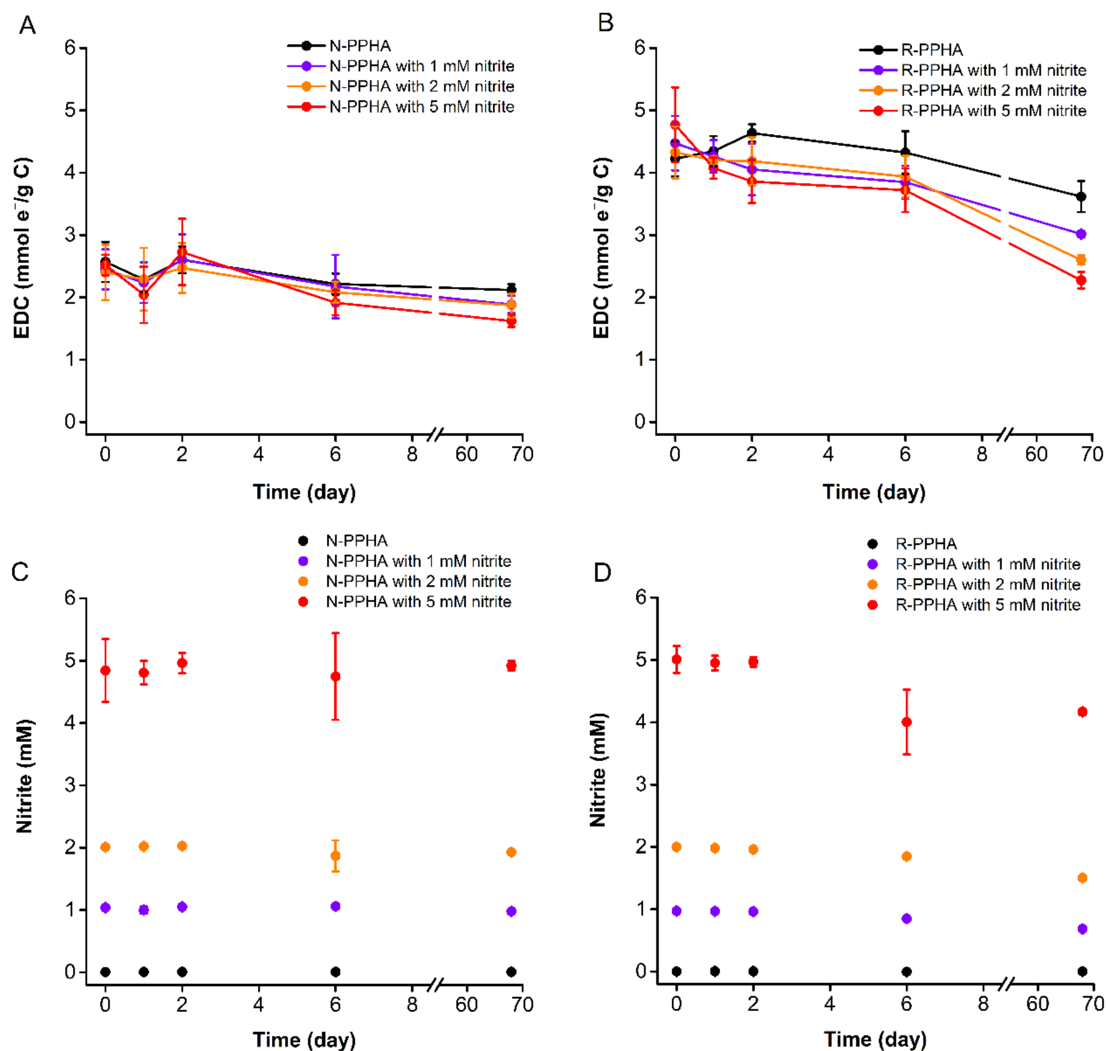



**Figure 3.S1.** Equilibrium of abiotic set-ups with PPHA and nitrite over 68 days. Changes in EDC of native (A) and reduced (B) PPHA samples in the presence of different concentrations of nitrite. Corresponding changes in nitrite of native (C) and reduced (D) PPHA samples. N-PPHA and R-PPHA represent native PPHA and reduced PPHA, respectively. All data are means ± SD.

# Chapter 4

## Do hydroquinones serve as electron donors for $N_2O$ reduction by non-denitrifying $N_2O$ reducers and complete denitrifiers?

**Abstract**

Humic substances (HS) oxidation coupled to nitrous oxide ($N_2O$) reduction is thermodynamically feasible. This process has been reported to be mediated by microbes, but mechanistic studies are rare. $N_2O$-reducing bacteria include non-denitrifying $N_2O$ reducers and complete denitrifiers. The former is recognized as key player in curbing global $N_2O$ emissions. However, it remains unclear whether non-denitrifying $N_2O$ reducer could use HS as electron donor for $N_2O$ respiration. This study evaluated the ability of three $N_2O$ reducing bacteria to couple $N_2O$ reduction with the oxidation of electrochemically reduced hydroquinone $AH_2QDS$ (anthrahydroquinone-2,6-disulfonate, a HS analog). The strains were the putative non-denitrifying $N_2O$ reducer *Desulfitobacterium hafniense* Y51 and the complete denitrifying strains *Paracoccus denitrificans* and *Thiobacillus denitrificans*. Using cell suspension experiments, we observed that all strains failed to take up electrons from the extracellular hydroquinone $AH_2QDS$ for $N_2O$ reduction. However, the strain *P. denitrificans* oxidized $AH_2QDS$ for denitrification with $N_2O$ as end product, i.e., without further reduction of $N_2O$ to $N_2$. It appeared that neither added (exogenic) $N_2O$ nor biogenic (endogenic) $N_2O$ was usable for hydroquinone oxidation. However, future studies could optimize the incubation strategies to further clarify the process of microbially mediated HS oxidation coupling to $N_2O$ reduction.

## 4.1 Introduction

Nitrous oxide ($N_2O$) is a powerful greenhouse gas and the major cause of stratospheric ozone destruction (Ravishankara et al., 2009). $N_2O$-reducing microbes, which possess $N_2O$ reductases (NosZ) encoding by *nosZ* genes, are crucial players in mitigating global $N_2O$ emissions (Jones et al., 2014). According to ecophysiology traits, microbial pathways of $N_2O$ reduction can be classified into complete denitrification ($NO_3^-/NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and non-denitrifying $N_2O$ reduction (without reducing $NO_2^-$ to nitrogenous gas) (Hallin et al., 2017). Denitrifying microorganisms often contain clade I nosZ genes. Non-denitrifying $N_2O$ respiring microorganisms often harbor cladeII *nosZ* genes, the prevalence of such genes has recently been reported in various nature habitats (Sanford et al., 2012). Unlike respiratory denitrification which could form $N_2O$ as an end-product, the process of non-denitrifying $N_2O$ reduction is thought to be the true sink of $N_2O$ (Domeignoz-Horta et al., 2016; Hallin et al., 2017). So far, the physiological function of non-denitrifying $N_2O$ reduction have only been validated in a few pure culture studies (Simon et al., 2006; Sanford et al., 2012; Yoon et al., 2016).

Humic substances (HS) are redox active as a bulk electron donor through mineralization but also as reversible electron donors and acceptors through their quinone moieties. Thus, they are involved in many biogeochemical processes under anoxic conditions (Lovley et al., 1996; Lovley et al.,1999; Roden et al., 2010). Despite $N_2O$ is among the most potent electron acceptor in nature, the coupling of microbial HS oxidation to $N_2O$ reduction has been scarcely reported. Aranda-Tamaura et al. (2007) have reported the $N_2O$ fueling $AH_2QDS$ (anthrahydroquinone-2,6-disulfonate, the reduced form of a HS analog) oxidation by a denitrifying sludge. More specifically, microbial HS oxidation coupled to non-denitrifying $N_2O$ has not been verified experimentally to date.

To elucidate the role that HS play in $N_2O$ mitigation, we performed laboratory incubations to study the ability to oxidize reduced HS by several microbial pure cultures. These strains include a potential non-denitrifying $N_2O$-reducing bacterium *Desulfitobacterium hafniense* Y51 (Sanford et al., 2012) possessing cladeII nosZ genes, as well as two well-studied complete denitrifiers containing clade I nosZ genes, i.e., heterotrophic denitrifying bacteria *Paracoccus denitrificans* and the autotrophic denitrifying bacteria *Thiobacillus denitrificans.* Strain Y51 is known as versatile organohalide-respiring bacteria (OHRB) (Suyama et al., 2001; Kruse et al., 2017). Notably, the Y51 genome lacks the *nirS* and *nirK* genes encoding $NO_2^-$ reductase but harbors the cladeII *nosZ* genes encoding $N_2O$ reductase (Sanford et al., 2012), indicating that this strain could be able to catalyze non-denitrifying $N_2O$ reduction. To

our knowledge, such a metabolism of Y51 has not been identified in previous studies. *P. denitrificans* was reported to oxidize $AH_2QDS$ and microbially reduced humic acid for nitrate reduction (Lovley et al.,1999). Yet, it remains unclear whether it can respire exogenous $N_2O$ with $AH_2QDS$ oxidation. *T. denitrificans* is a well-known obligate autotrophic denitrifier (Beller et al., 2006; Shao et al., 2010) and was shown to utilize humic acid as electron donor for nitrate reduction in syntrophy with *Geobacter sulfurreducens* (Zheng et al., 2018). Likewise, this raises the question whether *Thiobacillus denitrificans* can oxidize humic acid for autotrophic $N_2O$ reduction.

This study mainly primarily aimed to evaluate the ability of the potential non-denitrifying $N_2O$ reducer *Desulfitobacterium hafniense* Y51 to oxidize reduced AQDS with $N_2O$ reduction. Two well-defined complete denitrifying strains (*Paracoccus denitrificans* and *Thiobacillus denitrificans*) were also examined. To this end, the HS analog AQDS (anthraquinone-2,6-disulfonate) was electrochemical reduced to $AH_2QDS$ and subsequently used as potential electron donor for a suit of cell suspension experiments. $N_2O$ was supplied as a potential electron acceptor.

### 4.2 Materials and Methods

**Electrochemical Reduction of AQDS.** An AQDS (Sigma-Aldrich) stock solution of 25 mM was prepared in 0.1 M KCl as electrolyte. The electrochemical reduction of AQDS was performed inside an anoxic glovebox ($N_2$ atmosphere, MBRAUN UNIlab, Germany) using direct electrochemical reduction (DER) technique (Aeschbacher et al., 2010). The applied constant potential was −0.59V vs standard hydrogen electrode (SHE), controlled by a potentiostat (1000C Muti-potentiostat, CH Instruments, USA). After complete reduction of AQDS, the pH of the $AH_2QDS$ solution was adjusted to 7 and sterile filtered (0.22-µm) prior to reaction experiments.

