Microbial redox cycling of iron in Lake Grosse Fuchskuhle

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To my Parents, Deepak Chowdary Kanaparthi, Sai Rishitha Chowdary Murrakonda , Bavani Chowdary Murrakonda and Bianca Pommerenke

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Zusammenfassung

Obwohl Torfmoore lediglich 3% des Festlands der Erde ausmachen, enthalten sie jedoch annähernd ein Drittel der weltweiten gespeicherten organischen Kohlenstoffvorkommen. Torfmoore fungieren zwar als Senken für atmosphärischen Kohlenstoff, jedoch sind sie auch eine Netto-Quelle von athmosphärischen Treibhausgasen wie CH_4 und N_2O. Daher konzentrierten sich bisher die meisten Studien an Torfmooren auf Methanogenese und die Rolle von Umweltfaktoren, die diesen Prozess beeinflussen. Nur wenige Studien beschäftigten sich mit alternativen Prozessen zur Elektronenaufnahme. Neuere Studien liefern Hinweise darauf, das Fe(III) Reduktion eine wichtige Rolle bei der Mineralisierung von organischem Kohlenstoff in leicht sauren Torfmooren spielen könnte. Diese Prozesse sind jedoch bisher nicht genauer untersucht worden.

Im ersten Abschnitt dieser Arbeit wurde die Rolle von Fe(III) Reduktion und Methanogenese, als terminale Elektronen akzeptierende Prozesse, untersucht. Hingegen der früher vorherschenden Hypothese von sequenzieller Reduktion von Elektronenakzeptoren entsprechend ihres Redoxpotentials in Sedimenten, wurde simultan eine Reduktion von Fe(III) und Methanogenese im Sediment des Moorsees Große Fuchskuhle beobachtet. Ein quantitativer Vergleich dieser Prozesse über den Verlauf der Inkubation zeigte, dass Fe(III) Reduktion hier den vorherrschenden terminalen Prozess in der Mineralisierung organischen Materials darstellt. Nach initialer Fe(III) Reduktion konnte eine Schwankung in der Fe(II) Konzentration über Verlauf der Inkubation beobachtet werden, welche auf eine wiederholte anaerobe Fe(II)-Oxidation und Reduktion in diesem Sediment hindeutet.

Im Anschluß an die oben aufgeführten Ergebnisse konzentrierte sich der zweite Abschnitt dieser Arbeit auf die Identifizierung, Anreicherung und Charakterisierung von Mikroorganismen, welche an der anaeroben Nitrat-abhängigen Fe(II) Oxidation beteiligt sind. Diese Untersuchungen verdeutlichen die chemolithotrophe Nitrat-abhängige Fe(II) oxidierende Natur von TM3 *Actinobacteria* und das diese Organnismen an der anaeroben Oxidation von Fe(II) im Sediment beteiligt sein könnten. Kulturunabhängige Studien konnten bereits eine weite Verbreitung dieser Actinobacterien, was auf eine Beteiligung dieser Bakterien an wichtigen ökologischen Prozessen hindeuted, zeigen. Die physiologischen Fähigkeiten dieser Mikroorganismen blieben dennoch unbekannt. Soweit uns bekannt ist dies die erste Studie zur Untersuchung der autotrophen Nirat-abhängigen Fe(II) Oxidation von nicht kultivierten *Actinobacteria* der TM3 Gruppe.

Der dritte Abschnitt dieser Arbeit beschäftigt sich mit der Rolle von Huminstoffen in der abiotischen und mikrobiellen Fe(II)-Oxidation. Abgesehen von der Tatsache das Fe(II) in der

Umwelt vorwiegend an Huminstoffe gebunden vorkommt, konnte die Rolle von Huminstoffen in der Fe(II)-Oxidation noch nicht geklärt werden. Unsere Ergebnisse zeigen, dass die Anwesenheit von Huminstoffen einen Vorteil für Fe(II) oxidierende Mikroorganismen darstellen kann. Dies beruht auf der reduzierten abiotischen Fe(II)-Oxidation und einer möglicherweise erhöhten Energieausbeute durch eine Absenkung des Redoxpotentials von chelatiertem Fe(II) im Vergleich zu freiem Fe(II).

Eine MPN-basierte Quantifizierung der Nitrat-abhängige Fe(II)-oxidierenden Mikroorganismen im Sediment des Moorsees Große Fuchskuhle zeigte eine um zwei Größenordnung erhöhte Zellzahl von chemolithotrophen Nitrat-abhängige Fe(II)-oxidierenden Mikroorganismen bei Huminstoffzugabe zum Wachstumsmedium. Bei Sediment Inkubationen unter chemolithotrophen Nitrat-abhängigen Fe(II)-oxidierenden Bedingungen wurden Mikroorganismen der Gattung *Thiomonas* angereichert. Einen weitere Charakterisierung dieser Anreicherungen lieferte vorläufige Belege dafür, dass diese *Thiomonas* Stämme fähig zur chemolithotrophen Nitrat-abhängigen Fe(II)-Oxidation sind.

Zuletzt wurde der *Thiomonas arsenivorans* Stamm 3As auf die Fähigkeit zur chemolithotrophen Nitrat-abhängigen Fe(II)-Oxidation getestet, da alle dafür notwendigen Gene im Genom identifiziert werden konnten. Diese Versuche wurden sowohl unter Anwesenheit als auch in Abwesenheit von Huminstoffen durchgeführt. Es konnte ein stöchiometrischer Verbrauch von Fe(II) und Nitrat, wie für Nitrat-abhängige Fe(II)-Oxidation in Gegenwart von Huminstoffen unter autotrophen Wachstumsbedingungen erwarted, beobachtet werden. Demgegenüber konnte in Abwesenheit von Huminstoffen weder unter autothrophen noch unter heterotrophen Bedingungen Fe(II)-Oxidation beobachtet werden, was auf die Bedeutung von Huminstoffen bei der Vermittlung Nitrat-abhängiger Fe(II)-Oxidation hinweist.

Nach unserem Wissen ist dies die erste Studie, welche die chemolithotrophe Nitrat-abhängige Fe(II) oxidierende Physiologie in einer bakteriellen Reinkultur zeigt. Darüberhinaus konnte in dieser Arbeit gezeigt werden, dass Huminstoffe mikrobielle Fe(II)-Oxidation begünstigen.

Summary

Peatlands constitute >3% of the Earth's terrestrial area but store approximately one third of global soil organic carbon. Although peatlands act as sinks for atmospheric carbon, they are net emitters of greenhouse gasses, like CH_4 and N_2O, into the atmosphere. Hence, most of the studies conducted on peatlands focused on methanogenesis and the role of environmental factors influencing this process and very few studies focused on other electron-accepting processes. Recent studies have shown indications that Fe(III) reduction could be playing an important role in the mineralization of organic carbon in mildly acidic peat bogs. However, this process in peatlands has not been well investigated.

In the first part of the work the role of Fe(III) reduction and methanogenesis as electron-accepting processes was investigated. Unlike the earlier hypothesis of sequential reduction of electron acceptors according to their redox potentials in sediments, a simultaneous reduction of Fe(III) and methanogenesis was observed in the sediment of Lake Grosse Fuchskuhle. Quantitative comparison of these processes showed that Fe(III) reduction is the dominant organic matter mineralization process compared to methanogenesis during the course of the incubations. After an initial Fe(III) reduction a fluctuating Fe(II) concentration was observed during the course of our incubation indicating a continuous anaerobic Fe(II) oxidation and reduction in this sediment.

Following the above results, the second part of the work focused on identifying, enriching and characterizing microorganisms involved in anaerobic nitrate-dependent Fe(II) oxidation. These investigations indicated the chemolithotrophic nitrate-dependent Fe(II)-oxidizing nature of TM3 *Actinobacteria* and that these organisms could be involved in mediating anaerobic oxidation of Fe(II) in the sediment. Previous culture-independent studies had shown a widespread distribution of these *Actinobacteria* in natural environments and were hypothesized to be contributing to ecologically important processes; however, the physiological capabilities of these microorganisms remained unknown. To the best of our knowledge this is the first study to show the autotrophic nitrate-dependent Fe(II)-oxidizing nature of TM3 group of uncultured *Actinobacteria*.

The third part of the thesis deals with the role of humic substances in abiotic and microbial Fe(II) oxidation. Despite the fact that Fe(II) is predominantly present in natural environments as chelated to humic substances, the role of humic substances in mediating Fe(II) oxidation has not been elucidated. Our findings indicate that the presence of humic substances could be beneficial for microorganisms oxidizing Fe(II) due to reduced abiotic Fe(II) oxidation and also possibly due to an

increased energy yield caused by a lowering of the redox potential of chelated Fe(II) compared to free Fe(II). Estimations of nitrate-dependent Fe(II)-oxidizing microorganisms from Lake Grosse Fuchskuhle sediment using a cultivation-based approach showed a two-order of magnitude higher number of chemolithotrophic nitrate-dependent Fe(II)-oxidizing microorganisms when including humic substances in the growth medium. The incubations of sediment under chemolithotrophic nitrate-dependent Fe(II)-oxidizing conditions showed the enrichment of microorganisms belonging to the genus *Thiomonas*. Further characterization of these enrichments provided preliminary evidence of a chemolithotrophic nitrate-dependent Fe(II)-oxidizing capability of these *Thiomonas* strains.

Lastly, *Thiomonas arsenivorans* strain 3As was tested for chemolithoautotrophic nitrate-dependent Fe(II) oxidation since the presence of all the genes required for mediating this physiological process were identified in the genome. These assays were performed both in the presence and absence of humic substances. A stoichiometric consumption of Fe(II) and nitrate consistent with nitrate-dependent Fe(II) oxidation was observed in the presence of humic substances under autotrophic growth conditions. In contrast, no Fe(II) oxidation either under autotrophic or heterotrophic conditions was observed in the absence of humic substances, indicating the importance of humic substances in mediating nitrate-dependent Fe(II) oxidation. To the best of our knowledge this is the first study to show a chemolithotrophic nitrate-dependent Fe(II)-oxidizing physiology in a bacterial pure culture. Furthermore, the findings of the study indicate that humic substances are beneficial for microbial Fe(II) oxidation.

Chapter 1

1.1 Introduction

Iron is the most abundant element on Earth by weight and is a major component of the Earth's inner core. It is also one of the most abundant and ubiquitously distributed metals on the Earth´s crust constituting about 5% of the total mass (Schwertmann and Cornell, 2008). Like the other transition metals, iron exhibits a wide range of oxidation states (-2 to +6), but predominantly present in the Earth's crust in either ferrous (Fe^{2+}) or ferric forms (Fe^{3+}). Fe(III) is mostly insoluble and a stable form of iron under environmental conditions (Stumm and Morgan, 1996). Fe(II) is soluble, highly reactive and can form complexes with other elements like sulfur, aluminium, phosphate and is considered to play an important role in the biogeochemical cycling of these elements (Davidson et al., 2003). Iron is mostly present on Earth's crust as complexes with other elements in the form of minerals like magnetite (Fe_3O_4), hematite (Fe_2O_3), pyrite (FeS_2), siderite ($FeCO_3$) and vivianite ($Fe_3(PO_4)_2.8H_2O$). A large amount of iron in the Earth crust is present in the form hematite in banded iron deposits, formed during the Archaean and Precambrian eras (Goldich, 1973). These formations are primarily composed of alternating layers of ferric iron complexes like hematite or magnetite and silica (Drever, 1974). These formations are spread over hundreds or thousands of square kilometers and are a major source of commercial iron ore (Morris et al., 1980).

Banded iron formations (BIF) were known to be formed during the late Archaean to early Proterozic period, during the transition of Earth from a reducing to oxidizing atmosphere (Goldich, 1973; Holland, 2006). Despite several theories explaining the formation of these vast deposits, the exact mechanism of their formation remains highly debated (Simonson, 1985). During the Archaean period oceans of Earth were known to contain a high concentration of Fe(II) (Rouxel et al., 2005), which either entered the oceans through erosion from the continental rocks or due to the continuous input of Fe(II) in the form of lava from hydrothermal vents and drifts in the continental shelves (Veizer et al., 1982). Fe(II) released from these sources is highly soluble in water and could remain stable under anoxic conditions (Stumm and Morgan, 1996). During the early Proterozic period the oxygen concentrations steadily increased due to the evolution of photosynthetic cyanobacteria (Anbar and Knoll, 2002; Saito et al., 2003) and the reaction between Fe(II) and molecular oxygen have led to the formation of insoluble Fe(III) hydroxides like magnetite and hematite (Cloud, 1973). The precipitation of these minerals on the ocean floor led to the formation of BIF. Even though this

theory could explain successfully the formation of these deposits during the proteozoic period, geological studies have shown that BIFs started forming during the late Archaean period (Goodwin et al., 1985; Manikyamba et al., 1993), before the evolution cyanobacteria and release of free oxygen into the atmosphere. Recent studies have postulated that late Archaean BIFs could have formed either due to abiotic Fe(II) oxidation mediated by UV radiation (Konhauser et al., 2002) or by microbially mediated Fe(II) oxidation mediated either photoautotrophically (Ehrenreich and Widdel, 1994) or by reducing nitrate (Kappler and Newman, 2004) formed by disproportionation of nitric oxide produced during lightening (Kasting and Siefert, 2001).

The alternate iron rich and iron poor layers observed in BIFs were hypothesized to be due to seasonal precipitation of Fe(III) by abiotic or microbial Fe(II) oxidation and removal of precipitated Fe(III) by reduction reactions, hypothesized to be mediated by Fe(III)-reducing bacteria (Konhauser et al., 2002; Nealson and Myers, 1990). Millimeter scale variations of stable isotope abundances in carbonates from BIFs have showed alternating layers of isotopically light carbon and oxygen correlating with increasing Fe(III) concentrations and heavy carbon and oxygen correlating with removal of Fe(III) (Baur et al., 1985). These observations were in accordance with the postulated hypothesis that the formation of Fe(III) was associated with photoautotrophic Fe(II) oxidation resulting in the formation of light carbon followed by the removal of formed Fe(III) by Fe(III)-reducing bacteria utilizing reduced organic carbon (Nealson and Myers, 1990). These studies have led to the hypothesis that iron metabolism could be one of the first microbial metabolisms, predating nitrate and sulfate metabolisms which in turn predate cyanobacterial photosynthesis (Canfield et al., 2006). The quantitative estimations of primary production during this geological time period have also indicated that iron-based metabolism could have contributed largely to the CO_2 fixation process during the Archaean and Precambrian periods (Canfield et al., 2000; Canfield, 2005; Kharecha et al., 2005).

On the present day Earth, iron redox transformations are known to be happening ubiquitously in natural environments, at several orders of magnitude lower that those observed during the Archaean and Precambrian periods (Canfield et al., 2006). Apart from anaerobic iron oxidation and reduction reactions, the presence of oxygen also led to the evolution of microorganisms capable conserving energy by aerobic Fe(II) oxidation (Weber et al., 2006a). These are one of the first microorganisms to be observed in late 19^{th} century along with other physiological groups of bacteria like denitrifers, sulfate-reducers and methanogens; however the physiology and biochemistry of aerobic iron-oxidizers is not well studied due to difficulties associated with obtaining these organisms as pure cultures. Anaerobic Fe(II)-oxidizing microorganisms, which are considered to have played an

important ecological role in the Archaean and Precambrian periods have only been discovered recently (Widdel et al., 1993) and the ecological role of these organisms has not been well elucidated. Although Fe(III) reduction as electron-accepting process has been recognized for a long time, microorganisms capable of conserving energy by this physiological process have only been isolated in 1991 (Lovley et al., 1993). Subsequent studies have led to an understanding of the role of these organisms in mediating organic matter degradation (Coates et al., 1997), environmental factors influencing this process (Lovley et al., 1996) and isolation of several microorganisms capable of this physiological process (Lonergan et al., 1996).

At the current state of knowledge, biological redox cycling of iron involves oxidation of Fe(II) aerobically, anaerobically mediated by light, or by coupling to denitrification processes and reduction of Fe(III) (Figure 1).

1.2 Fe(II) oxidation

Aerobic Fe(II)-oxidizing bacteria were one of the first discovered microorganisms (Ehrenberg, 1836). Stalk forming microorganisms like *Gallionella ferruginea* were routinely observed in underground drainages and fresh water circulation systems and are hypothesized to be capable of Fe(II) oxidation due their high abundance in iron-rich environments and the presence of Fe(III) hydroxides in their stalks (Hanert, 1974). Apart from the initial observations, not much progress has been made in understanding this physiological guild of microorganisms due to the difficulties associated in culturing them. Moreover, the ecological role of these organisms was underestimated as Fe(II) oxidation was considered primarily to be an abiotic process in natural environments. Fe(II) is highly reactive with oxygen and has a half-life of less than a minute in well-oxygenated waters at circumneutral pH (Stumm and Morgan, 1996). The oxidation of Fe(II) follows first order kinetics with oxygen and a second order dependence on the concentration of OH^-, therefore the stability of Fe(II) greatly increases with decreasing concentration of OH^- or pH. A decrease of one pH unit increases the stability of Fe(II) by 100-fold, making Fe(II) totally unreactive with oxygen at pH values below 4 (Stumm and Morgan, 1996). Hence, low pH conditions (Singer and Stumm, 1970) or micro-oxic environments (Frenzel et al., 1999; Neubauer et al., 2002a) could provide a favorable habitat for the growth of Fe(II)-oxidizing bacteria. Due to the absence of abiotic Fe(II) oxidation in low pH environments, most of the initial studies on microbial Fe(II) oxidation were focused on environments like acid mine drainages (Weber et al., 2006a). However, an increasing body of literature over the last decade has shown that biological Fe(II) oxidation could play a more prominent role in Fe cycling than considered previously.

Although geochemists have hypothesized that anaerobic Fe(II) oxidation could be a prominent Fe(II) oxidation process on early Earth during the Archaean and Precambrian periods (Canfield et al., 2006), experimental evidences of such processes were not available till recently. Over the last few decades several mechanisms of anaerobic Fe(II) oxidation mediated by light (Ehrenreich and Widdel, 1994; Widdel et al., 1993) or coupled to denitrification (Straub et al., 1996) or reduction of per-chlorates (Chaudhuri et al., 2001) have been experimentally demonstrated. Due to the nature of

the present work, I would like to limit the scope of this section only to nitrate-dependent Fe(II) oxidation.

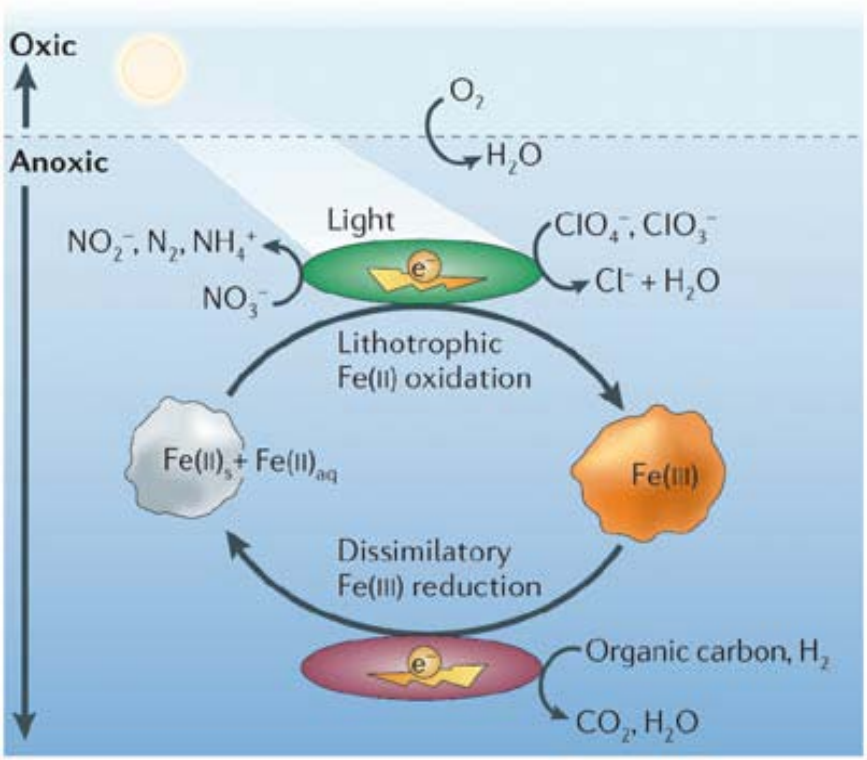

Figure 1: Microbially mediated iron redox cycle.

(Courtesy: Weber et al., 2006; Nature reviews Microbiology)

Nitrate-dependent Fe(II) oxidation

Compared to the redox potential of +770mV for Fe(III)/Fe(II) couple in low pH environments (Langmuir et al., 1997), the redox potential of Fe(II) decreases to 100mV under circumneutral pH due to formation of insoluble Fe(III) hydroxides as end products of Fe(II) oxidation (Garrels and Christ, 1990). Due to this lowering of redox potential, it was hypothesized that several redox pairs of higher redox potential like NO_3/NO_2 (0.43 V), NO_2/NO (0.35 V), NO/N_2O (1.18 V) and N_2O/N_2 (1.35 V) (Stumm and Morgan, 1996) could potentially be capable of Fe(II) oxidation under anoxic conditions. Subsequent studies done in this regard had led to the isolation or enrichment and characterization of microorganisms capable of these physiological processes (Straub et al., 1996).

Microbial nitrate-dependent Fe(II) oxidation was first reported by Straub et al. in 1996. This study had led to the characterization of several microorganisms capable of nitrate-dependent Fe(II) oxidation growing either autotrophically or organotrophically. Subsequent studies had shown that this physiological process was widespread among bacteria, but more commonly observed in bacterial groups involved in denitrification processes (Muehe et al., 2009; Straub et al., 2004; Straub and Buchholz-Cleven, 1998a).

Studies conducted on several natural environments have shown that this physiological process was widespread in natural environments and have been reported from several environments like lake sediments (Straub and Buchholz-Cleven, 1998a), marine environments (Edwards et al., 2004), rice paddies (Ratering and Schnell, 2001), hydrothermal vents (Hafenbradl et al., 1996) and brackish lagoons (Straub et al., 1996), indicating the ubiquitous distribution of this physiological process. Quantification of these physiological groups of organisms in natural environments have also indicated the presence of these organisms in the range of 10^2 to 10^5 cells g^{-1} of soil or sediments (Muehe et al., 2009; Straub and Buchholz-Cleven, 1998a). This physiological process is considered to play an important role in global Fe(II) oxidation due to the absence of light and predominantly anaerobic nature of soils and sediments, however the quantitative and ecological significance of this physiological process is not known. Despite several recent studies focused on this physiological process neither the physiology, nor the biochemistry or the influence of environmental factors on this process are known.

The nitrate-dependent Fe(II) oxidation process is known to be mediated by both autotrophically and organotrophically growing organism (Straub et al., 1996). Several organotrophic organisms capable of this physiological process were isolated and characterized (Muehe et al., 2009). Microorganisms capable of autotrophic growth under these physiological conditions have not been available in pure cultures. Attempts to isolate autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms have led to several isolates which were initially considered to be growing autotrophically, but lost the ability of autotrophic growth after repeated subculturing (Weber et al., 2006b). Several organisms capable of this physiological process were isolated from marine environments and were reported to be capable of autotrophic growth; however, subsequent studies confirming the autotrophic growth have not been done (Edwards et al., 2004). To date this physiological group of organisms capable of autotrophic growth were only available as enrichment cultures (Kanaparthi et al., 2013; Straub et al., 1996).