**Bacteria Strain and Cultivation.** All strains were cultured anaerobically in 30 mM bicarbonate-buffered mineral medium (pH 7.0). The basal medium consisted of (per liter) 0.3 g $KH_2PO_4$, 0.3 g $NH_4Cl$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, and 0.85 g $MgCl_2 \cdot 6H_2O$. After autoclaving and cooling, the medium was supplemented with 1 mL (per liter) trace elements solution SL10 (Tschech and Pfennig, 1984), selenate–tungstate (Widdel, 1980) and vitamins (Widdel and Pfennig, 1981). The medium was distributed into 58 mL serum bottles contained 25ml medium and a $N_2/CO_2$ (80/20%, v/v) headspace. All bottles were sealed with butyl rubber stoppers. *Desulfitobacterium hafniense* strain Y51 was grown on 20 mM Na-lactate and 20 mM Na-fumarate. *Paracoccus denitrificans* was grown on 20 mM Na-acetate and 40 mM Na-nitrate. *Thiobacillus denitrificans* was grown on 20 mM Na-thiosulfate and 40mM Na-nitrate.

All culture bottles were incubated at 28 °C in the dark.

**$N_2O$ Reduction Experiments.** Cells ware harvested anaerobically at late exponential phase by centrifugation (10000 rpm; 5 min), washed three times with 30 mM $NaHCO_3$ buffer (pH 7, flushed with $N_2/CO_2$, 80/20%, v/v), and resuspended in the buffer. The resultant cell density ($OD_{600}$) of *D. hafniense* Y51, *P. denitrificans*, *T. denitrificans* was around 0.49, 1.70, 1.31 respectively. A complete removal of residual metabolites from pre-culture was confirmed by determining nitrate or lactate content as described later.

For each strain, 1 mL of the washed cell suspension was immediately inoculated into a 58 mL brown serum bottle containing 25 mL $NaHCO_3$ buffer (30 mM), amended with 1.8 mM $AH_2QDS$ as potential electron donor and 0.5 mL sterilized $N_2O$ (99%) as potential electron acceptor. Additional biological treatments were performed as controls: cells with $AH_2QDS$ (no $N_2O$), cells with $N_2O$ (no $AH_2QDS$). An abiotic control was also set up: $AH_2QDS$ with $N_2O$ (no cells). All treatments were carried out in independent triplicates. All serum flasks were incubated with gentle shaking at 28 °C in the dark. For biological treatments of strain Y51, after sampling at 240 hours of incubation fumarate (4mM) was immediately added as an alternative electron acceptor to evaluate whether strain Y51 can oxidize $AH_2QDS$.

An extra treatment was conducted to examine the ability of strain Y51 to respire $N_2O$ with lactate as electron donor: cells with 6 mM lactate and $N_2O$. This treatment was run in duplicates and one cell-free control was set up. By replacing the $N_2O$ with nitrate (6 mM) as electron acceptor we performed similar treatments to check the nitrate respiration of strain Y51.

**Nitrate Reduction Experiments of Complete Denitrifiers.** To confirm the electrochemically reduced AQDS prepared in this study is microbially utilized, we separately examined its oxidation by cells suspension of *P. denitrificans* but with nitrate as terminal electron acceptor. The strain *T. denitrificans* was also studied. In brief, the strains *P. denitrificans* and *T. denitrificans* were grown, harvested, and washed in the same way as described above, and eventually resuspended to a density with $OD_{600}$ of around 2.14 and 1.66 respectively. Afterwards, 1 mL of the washed cell suspension of *P. denitrificans* was immediately injected into a 58 mL brown serum bottle containing 25 mL $NaHCO_3$ buffer (30 mM), amended with 1.8 mM $AH_2QDS$ and 6 mM nitrate (excess in electron equivalents) or 0.6 mM nitrate (limited in electron equivalents) respectively. For the strain *T. denitrificans*, two mL of the washed cell suspension were added into the same buffer containing 1.8 mM $AH_2QDS$ and 6 mM nitrate. The three treatments were all run in triplicates. One cell-free control with 1.8 mM $AH_2QDS$ and 0.6 mM nitrate was also set up. All serum bottles were incubated at 28 °C in the dark.

Changes in $AH_2QDS$ and AQDS, nitrate, nitrite and ammonium over incubation time as well as headspace $N_2O$ at 24 hours of incubation were quantified as described below.

**Analytical Methods.** The $OD_{600}$ (optical density at a wavelength of 600 nm) of the cells was determined by a UV/VIS spectrophotometer (photoLab 6600, WTW, Germany). All sampling procedures as well as $AH_2QDS$ and AQDS measurements were performed inside an anoxic glovebox.

At specified time intervals, 1 ml culture solution was collected and centrifuged anaerobically (10000 rpm; 10 min) in the glove box. After removal of cell pellets, the supernatant was used for subsequent aqueous metabolites analysis. $AH_2QDS$ and AQDS was determined inside the glove box using the above described UV/VIS approach at the wavelength of 400 nm and 328 nm (Orsetti et al., 2013), respectively. Nitrate, nitrite and ammonium were quantified using a flow injection analysis (FIA) system containing a dialysis membrane for organic ligands removal (Seal Analytical, Norderstedt, Germany). Organic acids including lactate was detected by a high-performance liquid chromatography (HPLC).

At different time points, 0.15 or 0.5 ml of headspace gas was sampled and injected into 20.6 mL crimped vials for storage inside the glove box. $N_2O$ was quantified by TRACE™ 1310 Gas Chromatograph (Thermo Scientific, USA) equipped with a PDD detector. Dilution by sampling at each time was taken into account for calculating the headspace $N_2O$ content. The gas solubility of $N_2O$ at 28°C was $4.026 \bullet 10^{-4}$ (mol gas per mol water at 1 atm partial pressure) calculated according to Wilhelm et al. (1977). It was further expressed as Ostwald coefficient with a value of 0.5502 (L gas per L water at 1 atm partial pressure). Dissolved $N_2O$ in aqueous phase was calculated from the headspace $N_2O$ using the Ostwald coefficient. The sum of headspace and aqueous $N_2O$ represented the total $N_2O$ in the vessel.

## 4.3 Results and Discussion

### 4.3.1 $N_2O$ Reduction Experiments of *D. hafniense* Y51

Initially, the cell suspension experiment contained around 1.8 mM $AH_2QDS$ and 25 µmoles $N_2O$, thus $AH_2QDS$ was 2-fold in excess with regard to reducing capacity. However, strain *D. hafniense* Y51 did not oxidize $AH_2QDS$ with $N_2O$ reduction over incubation (Figure 4.1A and B). After 240 hours of incubation, fumarate (4mM) was supplied to all treatments as an alternative electron acceptor to examine the ability of strain Y51 to oxidize $AH_2QDS$. Figure 4.1A shows that in the presence of $AH_2QDS$ and strain Y51 with or without $N_2O$, the addition of fumarate caused a significant decrease

of $AH_2QDS$ . These results revealed that $AH_2QDS$ could serve as electron donor for fumarate reduction by strain Y51. To the best of my knowledge, the ability of strain Y51 to take up electrons from extracellular hydroquinone has not been reported so far. However, this metabolic activity warrants further confirmation with explicit control treatments.

In parallel, we evaluated whether strain Y51 is capable of performing $N_2O$ reduction using intracellular donor such as lactate instead of the extracellular donor hydroquinone. Unexpectedly, no significant changes in lactate and $N_2O$ was found in the biological treatments containing cell suspension of strain Y51 (Figure 4.1C and D). In the treatment with lactate and nitrate, 0.4 mM lactate was consumed at 240 hours of incubation (Figure 4.S1A) and 0.26 mM nitrite concomitantly accumulated without further decrease even after a prolonged incubation of 672 hours (Figure 4.S1B). Moreover, $N_2O$ was not detectable over the entire incubation period (not shown). These results suggested that strain Y51 oxidized lactate for nitrate reduction to nitrite but not for denitrification (reduce nitrite to nitrogenous gas). However, the extent and rate of lactate oxidation coupled to nitrate reduction is limited. It seemed that compared to pyruvate (Nonaka et al., 2006; Peng et al., 2012), lactate may not be a preferable electron donor for N-oxide respiration by strain Y51. In this sense, the obtained results do not necessarily imply that strain Y51 in unable to catalyze $N_2O$ reduction.

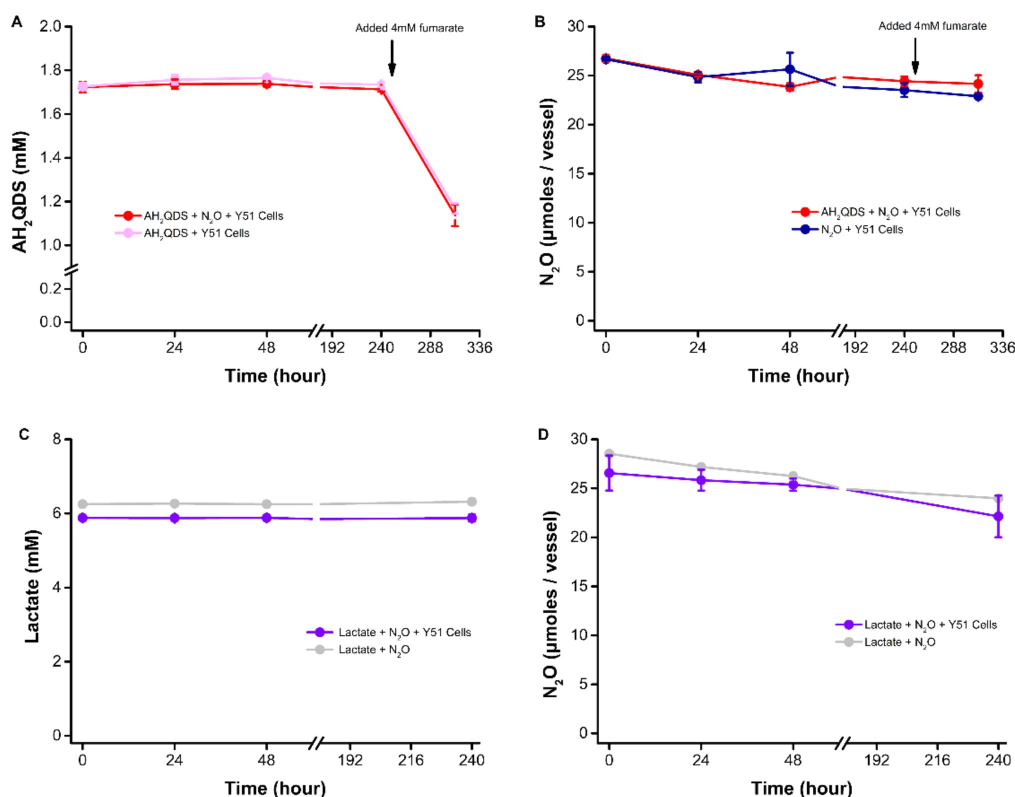


**Figure 4.1.** Changes in $AH_2QDS$ (A) and total $N_2O$ (B) in treatments for strain Y51 over incubation time. Changes in lactate (C) and total $N_2O$ (D) in treatments for strain Y51 over incubation time. Samples were taken at 0, 24, 48, and 240 or plus 312 hours of incubation. Data are means ± SD (A and B) and means ± ranges of duplicates (C and D) except the one cell-free controls.

### 4.3.2 $N_2O$ Reduction Experiments of *P. denitrificans* and *T. denitrificans*.

We did not observe clear evidence for coupling of $N_2O$ reduction to oxidation of $AH_2QDS$ by *P. denitrificans* (Figure 4.2A and C) and *T. denitrificans* (Figure 4.2B and D) over 240 hours of incubation. When comparing the biological treatments of the strain *P. denitrificans*, we found that the presence of $N_2O$ slightly promoted the decrease in $AH_2QDS$ (Figure 4.2A) over the entire incubation. However, no difference in $N_2O$ content was observed between the two cell-containing treatments. The observed $N_2O$ reduction to a limited extent could be derived from the internally stored electron donor of *P. denitrificans*. Nonetheless, we cannot conclude that *P. denitrificans* is able to oxidize $AH_2QDS$ coupling to exogenous $N_2O$ reduction under our experimental conditions.

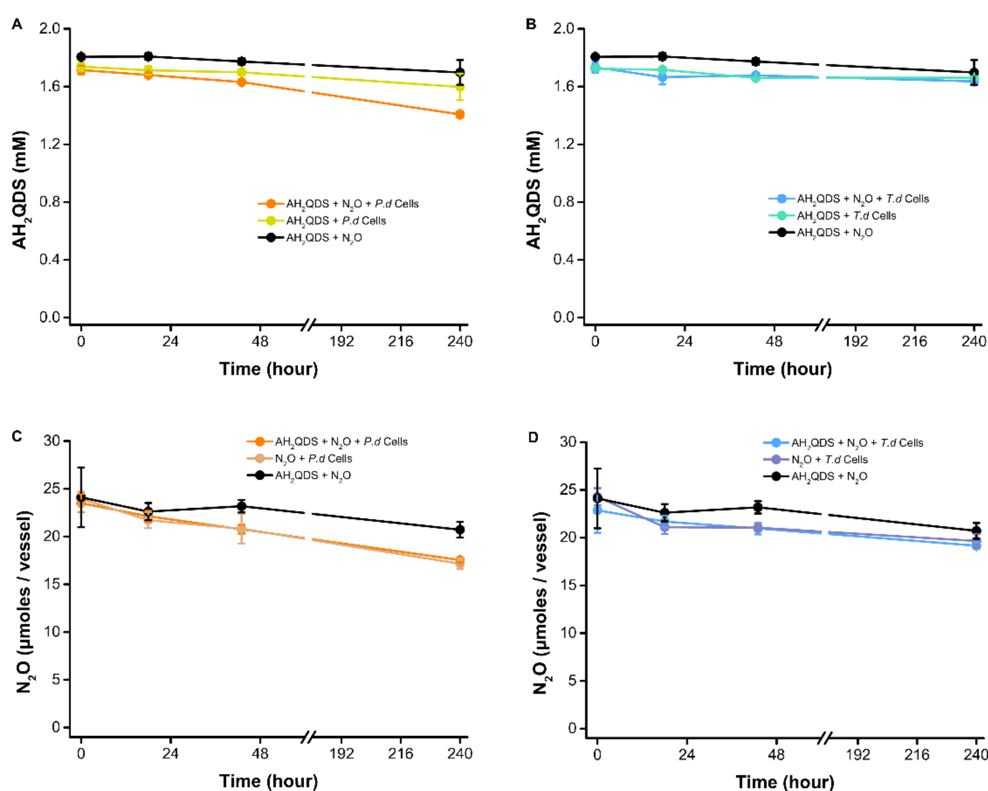


**Figure 4.2.** Changes in $AH_2QDS$ (A) and total $N_2O$ (C) in treatments for *P. denitrificans* over incubation time. Changes in reduced AQDS (B) and total $N_2O$ (D) in treatments for *T. denitrificans* over incubation time. Samples were taken at 0, 18, 43 and 240 hours of incubation. *P. d* and *T. d* are abbreviations of *P. denitrificans* and *T. denitrificans*, respectively. All data are means ± SD.

### 4.3.3 Nitrate Reduction Experiments of *P. denitrificans* and *T. denitrificans*.

*P. denitrificans* did oxidize $AH_2QDS$ coupled to denitrification. As shown in Figure 4.3A, $AH_2QDS$ was oxidized rapidly within 24-hour incubation under both the nitrate-excess (5mM) and nitrate-limited (0.6 mM) conditions. This observation was in line with Lovley et al. (1999) that reported *P. denitrificans* can oxidize $AH_2QDS$ produced by $H_2$/Pd catalyst coupled to nitrate reduction. Despite nitrate reduction products were not examined by Lovley et al. (1999), the stoichiometry indicated $N_2$ as the denitrifying end product. In our study, after 24-hour incubation the ratio of $AH_2QDS$ oxidation to nitrate reduction was 1.69 ± 0.13 (mean ± SD; n=3) under nitrate-excess and 1.75 ± 0.11 (mean ± SD; n=3) under nitrate-limitation conditions (Figure 4.3A and B). Since the cells likely used internally stored electron donor for nitrate reduction, the observed ratio should be understoichiometric. However, the theoretical ratio of $AH_2QDS$ oxidation to nitrate reduction with NO, $N_2O$ or $N_2$ as the sole products are 1.5, 2 or 2.5 respectively. Therefore, we hypothesize that in our treatments nitrate has been denitrified to nitrogenous gas with $N_2O$ as the predominant end product at 24 hours of incubation. This was further supported by the observations that at 24 hours of incubation under both nitrate-excess and nitrate-limitation conditions i) no nitrite and ammonium was detected; ii) $N_2O$ was accumulated (Figure 4.3C) and accounted for 88.1 ± 8.2% (mean ± SD; n=3) and 115.4 ± 11.8% (mean ± SD; n=3) of N loss from nitrate, respectively. Hence, the formation of nitrogenous gas differed between our study and Lovley et al. (1999) for so far unknown reasons. One difference between these studies was the method of AQDS reduction which involved $H_2$ in Lovely et al. (1999) and thus potentially in that study molecular $H_2$ gas might have been present in the experimental setups. Taken together, *P. denitrificans* can use the electrochemically reduced AQDS as electron donor for denitrification with $N_2O$ rather than $N_2$ as the main end product.

Of the *T. denitrificans* examined in nitrate reduction experiments, no significant decrease in $AH_2QDS$ concentration was observed in the presence of excess nitrate (5mM) over the 48-hour incubation (Figure 4.3A). In contrast to the cell-free control, however, there was a dramatic loss in $AH_2QDS$ at the beginning of the incubation even if considering the addition of 2 ml cell suspension into the 25 ml $NaHCO_3$ buffer caused

a slight dilution of $AH_2QDS$ (Figure 4.3A). The reason for this remains unclear and needs to be studied explicitly. In spite of $N_2O$ was detected at 24 hours of incubation and accounted for 37.0 ± 3.8% (mean ± SD; n=3) of N loss (Figure 4.3C), we cannot exclude the possibility of *T. denitrificans* utilizing internally stored electron donor for denitrification. In general, whether *T. denitrificans* can truly oxidize $AH_2QDS$ coupling to denitrification warrants further investigation.

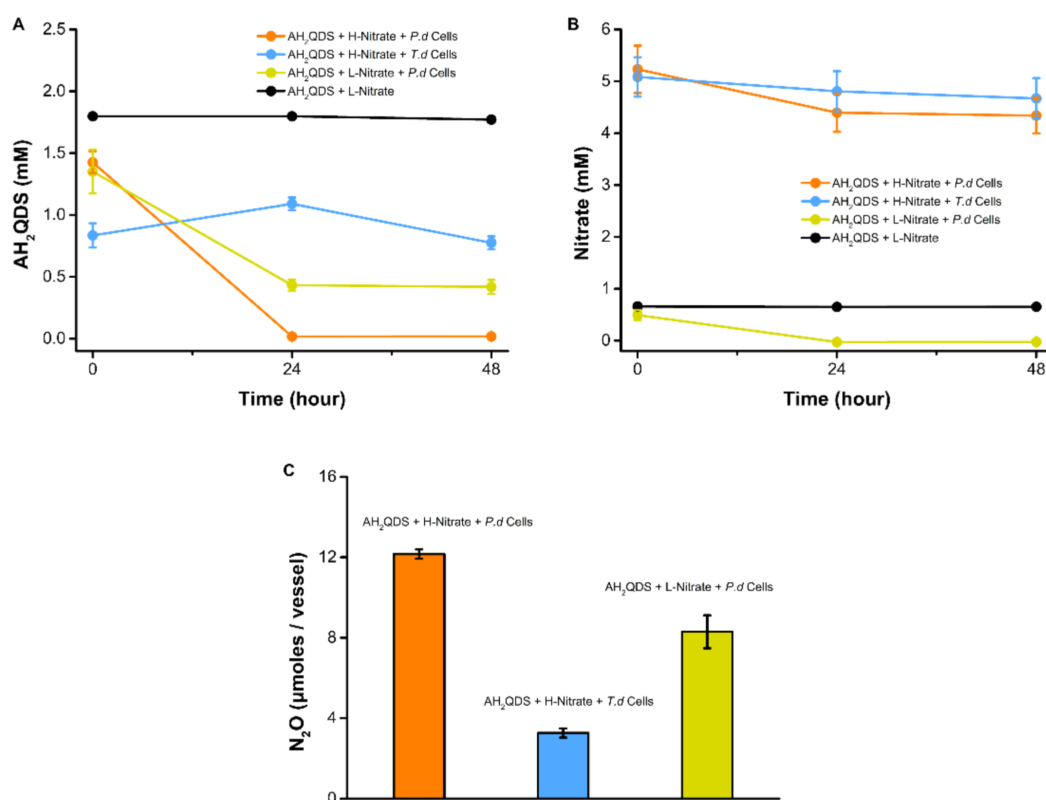



**Figure 4.3.** Changes in $AH_2QDS$ (A) and nitrate (B) in treatments over incubation time. Samples were taken at 0, 24 and 48 hours of incubation. Total $N_2O$ (C) in treatments at 24 hours of incubation. H-Nitrate and L-Nitrate represent higher (excess) nitrate addition and lower (limited) nitrate addition, respectively. *P. d* and *T. d* are abbreviations of *P. denitrificans* and *T. denitrificans*, respectively. All data are means ± SD except the one cell-free control.