Most probable number studies conducted on several environments have shown the ubiquitous presence of both autotrophic and organotrophic nitrate-dependent Fe(II)-oxidizing bacteria

(Kanaparthi et al., 2013; Muehe et al., 2009; Straub and Buchholz-Cleven, 1998a). The comparison of the numbers of this physiological group has consistently shown at least two-orders of magnitude higher organotrophic nitrate-dependent Fe(II)-oxidizing bacteria in natural environments (Kanaparthi et al., 2013; Muehe et al., 2009; Straub and Buchholz-Cleven, 1998a). These findings indicate that organotrophic nitrate-dependent Fe(II) oxidation contributes significantly to total Fe(II) oxidation in natural environments. This dominance of organotrophic nitrate-dependent Fe(II)-oxidizing microorganisms in natural environments was considered to be due to the energetic benefit of their mixotrophic growth (Muehe et al., 2009). Autotrophic growth requires input of energy in the form of ATP and reducing equivalents in the form of NAD(P)H (E'= -320mV). Although ATP is generated during the reduction of nitrate, Fe(II) oxidation could not directly mediate the production of NAD(P)H by reduction of NAD(P) due its low redox potential (Garrels and Christ, 1990). Hence, the electrons released from oxidation of Fe(II) have to be pumped uphill against the redox gradient for reduction of NAD(P) (Ferguson, 1988). This process requires input energy in the form of ATP (Ferguson and Ingledew, 2008). Hence, from these observations it was hypothesized that energy released from Fe(II) oxidation may not be sufficient to meet the requirements of both the cells energy needs and reduction of CO_2 (Muehe et al., 2009). Experiments conducted on Strain BoFeN1, which was initially considered to be capable of autotrophic nitrate-dependent Fe(II) oxidation have also shown a slow growth rate and only a few doubling under chemolithoautotrophic conditions (Weber et al., 2009).

Although studies conducted on several environments indicated that chemolithoautotrophic nitrate-dependent Fe(II) oxidation may not be a dominant physiological process based on MPN studies, these studies have shown evidence of this process in natural environments. Subsequent enrichment experiments conducted have also shown the possibility of autotrophic growth under these physiological conditions, despite the inability to isolate these organisms in pure cultures. Even though this physiological process may not be contributing dominantly to the total Fe(II) oxidation in the above tested environments, this process could play an important role in habitats containing low amounts of organic carbon like groundwater aquifers (Pauwels et al., 2000). Geochemical studies conducted on these environments have shown the presence of Fe(II) oxidation coupled to denitrification process and isotopic and field tracer studies conducted on these environments have shown that this process is being mediated autotrophically (Pauwels et al., 1998a; Pauwels et al., 2000). Even though no microbial studies have been conducted on such environments to date, these findings provide further evidence of the possibility of chemolithoautotrophic nitrate-dependent Fe(II) oxidation in natural environments. These studies also suggest the possibility that the

difficulties associated with culturing these organisms could be due to the differences between artificial media and the conditions observed under natural conditions.

1.3 Microbial Fe(III) reduction

Microbial iron reduction in natural environments was first reported in late 19th century and subsequent studies have reported that several bacteria, archaea and fungi are capable of dissimilatory Fe(III) reduction. This physiological process is considered to be widespread among bacteria and archaea (Jones et al., 1983; Starkey and Halvorson, 1927), however most of these organisms observed to be capable of Fe(III) reduction were shown mediating this process as a metabolic side-reaction and are not capable of utilizing Fe(III) as an important electron acceptor in anaerobic respiration (Hammann and Ottow, 1974). This phenomenon of Fe(III) reduction was also reported in several sulfate-reducing bacteria, however earlier studies have hypothesized that this transformation of Fe(III) is non-enzymatic (Ghiorse, 1988; Zehnder and Stumm, 1988). The first evidences of energy conservation by dissimilatory Fe(III) reduction coupled to complete oxidation of organic carbon was shown in *Geobacter metallireductans* (Lovley et al., 1993). Apart from Fe(III) reduction coupled to degradation of organic carbon, bacteria capable of reducing Fe(III) with molecular hydrogen were also isolated and characterized. This physiological process was reported from bacteria *Pseudomonas* and *Schawanella* sp. (Semple and Westlake, 1987), hyperthermophilic (Lovley et al., 2000) and mesophilic archaea like hydrogenotrophic methanogens (Vargas et al., 1998). Studies conducted on these organisms have showed a stoichiometric conversion of H_2 and Fe(III) (Balashova, 1980). Although this physiology has been reported from several pure cultures and was shown to be happening in natural environments, the ecological role of this process was not well understood.

The capability to reduce Fe(III) was observed in a wide range of bacteria and archaea. Several hyperthermophilic, thermophilic, mesophilic and psychrophilic archaea are known to be capable of Fe(III) reduction (Lonergan et al., 1996; Vargas et al., 1998). In bacteria, *Geobacter* was the first reported genus capable of Fe(III) reduction (Lovley et al., 1993) and later studies have revealed that members of closely genus like this *Pelobacter*, *Desulfuromonas* and *Desulfuromusa* are also capable of the physiological process and are classified under the novel family *Geobacteriaceae* within the δ-proteobacteria (Lonergan et al., 1996). Apart from the family *Geobacteriaceae*, members of the γ-proteobacteria belonging to genus *Shewanella* are known to be capable of Fe(III)

reduction and are well studied for this physiological process (Heidelberg et al., 2002). Recent studies have shown the capability of Fe(III) reduction among the members of the phylum *Acidobacteriaceae* like *Geothrix fermentas* (Coates et al., 1999) and *Acidobacterium capsulatum* (Kishimoto et al., 1991). These findings along with the studies conducted on iron-rich sediments have shown a higher abundance of members of the phylum *Acidobacteriaceae*, indicating that this physiological process could be widespread among the members of this genus (Reiche et al., 2008). Unlike the progress made in terms of understanding the diversity of other ghysiological guilds of microorganisms like denitrifiers, sulfate-reducing bacteria and methanogens due to the developments in 16S rRNA and functional gene-based culture-independent techniques in last decades, the diversity of Fe(III)-reducing bacteria is not well elucidated because of the lack of a specific functional gene. Hence, the exact diversity of this physiological group of organisms is not known as isolation or enrichment followed by characterization remains the only method of identifying Fe(III)-reducing microorganisms.

1.4 Introduction to Lake Grosse Fuchskuhle

Lake Fuchskuhle is located in Brandenburg-Mecklenburg Lake district in Germany. It is a *Sphagnum* bog lake with pH ranging from 3.9 to 6.1 (Koschel 1995). The lake is characterized by low phosphorus, nitrogen, inorganic carbon and less diverse planktonic community compared to other lakes in the region (Koschel *et al*, 1995). The lake was formed during the end of the post-glacial period along with the other lakes in Brandenburg-Mecklenburg region (Ginzel, 1999). The lake has no surface inlet or outlet and the water in the lake is derived from rain and the surrounding peat aquifer (Koschel, 1995). The lake is a relatively small with a catchment area of 5000m^2 and a mean depth of 3.3m. It is surrounded by a fen of *Ledo-Penetum* vegetation and the area of the fen is extensive to its west compared to that of its eastern side (Succow & Jeschke 1990). Moreover, the water-logged conditions of the southwest side of the lake lead to the formation of a more densely vegetated fen compared to the lower groundwater level in the north and eastern sides of the lake, which lead to the development of less densely vegetated fen (Sachse et al., 2001). Geological studies conducted on the lake have shown a groundwater divide between the main aquifer and the peat aquifer on the southwest side of the lake. This is caused due to the development of organogenic algal mud with an elastic consistency and low permeability over period of development of the lake below the peat aquifer, thereby causing a groundwater divide (Ginzel, 1999). To study the long-term ecological and limnological changes occurring due to artificially manipulating the lake ecosystem, Lake Grosse Fuchskuhle was divided into four basins. The first division of the lake in 1986 was done between the east and the west basins and in 1990 a second division of the lake was done dividing it into four basins of similar size with plastic curtains (Kasprzak, 1993; Koschel, 1995).

During the initial years of the division the limnological characteristics of the divided lake like nutrient levels, concentration of DOC and planktonic composition were similar to that of the undivided lake; however, from 1992 onwards, profound changes started appearing among the basins (Grossart et al., 2008; Koschel, 1995; Simek et al., 1998). The pH of the eastern basins started gradually increasing compared to a decreasing pH in the western basins. This change in the pH was considered to have mediated further differences in the microbial and planktonic community structure among the basins (Koschel, 1995). The reason for the change in pH among the basins could be explained by the hydrogeology of the region. The direction of the groundwater flow in the region is from the west to east, thus making the groundwater enter the lake from the west basins (SW & NW) through the peat aquifer and leave through the eastern basins (NE & SE) into the

surrounding peat aquifer towards the eastern and northern side of the lake (Sachse et al., 2001). The water balance studies conducted over years have shown that the SW and NW basins receive groundwater from the surrounding peat bog, making them acidic due to the influx of humic acids from the bog. The SW basin due to the larger area of surrounding bog receives a greater volume of water and subsequently DOC (and humic acid) input compared to the NW basin. The eastern compartments (NE & SE) do not receive water from the peat aquifer but water from these compartments enter the peat aquifer to the eastern side of the lake, thus receiving no input of humic acids from the peat aquifer (Sachse et al., 2001).

The nature of DOC among the basins also reflects the hydrogeology of the lake and the surrounding area. Quantification studies showed a similar concentration of DOC among the basins; however, the nature of DOC among the basins differed considerably. The hydrological influence of the peat aquifer on the western basins led to higher inputs of humic acids into these basins compared to the eastern basins receiving relatively low or no input of humic acids from the fen. A large fraction of DOC in the SW basin is composed of high molecular weight and highly aromatic humic substances, similar in nature to those observed in the surrounding peat aquifer (Sachse et al., 2001). The DOC in the NE basin is mainly composed of polysaccharides, low molecular weight compounds and a relatively low concentration and less aromatic humic acids compared to the SW basin (Sachse et al., 2001). These observations were in accordance with the studies conducted on primary productivity and bacterial abundances among these basins. The NE basin has higher bacterial biomass and phytoplankton represented by high chlorophyll content, indicating that the DOC in this basin is autochthonous in nature and derived from algae compared to the allochthonous nature of DOC in SW basin. Moreover, the NE basin also has favorable conditions for the growth of algae, like low humic acid concentration, neutral pH and a higher intensity of solar radiation compared to the SW basin (Simek et al., 1998).

1.5 Aims of this study

Northern peatlands constitute an important part of the global wetland ecosystem as they constitute 30% of the global soil organic carbon. These peatlands function as a sink for atmospheric CO_2 but are known to be net emitters of other greenhouse gases like CH_4 and N_2O. Hence, methanogenesis and denitrification processes in peatlands received considerable scientific attention in the past. Recent studies have shown importance of other electron-accepting processes like Fe(III) reduction, however these processes have not been well investigated from these environments.

In the present PhD work we investigated the role of iron redox cycling in the fixation and mineralization of organic carbon in Lake Grosse Fuchskuhle.

Chapter 2:

Fe(III)-reducing bacteria are shown to be capable of degrading high molecular weight organic carbon that is abundantly available in peatlands and bog lakes. Ecological conditions like slightly low pH and presence of humic substances is known to promote Fe(III) reduction process by increasing the biological availability of otherwise insoluble Fe(III) hydroxides. *What role does Fe(III) reduction play in degradation of organic matter in Lake Grosse Fuchskuhle?*

Chapter 3:

Results of Chapter 2 have shown the presence of prominent Fe(III) reduction in Lake Grosse Fuchskuhle littoral sediment. Fe(II) oxidation in sediments under anaerobic light-independent conditions is known to happen by coupling to denitrification processes, which is necessary for the long-term sustainment of Fe(III) reduction. *Is Fe(II) oxidation being mediated by nitrate reduction in the SW basin littoral sediment of Lake Grosse Fuchskuhle and which microorganisms are involved in mediating this process?*

Chapter 4:

Chelation to humic substances is known to affect the biological availability, toxicity, speciation and redox reactivity of metals. This phenomenon is known to be beneficial for Fe(III)-reducing bacteria. *Is this phenomenon also beneficial for Fe(II)-oxidizing bacteria?*

Chapter 5:

The results of Chapter 4 showed that chelation of Fe(II) to humic substances could inhibit abiotic Fe(II) oxidation and benefit Fe(II)-oxidizing microorganisms. *What differences could be observed by quantification and enrichment of nitrate-dependent Fe(II)-oxidizing microorganisms in the presence and absence of humic substances?*

Chapter 6:

The results of Chapter 5 along with several other studies provided preliminary evidence of the Fe(II)-oxidizing nature of members of the genus *Thiomonas*. The genome sequences of several members of this genus also revealed the presence of all the genes required for mediating nitrate-dependent Fe(II) oxidation under autotrophic conditions. *Are these organisms capable of this physiological process and what role do humic substances play in promoting this physiological process?*

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Chapter 2

Role of Fe(III) reduction as a terminal electron accepting process in organic matter degradation in the littoral sediment of Lake Grosse Fuchskuhle

2.1 Abstract

Earlier studies showed the low methanogenic potential of bog lake, Lake Grosse Fuchskuhle, despite the presence of large amount of organic carbon. This low conversion of organic matter to methane indicates either the presence of other electron acceptors mediating organic matter mineralization or resilient nature of organic carbon to microbial degradation. Hence the present study was conducted to elucidate the likely role of Fe(III) as a terminal electron accepting processes in this lake sediment. Sediment cores from the most acidic (SW) and neutral basins (NE) were collected, sectioned at 5cm depths till 30cm and incubated under anaerobic conditions. CO_2, CH_4 and Fe(II) was measured at weekly time points. Production of CO_2 and Fe(II) was intensive during the first week of incubation in both the basins of the lake indicating an active Fe(III) reduction process. Following weeks of incubation showed a simultaneous production of CO_2, CH_4 at a lower rate and an increase and decrease in concentrations of Fe(II), indicating a continuous reduction and oxidation of iron. None of the sediments at all depths have reached a steady state of methanogenesis during the course of our incubations. Comparison of the amount of electrons utilized for Fe(III) reduction and methanogenesis indicates Fe(III) reduction as a dominant electron accepting process in both the basins of lake Grosse Fuchskuhle. We hypothesize that availability of Fe(III), favorable limnological conditions, ability to utilize high molecular weight organic compounds as electron donors and higher affinity for substrates like acetate compared to methanogens could have favored Fe(III) reduction over methanogenesis as dominant electron accepting process during the course of our incubations.

2.2 Introduction

Under anoxic conditions the degradation of organic matter is coupled to the sequential reduction of electron acceptors in the decreasing order of their redox potentials (Ponnamperuma, 1983). Methanogenesis acts as a terminal electron accepting process in the degradation of organic matter leading to the release of methane, an important greenhouse gas into the atmosphere. Northern peatlands are an important part of the global wetland ecosystem as they constitute 30% of the global soil organic carbon and contribute significantly to global methane emissions (Aselmann and Crutzen, 1989; Fung et al., 1991). Hence understanding the process of methanogenesis and the factors regulating this process have been a subject of several scientific investigations over the past decades (Brauer et al., 2004; Kotsyurbenko et al., 2004; Küsel et al., 2008; Metje and Frenzel, 2007; Reiche et al., 2008). Although, methanogenesis from these environments has been relatively well studied, the roles of other electron accepting processes contributing to organic matter degradation have not been well understood.

The present study was conducted on an acidic bog lake, Lake Grosse Fuchskuhle. The lake was artificially divided into four basins to study the flow of organic matter and limnological changes that happen under artificial manipulation of the lake ecosystem (Koschel, 1995). Due to this artificial division and hydrogeology of the lake and the surrounding region, the western basins (NW & SW) receive a large input of humic acids from the surrounding peat aquifer (Sachse et al., 2001) compared to that of eastern basins (NE & SE). This differential input of humic acids among the basins has lead to the gradual reduction in pH and penetration of light into the water column in western basins, which in-turn reduced the primary production (Sachse et al., 2001). On the other hand pH and algal primary production in eastern basins have gradually increased due to low input of humic acids and a higher penetration of light into water column (Sachse et al., 2001). Despite the differences in the nature of organic carbon among the basins, all the basins in the lake are of similar nutritional status as they derive water from the same groundwater aquifer.

Earlier studies conducted on Lake Grosse Fuchskuhle have shown a low rate of methanogenesis compared to other lakes in the region (Casper et al., 2003; Chan et al., 2002a; Conrad et al., 2010). A more detailed analysis has shown differences in the rate of methanogenesis among the basins despite a similar methanogenic community composition and other parameters influencing methanogenesis (Casper et al., 2003; Chan et al., 2002). Stable isotope fractionation studies conducted on the profoundal sediment of SW basin have shown that a major fraction of methane produced in this basin is through hydrogenotrophic pathway (Conrad et al., 2010). These studies

(Conrad et al., 2010; Casper et al., 2003) have observed a lower rate of conversion of sediment organic matter to methane, indicating either the presence of other electron accepting processes or a reduced rate of methanogenesis. This low rate of methanogenesis could be due to a slow primary and secondary fermentations (Billen, 1982) caused either by low pH (Rao et al., 1984) or highly resilient nature of organic matter in the sediment (Sachse et al., 2001). The objective of the present study was to elucidate the role of Fe(III) reduction in organic matter degradation in comparison to methanogenesis among the NE and SW basins of the Lake Grosse Fuchskuhle.

2.3 Experimental procedure

Lake sediment samples were collected in October 2011 from acidic dystrophic Lake Grosse Fuchskuhle in the Brandenburg-Mecklenburg Lake District (Germany). Sediment samples were taken by a gravity corer (Uwitec, Mondsee, Austria) from the littoral of both the southwest (SW) and northeast (NE) basins. The sediments were collected using 6 cm diameter plexiglass cores and sectioned at 5 cm interval to 30 cm depth. The SW basin sediment had a high concentration of humic acids, which were visible from the dark brown colour of the sediment and half decayed organic matter was observed at all depths. The pH of the SW littoral sediment was 4.8. Similar procedure was used for collecting samples from NE basin littoral sediment. The pH of the SW littoral sediment was 6. Fe(II) and Fe(III) concentrations of the sediment from different depths were determined using Ferrozine assay (Stookey, 1970a) from two different cores collected from both the sediments.

The sediment samples were incubated under anaerobic conditions as follows. Oxygen was depleted from the sediment using a vacuum manifold by repeatedly flushing the headspace with N_2 gas. 5 ml of the sediment was dispensed into 25 ml glass tubes under a N_2 atmosphere in an anaerobic chamber (Mecaplex, Grenchen, Switzerland) and incubated at 4°C. CO_2, CH_4 and Fe(II) production was determined from all the incubations in duplicates at 1 week intervals for 7 weeks. CO_2 and CH_4 in the head space was measured by gas chromatography (GC) fitted with a methanizer and flame-ionizing detector (FID) and Fe(II) measurements were done using ferrozine assay (Stookey, 1970a). CO_2 and CH_4 production rates were estimated as the increase in their concentrations in the head space after 49 days of incubation. Amount of electrons accepted by Fe(III) reduction and methanogenesis was calculated according the procedure described earlier (Yao and Conrad, 2000).

The molar ratios of concentrations of Fe(II) and CO_2 produced were calculated using the net amount of CO_2 and Fe(II) produced during the course of our incubation.

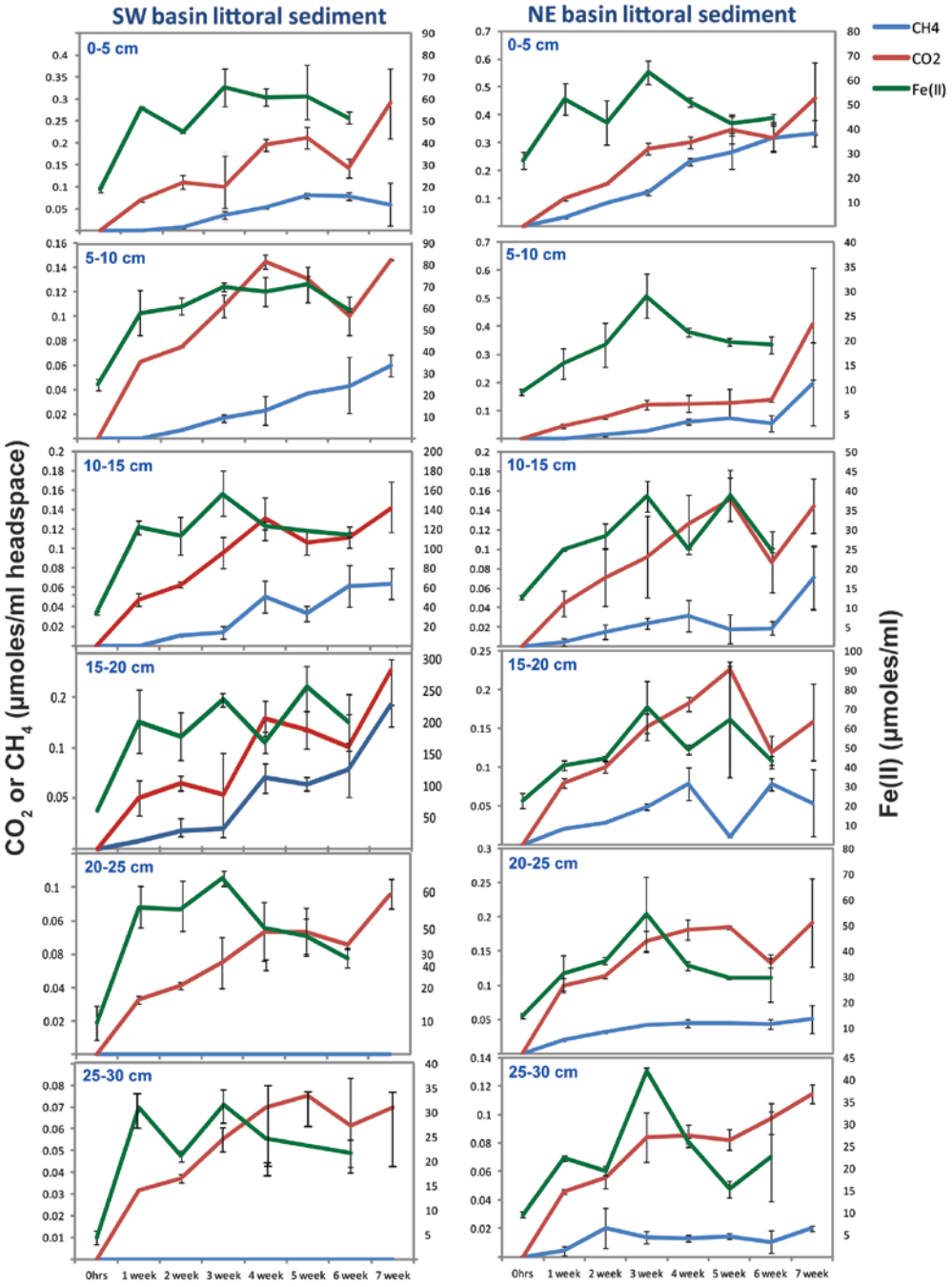

Figure.1: (A) Depth profile of Fe(II), CH_4 and CO_2 production during the time course of anaerobic incubation for seven weeks. Values are means of duplicates and bars represent standard deviation.

2.4 Results and Discussion

Incubation of the sediment from both the basins at all depths showed an immediate and continuous production of CO_2 (Figure.1). Methanogenesis could also be observed in both the basins at all depths with the exception of SW basin sediment from 20 to 30 cm depth; however significant differences could be observed among the basins (Figure.1). NE basin showed a continuous methanogeneis at all depths during the course of incubation compared to a delayed initiation of methane production in the SW basin. A high rate of methanogenesis was observed in the top 5cm of the NE basin followed by a decrease in the rate of methanogenesis with depth (Figure.2). In comparison an increasing rate of methanogenesis was observed in the SW basin with depth till 20cm (Figure.1&3), followed by no detectable methanogenesis below 20cm. The rate and depth profile of methanogenesis observed in the present study was similar to that of the past studies conducted on the profundal sediment (Casper *et al*., 2003; Chan *et al*., 2002), indicating the similarities among the sediments within the basins.