**Supporting information**

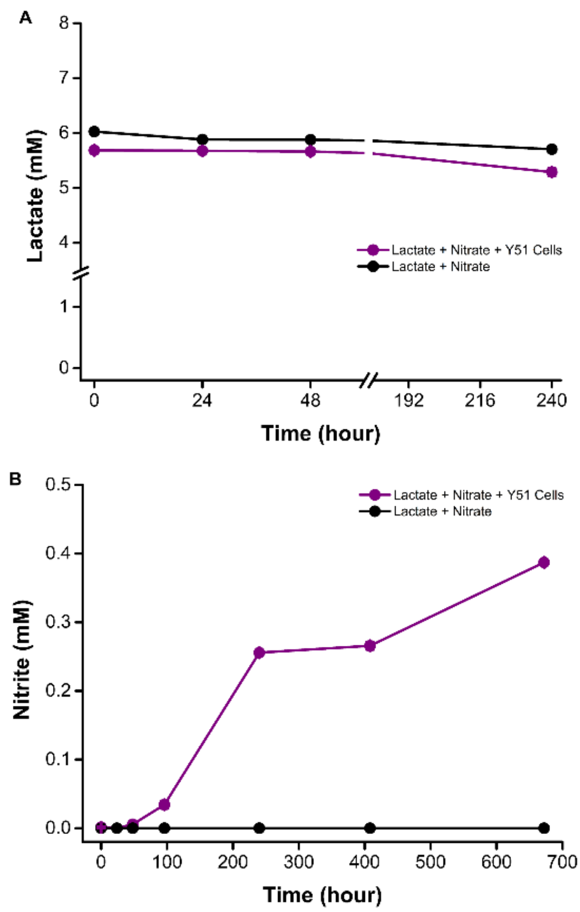



**Figure 4.S1.** Changes in lactate (A) and accumulation of nitrite (B) in treatments for strain Y51 over incubation time. Data are means ± ranges of duplicates except the one cell-free control.

# Chapter 5

## Conclusions and Outlook

## 5.1 Conclusions

The application of mediated electrochemical analysis on studying humics-driven anoxic C and N cycling is in its early stages. This PhD thesis advanced our understanding and methodology to use this approach in these research fields with emphasis on microbial N cycling. To do this, a synthesized perspective and multiple batch experiments were conducted, and the frontiers and results were presented in individual chapters. The major conclusions are as follows:

**Microbial redox cycling of natural organic matter linked to greenhouse gases mitigation and nitrogen loss: application of mediated electrochemical analysis**

By mimicking shuttle-based electron transfer processes occurring in nature, the application of the mediated electrochemical analysis technique allows for tracing the reversible electron flux to and from NOM during microbial extracellular respiration. Thus, mediated electrochemical analysis is a powerful yet underemployed tool in exploring the linkages between NOM redox cycling and gaseous C and N turnover driven by microbes. To address these research gaps, we anticipate that in forthcoming studies the prospects of mediated electrochemical analysis will be recognized and that this analytical technique will be utilized as a crucial and essential tool also in the fields of geomicrobiology and environmental microbiology.

**A denitrifier method for nitrite removal in electrochemical analysis of the electron accepting capacity of humic substances**

To summarize, we promoted the use of mediated electrochemical analysis in studying HS-driven anoxic N cycling that was so far not possible due to interference of (biogenic) nitrite in EAC quantification. We established a denitrifier assay to selectively eliminate such (biogenic) nitrite from HS sample without any alteration of the redox properties. The denitrifier assay was proven as robust method at circumneutral pH, thereby avoiding pH-dependent side reactions.

**Do hydroquinones serve as electron donors for $N_2O$ reduction by non-denitrifying $N_2O$ reducers and complete denitrifiers?**

In our study we did not observe reduction of added $N_2O$ coupled to hydroquinone oxidation by the potential non-denitrifying $N_2O$ reducer *Desulfitobacterium hafniense* Y51 and the complete denitrifiers *Paracoccus denitrificans* and *Thiobacillus denitrificans*. The strain *P. denitrificans*, however, oxidized electrochemically reduced AQDS for denitrification with $N_2O$ as the main end product without apparent further

reduction of $N_2O$ to $N_2$. It seemed that even reduction of biogenic $N_2O$ was not supported by the hydroquinone.

## 5.2 Outlook

Accordingly, the major outlook for each chapter are:

**Microbial redox cycling of natural organic matter linked to greenhouse gases mitigation and nitrogen loss: application of mediated electrochemical analysis**
Protocols to eliminate the interference from *Geobacter sulfurreducens* cells in mediated electrochemical analysis of solid NOM samples require to be developed. Intriguingly, mediated electrochemical analysis appears to be alternative technique to determine the redox state of cytochromes next to known methods such as fluorescence (Esteve-Núñez et al., 2008), electrochemical impedance spectroscopy (Malvankar et al., 2012) and Confocal Resonance Raman Microscopy (Virdis et al., 2014). Herein, I propose to apply mediated electrochemical analysis on studying redox chemistry of cytochromes in future investigations.

**A denitrifier method for nitrite removal in electrochemical analysis of the electron accepting capacity of humic substances**
Future investigations could reveal more nitrite reducers including denitrifiers and ammonifiers (catalyze DNRA process) which can neither oxidize nor reduce the tested HS sample for nitrite scavenge, not only limited to the strain *Pseudomonas nitroreducens* used in this study. In short, such bioassays will underpin the applications of mediated electrochemical analysis on expanding our understanding upon electron transfer mechanisms of HS-fueled biogeochemical N cycling.

**Do hydroquinones serve as electron donors for $N_2O$ reduction by non-denitrifying $N_2O$ reducers and complete denitrifiers?**
Next to non-growth conditions used in cell suspension experiments, future studies addressing such metabolic pathways should include growth mineral medium containing micronutrients required for synthesis of $N_2O$ reductase. For instance, $N_2O$ reductase is copper-dependent and its activity could be inhibited under copper deficient conditions (Richardson et al., 2009; Sullivan et al., 2013). Additionally, N-free mineral medium without inorganic N in the aqueous and without $N_2$ in the headspace (e.g., using an

Ar/$CO_2$ or He/$CO_2$ headspace) can be applied to provide a selective pressure to bacteria for $N_2O$ respiration (Conthe et al., 2018). Moreover, lowering the dose of added quinone model compounds (e.g., $AH_2QDS$) could be considered to minimize toxicity effects (if any) to cells (Nevin and Lovley, 2000). Further investigations of electron transfer mechanisms of HS-driven $N_2O$ reduction process are required to promote the development of strategies for climate change mitigation. Specifically, when HS samples rather than quinone model compounds are used in such studies, mediated electrochemical analysis can be used to track changes in redox state of HS samples with a wide range of standard reduction potentials.

# References

Aeschbacher, M., Graf, C., Schwarzenbach, R.P., Sander, M., 2012. Antioxidant properties of humic substances. Environ. Sci. Technol. 46, 4916–4925. https://doi.org/10.1021/es300039h

Aeschbacher, M., Sander, M., Schwarzenbach, R.P.,2010. Novel electrochemical approach to assess the redox properties of humic substances. Environ. Sci. Technol. 44, 87–93. https://doi.org/10.1021/es902627p

Aeschbacher, M., Vergari, D., Schwarzenbach, R.P., Sander, M., 2011. Electrochemical analysis of proton and electron transfer equilibria of the reducible moieties in humic acids. Environ. Sci. Technol. 45, 8385–8394. https://doi.org/10.1021/es201981g

Aranda-Tamaura, C., Estrada-Alvarado, M. I., Texier, A. C., Cuervo, F., Gómez, J., Cervantes, F. J., 2007. Effects of different quinoid redox mediators on the removal of sulphide and nitrate via denitrification. Chemosphere. 69 (11), 1722-1727. https://doi.org/10.1016/j.chemosphere.2007.06.004

Bauer, M., Heitmann, T., Macalady, D. L., Blodau, C., 2007. Electron transfer capacities and reaction kinetics of peat dissolved organic matter. Environ. Sci. Technol. 41 (1), 139-145. https://doi.org/10.1021/es061323j

Beal, E. J., House, C. H., Orphan, V. J., 2009. Manganese-and iron-dependent marine methane oxidation. Science. 325 (5937), 184-187. https://doi.org/10.1126/science.1169984

Beller, H. R., Chain, P. S., Letain, T. E., Chakicherla, A., Larimer, F. W., Richardson, P. M., Coleman, M. A., Wood, A. P., Kelly, D. P., 2006. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. J. Bacteriol. 188 (4), 1473-1488. https://doi.org/10.1128/JB.188.4.1473-1488.2006

Benz, M., Schink, B., Brune, A., 1998. Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. Appl. Environ. Microbiol. 64 (11), 4507-4512. https://doi.org/10.1016/S0027-5107(98)00209-7

Bernhardt, E. S., Schlesinger, W. H., 2013. Biogeochemistry: an analysis of glo bal change. Academic press.

Blodau, C., Bauer, M., Regenspurg, S., Macalady, D., 2009. Electron accepting capacity of dissolved organic matter as determined by reaction with metallic zi nc. Chem.Geol. 260 (3-4), 186-195. https://doi.org/10.1016/j.chemgeo.2008.12.01 6

Boetius, A., Ravenschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann R., Jörgensen, B. B., Witte, U., Pfannkuche, O., 2000. A marine m icrobial consortium apparently mediating anaerobic oxidation of methane. Natur e. 407 (6804), 623. https://doi.org/10.1038/35036572

Boone, D. R., Johnson, R. L., Liu, Y. T., 1989. Diffusion of the interspecies e lectron carriers $H_2$ and formate in methanogenic ecosystems and its implication s in the measurement of Km for $H_2$ or formate uptake. Appl. Environ. Microbi ol. 55 (7), 1735-1741. https://doi.org/10.1088/0032-1028/16/10/002

Böhlke, J. K., Smith, R. L., Hannon, J. E., 2007. Isotopic analysis of N and O in nitrite and nitrate by sequential selective bacterial reduction to $N_2O$. Anal. Chem. 79 (15), 5888-5895. https://doi.org/10.1021/ac070176k

Bryant, M. P., Wolin, E. A., Wolin, M. J., Wolfe, R. S., 1967. *Methanobacillu s omelianskii*, a symbiotic association of two species of bacteria. Archiv für M ikrobiologie. 59 (1-3), 20-31. https://doi.org/10.1007/BF00406313

Buffle, J., Cominoli, A., 1981. Voltammetric study of humic and fulvic substan ces: Part IV. Behaviour of fulvic substances at the mercury-water interface. J. Electroanal. Chem. Interfa. Electrochem. 121, 273-299. https://doi.org/10.1016/S0 022-0728(81)80585-2

Chen, J., Gu, B.H., Royer, R. A., Burgos, W. D., 2003. The roles of natural o rganic matter in chemical and microbial reduction of ferric iron. Sci. Total. En viron. 307 (1-3), 167-178. https://doi.org/10.1016/S0048-9697(02)00538-7

Chen, S.S., Rotaru, A. E., Liu, F.H., Philips, J., Woodard, T. L., Nevin, K. P., Lovley, D. R., 2014a. Carbon cloth stimulates direct interspecies electron transf er in syntrophic co-cultures. Bioresource Technol. 173, 82-86. https://doi.org/10.

1016/j.biortech.2014.09.009

Chen, S.S., Rotaru, A. E., Shrestha, P. M., Malvankar, N. S., Liu, F.H., Fan, W., Nevin, K.P., Lovley, D. R., 2014b. Promoting interspecies electron transfer with biochar. Sci. Rep. 4. 5019. https://doi.org/10.1038/srep05019

Clark, W. M., 1960. Oxidation-Reduction Potentials of Organic Systems. Baltim ore, Williams & Wilkins.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Ben der, K. S., Achenbach, L. A., 2001. Anaerobic benzene oxidation coupled to ni trate reduction in pure culture by two strains of Dechloromonas. Nature. 411 (6841), 1039. https://doi.org/10.1038/35082545

Coates, J. D., Cole, K. A., Chakraborty, R., O'Connor, S. M., Achenbach, L. A., 2002. Diversity and ubiquity of bacteria capable of utilizing humic substan ces as electron donors for anaerobic respiration. Appl. Environ. Microbiol. 68 (5), 2445-2452. https://doi.org/10.1128/AEM.68.5.2445-2452.2002

Conthe, M., Kuenen, J. G., Kleerebezem, R., van Loosdrecht, M. C., 2018. Ex ploring microbial $N_2O$ reduction: a continuous enrichment in nitrogen free medi um. Environ. Microbiol. Rep. 10 (1), 102-107. https://doi.org/10.1111/1758-2229.12615

Cruz-García, C., Murray, A. E., Klappenbach, J. A., Stewart, V., Tiedje, J. M., 2007. Respiratory nitrate ammonification by *Shewanella oneidensis* MR-1. J. Ba cteriol. 189 (2), 656-662. https://doi.org/10.1128/JB.01194-06

Dalsgaard, T., Thamdrup, B., 2002. Factors controlling anaerobic ammonium ox idation with nitrite in marine sediments. Appl. Environ. Microbiol. 68 (8), 380 2-3808. https://doi.org/10.1128/AEM.68.8.3802-3808.2002

Deutzmann, J. S., Stief, P., Brandes, J., Schink, B., 2014. Anaerobic methane o xidation coupled to denitrification is the dominant methane sink in a deep lake. Proc. Natl. Acad. Sci. U.S.A. 111 (51), 18273-18278. https://doi.org/10.1073/pnas.1411617111

Ding, L. J., An, X. L., Li, S., Zhang, G. L., Zhu, Y. G., 2014. Nitrogen loss

through anaerobic ammonium oxidation coupled to iron reduction from paddy s oils in a chronosequence. Environ. Sci. Technol. 48 (18), 10641-10647. https://doi.org/10.1021/es503113s

Domeignoz-Horta, L. A., Putz, M., Spor, A., Bru, D., Breuil, M. C., Hallin, S., Philippot, L., 2016. Non-denitrifying nitrous oxide-reducing bacteria-An effective $N_2O$ sink in soil. Soil Biol. Biochem. 103, 376-379. https://doi.org/10.1016/j.soilbio.2016.09.010

Esteve-Núñez, A., Sosnik, J., Visconti, P., Lovley, D. R., 2008. Fluorescent properties of c‐type cytochromes reveal their potential role as an extracytoplasmic electron sink in *Geobacter sulfurreducens*. Environ. Microbiol. 10 (2), 497-505. https://doi.org/10.1111/j.1462-2920.2007.01470.x

Estevez-Canales, M., Kuzume, A., Borjas, Z., Füeg, M., Lovley, D., Wandlowski, T., Esteve-Núñez, A., 2015. A severe reduction in the cytochrome C content of *Geobacter sulfurreducens* eliminates its capacity for extracellular electron transfer. Environ. Microbiol. Rep. 7 (2), 219-226. https://doi.org/10.1111/1758-2229.12230

Ettwig, K. F., Butler, M. K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M. M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J.,van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, HJ., Janssen-Megens, EM., Francoijs, KJ., Stunnenberg, H., Weissenbach, J., Jetten, M.,S., Strous, M., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature. 464 (7288), 543. https://doi.org/10.1038/nature08883

Ettwig, K. F., Zhu, B.L., Speth, D., Keltjens, J. T., Jetten, M. S., Kartal, B., 2016. Archaea catalyze iron-dependent anaerobic oxidation of methane. Proc. Natl. Acad. Sci. U.S.A. 113 (45), 12792-12796. https://doi.org/10.1073/pnas.1609534113

Falkowski, P. G., Fenchel, T., Delong, E. F., 2008. The microbial engines that drive Earth's biogeochemical cycles. Science. 320 (5879), 1034-1039. https://doi.org/10.1126/science.1153213

Figueroa, I. A., Barnum, T. P., Somasekhar, P. Y., Carlström, C. I., Engelbrekts

on, A. L., Coates, J. D., 2018. Metagenomics-guided analysis of microbial chemolithoautotrophic phosphite oxidation yields evidence of a seventh natural $CO_2$ fixation pathway. Proc. Natl. Acad. Sci. U.S.A. 115 (1), E92-E101. https://doi.org/10.1073/pnas.1715549114

Granger, J., Sigman, D. M., 2009. Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method. Rapid Commun. Mass Spectrom. 23 (23), 3753-3762. https://doi.org/10.1002/rcm.4307

Hallin, S., Philippot, L., Löffler, F. E., Sanford, R. A., Jones, C. M., 2017. Genomics and ecology of novel $N_2O$-reducing microorganisms. Trends Microbiol. 26, 43-55. https://doi.org/10.1016/j.tim.2017.07.003

Haroon, M. F., Hu, S.H., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z.G., Tyson, G. W., 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature. 500 (7464), 567. https://doi.org/10.1038/nature12375

Heitmann, T., Blodau, C., 2006. Oxidation and incorporation of hydrogen sulfide by dissolved organic matter. Chem.Geol. 235 (1-2), 12-20. https://doi.org/10.1016/j.chemgeo.2006.05.011

Heitmann, T., Goldhammer, T., Beer, J., Blodau, C., 2007. Electron transfer of dissolved organic matter and its potential significance for anaerobic respiration in a northern bog. Global Change Biol. 13 (8), 1771-1785. https://doi.org/10.1111/j.1365-2486.2007.01382.x

Helburn, R. S., MacCarthy, P., 1994. Determination of some redox properties of humic acid by alkaline ferricyanide titration. Anal. Chim.Acta. 295 (3), 263-272. https://doi.org/10.1016/0003-2670(94)80231-9

Holler, T., Wegener, G., Niemann, H., Deusner, C., Ferdelman, T. G., Boetius, A., Brunner, B., Widdel, F., 2011. Carbon and sulfur back flux during anaerobic microbial oxidation of methane and coupled sulfate reduction. Proc. Natl. Acad. Sci. U.S.A. 108 (52), E1484-E1490. https://doi.org/10.1073/pnas.1106032108

Hu, Z.Y., Wessels, H. J., van Alen, T., Jetten, M. S., Kartal, B., 2019. Nitric

oxide-dependent anaerobic ammonium oxidation. Nat. Commun. 10, 1244. https://doi.org/10.1038/s41467-019-09268-w

IPCC., 2013. Climate change 2013: the physical science basis. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jelen, B. I., Giovannelli, D., Falkowski, P. G., 2016. The role of microbial electron transfer in the coevolution of the biosphere and geosphere. Annu. Rev. Microbiol. 70, 45-62. https://doi.org/10.1146/annurev-micro-102215-095521

Jiang, J., Kappler, A., 2008. Kinetics of microbial and chemical reduction of humic substances: implications for electron shuttling. Environ. Sci. Technol. 42 (10), 3563-3569. https://doi.org/10.1021/es7023803

Jones, C. M., Spor, A., Brennan, F. P., Breuil, M. C., Bru, D., Lemanceau, P., Griffiths, B., Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil $N_2O$ sink capacity. Nat. Clim. Change. 4 (9), 801-805. https://doi.org/10.1038/nclimate2301

Kaden, J., Galushko, A. S., Schink, B., 2002. Cysteine-mediated electron transfer in syntrophic acetate oxidation by cocultures of *Geobacter sulfurreducens* and *Wolinella succinogenes*. Arch. Microbiol. 178 (1), 53-58. https://doi.org/10.1007/s00203-002-0425-3

Kappler, A., Haderlein, S. B., 2003. Natural organic matter as reductant for chlorinated aliphatic pollutants. Environ. Sci. Technol. 37 (12), 2714-2719. https://doi.org/10.1021/es0201808

Kappler, A., Benz, M., Schink, B., Brune, A., 2004. Electron shuttling via humic acids in microbial iron (III) reduction in a freshwater sediment. FEMS Microbiol. Ecol. 47 (1), 85-92. https://doi.org/10.1016/S0168-6496(03)00245-9

Kato, S., Hashimoto, K., Watanabe, K., 2012a. Methanogenesis facilitated by electric syntrophy via (semi) conductive iron-oxide minerals. Environ. Microbiol.