The depth profile of the rate of methanogenesis was in agreement with the initial concentrations of Fe(III) and Fe(II) in the sediment. A high rate of methanogenesis (Figure.3) was observed at sediment depths containing an initial low ratio of concentrations of Fe(III) to Fe(II), in comparison to the other depths of the sediment (Figure.2). Studies have shown that a high concentration of Fe(II) could inhibit further Fe(III) reduction (Roden and Urrutia, 1999; Roden and Zachara, 1996), which was caused due to the formation of high affinity Fe(III)-O-Fe(II) bond. This phemenenon leads to the adsorbtion of Fe(II) to Fe(III) oxide surface there by making it unacessible for microbial reduction process (Roden and Zachara, 1996). We hypothesise that this high rate of methanogenesis observed at certain depths could be due to unfavourable conditions for Fe(III) reduction. However results of our study that Fe(III) reduction had accepted a large fraction of electrons released from oxidation of organic matter, compared to methanogenesis, indicating Fe(III) reduction as a dominant electron accepting process during the course of our incubations (Figure.4). Recent studies have shown that Fe(III) reduction could be playing an important role in the mineralization of organic carbon in mildly acidic peat bogs and sediments (Küsel et al., 2008; Lu et al., 2010; Ludecke et al., 2010; Reiche et al., 2008). The ecological conditions of these environments like slightly lower pH and a high concentration of humic acids are also known to favor Fe(III) reduction process by increasing the solubility of otherwise insoluble Fe(III) hydroxides, making them biologically more available (Kappler et al., 2006a; Lovley et al., 1996).

In sediments of both the basins a sharp increase in Fe(II) and a corresponding increase in CO_2 production was observed during the first week of incubation indicating a dominant Fe(III) reduction during this period (Figure.1). In subsequent weeks of incubation only a relatively smaller increase and decrease in concentration of Fe(II) was observed, which could be due to the anaerobic oxidation and reduction of iron (Figure.1). The results of our subsequent studies (chapter 3&4) had showed the presence of nitrate-dependent Fe(II) oxidation in both the basins of the lake which could have mediated the oxidation of Fe(II).

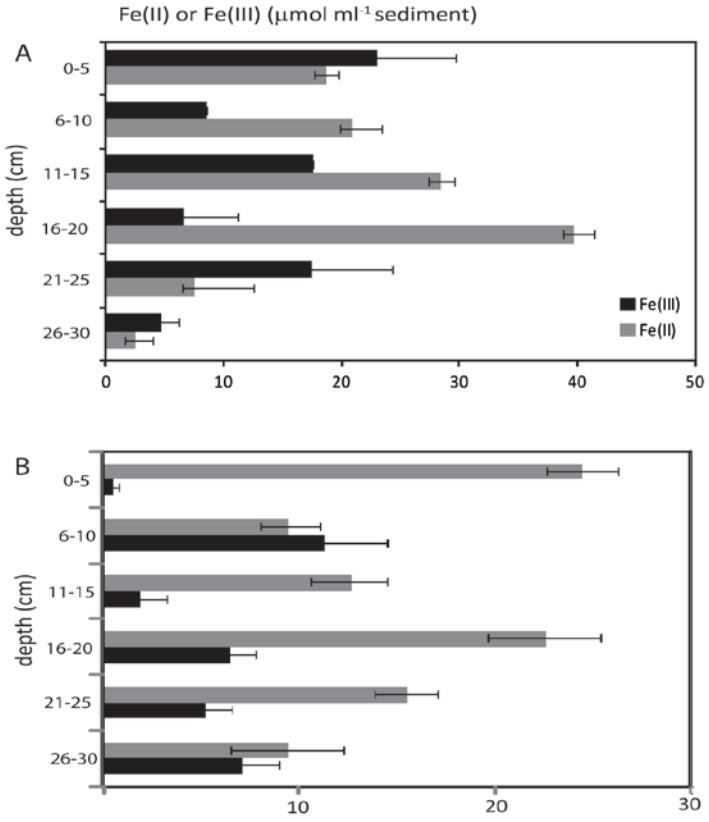

Figure.2: Depth profile of Fe(II) and Fe(III) concentrations in the (A) SW basin littoral sediment and (B) NE basin littoral sediment.

A higher ratio of Fe(II) to CO_2 production was observed at most of the depths, in comparison to steady state Fe(III) reducing conditions. Under a steady state of Fe(III) reduction coupled to organic matter degradation, the ratio of Fe(II) to CO_2 produced should be 4:1 (Roden and Wetzel, 1996). Ratios as high as 185:1 (Tab. 1) were observed in the present study indicating the reduction of Fe(III) without complete oxidation of organic carbon to CO_2. This could be due to the hydrolysis of higher molecular weight organic carbon like cellulose, hemicelluloses and lignin, which are known

to be present in high concentration in peat lands (Bland et al., 1968; Farmer and Morrison, 1964) to lower molecular weight compounds. Comparison of these ratios among the basins (Table.1) had shown that this process of Fe(III) reduction is more prevalent in SW basin compared to NE basin.

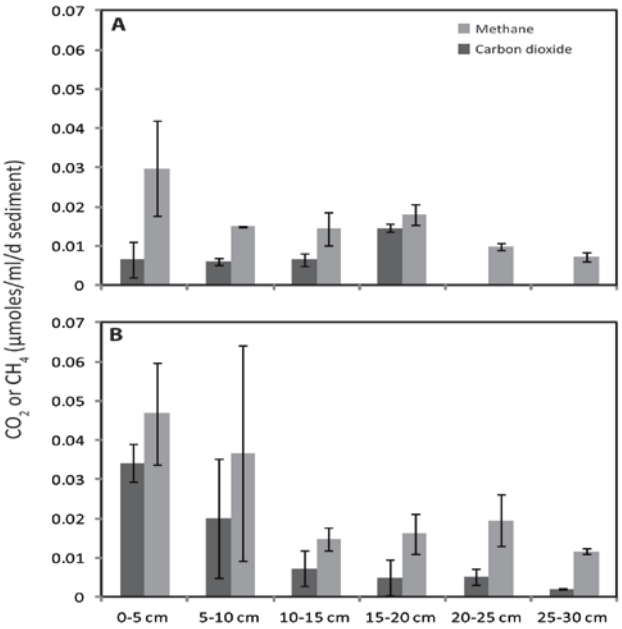

Figure.3: Methane production rate along the depth of the sediment in the (A) SW and (B) NE basin. Standard deviations of duplicates are indicated.

Studies conducted on the lake, have shown that a large fraction of organic carbon in NE basin is of algal origin (Sachse et al., 2001), this in combination with the more neutral conditions and formation of an anoxic hypolimnion could have provided favorable conditions for the hydrolysis of higher molecular weight organic carbon to simpler organic compounds like volatile fatty acids whose oxidation could have lead to the formation of a higher amount CO_2 compared to the SW basin. On the other hand a high concentration of resilient humic acids, low concentration of polysaccharides, lower bacterial numbers in combination with the low pH conditions of SW basin could have reduced the rate of hydrolysis of DOC in SW basin, which could have lead to the formation of lower concentration of volatile fatty acids. A large amount of partially degraded organic matter observed even in the deeper layers of the sediment also suggests a reduced rate of primary and secondary fermentation processes in this sediment. These ecological could also favor Fe(III) reducing microorganisms compared to methanogens due to the ability of Fe(III) reducing

microorganisms to oxidize high molecular weight organic compounds compared to methanogens which are capable of utilizing only a limited range of low molecular weight volatile fatty acids.

Degradation of hydrocarbons coupled to Fe(III) reduction was observed in several natural environments like contaminated ground water aquifers; however the degradation of high molecular weight natural organic carbon like cellulose, hemicelluloses and lignin have not been shown in pure cultures. Physiological characterization of known Fe(III) reducing bacteria have shown, that these bacteria have

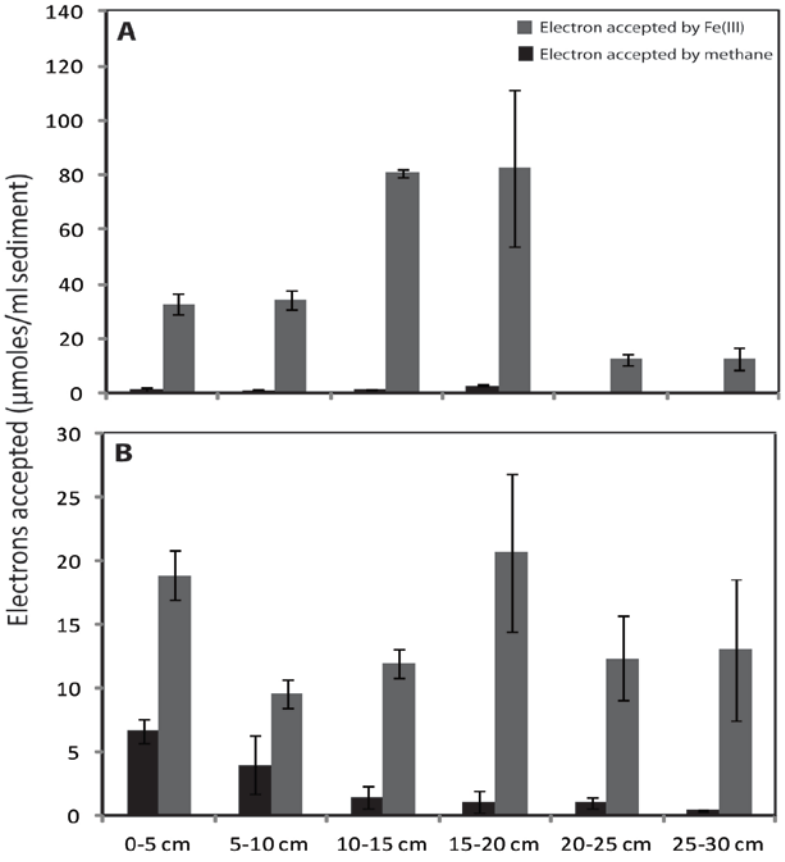

Figure.4: Amount of electrons accepted after 6 weeks of incubation (A) SW basin and (B) NE basin. The data are based on the net increase in concentration of Fe(II) and CH_4 during the course of our incubation.

Depth	SW basin	NE basin
0-5 cm	20.66	26.57
6-10 cm	49.53	19.06
11-15 cm	185.74	21.42
16-20 cm	138.60	40.99
21-25 cm	18.53	22.42
26-30 cm	25.75	42.66

Table 1: Depth profile of molar ratios of increase in concentrations of Fe(II) to CO_2 after 6 weeks of incubation from NE and SW basin sediments of Lake Grosse Fuchskuhle.

a diverse pattern in utilizing carbon substrates (Lonergan et al., 1996), indicating the preferential nature of carbon source among Fe(III) reducing bacteria. To date the identity of Fe(III) reducing bacteria capable of oxidizing high molecular weight organic compounds present in peat bogs have not been studied. Several cultured Fe(III) reducing bacteria were also shown to incompletely oxidize organic matter (Laverman et al., 1995), which could lead to the production of acetate and H_2, indicating the possibility of these organisms to form syntrophic association with other physiological groups of bacteria. Such syntrophic association of Fe(III) reducing bacteria with nitrate and sulfate reducing bacteria (Cord-Ruwisch et al., 1998) have been experimentally shown. The possibility of similar syntrophic association between Fe(III) reducing bacteria and methanogenic archaea have also been proposed by earlier studies (Achtnich et al., 1995), however experimental evidences of such interactions were only shown recently (Kato et al., 2012; Liu et al., 2012) and the ecological implications of these interactions were yet to be elucidated. The presence of high molecular weight organic carbon which cannot be directly utilized by methanogens and simultaneous Fe(III) reduction and methanogenesis observed in the present study provide preliminary evidences of syntrophic associations between these two physiological groups of microorganisms.

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Chapter 3

Chemolithotrophic nitrate-dependent Fe(II)-oxidizing nature of actinobacterial subdivision lineage TM3

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3.1 Abstract

Anaerobic nitrate-dependent Fe(II) oxidation is widespread in various environments and is known to be performed by both heterotrophic and autotrophic microorganisms. Although Fe(II) oxidation is predominantly biological under acidic conditions, to date most of the studies on nitrate-dependent Fe(II) oxidation were from environments of circumneutral pH. The present study was conducted in Lake Grosse Fuchskuhle, a moderately acidic ecosystem receiving humic acids from an adjacent bog,with the objective of identifying, characterizing and enumerating the microorganisms responsible for this process. The incubations of sediment under chemolithotrophic nitrate-dependent Fe(II)-oxidizing conditions have shown the enrichment of TM3 group of uncultured *Actinobacteria*. A time-course experiment done on these *Actinobacteria* showed a consumption of Fe(II) and nitrate in accordance with the expected stoichiometry (1:0.2) required for nitrate-dependent Fe(II) oxidation. Quantifications done by MPN showed the presence of $1x10^4$ autotrophic and $1x10^7$ heterotrophic nitrate-dependent Fe(II) oxidizers per gram fresh weight of sediment. The analysis of microbial community by 16S rRNA gene amplicon pyrosequencing showed that these actinobacterial sequences correspond toapproximately 0.6% of bacterial 16S rRNA gene sequences. Stable isotope probing using $^{13}CO_2$ was performed with the lake sediment and showed labeling of these *Actinobacteria*. This indicated that they might be important autotrophs in this environment.Although these *Actinobacteria* are not dominant members of the sediment microbial community, they could be of functional significance due to their contribution to the regeneration of Fe(III), which has a critical role as an electron acceptor for anaerobic microorganisms mineralizing sediment organic matter. To our best knowledge this is the first study to show the autotrophic nitrate-dependent Fe(II)-oxidizing nature of TM3 group of uncultured *Actinobacteria*.

3.2 Introduction

Organic matter degradation in aquatic environments such as wetlands or lake sediments is mediated anaerobically according to the redox stratification of the sediment with methanogenesis being the final process (Ponamperuma *et al*., 1972). Studies have shown that Fe(III) reduction can suppress methanogenesis and could contribute to the mineralization of a large fraction of sediment organic matter (Kusel et al., 2008; Roden and Wetzel, 1996). Due to the high concentration of biologically available iron (Steinmann and Shotyk, 1995; Steinmann and Shotyk, 1997) and favorable limnological conditions in humic-rich habitats, Fe(III) reduction could act as a dominant terminal electron-accepting process (Reiche *et al*., 2008) given a continuous recycling of Fe(III) from Fe(II) in the sediment.

Fe(II) oxidation in natural environments occurs at the oxic-anoxic interface by chemically reacting with atmospheric O_2 or by aerobic Fe(II)-oxidizing bacteria (Edwards *et al*., 2004; Emerson and Revsbech, 1994). In the deeper layers of the sediment, Fe(II) oxidation is known to happen in the rhizosphere of plants by root released O_2(Frenzel et al., 1999; Neubauer et al., 2002b; Neubauer et al., 2007; Weiss et al., 2007) or in the sediment by nitrate-dependent Fe(II) oxidation by organisms growing either autotrophically or heterotrophically (Straub et al., 1996). An increasing body of literature over the last decade has shown that biogenic Fe(II) oxidation could play a more prominent role in iron cycling than considered previously, especially in low pH environments (Lu et al., 2010; Ludecke et al., 2010).

Chemolithotrophic nitrate-dependent Fe(II) oxidation was first reported by Straub *et al.* (1996) and has been reported in various environments since, primarily in lake sediments (Emmerich et al., 2012; Hauck et al., 2001; Muehe et al., 2009; Straub and Buchholz-Cleven, 1998a). Although autotrophic nitrate-dependent oxidation of Fe(II) was observed in many natural habitats, to date there are only two pure cultures available: the hyperthermophilic archaeon *Ferroglobus placidus*(Hafenbradl *et al*., 1996) and the betaproteobacterium *Pseudogulbenkiania sp.* strain 2002, which can alternatively grow autotrophically or heterotrophically (Weber et al., 2006b). Autotrophic nitrate-dependent Fe(II) oxidation is a poorly understood process but could be of major significance in anoxic cycling of iron in lake sediments; however, due to the difficulty in culturing these organisms and lack of either functional gene or 16S rRNA gene-based primers for this functional guild, the ecology of these microorganisms is difficult to investigate.

Lake Grosse Fuchskuhle is an acidic bog lake with a high concentration of recalcitrant, high molecular weight humic acids (Sachse *et al*., 2001). The low pH of the sediment (pH 4.5), complexation of Fe(II) by organic matter (Theis and Singer, 1974) and the ability of oxygen to penetrate only the top few millimeters of the sediment could greatly reduce the rate of abiotic Fe(II) oxidation (Stumm and Morgan, 1996). As a result, we hypothesize that microbially-mediated anaerobic nitrate-dependent Fe(II) oxidation in the sediment could be contributing significantly to Fe(II) oxidation. The objective of this study was to identify microbial groups involved in nitrate-dependent Fe(II) oxidation in the littoral sediment of Lake Grosse Fuchskuhle.

3.3 Experimental procedure

Sampling

Lake sediment samples were collected in April 2010 from acidic dystrophic Lake Grosse Fuchskuhle in the Brandenburg-Mecklenburg Lake District (Germany). Lake Grosse Fuchskuhle is an artificially divided lake with different pH values in the four compartments. A main divergent factor is the inflow of humic acids from an adjacent bog; the southwest basin is the most acidic and the northeast is the least (Koschel, 1995). Sediment samples were taken by a gravity corer (Uwitec, Mondsee, Austria) from the profundal and littoral of both the southwest (SW) and northeast basins, but most experimental work in this study focused on the SW littoral samples. The top 10 cm of the sediments were collected using 6 cm diameter plexiglass cores. The sediment had a high concentration of humic acids, which were visible from the dark brown colour of the sediment and overlying water. The collected sediment was composed mainly of coarse particulate organic material (mainly half-decayed leaves and small pieces of wood). The pH of the SW littoral sediment was 4.5. The concentration of Fe(II) and Fe(III) in the sediment was determined using the ferrozine assay (Stookey, 1970a) and nitrate concentrations were measured by flow injection analysis (Tecator, Rellingen, Germany).

Enrichment of nitrate-dependentFe(II)-oxidizing bacteria

The enrichment of nitrate-dependent Fe(II)-oxidizing bacteria was done according to the procedure described by Straub et al., (1996) with the exception of using phosphate buffer, a pH of 4.5 and $FeCl_2$ instead of $FeSO_4$ to prevent the growth of sulfate reducing bacteria. The freshwater medium

was prepared as follows: NH_4Cl (0.3 g), $MgSO_4.7H_2O$ (0.05 g), $MgCl_2.6H_2O$ (0.4 g) and $CaCl_2$ (0.1 g) buffered by the addition of 100 ml of 0.5 M KH_2PO_4 to 900 ml of the above medium (final pH 4.5) added after autoclaving and cooling to room temperature to avoid precipitation. Filter sterilized vitamins and trace elements (Widdel and Bak, 1992) were added to the medium after autoclaving. Oxygen was depleted using a vacuum manifold and repeatedly flushing the headspace with N_2 gas. 20 ml of the medium was dispensed into 120 ml serum bottles under a N_2 atmosphere in an anaerobic chamber (Mecaplex, Grenchen, Switzerland). Different combinations of $FeCl_2$(10 mM), sodium nitrate (4 mM), sodium acetate (2.5 mM) and CO_2(5% headspace) were included in the enrichment medium. The Fe(II)-EDTA stock was prepared by mixing 50 mM EDTA with 100 mM $FeCl_2$. One set of the incubations was done without phosphate buffered medium and the pH adjusted to 4.5 with HCl. 1.5 ml of Lake Grosse Fuchskuhle SW littoral sediment was used as an inoculum. All incubations were performed in triplicate and incubated in the dark on a shaker (150 rpm) for ten days.

A time course experiment was performed to determine the ratio of Fe(II) to nitrate consumed over a period of 14 days. The experimental set up containedthe above medium with nitrate and Fe(II) with 1% of the actinobacterial enrichment as inoculum. 5% CO_2 was added to the headspace as the sole carbon source. Samples for T-RFLP as well as for Fe(II) and nitrate measurements were taken every 24 hours. The sediment filtrate was used for ammonia determinations (Kandler and Gerber, 1988).Nitrate measurements were done colorimetrically as described previously (Hart *et al*., 1994). N_2O was measured by gas chromatography (Carlo Erba Instruments, GC 8000) using a ^{63}Ni-electron capture detector (ECD).

Enumeration of Fe(II)-oxidizing bacteria by most probable number (MPN)

An MPN method was used to enumerate the Fe(II)-oxidizing bacteria according to the procedure described previously (Straub and Buchholz-Cleven, 1998a) with the above mentioned modifications. Two sets of MPN tubes were incubated each in triplicate. One set of tubes contained the phosphate buffered freshwater medium (pH 4.5) with Fe(II) and nitrate to enumerate autotrophic Fe(II)-oxidizing bacteria and the other set of tubes contained phosphate buffered freshwater medium, nitrate, Fe(II) and acetate for enumeration of heterotrophic Fe(II)-oxidizing bacteria. The tubes were incubated for 12 weeks in the dark at 25°C and gently inverted daily. Tubes were scored positive based on the reduction in the amount of Fe(II) and acetate in the respective tubes compared to the uninoculated controls. The Fe(II) estimations were done using the ferrozine assay (Stookey,

1970b) and a standard MPN table was used to calculate the cell numbers. DNA was extracted from the positive tubes and terminal restriction fragment length polymorphism (T-RFLP) profiling was performed as described below for the identification of the bacterial groups growing in the tubes.

Stable isotope probing (SIP) experimental setup and gradient fractionation

Time series SIP incubations were performed in duplicate. Equal volumes of sterile deionized water and sediment were mixed and 40 ml was dispensed into 120 ml serum bottles capped with black butyl stoppers. The bottles were made anaerobic by flushing with N_2 and $^{13}CO_2$ (Campro Scientific, Berlin, Germany) was added to the headspace to a final concentration of 5%. Control bottles contained 5% unlabeled CO_2. All the bottles were incubated at 25°C on a shaker (150 rpm) in the dark. The headspaces were renewed every 4 days. 4 ml samples were collected from all the incubation bottles after 3, 6 and 12 weeks. CO_2 and CH_4 measurements were done by gas chromatography (GC) fitted with a methanizer and flame-ionizing detector (FID). The ratios of ^{12}C and ^{13}C were determined weekly using GC-IRMS. The collected sediment was centrifuged and the pore water was used for the analysis of cations by ion chromatography and volatile fatty acids by HPLC. The centrifuged sediment was frozen in liquid nitrogen and stored at -80°C until the extraction of DNA. The pore water was used for the analysis of volatile fatty acids. Nucleic acids were extracted from the sediment using Nucleospin soil kit (Macherey-Nagel, Düren, Germany).

Density gradient centrifugation of DNA (5.0 µg) extracted from the incubated samples was performed with a cesium chloride (CsCl) buoyant density of 1.72 g ml^{-1} subjected to centrifugation at 177 000 *g* for 36 h at 20°C (Lueders *et al.*, 2004). CsCl gradients were fractionated from bottom to top by displacing the gradient medium with nuclease-free water using a syringe pump (Kent Scientific, Torrington, CT, USA) at a flow rate of 0.45 ml min^{-1}, generating 12 fractions per density gradient. The density of each fraction was determined by refractometry (Reichert, Depew, NY, USA). DNA was recovered by PEG 6000 precipitation and dissolved in 30 µl of nuclease free water (Applied Biosystems, Darmstadt, Germany).

The relative abundance of 16S rRNA genes within gradients was determined by real-time PCR using the SYBR Green JumpStart ReadyMix System (Sigma, Taufkirchen, Germany) as described previously (Stubner, 2002). The assays were performed using an iCycler instrument (Bio-Rad, Munich, Germany) and the associated software.