14 (7), 1646-1654. https://doi.org/10.1111/j.1462-2920.2011.02611.x

Kato, S., Hashimoto, K., Watanabe, K., 2012b. Microbial interspecies electron t ransfer via electric currents through conductive minerals. Proc. Natl. Acad. Sci. U.S.A. 109 (25), 10042-10046. https://doi.org/10.1073/pnas.1117592109

Klüpfel, L., Piepenbrock, A., Kappler, A., Sander, M., 2014. Humic substances as fully regenerable electron acceptors in recurrently anoxic environments. Nat. Geosci. 7, 195–200. https://doi.org/10.1038/ngeo2084

Kouzuma, A., Kato, S., Watanabe, K., 2015. Microbial interspecies interactions: recent findings in syntrophic consortia. Front. Microbiol. 6, 477. https://doi.org/10.3389/fmicb.2015.00477

Kruse, T., Goris, T., Maillard, J., Woyke, T., Lechner, U., de Vos, W., Smidt, H., 2017. Comparative genomics of the genus Desulfitobacterium. FEMS Micro biol. Ecol. 93 (12), fix135. https://doi.org/10.1093/femsec/fix135

Kuzyakov, Y., 2010. Priming effects: interactions between living and dead orga nic matter. Soil Biol. Biochem. 42 (9), 1363-1371. https://doi.org/10.1016/j.soilbio.2010.04.003

Lai, C. Y., Zhong, L., Zhang, Y., Chen, J. X., Wen, L. L., Shi, L. D., Sun, Y. P., Ma, F., Rittmann, B.E., Zhou, C., Tang, Y., Zheng, P., Zhao, H. P., 2016. Bioreduction of chromate in a methane-based membrane biofilm reactor. Enviro n. Sci. Technol. 50 (11), 5832-5839. https://doi.org/10.1021/acs.est.5b06177

Lau, M.P., Hupfer, M., Grossart, H.P., 2017. Reduction-oxidation cycles of orga nic matter increase bacterial activity in the pelagic oxycline. Environ. Microbio l. Rep. 9, 257–267. https://doi.org/10.1111/1758-2229.12526

Lau, M.P., Sander, M., Gelbrecht, J., Hupfer, M., 2015. Solid phases as import ant electron acceptors in freshwater organic sediments. Biogeochemistry. 123, 4 9–61. https://doi.org/10.1007/s10533-014-0052-5

Lau, M.P., Sander, M., Gelbrecht, J., Hupfer, M., 2016. Spatiotemporal redox d ynamics in a freshwater lake sediment under alternating oxygen availabilities: c ombined analysis of dissolved and particulate electron acceptors. Environ. Che

m. 13 (5), 826-837. https://doi.org/10.1071/EN15217_AC

Lehmann, J., Kleber, M., 2015. The contentious nature of soil organic matter. Nature. 528 (7580), 60. https://doi.org/10.1038/nature16069

Liu, F.H., Rotaru, A. E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., Lovley, D. R., 2012. Promoting direct interspecies electron transfer with activated carbon. Energy Environ. Sci. 5 (10), 8982-8989. https://doi.org/10.1039/C2EE22459C

Liu, F.H., Rotaru, A. E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., Lovley, D. R., 2015. Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. Environ. Microbiol. 17 (3), 648-655. https://doi.org/10.1111/1462-2920.12485

Lovley, D. R., Blunt-Harris, E. L., 1999. Role of humic-bound iron as an electron transfer agent in dissimilatory Fe (III) reduction. Appl. Environ. Microbiol. 65 (9), 4252-4254.

Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J., Woodward, J. C., 1996. Humic substances as electron acceptors for microbial respiration. Nature. 382 (6590), 445. https://doi.org/10.1038/382445a0

Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., Coates, J. D., 1998. Humic substances as a mediator for microbially catalyzed metal reduction. Acta Hydroch. Hydrob. 26 (3), 152-157.

Lovley, D. R., Fraga, J. L., Coates, J. D., Blunt-Harris, E. L., 1999. Humics as an electron donor for anaerobic respiration. Environ. Microbiol. 1 (1), 89-98. https://doi.org/10.1046/j.1462-2920.1999.00009.x

Lovley, D. R., 2012. Electromicrobiology. Annu. Rev. Microbiol. 66, 391-409. https://doi.org/10.1146/annurev-micro-092611-150104

Lovley, D. R., 2017. Syntrophy goes electric: direct interspecies electron transfer. Annu. Rev. Microbiol. 71, 643-664. https://doi.org/10.1146/annurev-micro-030117-020420

Luo, J. H., Chen, H., Hu, S. H., Cai, C., Yuan, Z. G., Guo, J. H., 2018. Microbial Selenate Reduction Driven by a Denitrifying Anaerobic Methane Oxidation Biofilm. Environ. Sci. Technol. 52, 4006-4012. https://doi.org/10.1021/acs.est.7b05046

Luo, J. H., Wu, M. X., Yuan, Z. G., Guo, J. H., 2017. Biological Bromate Reduction Driven by Methane in a Membrane Biofilm Reactor. Environ. Sci. Technol. Lett. 4 (12), 562-566. https://doi.org/10.1021/acs.estlett.7b00488

Luo, Y. H., Chen, R., Wen, L. L., Meng, F., Zhang, Y., Lai, C. Y., Rittmann, B. E., Zhao, H. P., Zheng, P., 2015. Complete perchlorate reduction using methane as the sole electron donor and carbon source. Environ. Sci. Technol. 49 (4), 2341-2349. https://doi.org/10.1021/es504990m

Mahne, I., Tiedje, J. M., 1995. Criteria and methodology for identifying respiratory denitrifiers. Appl. Environ. Microbiol. 61 (3), 1110-1115.

Malvankar, N. S., Mester, T., Tuominen, M. T., Lovley, D. R., 2012. Supercapacitors based on c-type cytochromes using conductive nanostructured networks of living bacteria. Chemphyschem. 13 (2), 463-468. https://doi.org/10.1002/cphc.201100865

Martinez, C. M., Alvarez, L. H., Celis, L. B., Cervantes, F. J., 2013. Humus-reducing microorganisms and their valuable contribution in environmental processes. Appl. Microbiol. Biot. 97 (24), 10293-10308. https://doi.org/10.1007/s00253-013-5350-7

Matthiessen, A., 1995. Determining the redox capacity of humic substances as a function of pH. Vom Wasser. 84, 229-235.

McGlynn, S. E., Chadwick, G. L., Kempes, C. P., Orphan, V. J., 2015. Single cell activity reveals direct electron transfer in methanotrophic consortia. Nature. 526 (7574), 531. https://doi.org/10.1038/nature15512

Milucka, J., Ferdelman, T. G., Polerecky, L., Franzke, D., Wegener, G., Schmid, M., Lieberwirth, I., Wagner, M., Widdel, F., Kuypers, M. M., 2012. Zero-valent sulphur is a key intermediate in marine methane oxidation. Nature. 491 (7425), 541. https://doi.org/10.1038/nature11656

Mota, A. M., Pinheiro, J. P., Gonçalves, M. L., 1994. Adsorption of humic aci d on a mercury/aqueous solution interface. Water Res. 28 (6), 1285-1296. https://doi.org/10.1016/0043-1354(94)90293-3

Motheo, A. J., Pinhedo, L., 2000. Electrochemical degradation of humic acid. Sci. Total. Environ. 256 (1), 67-76. https://doi.org/10.1016/S0048-9697(00)00469-1

Nevin, K. P., Lovley, D. R., 2000. Potential for nonenzymatic reduction of Fe (III) via electron shuttling in subsurface sediments. Environ. Sci. Technol. 34 (12), 2472-2478. https://doi.org/10.1021/es991181b

Nie, S. A., Li, H., Yang, X. R., Zhang, Z. J., Weng, B. S., Huang, F. Y., Zhu, G. B., Zhu, Y. G., 2015. Nitrogen loss by anaerobic oxidation of ammonium in rice rhizosphere. ISME J. 9 (9), 2059-2067. https://doi.org/10.1038/ismej.2015.25

Nonaka, H., Keresztes, G., Shinoda, Y., Ikenaga, Y., Abe, M., Naito, K., Inatomi, K., Furukawa, K., Inui, M., Yukawa, H., 2006. Complete genome sequence of the dehalorespiring bacterium *Desulfitobacterium hafniense* Y51 and comparison with *Dehalococcoides ethenogenes* 195. J. Bacteriol. 188 (6), 2262-2274. https://doi.org/10.1128/JB.188.6.2262-2274.2006

Nurmi, J. T., Tratnyek, P. G., 2002. Electrochemical properties of natural organic matter (NOM), fractions of NOM, and model biogeochemical electron shuttles. Environ. Sci. Technol. 36 (4), 617-624. https://doi.org/10.1021/es0110731

Orsetti, S., Laskov, C., Haderlein, S. B., 2013. Electron transfer between iron minerals and quinones: estimating the reduction potential of the Fe (II)-goethite surface from AQDS speciation. Environ. Sci. Technol. 47 (24), 14161-14168. https://doi.org/10.1021/es403658g

Osterberg, R., Shirshova L., (1997). Oscillating, nonequilibrium redox properties of humic acids. Geochim. Cosmochim. Ac. 61 (21), 4599-4604. https://doi.org/10.1016/S0016-7037(97)00266-4

Page, S. E., Kling, G. W., Sander, M., Harrold, K. H., Logan, J. R., McNeill,

K., Cory, R. M., 2013. Dark formation of hydroxyl radical in arctic soil and s urface waters. Environ. Sci. Technol. 47 (22), 12860-12867. https://doi.org/10.10 21/es4033265

Page, S. E., Sander, M., Arnold, W. A., McNeill, K., 2012. Hydroxyl radical f ormation upon oxidation of reduced humic acids by oxygen in the dark. Envir on. Sci. Technol. 46 (3), 1590-1597. https://doi.org/10.1021/es203836f

Pasek, M. A., 2008. Rethinking early Earth phosphorus geochemistry. Proc. Nat l. Acad. Sci. U.S.A. 105 (3), 853-858. https://doi.org/10.1073/pnas.0708205105

Peng, X., Yamamoto, S., Vertès, A. A., Keresztes, G., Inatomi, K. I., Inui, M., Yukawa, H., 2012. Global transcriptome analysis of the tetrachloroethene-dechlo rinating bacterium *Desulfitobacterium hafniense* Y51 in the presence of various electron donors and terminal electron acceptors. J. Ind. Microbiol. Biot. 39 (2), 255-268. https://doi.org/10.1007/s10295-011-1023-7