PCR, cloning and sequence analysis

For cloning and sequencing 16S rRNA genes from SIP experiments, PCR amplification was done using the Ba27fand Ba907r primers, which generates a product of approximately 900 bp (Lueders *et al*. 2004). All the PCR reactions were performed in 50 µl volume with the following composition: 1x PCR buffer (Promega, Mannheim, Germany), 0.2 mM $MgCl_2$, 10 pmol of each primer, 10 µg of BSA (Roche, Mannheim,Germany), 2U of GoTaq (Promega), 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany) and 1 µl of template DNA. The PCR was performed on a GeneAmp PCR system 9700 instrument (Applied Biosystems) with the following cycling conditions: 94°C for 4 min, 35 cycles of 94°C for 1 min, 52°C for 40 sec and 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were cloned using the pGEM-T Easy Vector System (Promega) and transformed into *E. coli* JM109 competent cells according to the manufacturer's instructions. 21 clones each were randomly picked from heavy and light fractions and sequenced. The phylogenetic affiliation of the clones was done using the ARB software package (Ludwig *et al*., 2004) and trees were constructed using the neighbor joining method. Sequences were deposited in GenBank with accession numbers KC540872 to KC540892.

Terminal restriction fragment length polymorphism (T-RFLP)

The PCR amplification of bacterial 16S rRNA genes for T-RFLP analysis was performed as described above, except that the Ba27F primer was labeled with FAM (6-carboxyfluorescein). PCR products were purified using Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). Approximately 100 ng of purified PCR product was used for restriction digestion. Digestions were performed in a reaction volume of 20 µl containing 1x Tango buffer and 5U of MspI enzyme (Fermentas); reactions were incubated at 37°C incubator overnight. The reactions were processed using SigmaSpin™ Post Reaction Clean-Up Columns (Sigma) and 2 µl of the processed fragments were mixed with 11µl of Hi-Di™ formamide (Applied Biosystems), 0.3 µl of ROX-labeled MapMarker 1000+30, 40 (BioVentures, Murfreesboro, TN, USA) and incubated at 94°C for 3 min and cooled on ice. The size separation was performed using 3130 Genetic Analyzer (Applied Biosystems). T-RFs shorter than 50 bp were not considered to avoid the detection of primers and primer-dimers.

Pyrosequencing of bacterial 16S rRNA genes

DNA was extracted from littoral and profundal sediment from both the southwest and northeast basins of Lake Grosse Fuchskuhle using the Nucleospin soil kit (Macherey-Nagel). Bacterial 16S rRNA gene PCR products were amplified using primers 343Fmod and 784Rmod as described previously (Köhler *et al*., 2012). The PCR products were sequenced using a Roche 454 GS Junior instrument. Sequence analysis was performed using the Mothur software v1.25.0 (Schloss *et al*., 2009). Processing of sequences within Mothur, including denoising and chimera removal, was performed according to the standard operating procedure of the software developer. Briefly, sequences were screened by allowing 1 mismatch to the barcode, 2 mismatches to the primer and a maximum homopolymer length of 8 bases. Sequences shorter than 200 bp were removed. Chimeras were removed using uchime within Mothur. Between 1047 and 3444 high quality sequences were obtained from each sample. Identification of *Actinobacteria* was performed using the SILVA taxonomy and the classification was verified by adding these sequences into the SILVA108 reference tree (Pruesse *et al*., 2007) by parsimony within ARB (Ludwig *et al*., 2004).

3.4 Results

Fe(II), Fe(III), nitrate and methanogenesis potential in Lake Fuchskuhle littoral sediment

Duplicate sediment cores were collected from the littoral zone of Lake Grosse Fuchskuhle and partitioned into 5 cm sections to a depth of 30 cm. The concentration of Fe(II) and Fe(III) was determined at each depth (Figure 1A). Although insufficient replication of the data was available to calculate significance, the observed Fe(II) concentration was higher than Fe(III) only between 5 to 20 cm. The concentration of CH_4 and CO_2 after anaerobic incubation for 7weeks was determined (Figure 1B) and greatest methanogenesis potential was observed at the 15-20 cm depth. Nitrate was detectable in small amounts in the hypolimnion (4.0 m; < 2 µmol/l) and also in sediment pore-water (2 µmol/l)(results not shown).

Enumeration and enrichment of nitrate-dependent Fe(II)-oxidizing microorganisms

A most probable number enrichment assay was performed to estimate the abundance of readily cultivable anaerobic nitrate-dependent Fe(II)-oxidizing microorganisms in the littoral sediment of

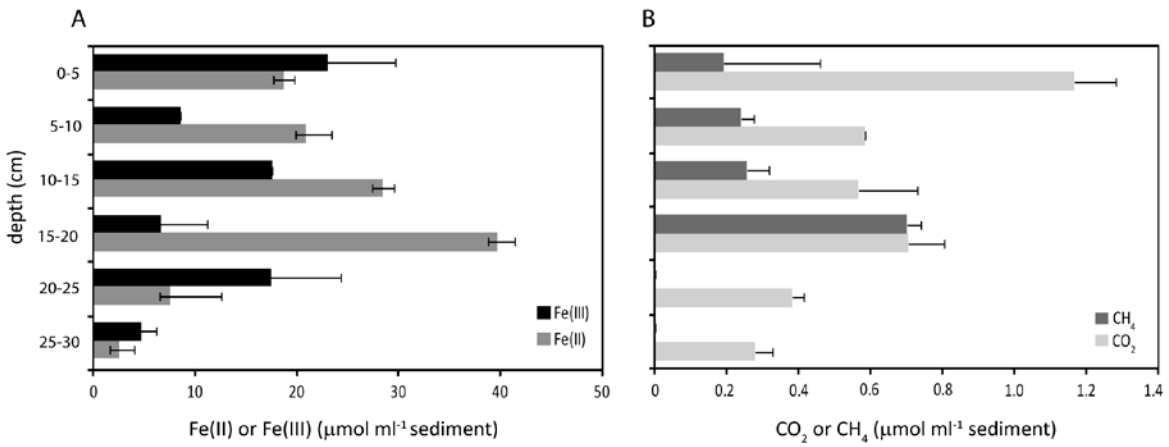

Figure 1. (A) Depth profile of Fe(II) and Fe(III) concentrations in Lake Grosse Fuchskuhle SW basin littoral sediment. (B) CH_4 and CO_2 production during anaerobic incubation for seven weeks. Values are means of duplicates and bars represent standard deviation.

Lake Grosse Fuchskuhle. The assay was performed with either CO_2 or acetate as carbon source to estimate potential autotrophic and heterotrophic microorganisms. The assays indicated the presence of $1x10^4$ autotrophic and $1x10^7$ heterotrophic nitrate-dependent Fe(II)-oxidizers per gram fresh weight of sediment.

A freshwater medium was used to enrich potential nitrate-dependent Fe(II)-oxidizing microorganisms from Lake Grosse Fuchskuhle littoral sediment. After ten days of incubation the enrichments were characterized by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting (Figure 2). The incubation with added Fe(II), nitrate and CO_2 (Figure 2B) resulted in an enrichment culture characterized by a dominant T-RF of 146 bp. When Fe(II) or both Fe(II) and nitrate were omitted, enrichments characterized by a dominant T-RF of 429 bp were obtained (Figure 2C and 2D). When nitrate alone was omitted, the dominant T-RF was 97 bp (Figure 2E). Finally, an enrichment characterized by a 437 bp peak was obtained when acetate was provided. The results of the various enrichments were reproducible across triplicates (results not shown). 16S rRNA gene cloning and sequencing were performed to identify the 146 bp T-RF in the enrichment. A total of 86% of the clones obtained had the 146 bp T-RF and a phylogenetic analysis (Figure 3) indicated they belong to the uncultivated TM3 group *Actinobacteria* (Rheims *et al*., 1996). To our knowledge, no isolates from this group have been identified and their physiology is unknown.

Characterization of the *Actinobacteria* enrichment culture

An enrichment of the TM3 *Actinobacteria* could be reproducibly obtained by inoculation of the sediment into the enrichment medium. Dilution series done from the enrichment showed the presence of *Actinobacteria* and the oxidation of Fe(II) until the 10^{-4} dilution, beyond which neither

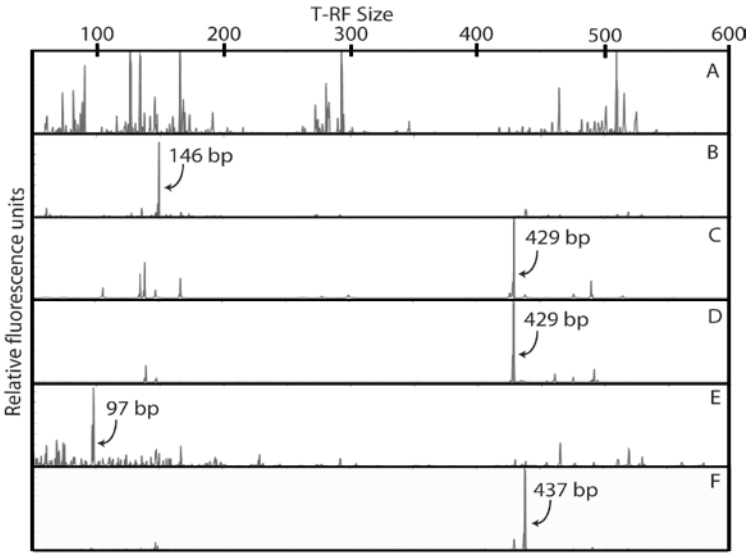

Figure 2. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA genes from different enrichment incubations. The enrichment conditions are described in detail in the materials and methods section. All enrichments were performed in triplicate and each produced similar T-RFLP profiles, therefore a single representative is shown. (A) T-RFLP from the unincubated sediment; (B) freshwater medium containing added Fe(II) as electron donor, NO_3^- as terminal electron acceptor and CO_2 as carbon source; (C) as (B), but without added Fe(II) and NO_3^-; (D) as (B), but without added NO_3^-; (E) as (B), but without added Fe(II); (F) as (B), but with acetate instead of CO_2 as carbon source.

the growth of *Actinobacteria* nor Fe(II) oxidation was observed. Modifying the medium, for example by omitting the phosphate buffer to avoid formation of white precipitate (presumably iron phosphates), or the addition of EDTA-chelated Fe(II) to simulate the chelation by humic acids, failed to enrich the TM3 *Actinobacteria*.

The ratio of Fe(II) oxidized to nitrate reduced by a 10^{-2} dilution of the enrichment was determined (Figure 4). Both Fe(II) and nitrate were consumed during the incubation with a molar

ratio of Fe(II) to nitrate of 1:0.23 at each time point measured. No N_2O production was observed in these incubations. Uninoculated and killed controls did not show Fe(II) or nitrate consumption. The T-RFLP profiling done at all time points showed the presence of the single dominant 146 bp T-RF.

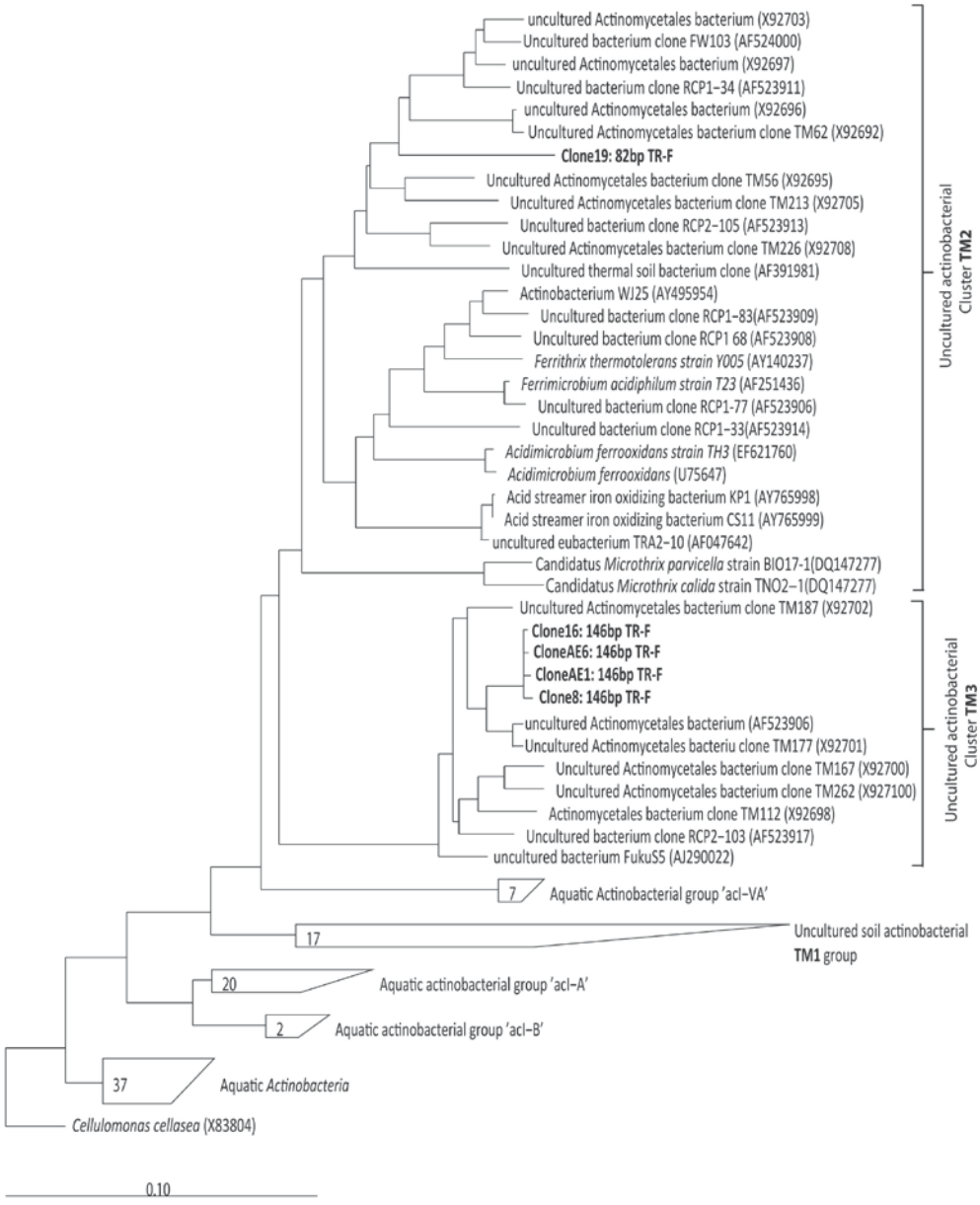

Figure 3. Neighbor-joining phylogenetic tree of *Actinobacteria*16S rRNA gene sequences. Representative sequences obtained in this study are shown (bold type). The T-RF sizes of the sequences are indicated.

Estimation of TM3 *Actinobacteria* abundance in sediment

Cloning and Sanger sequencing as well as pyrosequencing of 16S rRNA genes from Lake Grosse Fuchskuhle sediment was performed to estimate the abundance of TM3 *Actinobacteria* in the sediment. Sequencing was performed from four samples taken from four different zones: southwest littoral (SWL), southwest profundal (SWP), northeast littoral (NEL) and northeast profundal (NEP). 96 clone sequences from each sample were obtained and none were found to match closely to the TM3 *Actinobacteria*. Many of the sequences had T-RFs of 146 bp, but these did not belong to *Actinobacteria* (results not shown). Sequences related to TM3 *Actinobacteria* were obtained from each sample by pyrosequencing: 11 of 1047 sequences from SWL; 23 of 3444 sequences from SWP; 6 of 2743 sequences from NEL; and 10 of 2369 sequences from NEP sediment.Assuming average 16S rRNA gene copy numbers of these organisms compared with the other community members, this corresponds to an average abundance of 0.57% of bacteria in the sediment.Quantification of total bacterial 16S rRNA genes from SWL, SWP and NEP sediment was performed by real-time PCR and found to be 6.44×10^8 ($\pm 2.35 \times 10^8$), 1.97×10^8 ($\pm 3.99 \times 10^7$) and 7.53×10^7 ($\pm 5.55 \times 10^7$) per gram fresh sediment respectively. Assuming the TM3 *Actinobacteria* sequences are 0.57% of the total, this would correspond to between4.3×10^5 and 3.7×10^6TM3 actinobacterial 16S rRNA genes per gram of fresh sediment.

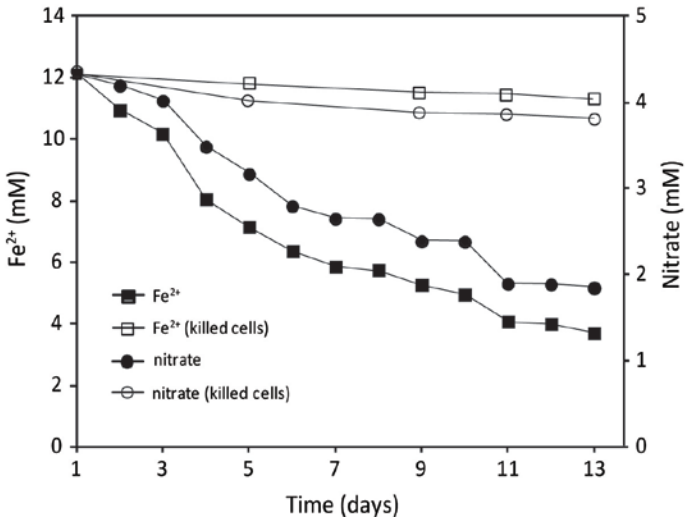

Figure 4. Characterization of Fe(II) and NO_3^- consumption by the actinobacterial enrichment culture.

Stable isotope probing

DNA-based stable isotope probing (DNA-SIP) was performed to determine if the TM3 *Actinobacteria* obtained in the enrichment cultures could be detected by SIP in the sediment using $^{13}CO_2$. The sediment was incubated with added CO_2 in the headspace with no additional supply of nutrients to the sediment samples. A time-course was performed and subsamples of the sediment were taken after 3, 6 and 12 weeks. Volatile fatty acid (VFA) analysis by HPLC never showed an accumulation of VFAs and the incubations showed a delayed initiation of methanogenesis (~10 weeks), which subsequently proceeded at a low rate (results not shown).

The DNA was isolated from the samples taken at 3-, 6- and 12-weeks and subjected to CsCl centrifugation. The quantification of bacterial 16S rRNA genes from the SIP gradient fractions showed the largest abundance in the light gradient fractions corresponding to densities between 1.71 and 1.75 g ml^{-1} in both the labeled and control incubations (Figure 5). Relatively low copy numbers of 16S rRNA genes were observed in the heavy gradient fractions ranging from 1.8×10^5 to 2.2×10^6 in labeled CO_2 incubations and 1.6×10^3 to 5.0×10^4 in the corresponding gradient fractions of the controls. A similar trend was observed in all the time points, indicating that the rate of growth and labeling by the autotrophic microorganisms was very low. T-RFLP fingerprinting was performed and peaks of 69 bp, 119 bp, 146 bp and 487 bp were found in heavy fractions only in the incubations with $^{13}CO_2$ and unlabeled CO_2 (Figure 5). A more diverse T-RFLP profile was observed from the light gradient fractions. A similar pattern was found after 3-, 6- and 12-weeks (results not shown).

Cloning and sequencing of 16S rRNA genes were performed from light and heavy gradient fractions. Clones from the heavy fraction with T-RF sizes corresponding to the 119 bp, 146 bp and 487 bp fragments observed in the T-RFLP were obtained (Figure 6). The clones corresponding to 119 bp T-RFs grouped among sequences from *Gallionella* and *Sideroxydans*. The clones characterized by a 146 bp T-RFs clustered with the *Actinobacteria* TM3 sequences obtained from the enrichment cultures and those characterized by a 487 bp T-RF grouped among sequences from the genus *Thiomonas*. A more diverse set of sequences were obtained from the light gradient fractions, including *Planctomyces*, *Verrucomicrobia* and *Chloroflexi*, which are all previously reported to be abundant in peat bogs (Dedysh *et al*., 2006; Kulichevskaya *et al*., 2006; Kulichevskaya *et al*., 2007). Several *Methylocystis* clones were obtained from the light fractions and also had a T-RF length of 146 bp.

A similar T-RFLP analysis of archaeal 16S rRNA gene did not show differences between light and heavy fractions, indicating that *Archaea* were not labeled during the SIP incubation (results not shown)

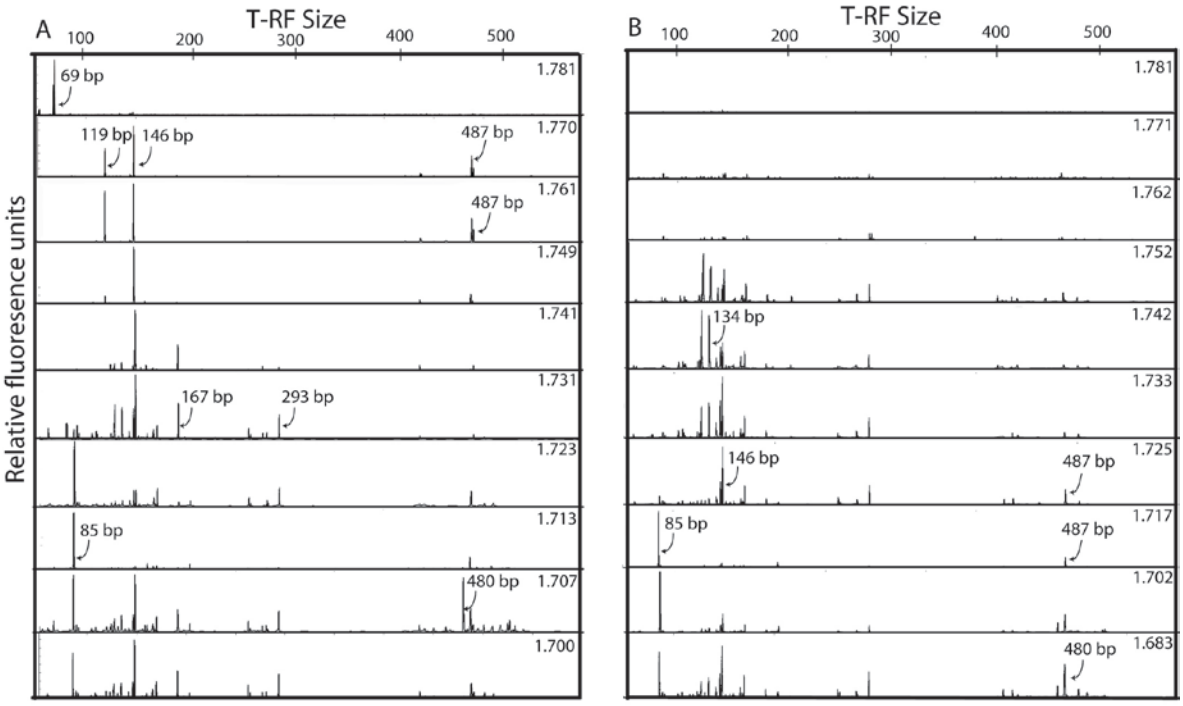

Figure 5. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of density resolved bacterial 16S rRNA from the 6-week time point. (A) Incubation with $^{13}CO_2$ and (B) control with unlabeled CO_2. The densities of the fractions are given in right top of each fraction as g ml^{-1} values. The fractions with densities greater than 1.76 g ml^{-1} were considered heavy. The sizes of the major T-RFs are indicated.

3.5 Discussion

Previous studies on the profundal sediment of Lake Grosse Fuchskuhle have shown a relatively low rate of methanogenesis and a less diverse methanogenic archaeal community compared with other freshwater lakes (Casper et al., 2003; Chan et al., 2002b). A similar trend was observed in this study with the littoral sediment showing a relatively poor methanogenic potential (Figure 1). The low rate of methanogenesis in this sediment might be attributed to favorable conditions for Fe(III) reduction. Indeed, the iron depth-profile of the sediment (Figure 1) showed

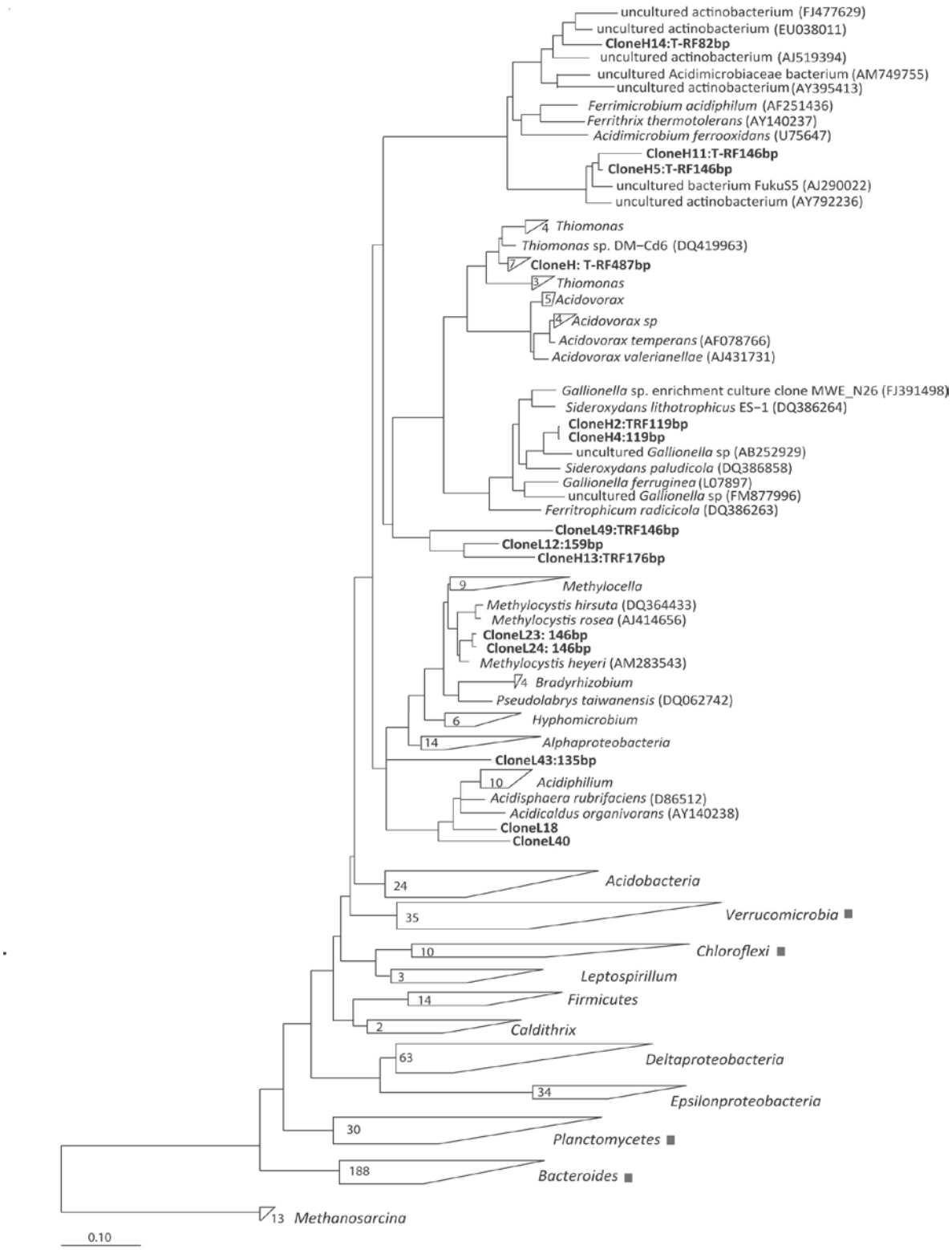

Figure 6. Neighbor-joining phylogenetic tree of the 16S rRNA gene clone sequences from the heavy (CloneH) and light (CloneL) SIP gradient fractions. Square symbols next to collapsed clades indicate that clones from the light fractions are contained within.

the presence of biologically available Fe(III) in the top 15 cm of the sediment at concentrations sufficient to suppress methanogenesis (Roden and Wetzel, 1996). A maximum rate of methanogenesis was found at the sediment depth of 15-20 cm, which is similar to an earlier study (Chan *et al*., 2003) and might be attributed to a relatively high concentration of Fe(II), which is known to be capable of inhibiting Fe(III) reduction (Roden and Urrutia, 1999; Roden and Zachara, 1996). Although our results have suggested that Fe(III) reduction could be playing a role in the mineralization of sediment organic matter, the long term sustainment of Fe(III) reduction in anoxic zones requires a continuous recycling of Fe(III) from Fe(II) by anaerobic Fe(II)-oxidizing bacteria.

In order to isolate and characterize the chemolithotrophic Fe(II)-oxidizing bacteria in Lake Grosse Fuchskuhle, we performed incubations according to the procedure described by Straub and Buchholz-Cleven (1998), which resulted in the enrichment of TM3 group of uncultured *Actinobacteria*, phylogenetically close to the sequences previously reported from the same basin of this lake (Glockner et al., 2000). Previous culture-independent studies have revealed the presence of deeply-branching phylogenetic groups of *Actinobacteria* from various terrestrial and marine environments (Liesack and Stackebrandt, 1992; Colquhoun *et al*., 1998; Wohl and McArthur, 1998). These actinobacterial groups from soil have been designated TM, forming three major clusters TM1, TM2 and TM3 (Rheims *et al*., 1996). *Actinobacteria* belonging to groups TM2 and TM3 in particular have been reported from various environments worldwide and with a greater abundance in low pH environments such as peat bogs (Rheims *et al*., 1999). Due to their ubiquitous distribution, they are believed to be contributing to ecologically important processes (Rheims *et al*., 1999; Felske *et al*., 1997). Studies have shown that these *Actinobacteria* are metabolically active (Felske *et al*., 1997), slow-growing and easily overgrown under enrichment conditions (Rheims *et al*., 1999). Cultivation attempts have led to the isolation of representatives related to the TM2 group, namely*Ferrithrix thermotolerans* (Johnson *et al*., 2009), *Ferrimicrobium acidiphilum* (Johnson *et al*., 2009) and *Acidimicrobium ferrooxidans* (Clark and Norris, 1996), all of which are autotrophic iron-oxidizers; however, until now the TM3 *Actinobacteria* lacked cultivated representatives.

Characterization of the TM3 group *Actinobacteria* by incubations with different combinations of Fe(II), nitrate, acetate and CO_2 showed that a combination of Fe(II), nitrate and CO_2 are required for growth. Repeated attempts to isolate the *Actinobacteria* in pure culture by serial dilution of the enrichment neither showed growth nor a reduction in Fe(II) in the dilutions greater than 10^{-4}. Other attempts to modify the medium like using EDTA-chelated Fe(II) or removal of phosphate buffer, to prevent the formation of white precipitate, did not lead to an enrichment of

the *Actinobacteria*. The role of ferrous phosphates on the growth of Fe(II)-oxidizing bacteria is currently unknown, however a similar phenomenon was reported earlier (Straub*et al*., 2004).

A time course of Fe(II) oxidation and nitrate consumption performed with the actinobacterial enrichment was in good agreement with the expected stoichiometry required for nitrate-dependent Fe(II) oxidation (Figure 4). The molar ratio of Fe(II) to nitrate consumed was 1.00:0.23 at all time points compared to the ideal ratio of 1.00:0.20. This slightly higher ratio of Fe(II) to nitrate consumed could be due to utilization of some nitrate for growth. Abiotic Fe(II) oxidation with nitrate, nitrite or nitrous oxide would not be possible under the incubation conditions due to acidic pH and absence of copper in high concentrations (Buresh and Moraghan, 1976). Denitrification and abiotic oxidation of Fe(II) by nitrate was unlikely because of the lack of any nitrous oxide in the incubations. No accumulation of ammonia was observed indicating no reduction of nitrate to ammonia. These results indicate that the TM3 actinobacterial enrichment was capable of Fe(II) oxidation coupled to nitrate reduction.

The quantification of nitrate-dependent Fe(II)-oxidizing microorganisms in the sediment could not be done by molecular methods due to the absence of a suitable functional marker gene or 16S rRNA primers for this group. For this reason, an MPN method was used and the results showed the presence of $1x10^4$ autotrophic and $1x10^7$ heterotrophic nitrate-dependent Fe(II) oxidizers per gram fresh weight of sediment. These results are in accordance with previous findings that the heterotrophic outnumber the autotrophic Fe(II)-oxidizers by several orders of magnitude in lake sediments (Hauck *et al*., 2001; Muehe *et al*., 2009; Straub and Buchholz-Cleven, 1998b); however the quantification by this approach will be an underestimation due to the selective nature of enrichment media and incubation conditions. The different microbial community composition in both autotrophic and heterotrophic MPN tubes suggests that these processes are mediated by different groups of organisms.

In addition to MPN assays, we used a combination of real-time PCR and amplicon pyrosequencing to quantify TM3 *Actinobacteria* in Lake Grosse Fuchskuhle sediment. This indicated TM3 group *Actinobacteria* 16S rRNA genes could account for approximately0.6% of total bacterial sequences in the lake sediment. Based on the comparison of these results with the bacterial quantifications by real-time PCR, the number of these *Actinobacteria* would correspond to 10^6 cells per g of wet sediment. According to the rate of nitrate-dependent Fe(II)-oxidation per cell

estimated by Melton *et al*., (2012), these organisms could be contributing significantly to the regeneration of Fe(III) in the sediment.

Stable isotope probing (SIP) under anoxic conditions using $^{13}CO_2$ was performed in order to investigate the potential importance of TM3 *Actinobacteriain situ*. The same *Actinobacteria* TM3 organisms that were obtained in the enrichment cultures were labeled in the $^{13}CO_2$ SIP incubations, indicating that these are important autotrophic organisms in this sediment. In addition, organisms related to *Gallionella* and *Sideroxydans* species as well as *Thiomonas* were labeled. The fact that the T-RFLP profiles in the heavy fractions did not change over the time-course suggests that cross-feeding of the ^{13}C did not occur, which is a common phenomenon observed in many SIP studies involving long incubation times (Lueders et al., 2004). Probably, the relatively low abundance of labeled organisms was insufficient to provide carbon to the community and observe cross-feeding of heterotrophic organisms. The quantification of bacterial 16S rRNA genes from the SIP gradient fractions showed a large abundance in the light fractions compared to the heavy gradient fractions. A similar trend was observed in all the time points, indicating the labeling of a relatively small fraction of the sediment microbial community and a slow rate of growth of these organisms.

Gallionella were the first microorganisms shown to be Fe(II) oxidizers (Ehrenberg, 1836). They are known to be prevalent in groundwater systems and mineral springs (Hanert *et al*., 2006) and are capable of both autotrophic and mixotrophic growth (Hallbeck and Pedersen, 1991). *Sideroxydans* spp. are prevalent in iron-rich environments and prefer microaerophilic conditions (Weiss *et al*., 2007; Emerson and Moyer, 1997); furthermore, they have been detected in acidic peatlands indicating that some species are acidophilic or acid-tolerant (Ludecke et al., 2010). A previous study indicated that *Gallionella ferruginea* could perform autotrophic nitrate-dependent Fe(II) oxidation (Gouy *et al.*, 1984), but no further studies with *Gallionella* have investigated this capability; however, studies have shown a complex distribution of *Gallionella* in relationship to the redox zonation in wetland soils (Wang *et al*., 2009), which is consistent with the existence of anaerobic phylotypes.

Members of genus *Thiomonas* are metabolically versatile with autotrophic, mixotrophic and heterotrophic physiologies and are capable of deriving energy by oxidation of reduced inorganic sulfur compounds or As(III) (Battaglia-Brunet et al., 2002; Duquesne et al., 2007; Gonzalez-Toril et al., 2003a). They are common inhabitants of extreme environments such as acid mine drainage, which have a low pH and high concentrations of sulfur and metals, such as iron and arsenite

(Bruneel *et al*., 2003; Duquesne *et al*., 2008). The labeling of *Thiomonas* in our SIP experiment indicates that they also might be important anaerobic autotrophs in Lake Grosse Fuchskuhle sediment.

The fast growth of TM3 *Actinobacteria* under enrichment conditions compared to the SIP incubations could be due to the higher concentration of nitrate provided in the enrichments compared to that naturally present in the sediment. Our measurements have shown the presence of a low concentration of nitrate in the sediment, but the source of nitrate is not clear. Studies have indicated a reduced rate of denitrification (Nagele and Conrad, 1990a; Nagele and Conrad, 1990b; Simek and Cooper, 2002) and a longer retention of nitrate (Muller et al., 1980) in low pH environments. Moreover, ammonia oxidation within the rhizosphere of macrophytes could be a continuous source in the top 10 cm of the sediment (Herrmann et al., 2009; Herrmann et al., 2011). Due to these factors, we assume that there could be a continuous supply of nitrate in low concentrations to facilitate nitrate-dependent Fe(II) oxidation in Lake Grosse Fuchskuhle.

In summary, our results have indicated the chemolithotrophic nitrate-dependent Fe(II)-oxidizing nature of the TM3 group of uncultured soil *Actinobacteria,* which are widely distributed and whose function was previously unknown. The labeling of species related to the genera *Gallionella*, *Sideroxydans* and *Thiomonas,* which are capable of Fe(II) oxidation, suggests that Fe(II) oxidation is an important process in Lake Grosse Fuchskuhle sediment and may explain the relatively low methanogenic potential of this lake. Although TM3 *Actinobacteria* may represent less than 1% of the bacterial community in the sediment, they could be of great functional significance in this environment due to their contribution to the regeneration of Fe(III) that is essential for iron-reducing microorganisms, which play an important role in the mineralization of sediment organic matter.

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Chapter 4

Effect of chelation of Fe(II) to humic substances in mediating abiotic and microbial Fe(II) oxidation in Lake Grosse Fuchskuhle

4.1 Abstract

Chelation of metals to humic substances is known to alter their reactivity in natural environments. This study investigates the role of humic substances in mediating abiotic and microbial Fe(II) oxidation. To understand these processes in this specific environment and also due to lack of uniformity in the nature of humic substances from different environments, lake water rich in humic substances was used in the present study instead of artificial humic substances. Different concentrations of water from Lake Grosse Fuchskuhle were incubated with added Fe(II) and the formation of Fe(III) by abiotic oxidation was monitored at daily time points for 10 days. To understand the role of humic substances in promoting nitrate-dependent Fe(II) oxidation, incubations were done on North east basin littoral sediment containing low concentration of humic substances with added humic substances, Fe(II) and nitrate. The results indicated that in South West basin water column Fe(II) concentrations below 6mM remain stable under environmental conditions during the course of our incubations, without reacting with atmospheric O_2. Under anaerobic nitrate-dependent Fe(II)-oxidizing conditions a faster depletion of Fe(II) was observed in the presence of humic substances compared to their absence. These findings indicate that limnological conditions of Lake Grosse Fuchskuhle could inhibit abiotic Fe(II) oxidation and promote microbial Fe(II) oxidation due to chelation of Fe(II) to humic substances.

4.2 Introduction

Humic substances (HS) are complex high molecular weight organic compounds produced by degradation of dead organic matter (Stevenson, 1994). They form a major fraction of soil organic carbon and are resistant to microbial degradation (Ford and Lock, 1987). The structure of HS is known to contain randomly coiled macromolecular structures comprising a high number of aromatic ring structures. Although structure and composition of HS is determined by soil conditions, all HS exhibit similar properties due to the presence of similar oxygen, nitrogen and sulfur-containing functional groups (Barancikova et al., 1997). Due to the presence of these charged functional groups, HS are capable of forming complexes with metal ions like Cu^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} (Kerndorff and Schnitzer, 1980; Millero et al., 1995). These organo-metallic complexes are known to influence the biological availability (Gress et al., 2004), toxicity (Hutchins et al., 1999), solubility (Rashid and Leonard, 1973; Sholkovitz and Copland, 1981) and redox potentials of metals (Strathmann, 2011).

Iron is one of the micronutrients required for all life-forms and several microorganisms are also known to conserve energy by mediating oxidation and reduction of iron (Weber et al., 2006a). Due to the high affinity between positively charged metals and HS, Fe^{2+} and Fe^{3+} are known to be predominantly present in soil as HS chelates (Kerndorff and Schnitzer, 1980; Rashid, 1974). The role of Fe-humic complexes in regulating the rate of redox reactions and biological availability has received considerable scientific attention in recent years. These studies have elucidated the role of humic acids in mediating biotic (Lovley et al., 1998) and abiotic (Shikha et al., 2011) reduction and speciation of Fe in natural environments. Although the role of humic acids in mediating Fe(III) reduction in natural environments is relatively well understood (Lovley et al., 1998), their role in mediating the oxidation of Fe has not been elucidated. Studies conducted in this regard have led to contradicting hypotheses regarding the role played by HS in mediating abiotic Fe(II) oxidation. Earlier studies have shown that complexation of Fe(II) to HS could considerably reduce the reactivity with environmental O_2 and increase the half-life thereby making significant quantities of Fe(II) available for microbial Fe(II) oxidation (Theis and Singer, 1974). More recent studies have shown that chelation to HS could either increase or decrease the reactivity of Fe(II) with environmental O_2 depending on the nature of the functional group involved in chelation and ratio of Fe(II) and humic acid concentration in the environment (Gaffney et al., 2008; Strathmann, 2011). Despite the similarities in several properties among HS from different environments, the precise structure and composition of HS depends on the nature of the organic carbon and ecological

conditions of the specific environment (Stevenson, 1994). Lack of uniformity in the structure of HS, the absence of quantitative information of specific functional groups and technical difficulties in differentially quantifying chelated and unchelated Fe(II) have made the generalization of the above results difficult. Hence, the experiments of the present study were designed to study the role of Lake Grosse Fuchskuhle HS in mediating both abiotic and microbial Fe(II) oxidation processes.

4.3 Experimental procedure

Lake sediment samples were collected from acidic dystrophic Lake Grosse Fuchskuhle in the Brandenburg-Mecklenburg Lake District (Germany). Lake Grosse Fuchskuhle is an artificially divided lake with different pH values in the four compartments (Chapter 1). A main divergent factor is the inflow of HS from an adjacent bog; the southwest basin contains a high concentration of HS and the northeast contains the least (Koschel, 1995). Sediment samples were taken by a gravity corer (Uwitec, Mondsee, Austria) from the littoral of both the southwest (SW) and northeast (NE) basins. The top 10 cm of the sediments were collected using 6 cm diameter plexiglass cores. The water from the littoral region of SW basin was collected and stored at room temperature for a period of 10 months to deplete the readily utilizable organic substrates.

Different concentrations of HS were obtained by diluting the SW basin water with sterile distilled water. 1X, 0.5X and 0.1X dilutions were used in the present study. A 2X concentration of SW basin water was prepared by lyophilizing 100ml of SW basin water and finally dissolving in 50ml distilled water. 9.9 ml of the above preparations were transferred into respective 25ml test tubes and 100µl of $FeCl_2$ was added from a 1M $FeCl_2$ stock solution. The tubes were capped using aluminium foil allowing air exchange with the atmosphere and incubated at room temperature. Fe(II) measurements were done using ferrozine reagent at every 24 hour time point as described before (Chapter 2&3). All the incubations were done in duplicate.

To study the role of HS on microbial nitrate-dependent Fe(II) oxidation, three sets of incubations were done with the sediment collected from the NE basin littoral region. The first set of incubations was done by incubating 5ml of NE basin littoral sediment with 5ml of overlying water. The second set of incubations wasdone by centrifuging 5ml of NE basin littoral sediment at 5000rpm for 5min followed by re-suspending it in equal volume of water collected from SW basin littoral region containing high concentrations of HS. 0.5ml of minimal salts medium was added to both the incubations (Widdel and Bak, 1992). The third set of incubations was done using the same

procedure (as second set of incubations) with the exception of using minimal salt medium instead of lake water. The incubations were done according to the same procedure with added Fe(II), nitrate and CO_2 as described in Chapter 3. Fe(II) measurements were done at every 24 hour time point to determine the rate of anaerobic Fe(II) oxidation.

4.4 Results and discussion

The conventional method of humic acid isolation involves alkaline extraction, which is known to alter the properties of HS by co-precipitating ash and silica fractions. Moreover, this method involves fractionation of humic substances into fulvic acids, humic acids and humus (Livens, 1991). As the objective of the present study was to understand the role of HS in mediating Fe(II) oxidation in natural environments, fractionation or modification of humic acids from their natural state was undesirable. Due to the differences in the nature of HS from different environments (Barancikova et al., 1997) and limitations of using commercially available humic acids (Malcolm and MacCarthy, 1986), we employed the use of lake water containing natural concentrations of HS. Dilution of lake water or concentration using lyophilization was done for obtaining different concentrations of HS.

An inverse relationship was observed between the rate of abiotic Fe(II) oxidation and the concentration of HS (Figure. 1), indicating that abiotic Fe(II) oxidation was greatly reduced by the presence of HS. Concentrations of Fe(II) below 6mM seemed to be completely resistant to chemical oxidation with atmospheric oxygen in water of SW basin, during the course of our incubation (Figure. 1). In comparison, several orders of magnitude lower concentrations of Fe(II) was observed in the lake sediment (Chapter 2, Figure.1). Hence, we hypothesize that the high concentration of HS and probably low pH (4.8) of the lake water in this basin will be able to greatly reduce the abiotic Fe(II) oxidation.

Earlier studies have reported the role of HS in promoting reduction of Fe(III) by dissimilatory iron-reducing bacteria (Lovley et al., 1998) and methanogenic archaea (Bond and Lovley, 2002). However, the role of HS in mediating microbial Fe(II) oxidation has not been elucidated. Chelated Fe(II) is known to exhibit a wide range of redox potentials depending on the nature of the ligand involved in chelation (Strathmann, 2011). Chelation of Fe(II) to HS is known to lower the redox potential of Fe(II) due to the saturation of the inner co-ordinate metal shell by forming multi-dentate complexes that increase access of oxidants (Rush et al., 1990; Strathmann and Stone, 2002a). The enhanced reactivity of Fe(II) in the

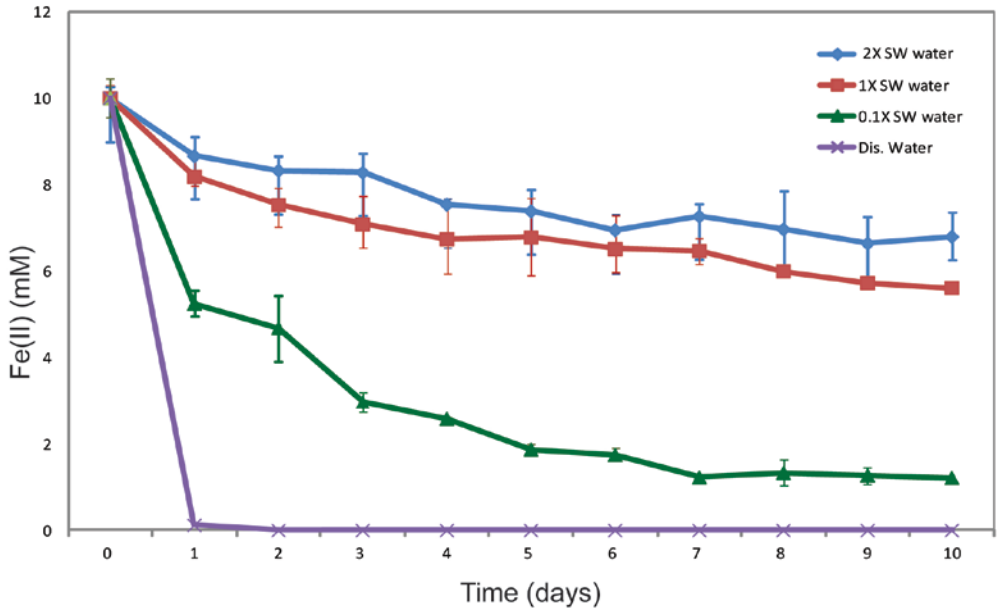

Figure 1: Effect of humic substance concentration on abiotic Fe(II) oxidation.Lake Grosse Fuchskuhle southwest basin (SW) water wasincubated with added 10 mMFe(II) under an atmosphere of air.Different concentrations of SW water were obtained by diluting (0.1X) or concentrating by lyophilization (2X), as indicated.A control incubation consisting of only distilled (Dis.) water and Fe(II) was included.

presence of HS is also due to the formation of Fe(III) stabilizing ligand complexes that reduce the standard redox potential of the Fe(III)/Fe(II) redox couple (Strathmann, 2011). Recent studies have shown that this lowering of the redox potential could increase the reactivity of Fe(II) in anaerobic environments and could be playing an important role in contaminant degradation in aquifers (Kim et al., 2009a). The lowering of the redox potential could also provide favorable thermodynamic conditions for microbial Fe(II) oxidation and the high affinity of HS to Fe(III) also reduces the risk of cell encrustation (Kappler et al., 2006b; Kappler and Newman, 2004). Although in theory Fe(II) chelation to HS could be beneficial for microbial Fe(II) oxidation, this phenomenon has not been experimentally shown.