Peretyazhko, T., Sposito, G., 2006. Reducing capacity of terrestrial humic acids. Geoderma. 137 (1-2), 140-146. https://doi.org/10.1016/j.geoderma.2006.08.004

Porter, L. K., 1969. Gaseous Products Produced by Anaerobic Reaction of Sodi um Nitrite with Oxime Compounds and Oximes Synthesized from Organic Mat ter 1. Soil Sci.Soc. Am. J. 33 (5), 696-702. https://doi.org/10.2136/sssaj1969.03 615995003300050023x

Raghoebarsing, A. A., Pol, A., Van de Pas-Schoonen, K. T., Smolders, A. J., Ettwig, K. F., Rijpstra, W. I. C., Schouten, S., Damsté, J.S.S., Op den Camp, H.J.M., Jetten, M.S., Strous, M., 2006. A microbial consortium couples anaerob ic methane oxidation to denitrification. Nature. 440 (7086), 918. https://doi.org/ 10.1038/nature04617

Ratasuk, N., Nanny, M. A., 2007. Characterization and quantification of reversi ble redox sites in humic substances. Environ. Sci. Technol. 41 (22), 7844-7850. https://doi.org/10.1021/es071389u

Rakshit, S., Sarkar, D., 2017. Assessing redox properties of standard humic sub stances. Int. J. Environ. Sci. Technol. 14 (7), 1497-1504. https://doi.org/10.1007/ s13762-017-1263-9

Rakshit, S., Uchimiya, M., Sposito, G., 2009. Iron (III) bioreduction in soil in the presence of added humic substances. Soil Sci.Soc. Am. J. 73 (1), 65-71. https://doi.org/10.2136/sssaj2007.0418

Ravishankara, A. R., Daniel, J. S., Portmann, R. W., 2009. Nitrous oxide ($N_2O$): the dominant ozone-depleting substance emitted in the 21st century. Science. 326 (5949), 123-125. https://doi.org/10.1126/science.1176985

Reguera, G., McCarthy, K. D., MEhta, T., Nicoll, J. S., Tuominen, M. T., Lovley, D. R., 2005. Extracellular electron transfer via microbial nanowires. Nature. 435 (7045), 1098-1101. https://doi.org/10.1038/nature03661

Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of the potent greenhouse gas $N_2O$ from the nitrogen cycle–could enzymic regulation hold the key ?. Trends Biotechnol. 27 (7), 388-397. https://doi.org/10.1016/j.tibtech.2009.03.009

Rios-Del Toro, E. E., Valenzuela, E. I., Ramírez, J. E., López-Lozano, N. E., Cervantes, F. J., 2018. Anaerobic ammonium oxidation linked to microbial reduction of natural organic matter in marine sediments. Environ. Sci. Technol. Lett. 5 (9), 571-577. https://doi.org/10.1021/acs.estlett.8b00330

Roden, E. E., Kappler, A., Bauer, I., Jiang, J., Paul, A., Stoesser, R., Konishi, H., Xu, H. F., 2010. Extracellular electron transfer through microbial reduction of solid-phase humic substances. Nat. Geosci. 3 (6), 417. https://doi.org/10.1038/ngeo870

Rotaru, A. E., Shrestha, P. M., Liu, F.H., Markovaite, B., Chen, S., Nevin, K. P., Lovley, D. R., 2014. Direct interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*. Appl. Environ. Microbiol. 80 (15), 4599-4605. https://doi.org/10.1128/AEM.00895-14

Sander, M., Hofstetter, T. B., Gorski, C. A., 2015. Electrochemical analyses of redox-active iron minerals: a review of nonmediated and mediated approaches. Environ. Sci. Technol. 49 (10), 5862-5878. https://doi.org/10.1021/acs.est.5b00006

Sanford, R. A., Wagner, D. D., Wu, Q.Z., Chee-Sanford, J. C., Thomas, S. H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E., 2012. Unexpected nondenit rifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U.S.A. 109 (48), 19709-19714. https://doi.org/10.1073/pnas.12112381 09

Schaeffer, A., Nannipieri, P., Kästner, M., Schmidt, B., Botterweck, J., 2015. Fr om humic substances to soil organic matter–microbial contributions. In honour of Konrad Haider and James P. Martin for their outstanding research contributi on to soil science. J. Soil. Sediment. 15 (9), 1865-1881. https://doi.org/10.1007/ s11368-015-1177-4

Scheller, S., Yu, H., Chadwick, G. L., McGlynn, S. E., Orphan, V. J., 2016. A rtificial electron acceptors decouple archaeal methane oxidation from sulfate red uction. Science. 351 (6274), 703-707. https://doi.org/10.1126/science.aad7154

Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E., Lovley, D. R., 1998. Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ. Sci. Technol. 32 (19), 2984-2989. https://doi.org/10.1021/es980272q

Shao, M. F., Zhang, T., Fang, H. H. P., 2010. Sulfur-driven autotrophic denitrif ication: diversity, biochemistry, and engineering applications. Appl. Microbiol. B iot. 88 (5), 1027-1042. https://doi.org/10.1007/s00253-010-2847-1

Sharpless, C. M., Aeschbacher, M., Page, S. E., Wenk, J., Sander, M., McNeill, K., 2014. Photooxidation-induced changes in optical, electrochemical, and phot ochemical properties of humic substances. Environ. Sci. Technol. 48 (5), 2688-2696. https://doi.org/10.1021/es403925g

Shi, L., Dong, H. L., Reguera, G., Beyenal, H., Lu, A. H., Liu, J., Yu, H.Q., Fredrickson, J. K., 2016. Extracellular electron transfer mechanisms between mi croorganisms and minerals. Nat. Rev. Microbiol. 14 (10), 651-662. https://doi.or g/10.1038/nrmicro.2016.93

Sieber, J. R., Le, H. M., McInerney, M. J., 2014. The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. En

viron. Microbiol. 16 (1), 177-188. https://doi.org/10.1111/1462-2920.12269

Simon, J., Einsle, O., Kroneck, P. M., Zumft, W. G., 2004. The unprecedented nos gene cluster of *Wolinella succinogenes* encodes a novel respiratory electron transfer pathway to cytochrome c nitrous oxide reductase. FEBS Lett. 569 (1-3), 7-12. https://doi.org/10.1016/j.febslet.2004.05.060

Simon, J., Gross, R., Klimmek, O., Kröger, A., 2006. The genus *Wolinella*. The Prokaryotes: Volume 7: Proteobacteria: Delta, Epsilon Subclass. 178-191. https://doi.org/10.1007/0-387-30747-8_6

Smith, J. A., Nevin, K. P., Lovley, D. R. (2015). Syntrophic growth via quinone-mediated interspecies electron transfer. Front. Microbiol. 6, 121. https://doi.org/10.3389/fmicb.2015.00121

Stams, A. J., Plugge, C. M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7 (8), 568-577. https://doi.org/10.1038/nrmicro2166

Stein, L. Y., Klotz, M. G., 2016. The nitrogen cycle. Curr. Biol. 26 (3), R94-R98. https://doi.org/10.1016/j.cub.2015.12.021

Stevenson, F. J., 1994. Humus chemistry: genesis, composition, reactions. John Wiley & Sons.

Stevenson, F. J., Harrison, R. M., Wetselaar, R., Leeper, R. A., 1970. Nitrosation of Soil Organic Matter: III. Nature of Gases Produced by Reaction of Nitrite with Lignins, Humic Substances, and Phenolic Constituents Under Neutral and Slightly Acidic Conditions 1. Soil Sci. Soc. Am. J. 34 (3), 430-435. https://doi.org/10.2136/sssaj1970.03615995003400030024x

Storck, T., Virdis, B., Batstone, D. J., 2016. Modelling extracellular limitations for mediated versus direct interspecies electron transfer. ISME J. 10 (3), 621-631. https://doi.org/10.1038/ismej.2015.139

Struyk, Z., Sposito, G., 2001. Redox properties of standard humic acids. Geoderma. 102 (3-4), 329-346. https://doi.org/10.1016/S0016-7061(01)00040-4

Sullivan, M. J., Gates, A. J., Appia-Ayme, C., Rowley, G., Richardson, D. J., 2013. Copper control of bacterial nitrous oxide emission and its impact on vita min $B_{12}$-dependent metabolism. Proc. Natl. Acad. Sci. U.S.A. 110 (49), 19926-19931. https://doi.org/10.1073/pnas.1314529110

Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S., Lovley, D. R., 2010. Direct exchange of electrons within aggregates of an evol ved syntrophic coculture of anaerobic bacteria. Science. 330 (6009), 1413-1415. https://doi.org/10.1126/science.1196526

Suyama, A., Iwakiri, R., Kai, K., Tokunaga, T., Sera, N., Furukawa, K., 2001. Isolation and characterization of Desulfitobacterium sp. strain Y51 capable of e fficient dehalogenation of tetrachloroethene and polychloroethanes. Biosci. Biote ch. Bioch. 65 (7), 1474-1481. https://doi.org/10.1271/bbb.65.1474

Tan, W.B., Xi, B. D., Wang, G. A., Jiang, J., He, X. S., Mao, X. H., Gao, R. T., Hang, C.H., Zhang, H., Li, D., Jia, Y. F., Yuan, Y., Zhao, X. Y., 2017. Inc reased electron-accepting and decreased electron-donating capacities of soil hum ic substances in response to increasing temperature. Environ. Sci. Technol. 51 (6), 3176-3186. https://doi.org/10.1021/acs.est.6b04131

Timmers, P. H., Suarez-Zuluaga, D. A., van Rossem, M., Diender, M., Stams, A. J., Plugge, C. M., 2016. Anaerobic oxidation of methane associated with su lfate reduction in a natural freshwater gas source. ISME J. 10 (6), 1400-1412. https://doi.org/10.1038/ismej.2015.213

Tratnyek, P. G., Grundl, T. J., Haderlein, S. B. (Eds.). 2011. Aquatic redox ch emistry. American Chemical Society. Washington.