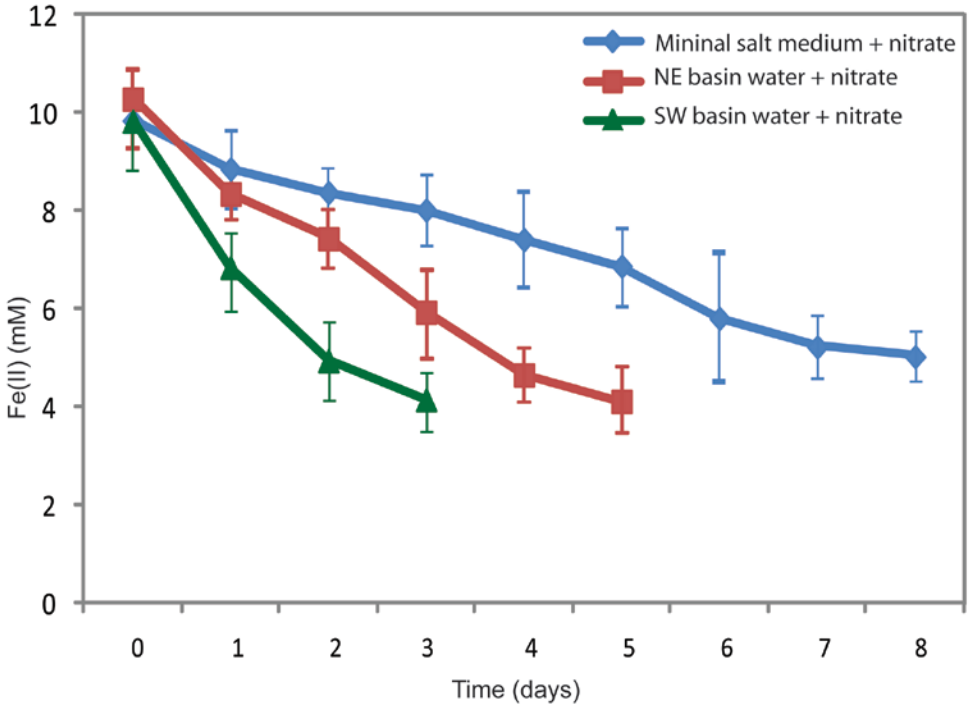

Figure 2: Effect of humic substances on microbial nitrate-dependent Fe(II) oxidation. Lake Grosse Fuchskuhle north east (NE) basin sediment was incubated with water collected from NE basin, south west (SW) basin and minimal salt medium with added nitrate and Fe(II).

The present study was conducted on the NE basin sediment of Lake Grosse Fuchskuhle, which is known to contain low concentration of HS (Sachse et al., 2001). The incubations done from this sediment have shown a higher rate of Fe(II) oxidation when incubated with SW basin water compared to the incubations done with NE basin water or minimal salts medium (Figure 2). The water from the NE and SW basin should be of similar nutritional status as both the basins receive water from same groundwater aquifer (Chapter 1) with the exception of the nature and concentration of dissolved organic carbon (Sachse et al., 2001). Hence, we hypothesize that the differences observed in the rate of Fe(II) oxidation could be due to the differences in the concentration of HS between the incubations. Abiotic Fe(II) oxidation with nitrate, nitrite or nitrous oxide would not be possible under the incubation conditions due to the acidic pH and absence of copper in high concentrations (Buresh and Moraghan, 1976).

4.5 Conclusion and environmental implications

A growing body of literature indicates the importance of Fe(III) reduction in mediating organic carbon mineralization in peatlands. This process leads to an accumulation of Fe(II) in anoxic parts of the sediment. Oxidation of this Fe(II) to Fe(III) is the rate limiting step for the continuous sustainment of iron reduction in sediments and soils. However, due to a high redox potential, Fe(II) is considered to be un-reactive under anoxic conditions. Recent studies have elucidated several pathways of Fe(II) oxidation under anaerobic conditions, but the significance for soil biogeochemistry, the effect on the microbial ecology or the environmental factors regulating the process are not well understood. Recent studies have shown that HS-Fe(II) chelates play an important but previously unrecognized role in Fe(II) oxidation in anoxic environments. The findings of our study show that the presence of HS accelerated the oxidation of Fe(II) indicating a beneficial role in microbial Fe(II) oxidation. We hypothesize that a reduction of the redox potential of Fe(II) caused by chelation to HS was responsible for the observed effect. These findings could be of significance in understanding environmental Fe(II) oxidation under anoxic conditions as most of the Fe(II) present in soils and sediments is present as chelated to HS, which was not taken into consideration in the earlier microbiological studies. The results of our study and those of the earlier studies also indicate the beneficial effect of chelation for Fe(II)-oxidizing microorganisms as chelated Fe(II) is resistant to environmental Fe(II) oxidation.

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Chapter 5

Chemolithoautotrophic nitrate-dependent Fe(II) oxidizing nature of members of genus *Thiomonas* and the role of humic acids in microbial Fe(II) oxidation

5.1 Abstract

Microbial Fe(II) oxidation in anoxic environments has been shown to be mediated by coupling to denitrification processes. Studies conducted on a diverse range of soils have indicated that this process is being mediated by autotrophically growing microorganisms. Although several studies have shown the ubiquity of chemolithoautotrophic nitrate-dependent Fe(II) oxidation, the organisms responsible are not been available as pure cultures. The present study was conducted on the littoral sediment of an acidic bog lake, Lake Grosse Fuchskuhle, with the objective of identifying, characterizing and enumerating the microorganisms responsible for autotrophic nitrate-dependent Fe(II) oxidation. Following the results of our earlier studies all the incubations were done using lake water containing a high concentration of humic substances. Quantifications done by MPN showed the presence of $1x10^6$ autotrophic and $1x10^7$ heterotrophic nitrate-dependent Fe(II)-oxidizers per gram fresh weight of sediment. A two-order of magnitude higher autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms was observed in the present study in comparison to our earlier study done on the same sediment using an artificial medium. The incubations of sediment under chemolithotrophic nitrate-dependent Fe(II)-oxidizing conditions have shown the enrichment of microorganisms belonging to genus *Thiomonas*. A time-course experiment done on this *Thiomonas* enrichment showed a consumption of Fe(II) and nitrate in accordance with the expected stoichiometry (1:0.2) required for nitrate-dependent Fe(II) oxidation. Quantification of RuBisCO (*cbbL*) gene copy numbers by quantitative real-time PCR showed a logarithmic increase of these genes under the incubation conditions over the course of the incubations. Unlike the earlier isolates of this physiological group, the *Thiomonas* species, enriched in our incubations could be repeatedly sub-cultured under nitrate-dependent Fe(II)-oxidizing conditions without losing their ability to grow autotrophically when Fe(II) was provided as chelated to humic substances. In summary this study

showed profound differences in enrichment and quantification of autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms in the presence and absence of humic substances. This higher numbers of autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms and continuous sustainment of autotrophic growth observed in the present study indicates the beneficial role of humic substances to this physiological group of microorganisms. We hypothesize that this beneficial role of humic acids for autotrophic nitrate-dependent Fe(II)-oxidizing bacteria could be due to an energetic benefit of the lowering of the redox potential of Fe(II) caused by chelation to humic acids.

5.2 Introduction

Chemolithotrophic nitrate-dependent Fe(II) oxidation was first reported by Straub et al., 1996 and has been reported in several environments ever since (Muehe et al., 2009; Straub et al., 1996; Straub et al., 2004; Straub and Buchholz-Cleven, 1998a). This process was shown to be mediated by both autotrophically and organotrophically growing microorganisms. Although organotrophic nitrate-dependent Fe(II) oxidation was shown to be mediated by several bacterial isolates, autotrophic nitrate-dependent Fe(II) oxidation is not well understood due to the lack of bacterial isolates. Attempts to isolate these organisms have led to few bacterial isolates which were shown to be incapable of autotrophic growth after successive sub-culturing (Weber et al., 2006b). Earlier studies have hypothesized that a high redox potential and low amount of energy released by oxidation of Fe(II) may not be energetically favorable for supporting autotrophic growth under nitrate-dependent Fe(II)-oxidizing conditions (Muehe et al., 2009). However, both geochemical and microbial studies conducted on a diverse range of soils have reported evidence of chemolithoautotrophic nitrate-dependent Fe(II) oxidation (Pauwels et al., 1998b; Pauwels et al., 2000; Postma et al., 1991). These studies have also shown the absence of abiotic nitrate-dependent Fe(II) oxidation in these soils (Colman et al., 2007) indicating a greater contribution of microorganisms in mediating this process in natural environments.

Lake Grosse Fuchskuhle is a *Sphagnum* bog lake with pH ranging from 3.9 to 6.1. The south west (SW) basin is the most acidic due to large inputs of humic acids from the surrounding peat bog (Koschel, 1995). A low abundance of phytoplankton is observed in this basin due to high concentrations of humic acids (Hehmann et al., 2001), indicating a greater contribution of chemolithoautotrophic microorganisms to the total CO_2 uptake compared to photoautotrophic CO_2 fixation. $^{13}CO_2$ stable isotope probing done on the littoral sediment of the lake to elucidate the chemolithoautotrophic microbial community has shown that Fe(II) and inorganic sulfur-oxidizing microorganisms could be the major autotrophic microbial community in this sediment (Chapter 3). Attempts to isolate these Chemolithotrophic Fe(II)-oxidizing microorganisms using a defined medium led to the enrichment of TM3 *Actinobacteria*, which were shown to be capable of chemolithotrophic nitrate-dependent Fe(II) oxidation (Chapter 3). Observations made from this study indicated faster growth and more rapid oxidation of Fe(II) in the growth medium containing humic substances (HS) compared to the artificial medium without HS, indicating a role played by HS in culturing Fe(II) oxidizing microorganisms.

The growth rate and adaptability of bacteria in artificial medium depends on various factors like available form of nutrients, temperature, acidity and HS. Studies have reported the phenomenon of low adaptability of the microbial communities in humic acid lakes to growth on media containing low amounts of HS (Langenheder et al., 2005), indicating the role of HS in culturing or enriching these microorganisms. Moreover HS could play a more significant role in culturing iron metabolizing microorganisms due to the chelation of iron by HS. The chelation of iron by HS is a well reported phenomenon (Kerndorff and Schnitzer, 1980) and the role of this phenomenon in improving the solubility of Fe(III) (Cameron and Liss, 1984; Shapiro, 1964), effects on speciation of iron (Millero et al., 1995; Pitzer, 1973; Pitzer and Mayorga, 1974) and inhibition of abiotic oxidation of Fe(II) (Theis and Singer, 1974) have been well studied.

Chelated Fe(II) exhibits a wide range of redox potentials depending on the nature of the organic ligand involved in the complex (Buerge and Hug, 1998; Schwarzenbach et al., 2002; Strathmann, 2011; Stumm and Morgan, 1996). Studies have shown that chelation of Fe(II) by HS could reduce the redox potential from +100mV to -380mV, increasing the reactivity of Fe(II) in natural environments (Kim et al., 2009b; Naka et al., 2006; Strathmann, 2011). The decrease in the redox potential of Fe(II) combined with the higher affinity of humic acids towards Fe(III) compared to Fe(II) (Martell and Hancock, 1996) could provide thermodynamically favorable conditions for oxidation of Fe(II) in humic-rich environments (Strathmann and Stone, 2002b). Even though the effect of chelation on abiotic cycling of iron in natural environments have been well studied, the role of this phenomenon on microbial Fe(II) oxidation is not well understood.

The present study was conducted on the littoral sediment of Lake Grosse Fuchskuhle to isolate, characterize and quantify the chemolithoautotrophic nitrate-dependent Fe(II)-oxidizing microorganisms in growth media containing natural concentrations of HS. A medium containing natural concentrations of HS was used for this purpose to test the effect of humic acids on the growth of Fe(II)-oxidizing bacteria. The results of our study have provided the evidence of a positive role played by HS in the growth and cultivation of Fe(II) oxidizing microorganisms.

5.3 Experimental procedure

Sampling

Lake sediment samples were collected in April 2010 from acidic dystrophic Lake Grosse Fuchskuhle in the Brandenburg-Mecklenburg Lake District (Germany). The top 10 cm of the sediments were collected using 6 cm diameter plexiglass cores. The sediment has a high concentration of humic acids, which were visible from the dark brown colour of the sediment and overlying water. The collected sediment was composed mainly of coarse particulate organic material. The pH of the sediment was 4.5. The concentration of Fe(II) and Fe(III) in the SW littoral sediment was determined using the ferrozine assay (Stookey, 1970a) and nitrate concentrations were measured by flow injection analysis (Tecator, Rellingen, Germany).

Enrichment of nitrate-dependentFe(II)-oxidizing bacteria

The enrichment of nitrate-dependent Fe(II)-oxidizing bacteria was done according to the procedure described by Straub et al. (1998) with the exception of using water collected from the lake instead of fresh water medium and $FeCl_2$ instead of $FeSO_4$ to prevent the growth of sulfate-reducing bacteria. The water from the littoral region of the SW basin was collected and stored at room temperature for a period of 6 months to deplete the readily utilizable organic substrates. The presence of volatile fatty acids and anions like nitrate and sulfate were determined using HPLC and IC analysis. The medium for enrichment and subsequent SIP experiments was prepared as follows: water from Lake Grosse Fuchskuhle was filter sterilized through 0.22µm filter and filter sterilized vitamins and trace elements were added to the medium (Widdel, 1992). Oxygen was depleted using a vacuum manifold and repeatedly flushing the headspace with N_2 gas. Nitrate and $FeCl_2$ were added from the stock solutions to the medium under a N_2 atmosphere in an anaerobic chamber (Mecaplex, Grenchen, Switzerland). 40 ml of the medium was dispensed into 120 ml serum bottles and bottles were sealed with butyl stoppers. 5ml CO_2 was added to the headspace using a syringe.

A time course experiment was done to elucidate the ratio of Fe(II) to nitrate consumed over a period of 10 days. The experimental set-up contained the above mentioned medium with nitrate and Fe(II) with 10% of the *Thiomonas* enrichment obtained from above incubations as inocula. 5% CO_2 was added to the headspace as the sole carbon source. Samples for T-RFLP as well as for Fe(II) and

nitrate measurements were taken once at 10 day and 14 day time points. N_2O was measured by gas chromatography (Carlo Erba Instuments, GC 8000) using a ^{63}Ni-electron capture detector (ECD).

Enumeration of Fe(II)-oxidizing bacteria by most probable number (MPN)

An MPN method was used to enumerate the Fe(II)-oxidizing bacteria according to the procedure described previously (Straub and Buchholz-Cleven, 1998a) with the exception of using water collected from SW basin instead of fresh water medium. Two sets of MPN tubes were incubated each in triplicate. One set of tubes contained SW basin water with Fe(II), nitrate and 5% CO_2 in the headspace to enumerate autotrophic Fe(II)-oxidizing bacteria. The second set of tubes contained SW basin water with nitrate, Fe(II) and acetate for enumeration of organotrophic Fe(II)-oxidizing bacteria. The tubes were incubated for 5 days in the dark at 25°C and gently inverted daily. Tubes were scored positive based on the reduction in the amount of Fe(II) and acetate in the respective tubes compared to the uninoculated controls. The Fe(II) estimations were done using the ferrozine assay (Stookey, 1970b) and a standard MPN table was used to calculate the cell numbers.

Relative quantification of cbbL gene copy numbers

The relative abundance of 'red-like' RuBisCO genes at different time points of the enrichment was determined by real-time PCR using the JumpStart TaqReadyMix System (Sigma, Taufkirchen, Germany). The assays were performed as described before (Selesi et al., 2007) using an iCycler instrument (Bio-Rad, Munich, Germany) and the associated software.

PCR, cloning and sequence analysis

For cloning and sequencing 16S rRNA genes from enrichment incubations, PCR amplification was done using the Ba27f and Ba907r primers (Lueders et al., 2004). All the PCR reactions were performed in 50 µl volumes with the following composition: 1x PCR buffer (Promega, Mannheim, Germany), 0.2 mM $MgCl_2$, 10 pmol of each primer, 10 µg of BSA (Roche, Mannheim, Germany), 2U of GoTaq (Promega), 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany) and 1 µl of template DNA. The PCR was performed on a GeneAmp PCR system 9700 instrument (Applied Biosystems) with the following cycling conditions: 94°C for 4 min, 35 cycles of 94°C for 1 min, 52°C for 40 sec and 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were cloned using the

pGEM-T Easy Vector System (Promega) and transformed into *E. coli* JM109 competent cells according to the manufacturer's instructions. 11 clones each were randomly picked and sequenced. The phylogenetic affiliation of the clones was done using the ARB software package (Ludwig *et al*., 2004) and trees were constructed using the neighbor-joining method.

Terminal restriction fragment length polymorphism (T-RFLP)

The PCR amplification of bacterial 16S rRNA genes for T-RFLP analysis was performed as described above, except the Ba27F primer was labeled with FAM (6-carboxyfluorescein). PCR products were purified using Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). Approximately 100 ng of purified PCR product was used for restriction digestion. Digestions were performed in a reaction volume of 20 µl containing 1x Tango buffer and 5U of MspI enzyme (Fermentas); reactions were incubated at 37°C incubator overnight. The reactions were processed using SigmaSpin™Post Reaction Clean-Up Columns (Sigma) and 2 µl of the processed fragments were mixed with 11µl of Hi-Di™ formamide (Applied Biosystems), 0.3 µl of ROX-labeled MapMarker 1000+30, 40 (BioVentures, Murfreesboro, TN, USA) and incubated at 94°C for 3 min and cooled on ice. The size separation was performed using 3130 Genetic Analyzer (Applied Biosystems).

5.4 Results

Fe(II), Fe(III) and nitrate in Lake Fuchskuhle littoral sediment

Duplicate sediment cores were collected from the littoral zone of Lake Grosse Fuchskuhle and the top 10cm sections were partitioned. The concentration of Fe(II), Fe(III) and nitrate were as mentioned Chapter 1.

Enumeration and enrichment of nitrate-dependent Fe(II)-oxidizing microorganisms

A most probable number assay was performed to estimate the abundance of readily cultivable anaerobic nitrate-dependent Fe(II)-oxidizing microorganisms in the littoral sediment of Lake Grosse Fuchskuhle. The assay was performed with either CO_2 or acetate as carbon source to estimate potential autotrophic and heterotrophic microorganisms. The assays indicated the presence of $1x10^6$ autotrophic and $1x10^7$ heterotrophic nitrate-dependent Fe(II)-oxidizers per gram fresh weight of sediment.

The incubations done with water collected from SW basin for the enrichment of potential autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms showed the enrichment of microorganisms characterized by a single dominant T-RF of 487bp (Figure 1). The enrichments done without the added nitrate or Fe(II) did not show a specific enrichment of microorganisms belonging to T-RF of 487bp, indicating the requirement of both nitrate and Fe(II) for the enrichment of these organisms. 16S rRNA gene cloning and sequencing were performed to identify the 487bp T-RF in the enrichment. A total of 95% of the clones obtained had the 487bp T-RF and the phylogenetic analyses (Figure 2) of these clones indicated they belong to the genus *Thiomonas* forming a single distinct cluster within this genus. Sequences were deposited in GenBank with accession numbers KC540879-KC540882.

Characterization of the *Thiomonas* enrichment culture

An enrichment of the *Thiomonas* sp. could be reproducibly obtained by inoculation of the sediment into the enrichment medium. Dilution series done from the enrichment showed the presence of *Thiomonas sp* and the oxidation of Fe(II) until the 10^8 dilution.

The ratio of Fe(II) oxidized to nitrate reduced by a 10^2 dilution of the enrichment was determined (Figure 3). Both Fe(II) and nitrate were consumed during the incubation with a molar ratio of Fe(II) to nitrate of 1:0.3 at each time point measured. No N_2O production was observed in these incubations. Uninoculated and killed controls did not show consumption of either Fe(II) or nitrate. The T-RFLP profiling done at all time points showed the presence of the single dominant 487 bp T-RF. The quantification of total red type *cbbL* gene copies was performed by real-time PCR and showed a logarithmic increase in the gene copy numbers over the incubation period of 2 weeks. The

cbbL gene copy numbers at zero hours, one week and two weeks were 6.45 x 10^1, 5.22 x 10^2 and 1.17 x 10^4 per ml respectively.

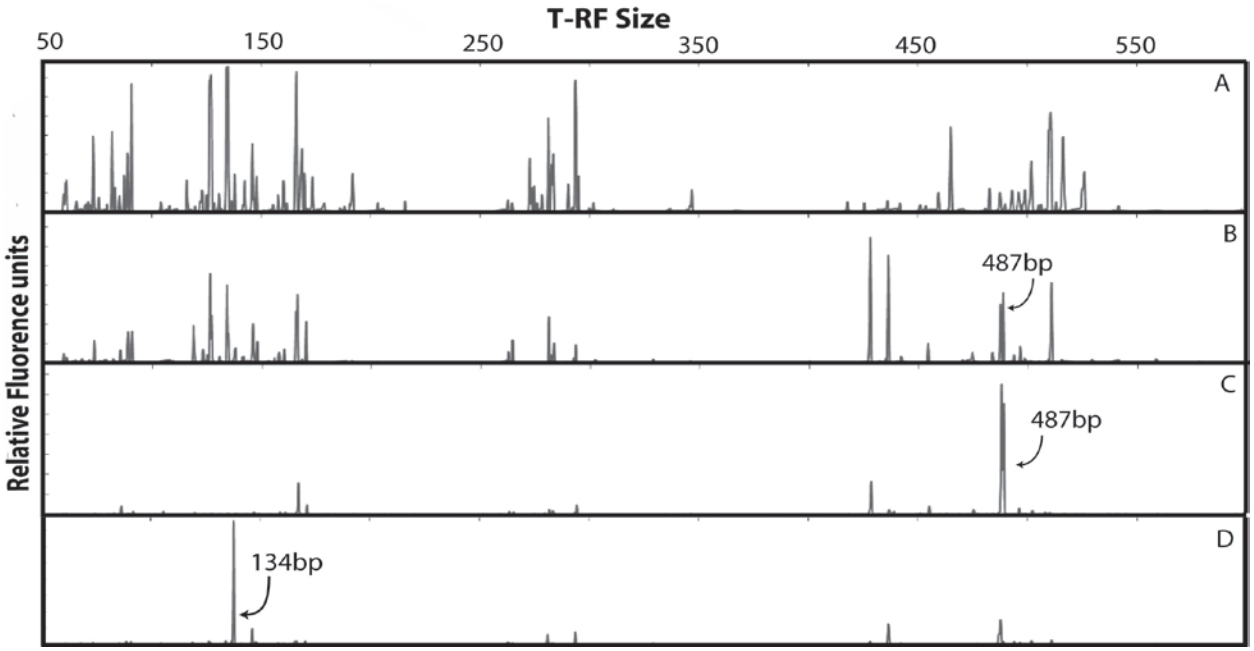

Figure 1: Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA genes from different enrichment incubations. The enrichment conditions are described in detail in the experimental procedures section. All enrichments were performed in triplicate and each produced similar T-RFLP profiles, therefore a single representative is shown. (A) T-RFLP from the unincubated sediment; (B) SW basin water medium containing added NO_3^- as terminal electron acceptor and CO_2 as carbon source (C) SW basin water medium containing added Fe(II) as electron donor, NO_3^- as terminal electron acceptor and CO_2 as carbon source; (D) as (B), but without added NO_3^-.

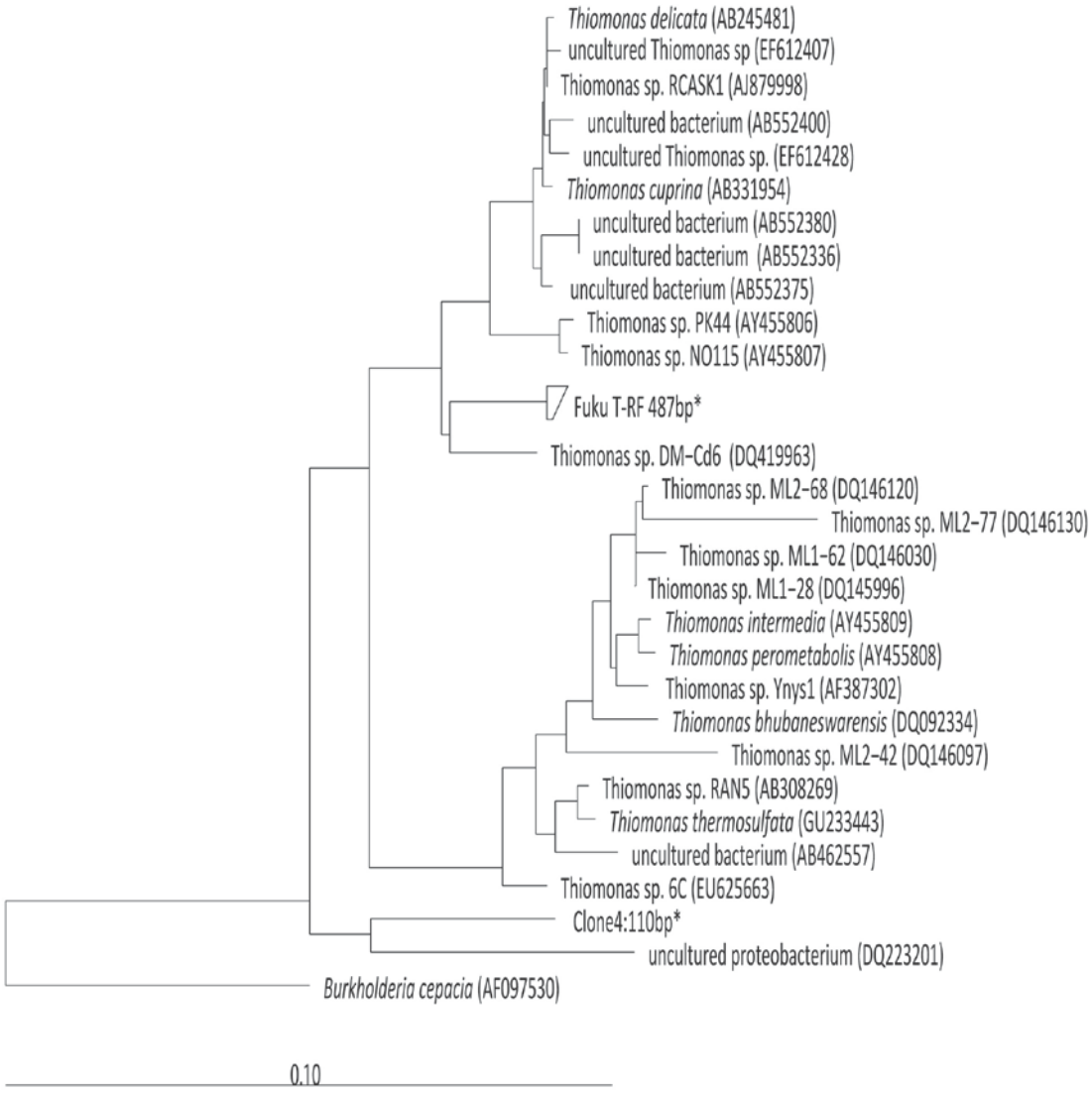

Figure 2. Phylogenetic analysis of 16S rRNA gene sequences from the enrichment (indicated by *) in comparision with the closely related sequences. The tree was constructed using neighbor-joining method. *Burkholderia cepacia* was used as an outgroup. The T-RF sizes of the sequences are indicated.

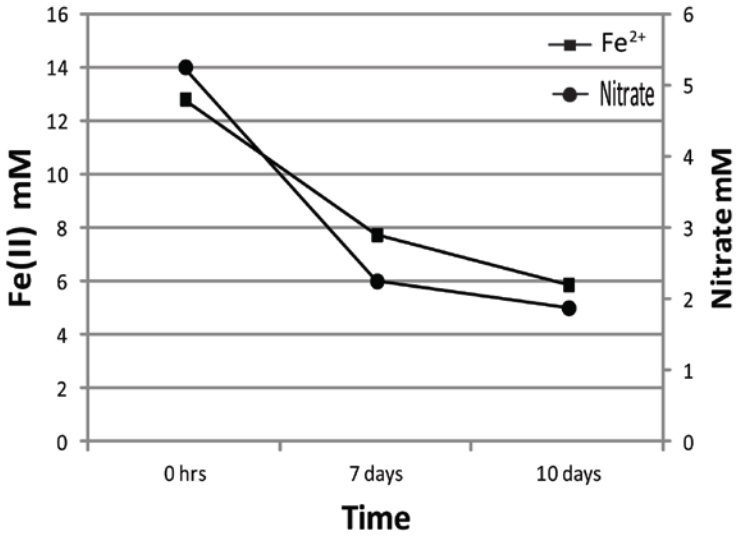

Figure 3. Characterization of Fe(II) and NO_3^- consumption by the *Thiomonas* enrichment culture under anaerobic conditions. Data shown are the average values from duplicate incubations. The enrichment had been grown 2 consecutive times before being used as inoculum for the experiment. 5% CO_2 was added to the headspace as the only utilizable carbon source. No reduction in the concentration of Fe(II) and nitrate was observed in the uninoculated controls.

5.5 Discussion

Studies have shown a low methanogenic potential of Lake Grosse Fuchskuhle compared to other lakes in the region (Casper et al., 2003; Chan et al., 2002a). Our previous study has shown that this phenomenon was due to a dominant Fe(III) reduction process, which could be outcompeting methanogenesis as the terminal electron accepting process for mineralization of sediment organic carbon (Chapters 2). A continuous Fe(III) reduction requires a constant regeneration of Fe(III) by oxidation of Fe(II), which is the rate limiting step for the longterm sustainment of Fe(III) reduction in sediments (Roden and Urrutia, 1999). Our experimental results from the previous study have also shown that chelation of Fe(II) by dissolved organic carbon could be preventing abiotic Fe(II) oxidation with atmospheric oxygen, indicating a greater role played by microbial Fe(II) oxidation in this sediment (Chapter 4). We assume that this absence of abiotic Fe(II) oxidation, reduced rate of aerobic Fe(II) oxidation using molecular oxygen due to the predominantly anoxic nature of the sediment and thermodynamically unfavorable conditions for denitrification due to low pH (Muller et al., 1980; Saleh-Lakha et al., 2009), could provide favorable conditions for nitrate-dependent

Fe(II) oxidation. Several field experiments done on mildly acidic groundwater systems have also suggested that nitrate could be playing an important role in the oxidation of Fe(II) and also provided evidence that this process is being mediated by autotrophically growing bacteria (Molenat et al., 2002; Pauwels et al., 1998b; Postma, 1990).

Due to the earlier reports of low adaptability of microbial communities from humic-rich environments to growth in artificial medium (Langenheder et al., 2005) and also due to the energetic benefits of oxidizing Fe(II) chelated to humic acid (Strathmann, 2011) for Fe(II)-oxidizing bacteria, the quantification of autotrophic and organotrophic nitrate-dependent Fe(II) oxidizing microorganisms was done using the medium containing natural concentration of HS. Results of our study have indicated a single order of magnitude difference between autotrophic and organotrophic nitrate-dependent Fe(II)-oxidizing microorganisms. These results are in contrast to all the earlier studies which have reported a difference of at least two orders of magnitude between these two physiological groups of Fe(II)-oxidizing bacteria (Muehe et al., 2009). Moreover, a two order of magnitude higher number of autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms were observed by this method compared to our earlier study done on the same sediment using an artificial medium (Chapter 3). These results indicate a positive role played by HS in consumption of Fe(II) or growth of Fe(II)-oxidizing bacteria. Even though our results (Chapter 3 & 4) demonstrate the enhancement of Fe(II) consumption by DOC, the possibility that these differences could be due to the differences in the growth rates and consumption of Fe(II) among the two organisms enriched in these incubations could not be totally ruled-out.

The incubations done for enrichment of chemolithoautotrophic nitrate-dependent Fe(II)-oxidizing bacteria under similar conditions showed an enrichment of microorganisms belonging to genus *Thiomonas*. The phylogenetic analysis of these sequences have shown that these are the same *Thiomonas* species labeled in our SIP incubations (Chapter 3) and are phylogenetically close to *Thiomonas* clone sequences previously reported from an old pyrite mine. These sequences form a distinct cluster among the genus *Thiomonas* indicating the possibility of a novel species within the genus. The absence of the enrichment of *Thiomonas* in the incubations done previously (Chapter 3) on a defined medium could be due to the selective nature of the enrichment media. No specific enrichment of these *Thiomonas* species could be observed in the control incubations done from the sediment without an addition of both Fe(II) and nitrate indicating the nitrate-dependent Fe(II)-oxidizing nature of these organisms (Figure 1). Subsequent dilution series done from the enrichment under autotrophic nitrate-dependent Fe(II) oxidizing conditions have shown a strong enrichment of

these *Thiomonas* species and a rapid reduction in the concentration of Fe(II) until the10^8 dilution, indicating a fast growth of these organisms under the incubation conditions.

The members of genus *Thiomonas* are common inhabitants of extreme environments like acid mine drainage, characterized by low pH, presence of high concentrations of metals like sulfur, iron, and arsenite (Bruneel *et al*., 2003; Duquesne *et al*., 2008). The members of this genus are metabolically versatile and are capable of deriving energy by oxidation of reduced inorganic sulfur compounds or As(III) (Battaglia-Brunet *et al*., 2002; Duquesne *et al*., 2007; Gonzalez-Toril *et al*., 2003a). Recent studies have shown the preliminary indications of Fe(II)-oxidizing physiology in *Thiomonas* strain 3As (Bruneel et al., 2003), however this physiological trait has not been well studied in the members of this genus. The genome sequences of several members of genus *Thiomonas* species have shown the presence of genes required for Fe(II) oxidation, such as nitrate reductase and a nitrite antiporter gene, suggesting the possibility that nitrate could be used as an electron acceptor under anaerobic conditions (Arsene-Ploetze et al., 2010) for Fe(II) oxidation. A time course of Fe(II) oxidation and nitrate consumption assay was performed with the *Thiomonas* enrichment showed a stoichiometric consumption of Fe(II) and nitrate required for nitrate-dependent Fe(II) oxidation (Figure. 3). The molar ratio of Fe(II) to nitrate consumed was 1.0:0.3 at all time points compared to the ideal ratio of 1.00:0.20. This slightly higher ratio of Fe(II) to nitrate consumed could be due to utilization of some nitrate for growth. Abiotic Fe(II) oxidation with nitrate could not be happening in the incubations due to low pH and absence of copper in high concentration required for catalyzing this reaction (Buresh and Moraghan, 1976; Langmuir et al., 1997). No formation of ammonia or N_2O in the headspace was observed indicating the absence of abiotic Fe(II) oxidation with nitrate. Moreover, no reduction in the concentration of Fe(II) or nitrate could be noticed in the uninoculation controls. These results are consistent with a nitrate-dependent Fe(II)-oxidizing physiology of these *Thiomonas* species.

The enumeration of RuBisCO (*cbbL*) gene copy numbers by quantitative real-time PCR showed the presence of high abundance and a logarithmic increase of copy numbers of these genes under the incubation conditions. Studies have shown that all the cultured members of the genus *Thiomonas* are capable of autotrophic growth; however, these organisms are metabolically versatile and are capable of mixotrophic and heterotrophic growth (Arsene-Ploetze et al., 2010; Panda et al., 2009; Slyemi et al., 2011). Although the results of our experiments do not disprove the mixotrophic growth of these organisms, the utilization of humic acids as carbon source to support the fast growth observed in our incubations would be unlikely. Earlier studies have shown the presence of high molecular weight humic acids in this basin (Sachse et al., 2001), which cannot be used as

carbon source directly and are resilient to degradation especially under anaerobic conditions (Martin and Haider, 1980; Schulten and Schnitzer, 1997) and absence of UV radiation (Allard et al., 1997). The presence of the Calvin cycle for the fixation of CO_2 has been reported from several members of the genus *Thiomonas.* This process requires NAD(P)H for the reduction of CO_2, which could be generated by reverse electron transport mechanism as shown in Fe(II)-oxidizing bacteria like *Acidithiobacillus ferrooxydans* (Appia-Ayme et al., 1999; Ingledew, 1982), which are phylogenetically closely related to the genus *Thiomonas*. Due to the high redox potential of Fe(II) (between +100 to -100 mV), the electrons released from its oxidation have to be pumped uphill by reversing the electron transport mechanism to a redox potential of -320mV, required for the synthesis of NAD(P)H (Ferguson, 1988; Ferguson and Ingledew, 2008). This process requires the input of energy in the form of proton motive force (PMF), which otherwise could be used for the synthesis of ATP. This diversion of PMF for the synthesis of NAD(P)H could lead to the limited amount of ATP synthesized per ferrous iron oxidized. Based in these observations, earlier studies have hypothesized that the nitrate-dependent Fe(II) oxidation process would be energetically unfavorable to support the autotrophic growth and could be the reason for low numbers of autotrophic compared to mixotrophic or organotrophic nitrate-dependent Fe(II)-oxidizing bacterial counts observed in several natural environments by the MPN method (Muehe et al., 2009).

Even though the above discussion is apt under laboratory conditions for growing organisms in a defined medium, under natural conditions Fe(II) is predominantly available as chelated to humic acids (Kerndorff and Schnitzer, 1980). Fe(II) exhibits a variable redox potential depending the type of Fe(III) oxide formed and on nature of organic ligand to which Fe(II) is chelated (Strathmann, 2011). This chelation to humic acids reduces the redox potential of Fe(II) to -380mV compared to +100mV in the unchelated form (Strathmann, 2011; Strathmann and Stone, 2002b). Under these conditions the difference between the redox potential of Fe(II) and nitrate could be increased to 800mV, which was similar to the difference in the redox potential of Fe(II) and O_2 observed for aerobic Fe(II) oxidation (Ferguson and Ingledew, 2008). Considering that autotrophic growth has been reported in aerobic Fe(II)-oxidizing bacteria under similar energetic conditions, we assume that humic acid chelated Fe(II) oxidation coupled to reduction of nitrate could also be able to support autotrophic growth. Hence, due to the energetic benefit of oxidizing humic acid chelated Fe(II), we assume that this phenomenon could be important for promoting autotrophic growth of nitrate-dependent Fe(II)-oxidizing bacteria.

5.6 References

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Chapter 6

Chemolithoautotrophic nitrate-dependent Fe(II) oxidizing nature of *Thiomonas arsenivorans* strain 3As

6.1 Abstract

Thiomonas arsenivorans strain 3As (DSM-22701) was tested for chemolithoautotrophic nitrate-dependent Fe(II) oxidation ability due to the presence of all the genes required for mediating this physiological process. Incubation experiments were conducted with and without humic substances to understand their role in mediating Fe(II) oxidation. *Thiomonas arsenivorans* showed good growth under autotrophic conditions in the presence of humic substances in comparison to no growth under similar conditions in the absence of humic substances. We hypothesize that these differences were due to the differential gene expression caused by reduced availability of chelated-Fe(II) compared to free Fe(II).

6.2. Introduction

Microbial Fe(II) oxidation in anoxic environments was shown to be mediated by coupling to denitrification processes (Straub et al., 1996). Subsequent studies have shown this physiological capability in several microorganisms (Hauck et al., 2001; Muehe et al., 2009; Straub and Buchholz-Cleven, 1998a). Ecological studies conducted on several natural environments have also shown that this physiological process is widespread and hypothesized to be quantitatively more significant compared with other Fe(II) oxidation processes due to absence of light and predominantly anoxic nature of soils and sediments (Straub et al., 1996). This physiological process has been initially reported from both organotrophically and autotrophically growing bacteria (Straub et al., 1996); however, to date only organotrophically growing bacteria are available as pure cultures (Muehe et al., 2009). Despite several attempts to isolate chemolithoautotrophic nitrate-dependent Fe(II)-oxidizing microorganisms, these organisms are not yet available as pure cultures. However, enrichment and quantification studies conducted on soils and sediments have shown the presence of

this physiological group of microorganisms in natural environments (Straub et al., 2004). Geochemical studies conducted on aquifers and soils have not only reported the widespread nature of this physiological process, but also indicated that this process is being mediated predominantly by autotrophically growing microorganisms (Pauwels et al., 1998b; Pauwels et al., 2000; Postma, 1990).

Studies conducted on groundwater aquifers showed that in the presence of chelating agents, Fe(II) could mediate the degradation of halogenated hydrocarbons (Strathmann and Stone, 2002a). Subsequent studies showed that chelation of Fe(II) to humic substances could lower its redox potential and this could be the possible reason for the observed increase in its reactivity (Kim et al., 2009a; Strathmann, 2011). Due to a high affinity of humic substances for charged metal ions, Fe(II) is predominantly present in natural environments as chelated to humic substances (Kerndorff and Schnitzer, 1980). Although recent studies have reported the importance of the form of Fe(II) in culturing Fe(II)-oxidizing microorganisms (Kopf and Newman, 2012; Straub and Buchholz-Cleven, 1998a), microbial cultivation and enrichment experiments done on Fe(II)-oxidizing bacteria to date have largely ignored this consideration.

Due to this lack of knowledge on the role of humic substances in culturing Fe(II)-oxidizing bacteria, the present study was conducted to elucidate whether the hypothesized energetic benefits of chelation could promote autotrophic nitrate-dependent Fe(II) oxidation, which was earlier considered to be energetically unfavorable. The present study was conducted using *Thiomonas arsenivorans* strain 3As, as the genome of this organism has shown the presence of all the genes required for Fe(II) oxidation, nitrate reduction and CO_2 fixation by the Calvin cycle (Arsene-Ploetze et al., 2010).

6.3 Experimental procedure

The strain *T. arsenivorans* was obtained from the DSMZ. The incubations were done using filter sterilized water collected from SW basin of Lake Grosse Fuchskuhle as described in Section 5.4. The medium was prepared by adding 1ml of minimal salts medium (without buffering) along with trace salts and vitamins (Widdel, 1992) to 29ml of filter sterilized lake water. $FeCl_2$ and sodium nitrate were added to the medium from stock solutions with final concentrations of 2mM and 0.4mM respectively. A lower concentration of Fe(II) compared to our earlier studies was used in the present study to ensure that all the Fe(II) present in the medium was chelated to humic substances. Control experiments were also done to check for abiotic Fe(II) oxidation and possible utilization of

humic substances as electron donors with reduction of nitrate. Similar incubations were also done using a defined medium under autotrophic and heterotrophic conditions, using 5mM sodium formate according to the procedure described earlier by Straub et al. (1998), to check the differences in growth and Fe(II) oxidation in the presence and absence of humic substances. All the incubations were inoculated with *T. arsenivorans* with the exception of abiotic control incubations. The above described setup was done under a N_2 atmosphere in an anaerobic chamber (Mecaplex, Grenchen, Switzerland) in triplicate and bottles were incubated on a shaker at 30°C. Fe(II) and nitrate measurements were done according to the procedure described earlier (Chapter 3) and OD_{600} was measured to determine the growth at every 2 day time point for 10 days.

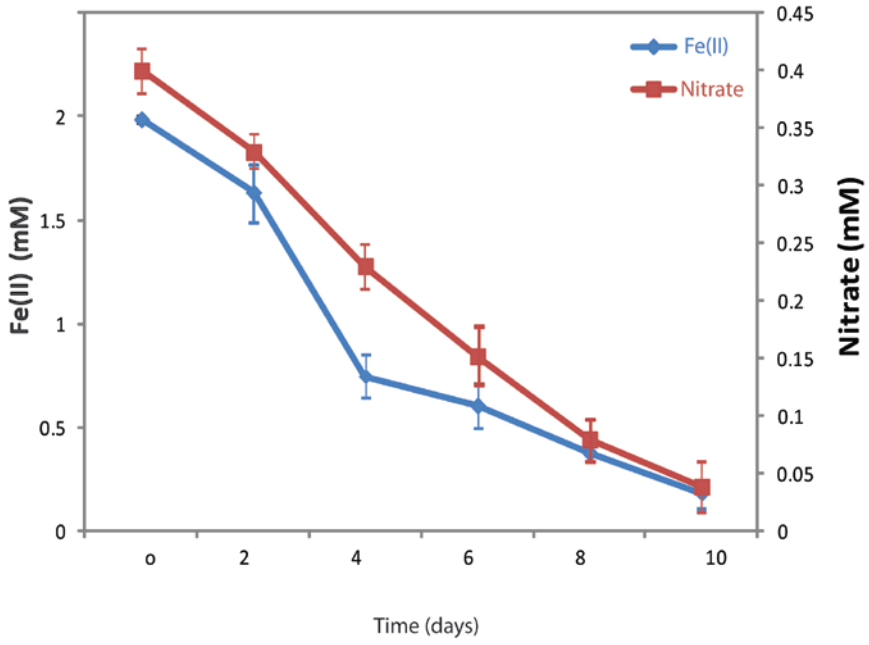

Figure 1: Characterization of Fe(II) and nitrate consumption by *T. arsenivorans* during growth in the presence of humic substances

6.4 Results and discussion

The incubations containing humic substances, Fe(II), nitrate and CO_2 showed visible growth and reduction in the concentration of Fe(II) and nitrate after 2 days of incubation (Figure. 1). The molar ratios of Fe(II) to nitrate consumed was 1:0.29 (Figure. 1), which was slightly higher than that of the expected ratio of 1:0.2 (Straub et al., 1996). This higher

ratio could be due to the utilization of nitrate for cell growth. No reduction in the concentrations of Fe(II) or nitrate was observed in un-inoculation controls indicating the absence of abiotic nitrate-dependent Fe(II) oxidation (results not shown). No growth was observed in incubations with only nitrate indicating the absence of both autotrophic nitrate reduction and capability of these organisms to degrade humic substances by reduction of nitrate (Figure. 2). No growth nor a decrease in the concentrations of Fe(II) and nitrate were observed in incubations done without the addition of humic substances under autotrophic and organotrophic conditions (results not shown).