Tremblay, P. L., Angenent, L. T., Zhang, T., 2017. Extracellular electron uptak e: among autotrophs and mediated by surfaces. Trends Biotechnol. 35(4), 360-371. https://doi.org/10.1016/j.tibtech.2016.10.004

Tschech, A, Pfennig, N., 1984. Growth yield increase linked to caffeate reducti on in *Acetobacterium woodii*. Arch Microbiol. 137, 163–167. https://doi.org/10.1007/BF00414460

Vairavamurthy, A., Zhou, W., Eglinton, T., Manowitz, B., 1994. Sulfonates: a n

ovel class of organic sulfur compounds in marine sediments. Geochim. Cosmoc him. Ac. 58 (21), 4681-4687. https://doi.org/10.1016/0016-7037(94)90200-3

Valenzuela, E. I., Avendaño, K. A., Balagurusamy, N., Arriaga, S., Nieto-Delga do, C., Thalasso, F., Cervantes, F. J., 2019. Electron shuttling mediated by hu mic substances fuels anaerobic methane oxidation and carbon burial in wetland sediments. Sci. Total. Environ. 650, 2674-2684. https://doi.org/10.1016/j.scitotenv.2018.09.388

Valenzuela, E. I., Prieto-Davó, A., López-Lozano, N. E., Hernández-Eligio, A., Vega-Alvarado, L., Juárez, K., García-González, A.S., López, M.G., Cervantes, F. J., 2017. Anaerobic methane oxidation driven by microbial reduction of natu ral organic matter in a tropical wetland. Appl. Environ. Microbiol. 83 (11), e00 645-17. https://doi.org/10.1128/AEM.00645-17

Van Trump, J. I., Wrighton, K. C., Thrash, J. C., Weber, K. A., Andersen, G. L., Coates, J. D., 2011. Humic acid-oxidizing, nitrate-reducing bacteria in agric ultural soils. Mbio. 2 (4), e00044-11. https://doi.org/10.1128/mBio.00044-11

Virdis, B., Millo, D., Donose, B. C., Batstone, D. J., 2014. Real-time measure ments of the redox states of c-type cytochromes in electroactive biofilms: A co nfocal resonance raman microscopy study. PloS one, 9 (2), e89918. https://doi.org/10.1371/journal.pone.0089918

Wallace, G.C., Sander, M., Chin, Y.P., Arnold, W.A., 2017. Quantifying the ele ctron donating capacities of sulfide and dissolved organic matter in sediment p ore waters of wetlands. Environ. Sci. Process Impacts. 19, 758–767. https://doi.org/10.1039/C7EM00060J

Walpen, N., Getzinger, G. J., Schroth, M. H., Sander, M., 2018. Electron-Donat ing Phenolic and Electron-Accepting Quinone Moieties in Peat Dissolved Organ ic Matter: Quantities and Redox Transformations in the Context of Peat Biogeo chemistry. Environ. Sci. Technol. 52 (9), 5236-5245. https://doi.org/10.1021/acs.est.8b00594

Wegener, G., Krukenberg, V., Riedel, D., Tegetmeyer, H. E., Boetius, A., 2015. Intercellular wiring enables electron transfer between methanotrophic archaea a nd bacteria. Nature. 526 (7574), 587-590. https://doi.org/10.1038/nature15733

Wenk, J., Aeschbacher, M., Salhi, E., Canonica, S., von Gunten, U., Sander, M., 2013. Chemical oxidation of dissolved organic matter by chlorine dioxide, chlorine, and ozone: Effects on its optical and antioxidant properties. Environ. Sci. Technol. 47, 11147–11156. https://doi.org/10.1021/es402516b

Widdel, F., 1980. Anaerober Abbau von Fettsäuren und Benzoesäure durchneu isolierte Arten Sufat reduzierender Bakterien. PhD thesis. Universität Göttingen, Göttingen, Germany.

Widdel, F, Pfennig, N., 1981. Studies on dissimilatory sulfate-reducing bacteria that compose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* g en. nov., sp. nov. Arch Microbiol. 129, 395–400. https://doi.org/10.1007/BF0040 6470

Wilhelm, E., Battino, R., Wilcock, R. J., 1977. Low-pressure solubility of gase s in liquid water. Chem. Rev. 77 (2), 219-262. https://doi.org/10.1021/cr60306a0 03

Wolf, M., Kappler, A., Jiang, J., Meckenstock, R. U., 2009. Effects of humic s ubstances and quinones at low concentrations on ferrihydrite reduction by *Geob acter metallireducens*. Environ. Sci. Technol. 43 (15), 5679-5685. https://doi.org/ 10.1021/es803647r

Xiao, Z. X, Awata, T., Zhang, D. D., Zhang, C. F., Li, Z. L., Katayama, A., 2016. Enhanced denitrification of *Pseudomonas stutzeri* by a bioelectrochemical system assisted with solid-phase humin. J. Biosci. Bioeng. 122 (1), 85-91. http s://doi.org/10.1016/j.jbiosc.2015.11.004

Xue, Z. R., 2018. Redox Reactions and Sorption of Quinones and Natural Org anic Matter at Iron Mineral/Fe(II) Interfaces. PhD Dissertation, University of T übingen. http://dx.doi.org/10.15496/publikation-24006

Yang, W. H., Weber, K. A., Silver, W. L., 2012. Nitrogen loss from soil throu gh anaerobic ammonium oxidation coupled to iron reduction. Nat. Geosci. 5 (8), 538-541. https://doi.org/10.1038/ngeo1530

Yoon, S., Nissen, S., Park, D., Sanford, R. A., Löffler, F. E., 2016. Nitrous ox

ide reduction kinetics distinguish bacteria harboring clade I NosZ from those harboring clade II NosZ. Appl. Environ. Microbiol. 82 (13), 3793-3800. https://doi.org/10.1128/AEM.00409-16

Yoshinari, T., 1980. $N_2O$ reduction by *Vibrio succinogenes*. Appl. Environ. Microbiol. 39, 81–84. https://doi.org/10.1016/0141-4607(80)90006-2

Yu, Z. G., Peiffer, S., Göttlicher, J., Knorr, K. H., 2015. Electron transfer budgets and kinetics of abiotic oxidation and incorporation of aqueous sulfide by dissolved organic matter. Environ. Sci. Technol. 49 (9), 5441-5449. https://doi.org/10.1021/es505531u

Zheng, Y., Kappler, A., Xiao, Y., Yang, F., Mahadeva, G. D., Zhao, F., 2019. Redox-active humics support interspecies syntrophy and shift microbial community. Sci. China Technol. Sci. 62, 1-8. https://doi.org/10.1007/s11431-018-9360-5

Zhu, B. L., Bradford, L., Huang, S. C., Szalay, A., Leix, C., Weissbach, M., Táncsics, A., Drewes, J.E., Lueders, T., 2017. Unexpected diversity and high abundance of putative nitric oxide dismutase (Nod) genes in contaminated aquifers and wastewater treatment systems. Appl. Environ. Microbiol. 83 (4), e02750-16. https://doi.org/10.1128/AEM.02750-16

Zhu-Barker, X., Cavazos, A. R., Ostrom, N. E., Horwath, W. R., Glass, J. B., 2015. The importance of abiotic reactions for nitrous oxide production. Biogeochemistry. 126 (3), 251-267. https://doi.org/10.1007/s10533-015-0166-4

Zhou, G. W., Yang, X. R., Li, H., Marshall, C. W., Zheng, B. X., Yan, Y., Su, J, Q., Zhu, Y. G., 2016. Electron shuttles enhance anaerobic ammonium oxidation coupled to iron (III) reduction. Environ. Sci. Technol. 50 (17), 9298-9307. https://doi.org/10.1021/acs.est.6b02077

Zhu, G. B., Wang, S. Y., Li, Y. X., Zhuang, L. J, Zhao, S. Y., Wang, C., Kuypers, M.M., Jetten, M.S., Zhu, Y. G., 2018. Microbial pathways for nitrogen loss in an upland soil. Environ. Microbiol. 20 (5), 1723-1738. https://doi.org/10.1111/1462-2920.14098

## Statement of Personal Contribution

This dissertation is based on the research concept proposed by Professor Stefan B. Haderlein. His wise guidance inspired me to conceive different ideas presented as chapters in this thesis. I performed all the research work and data analysis. This PhD work was supported by the China scholarship council (CSC) fellowship (File No. 201404910472) and the German Research Foundation (CRC 1253, CAMPOS, Subproject P4).

I conceived the first major idea of a perspective (a combination of review and experimental study) after doing the literature survey. I observed that mediated electrochemical analysis has rarely been applied on studying microbial NOM redox cycling in terms of electron transfer mechanisms. The intensive discussions with Professor Haderlein made me realize the need to promote the use of mediated electrochemical analysis to other researchers which we have presented as a perspective for submission to a peer-reviewed research journal. For review section, I summarized literatures and performed all calculations of redox potentials. Professor Yongguan Zhu made substantial contributions to the concepts and structure of this section. For experimental section, Professor Haderlein initially pointed out that the unknown electron-active substance in my denitrifying bacterium culture could be nitrite. The idea of using sulfamic acid method to remove nitrite from DOM samples was conceived by me. I designed and performed the experiments. In addition, I observed the unexpected current signals of *Geobacter sulfurreducens* cells in MER and MEO. We consulted with Professor Derek Lovley for explanation owing to his expertise on *Geobacter* species. Professor Lovley helped me to understand the electro-activity of *Geobacter sulfurreducens* and proposed a straightforward way to identify this behavior. And then I conducted the experiments. I performed all data analyses and wrote the manuscript. Professor Haderlein and Professor Zhu carefully revised it for multiple times.

I proposed the second major idea of denitrifier assay and developed it together with Professor Haderlein. I designed and conducted the experiments followed by the data analysis. These findings are presented in the form of an article prepared for a peer-reviewed research journal written by me in close consultation with Prof. Haderlein.

I proposed the third major idea of studying non-denitrifying $N_2O$ reduction. I did all the experimental work and wrote the manuscript after required data analysis. Again, this work was completed with full support from Professor Haderlein. Professor Andreas Kappler provided critical comments to improve the quality of this chapter.

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I sincerely thank Professor Andreas Kappler for providing me access to his lab facilities. Many thanks to Professor Kappler for offering me microbial cultures from their culture collection for study. Herein I appreciate lab technician Ellen Röhm from his group, for her tremendous help with the routine measurements.

I am extremely grateful to our lab technician Monika Hertel for regular lab assistance during the past years. I would like to thank our group members Jana, Tu Trinh, Edisson, Aaifa, Leyla, Shaojian, Zhengrong, Johannes and Philipp for mutual helps in the lab and office. I sincerely thank Dr. Silvia Orsetti and Dr. Daniel Buchner for joining in discussions of experimental results. Particularly, I sincerely appreciate Dr. Daniel Buchner for his great help on lab work and critical comments on my manuscripts. Big thanks to Dr. Usman Muhammad for sharing his wonderful experiences on doing sciences.

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Massive thanks to my parents and my younger sister for their everlasting support to my doctoral study abroad. Herein, I thank my fiancée Xinmei for her support and extreme

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# Curriculum Vitae

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