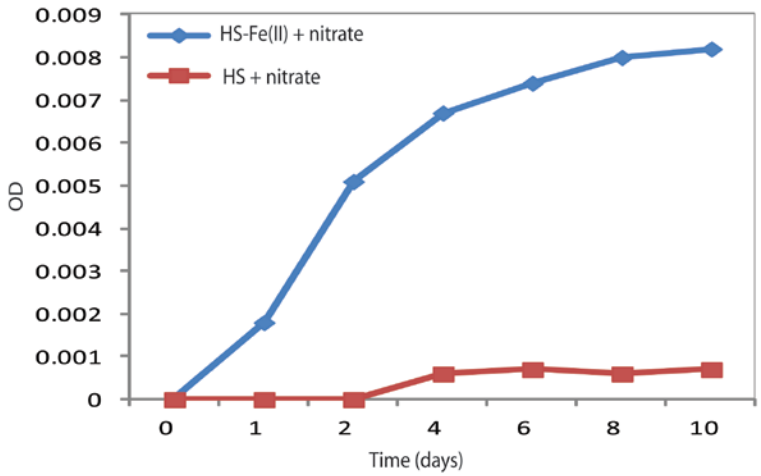

Figure 2: Anaerobic growth curve of *T. arsenivorans* with CO_2, nitrate and humic substances (HS) in the presence or absence of added Fe(II). The plotted values are average of triplicate incubations.

The results of our study show that *T. arsenivorans* is capable of chemolithoautotrophic nitrate-dependent Fe(II) oxidation. Our results also indicate that *T. arsenivorans* is capable of this physiological process only when Fe(II) is provided as chelated to humic substances. The absence of either autotrophic or heterotrophic Fe(II) oxidation when Fe(II) is provided in an unchelated form could be due to the differential regulation of genes in the presence and absence of Fe(II) chelators. The expression of several genes under anaerobic conditions is known to be mediated by the Fnr protein (Darwin et al., 1998). This regulation process is also known to regulate the expression of genes required for nitrate reduction like *narGHIJ* through the NarL and NarP regulatory system

(Darwin and Stewart, 1995). NarL proteins are known to activate genes required for nitrate reduction and is known to be upregulated under anoxic conditions along with Fnr. NarP is active under anaerobic conditions and is known to downregulate the genes required for nitrate reduction (Darwin et al., 1998). This Fnr, NarP and NarL regulation of nitrate reduction was shown in several microorganisms like *E. coli* (Darwin and Stewart, 1995), *Salmonella enterica* (Teixido et al., 2010) and *Haemophilus influenzae* (Stewart and Bledsoe, 2005).

Recent studies have shown that the expression of Fnr is regulated by the redox regulator Fur, which is known to regulate the expression of several other genes involved in oxidative stress responses and Fe(II) uptake (Teixido et al., 2010). In the presence of a high concentration of free Fe(II) in the medium, Fur downregulates genes responsible for Fe(II) intake to inhibit the oxidative damage to cell caused by the Fenton reaction (Kiley and Beinert, 2006). This process is also known to downregulate the expression of both Fnr and NarL required for the expression of nitrate reduction genes (Kiley and Beinert, 2006). However, chelation of Fe(II) in the medium is known to reduce the biological availability of Fe(II) (Hutchins et al., 1999; Imai et al., 1999). This process was experimentally shown to increase the expression of NarL and repression of NarP, which is known to upregulate the expression of genes required for nitrate reduction (Teixido et al., 2010). This Fur, Fnr, NarP and NarL regulation of the nitrate reduction system to date has been shown in several microorganisms. Although this regulation process was not shown in *T. arsenivorans*, all the genes involved in this processes are present in this organism (Arsene-Ploetze et al., 2010). Hence, we hypothesize the possibility that the presence of free-Fe(II) could have inhibited nitrate reduction, leading to the inhibition of nitrate-dependent Fe(II) oxidation.

In summary the results of our study indicate that *T. arsenivorans* is capable of chemolithoautotrophic nitrate-dependent Fe(II) oxidation only when Fe(II) is provided in a chelated form. Our results also indicate the importance of the chelation of Fe(II) in regulating microbial Fe(II) oxidation.

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Chapter 7

General discussion and outlook

Iron is one of the most abundant metals on Earth's crust that can undergo redox transformations. Over the course of evolution, microorganisms have developed physiological capabilities to conserve energy either by reducing or oxidizing iron . These microbial redox transformations of iron are known to play an important role in transformation of soil and sediment organic carbon (Kusel et al., 2008). A growing body of literature from geochemical investigations has also shown that redox cycling of iron could also play an important role in the biogeochemical cycling of other elements like nitrogen, sulfur and phosphorus (Davidson et al., 2003). Although microorganisms were known to play an important role in the transformation of iron, which subsequently influence the cycling of other elements, the microbial ecology of several of these interactions has not been investigated or experimentally shown.

Iron-metabolizing bacteria were one of the first microorganisms to be isolated (Ehrenberg, 1836); however, the ecology of these microorganisms remains less understood compared to that of microorganisms involved in other electron-accepting processes like nitrate reduction, sulphate reduction and methanogenesis due to the lack of a specific functional marker gene. Hence, most of the progress made in the last decades in terms of biological redox cycling of iron was largely obtained from culture-dependent studies like isolation or enrichment followed by characterization of the organisms. The absence of culture-independent methods in combination with difficulties associated with culturing both Fe(III)-reducing and Fe(II)-oxidizing bacteria led to the current lack of understanding of the diversity of iron-metabolizing microorganisms.

This PhD thesis was focused on iron reduction and oxidation processes and their roles in mineralization and fixation of inorganic carbon in an acidic bog lake, Lake Grosse Fuchskuhle. Peatlands constitute >3% of the Earth's terrestrial area but store approximately one third of global soil organic carbon. Although peatlands act as sinks for atmospheric carbon, they are net emitters of greenhouse gasses, like CH_4 and N_2O, into the atmosphere. Hence, most of the studies conducted on peatlands focused on methanogenesis and the role of environmental factors influencing this process and very few studies were focused on other electron-accepting processes. In the first part of the thesis the role of Fe(III) reduction in mediating soil organic carbon mineralization, its ability to suppress methanogenesis and the differences within the basins with depth and among the basins was investigated (Chapter 2). The second part was focused on the role of

autotrophic nitrate-dependent Fe(II) oxidation, which is important for long term sustainment of Fe(III) reduction. Finally, the role of chelation of Fe(II) by humic substances and its influence on microbial and abiotic Fe(II) oxidation was studied.

Fe(III) reduction process in Lake Grosse Fuchskuhle

Bog lakes are characterized by the presence of a high concentration of humic substances, acidic pH and low photosynthetic primary production compared to other fresh water lakes. Humic substances have a high affinity for positively charged metals like Fe(III) and are known to form chelates (Kerndorff and Schnitzer, 1980) that are biologically more available than insoluble Fe(III) hydroxides for microbial Fe(III) reduction. Humic substances are also shown to be capable of mediating the transfer of electrons between Fe(III)-reducing microorganisms and insoluble Fe(III) hydroxides (Lovley et al., 1998). Low pH conditions like those observed in bog lakes are also known to increase the solubility of Fe(III) hydroxides making them biologically more available. Due to these conditions, Fe(III) reduction could be the favored electron-accepting process in bog lakes, which could lead to the suppression of sulfate reduction and methanogenesis.

Studies conducted on Lake Grosse Fuchskuhle have shown low methanogenesis in the lake and hypothesized that this phenomenon could to be due to the presence of other electron-accepting processes, which could be suppressing methanogenesis (Casper et al., 2003; Conrad et al., 2010); however, these electron-accepting processes have not been elucidated. Due to a low concentration of sulphate (Peter Casper, personal communication) and thermodynamically unfavorable conditions for nitrate reduction due to low pH (Muller et al., 1980; Saleh-Lakha et al., 2009), the possibility of Fe(III) reduction as a dominant electron accepting process for degradation of organic carbon were tested in this environment. The results of our study (Chapter 2) have shown that Fe(III) reduction is prevalent in the sediments of both the basins in Lake Grosse Fuchskuhle, however differences in the rates of these processes were observed among the basins. Based on earlier findings on the role of humic substances in promoting Fe(III) reduction (Lovley et al., 1998), we assume that the differences in the concentration of humic substances is responsible for these difference in the observed rates of Fe(III) reduction.

Comparison of Fe(II) and CO_2 production showed a higher than expected Fe(II) production compared to CO_2 production (Roden and Wetzel, 1996), indicating the reduction of Fe(III) without complete oxidation of organic matter. Unlike lake sediments of circumneutral pH where a relatively fast hydrolysis and

fermentation organic matter lead to the formation of relatively low molecular weight organic carbon, , the degradation of organic matter is relatively slow in Lake Grosse Fuchskuhle due to low pH (Rao et al., 1984), the presence of a large fraction of organic matter in the form of resilient humic substances (Sachse et al., 2001) and lignin-rich *Sphagnum* material (Williams et al., 1998), which are recilient to degradation. Hence, the presence of organic matter undergoing degradation can be observed even at deeper layers (25-30 cm) of the sediment. Hence we hypothesize that the high ratio of Fe(II) to CO_2 produced could be due to partial degradation or hydrolysis of higher molecular weight organic carbon to lower molecular weight organic carbon without production of CO_2.

Physiological characterization of known Fe(III)-reducing bacteria have shown that these bacteria have a diverse pattern in utilizing carbon substrates (Lonergan et al., 1996). Several cultured Fe(III)-reducing bacteria were also shown to incompletely oxidize organic matter (Laverman et al., 1995), which could lead to the production of acetate and H_2. Culture-independent studies conducted on peatlands have also reported a high diversity of [Fe-Fe]-hydrogenases and indicated that Fe(III)-reducing bacteria are a key group of microorganisms responsible for H_2 production (Schmidt et al., 2010). These results indicate that Fe(III)-reducing bacteria are capable of forming syntrophic associations with other H_2 utilizing bacteria like sulfate reducers, methanogens and hydrogenotrophic Fe(III)-reducing bacteria. The syntrophic association of Fe(III)-reducing bacteria like *Geobacter sulfurreducens* strain PCA with nitrate and sulfate-reducing bacteria (Cord-Ruwisch et al., 1998) have been experimentally shown. However, the evidence of such syntrophic association between Fe(III)-reducing bacteria with hydrogenotrophic methanogenic archaea have only been reported in the past few years (Kato et al., 2012) and the ecological implications of such interactions remain completely unknown. Based on our results we assume that such syntrophic associations are prevalent in the sediment of Lake Grosse Fuchskuhle, which could have favored hydrogenitrophic methanogenesis.

Fe(II) oxidation in Lake Grosse Fuchskuhle

The first part of the thesis had shown that Fe(III) reduction is the dominant electron-accepting process in Lake Grosse Fuchskuhle and could be mediating the mineralization of a large fraction of sediment organic carbon. The second part of the thesis was focused on the Fe(II) oxidation and the effect of limnological conditions of Lake Grosse Fuchskuhle on this process. Fe(II) oxidation is of great significance in this environment as the rate and longterm sustainment of Fe(III) reduction in natural environments depends of the rate at which Fe(III) is recycled by Fe(II) oxidation (Roden and Urrutia, 1999).

Fe(II) oxidation in lakes could happen either aerobically in the water column or anaerobically within the sediment (Straub et al., 1996), both of which could be mediated either biologically or abiotically (Stumm and Morgan, 1996). Fe(II) is highly soluble in water and the upward flow of groundwater could continuously transport Fe(II) into the oxic parts of the lake like the water column or rhizosphere of macrophytes (Frenzel et al., 1999; Neubauer et al., 2007), where it can be oxidized either by reacting with molecular O_2 or by Fe(II)-oxidizing bacteria. Studies in the past have hypothesized that this process in natural environments is predominantly abiotic due to the high reactivity of Fe(II) with environmental O_2 at circumneutral pH (Stumm and Morgan, 1996). Microbially-mediated Fe(II) oxidation was considered to be restricted to few specific environments like acid mine drainage where low pH inhibits abiotic reaction between Fe(II) and O_2 (Johnson and Hallberg, 2003; Singer and Stumm, 1970). However, a growing body of literature over the past decade has shown the importance of microbial Fe(II) oxidation and the role of dissolved organic carbon in inhibiting abiotic Fe(II) oxidation (Emerson and Moyer, 1997; Moses and Herman, 1991).

Studies have hypothesized that the rate of abiotic Fe(II) oxidation in natural environments depends on environmental factors like pH (Stumm and Morgan, 1996) and concentration of dissolved organic carbon (Theis and Singer, 1974). Although the role of pH in mediating this process was well understood, the effect of dissolved organic carbon on this process has not been well elucidated. Studies conducted in this regard showed that the rate of abiotic Fe(II) oxidation depends on several factors like the ratio of concentration of DOC and Fe(II) (Gaffney et al., 2008), composition of DOC and nature of the functional group in DOC to which Fe(II) is chelated (Strathmann, 2011). As the structure and composition of DOC or humic substances is dependent on the ecological conditions of that specific environment, generalizations about the role of DOC in abiotic Fe(II) oxidation is difficult. Hence, experiments were conducted to determine the rate of abiotic Fe(II) oxidation in Lake Grosse Fuchskuhle. The results of our study suggests a reduced rate of abiotic Fe(II) oxidation in this lake (Chapter 4). The presence of high

concentrations of humic substances, possibly low ratio Fe(II) to DOC and slightly acidic pH of the lake could be likely reasons for the absence of abiotic Fe(II) oxidation. These results indicate that microbial processes are solely responsible for Fe(II) oxidation in this lake sediment.

The water column of Lake Grosse Fuchskuhle undergoes stratification and the sediment surface remains anoxic for most of the year (Burkert et al., 2004; Koschel, 1995). Even when the lake is not stratified, oxygen only penetrates the top few millimeters of the sediment leaving most of the sediment anoxic. Earlier studies have hypothesized that under these conditions, Fe(II) generated by Fe(III) reduction processes remains un-reactive due to its high redox potential (+770mV). However, recent studies showed that microorganisms are capable of Fe(II) oxidation under anaerobic conditions by coupling it to denitrification processes (Straub et al., 1996). Subsequent studies conducted on diverse environments have also reported the evidence of this physiological process (Pauwels et al., 1998b; Straub et al., 2004). As most of the sediments and soils are predominantly anaerobic and devoid of light, nitrate-dependent Fe(II) oxidation was hypothesized to be widespread and quantitatively more significant that other Fe(II) oxidation processes (Straub et al., 1996). Hence, the present study was focused on elucidating the identity and to the enrichment and characterization of microorganisms belonging to this physiological group in Lake Grosse Fuchskuhle.

The results of our study showed that *Actinobacteria* belonging to TM3 subdivision are capable of nitrate-dependent Fe(II) oxidation (Chapter 2). TM3 along with TM2 *Actinobacteria* are known to be ubiquitously distributed in natural environments, with a higher abundance in acidic environments like peatlands (Rheims et al., 1999). Although studies have reported the presence of these organisms from several environments, the physiological properties of these organisms was not known due to the lack of either enrichments or pure cultures. To our knowledge, this is the first study to assign a physiological function to this uncultured group of *Actinobacteria*. The growth of these *Actinobacteria* under autotrophic conditions and subsequent labeling of these organisms by $^{13}CO_2$ by stable isotope probing conducted on the sediment strongly suggests that these organisms are capable of autotrophc growth.

Actinobacteria are a morphologically diverse prokaryotic phylum and are distributed ubiquitously in both terrestrial and marine habitats (Hugenholtz et al., 1998; Stackebrandt et al., 1997). They are considered to be aerobic or microaerophilic organisms involved in the degradation of soil organic carbon (Boer et al., 2006). However, recent studies have shown that *Actinobacteria* are present in high abundance in anaerobic regions of the soils and sediments (Yongkyu Kim, personal communication). Genome sequencing of cultured *Actinobacteria* and culture-independent studies conducted on natural environments have shown that these organisms are capable of nitrate reduction (Bru et al., 2007; Philippot

et al., 2002), which could explain their presence in anoxic regions of the soils. These findings are of great significance as *Actinobacteria* are considered to be the ancestor of the clade Neomura and have evolved during the Archaean period (Cavalier-Smith, 2010). The Earth's atmospheric conditions during this time are considered to have been reducing due to the lack of free oxygen and anaerobic processes involving Fe(II), H_2 or denitrification processes are hypothesized to have played an important role in CO_2 fixation (Canfield et al., 2006). Oceans during this time period are considered to have had a high concentration of Fe(II) and microbial ferrous iron oxidation is considered to have led to the banded iron formations (Konhauser et al., 2002). This physiological process was hypothesized to be mediated either phototrophically (Ehrenreich and Widdel, 1994; Widdel et al., 1993) or by nitrate derived from disproportionation of nitric oxide, generated from lightening (Canfield et al., 2006). Although *Actinobacteria* were known to be present on Earth during the Archaean period, the role of these bacteria in mediating any of these anaerobic processes have not been experimentally shown. Although an earlier study conducted by Straub and Buchholz-Cleven et al. (1998) had indicated that *Actinobacteria* are capable of nitrate-dependent Fe(II) oxidation, to our knowledge ours is the first study to experimentally show this physiological process in these organisms.

Role of humic substances in mediating nitrate-dependent Fe(II) oxidation

Humic substances are the major organic constituents of soil and are ubiquitously distributed in terrestrial and marine habitats (Ertel and Hedges, 1984; Malcolm, 1990; Zech et al., 1992). Due to the presence of charged functional groups, humic substances have a high affinity for metal ions like Fe(II) and Fe(III) (Kerndorff and Schnitzer, 1980; Millero et al., 1995). Hence, most of the Fe(II) and Fe(III) present in natural environments is present as chelated to humic substances (Kerndorff and Schnitzer, 1980). The chelation of metal ions to humic substances could affect the solubility (Rashid and Leonard, 1973), toxicity (Gress et al., 2004), biological availability (Hutchins et al., 1999) and redox potentials (Strathmann, 2011) of metals in natural environments. Fe(II) exhibits a variable redox potential depending on the nature of the ligand involved in the chelation. Some chelating agents could increase its redox potential making it unreactive or reduce the redox potential making it more reactive (Strathmann, 2011). Chelation of Fe(II) to humic substances is known to reduce its redox potential to -380mV (from 770mV) making it more reactive (Strathmann and Stone, 2002c). However, this effect of chelation of Fe(II) has been largely neglected to date with respect to culturing Fe(II)-oxidizing microorganisms using a defined medium.

Lake Grosse Fuchskuhle is an acidic bog lake divided artificially into four basins (Koschel, 1995). Due to the hydrogeology of the lake and the surrounding region, the western basins of the lake receive a large input of humic substances and the division has largely reduced the input of humic substances into the eastern basins (Simek et al., 1998). The presence of different basins that are similar in their nutritional status with the exception in the nature of dissolved organic carbon makes this lake an ideal environment for determining the role of humic substances on several physiological processes. The experiments conducted on the NE basin littoral sediment by incubation with water collected from NE basin containing low concentrations of humic substances, SW basin water containing high concentrations of humic substances and mineral salt medium containing no humic substances have shown a rate of Fe(II) oxidation proportional to the concentration of humic substances. We hypothesize that this beneficial role of humic acids for Fe(II) oxidation was due to the lowering of the redox potential of Fe(II), which could have made the oxidation thermodynamically more favorable. Moreover, humic substances have a high affinity for Fe(III) and chelation of Fe(III) (formed by oxidation of ferrous iron) by humic substances could reduce the formation of insoluble Fe(III) hydroxides, which reduces the risk of cell encrustation (Kappler et al., 2006b). However, the exact mechanism by which humic substances promoted the oxidation of Fe(II) is currently unknown.

Due to the observed positive effect of humic substances on Fe(II) oxidation (Kim et al., 2009a) and reports of low adaptability of microorganisms from humic rich environments to artificial media without humic substances (Langenheder et al., 2005), all the quantification and enrichment experiments conducted earlier were repeated with the only exception of using the lake water instead of an artificial medium. These experiments showed a faster Fe(II) oxidation, enrichment of different microbial populations and higher numbers of autotrophic microorganisms and similar numbers of organotrophic nitrate-dependent Fe(II)-oxidizing bacteria compared to that observed in the earlier study done on the same sediment sample using a defined medium (Chapter 4). Earlier studies have hypothesized that lower numbers of autotrophic compared to organotrophic nitrate-dependent Fe(II)-oxidizing bacteria observed in different environments could be due to the energetic benefit of organotrophic growth in this physiological group of organisms (Muehe et al., 2009). Autotrophic growth requires the input of energy as ATP and reducing equivalents in the form of NAD(P)H. As the reduction potential of Fe(III)/Fe(II) couple is close 100 to -100mV compared to the -320mV required for the reduction of NAD(P) to NAD(P)H, the electrons released from oxidation of Fe(II) should be pumped uphill against the redox gradient by reversing the electron transport chain with input of energy in the form of ATP (Ferguson, 1988). Based on these observations it was hypothesized that oxidation of Fe(II) would not be capable of supporting both the energy requirements and CO_2 fixation of nitrate-dependent Fe(II)-oxidizing

microorganisms (Muehe et al., 2009). However, the chelation of Fe(II) to humic substances reduces the redox potential of Fe(III)/Fe(II) couple from 100mV to -380mV (Strathmann, 2011). Under these conditions, oxidation of Fe(II) could mediate the reduction of NAD(P) to NAD(P)H without the consumption of ATP. Hence, we hypothesize that this energetic benefit of oxidizing Fe(II) chelated to humic substances could support the autototrophic growth in nitrate-dependent Fe(II)-oxidizing microorganisms and could be the possible reason for the higher numbers of autotrophic nitrate-dependent Fe(II)-oxidizing microorganisms observed in the presence of humic substances.

Incubations done for the enrichment of nitrate-dependent Fe(II)-oxidizing microorganisms in the presence of humic substances showed the enrichment of *Thiomonas* species closely related to uncultivated members reported from an old pyrite mine. Unlike the enrichment reported earlier, this *Thiomonas* enrichment could be cultured under nitrate-dependent Fe(II)-oxidizing conditions without losing the capability to grow autotrophically after repeated subculturing (Chapter 4). Members of the genus *Thiomonas* are ubiquitously distributed in mining impacted soils and sediments containing high concentrations of iron and arsenate (Bruneel et al., 2003; Coupland and Johnson, 2004; Duquesne et al., 2007). The genus *Thiomonas* are phylogenetically closely related to several iron-oxidizing bacteria like *Acidithiobacillus ferrooxidans* (Clark and Norris, 1996) and *Thiobacillus denitrificans* (Straub et al., 1996); however, this physiological trait has not been well studied among the members of this genus.

Recent studies had reported that *Thiomonas arsenivorans* strain 3As, isolated from a mining impacted soil, is capable of chemolithotrophic Fe(II) oxidation under aerobic conditions (Battaglia-Brunet et al., 2006). Although this isolate had not been tested for nitrate-dependent Fe(II) oxidation, the genome sequence of this isolate and closely related strains have shown the presence of genes required for Fe(II) oxidation, nitrate reduction and CO_2 fixation (Arsene-Ploetze et al., 2010). Hence, incubations were done in order to elucidate if this organism is capable of chemolithoautotrophic nitrate-dependent Fe(II) oxidation and to test the hypothesis about the role of humic acids in promoting autotrophic growth under nitrate-dependent Fe(II)-oxidizing conditions. The results of our study suggests that this isolate is capable of autotrophic growth by nitrate-dependent Fe(II) oxidation. However, this strain is capable of mediating this physiological process only in the presence of humic substances. To our knowledge ours is the first study to report the chemolithaototrophic nitrate-dependent Fe(II) oxidation by *Thiomonas*.

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Pledge

I certify that the present thesis entitled:

"Microbial iron redox cycling in Lake Grosse Fuchskuhle"

was carried out without any unlawful means; no literature resources, reagents and technical devices were used other than those stated. This work has never been submitted before in this or similar format to any other university and has not been used before any examination.

Marburg, April 2013

Dheeraj Kanaparthi

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