Chemically enhanced biological NO_x removal from flue gases

Nitric oxide and ferric EDTA reduction in BioDeNOx reactors

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Onze grootste vrees is niet dat we onvolmaakt zijn,
onze grootste vrees is onze mateloze kracht te uiten.
Het is ons licht, niet onze schaduw, die ons het meest beangstigt.

Nelson Mandela

ABSTRACT

Van der Maas PMF (2005). **Chemically enhanced biological NO_x removal from flue gases – Nitric oxide and ferric EDTA reduction in BioDeNOx reactors**. Doctoral Thesis, Wageningen, The Netherlands, 224 pages.

The emission of nitrogen oxides (NO_x) to the atmosphere is a major environmental problem. To abate NO_x emissions from industrial flue gases, to date, mainly chemical processes like selective catalytic reduction (SCR) are applied. All these processes require high temperatures (>300 °C) and expensive catalysts. Therefore, biological NO_x removal techniques using denitrification may represent promising alternatives for the conventional SCR techniques. However, water based biofiltration requires relatively long scrubber/bioreactor retention times, i.e. big reactor volumes, due to the slow mass transfer of NO from the gas into the liquid phase.

BioDeNOx is in principle a welcome alternative for conventional NO_x removal techniques like SCR and water-based biofiltration, since it does not need high temperatures and catalysts, while scrubber retention times can be very short (< 10 seconds) due to the chemically enhanced NO absorption. In this thesis, the BioDeNOx concept was investigated with special attention to the bioreactor key conversions: NO and $Fe(III)EDTA^-$ reduction.

This study showed stable NO removal from the gas phase with efficiencies up to 80 %. It was found that the NO removal from the gas phase is primary determined by NO absorption kinetics. Therefore, a high $Fe(II)EDTA^{2-}$ concentration is required, i.e. the FeEDTA absorption liquor should be in the reduced state. However, a totally reduced system should be avoided, since this will induce sulfide accumulation. The latter process is unwanted, since already low sulfide concentrations showed an incomplete NO reduction due to inhibition of N_2O reduction to N_2. To achieve satisfying NO removal from the gas and to avoid sulfdogenic conditions, the redox potential of BioDeNOx reactors should be steered between -180 and -200 mV versus Ag/AgCl (pH 7.2±0.2). An ethanol dosing system that is controlled by the redox potential signal was shown to be a proper manner to do so.

The volumetric $Fe(III)EDTA^-$ reduction rate was found to be the limiting factor in the $Fe(II)EDTA^{2-}$ regeneration capacity in case the flue gas contained elevated O_2 concentrations (> 1%). An insufficiently high $Fe(III)EDTA^-$ reduction rate results in high redox potential (> 140 mV versus Ag/AgCl) and therefore, poor NO removal efficiencies from the gas phase. Therefore, high volumetric $Fe(III)EDTA^-$ reduction rates are of crucial importance for the successful application of the BioDeNOx technology. Batch experiments showed that the addition of low amounts (< 1 mM) of reduced sulfur compounds (e.g. sulfide, cysteine or elemental sulfur) accelerated the $Fe(III)EDTA^-$ reduction substantially. The linear relationship between the amount of sulfide added and the volumetric $Fe(III)EDTA^-$ reduction rate strongly suggests that $Fe(III)EDTA^-$ reduction in BioDeNOx reactors is mediated by an electron shuttle, presumably polysulfides. For continuous BioDeNOx reactors it is, therefore, impotant to maintain a certain amount of the electron mediation compound (polysulfides) in solution.

The observation that the specific rate of NO reduction in aqueous Fe(II)EDTA^{2-} solutions, e.g. the medium in BioDeNOx reactors, was independent of the presence of ethanol strongly suggests that, except absorbent, Fe(II)EDTA^{2-} is also an electron donor, i.e. it interferes with the biological NO reduction electron transfer chain via reduction of the Nitric Oxide Reductase (NOR), directly or indireactly via viz. Cytochrome c. In continuous reactors, the volumetric NO reduction rate depends on the activity and concentration of the denitrifying biomass fraction. The NO loading rate of the BioDeNOx reactor may also influence the volumetric NO reduction rate, since it directly determines the free NO (electron acceptor) concentration.

This study showed substantial EDTA degradation when treating an artificial flue gas containing elevated oxygen concentrations (> 3 %), probably due to radical formation via Haber-Weiss like reactions. Taking also the electron flow related to Fe oxidation and reduction into account, it is evident that the oxygen sensitivity of Fe(II)EDTA^{2-} leads to high ethanol and EDTA consumption and thus to high operation costs. In that scope, the development and application of a metal chelate that is less sensitive for oxidation by oxygen but with comparable NO absorption properties as Fe(II)EDTA^{2-} is highly welcome.

Contents

CHAPTER 1

GENERAL INTRODUCTION

NITROGEN OXIDES

Nitrogen oxides (NO_x) constitute a group of highly reactive gases, all containing nitrogen and oxygen in a variable ratio. The major nitrogen oxides comprise nitric oxide (NO), a colourless and odourless gas and nitrogen dioxide (NO_2), which is the reaction product of NO with oxygen (Jager, 2001). NO is the primary NO_x form in combustion products, typically 95% of total NO_x content (Bosch *et al.*, 1988; Fritz and Pitchon., 1997; Janssen, 1999).

Environmental impact of NO_x emissions

The ongoing emission of NO_x is a serious persistent environmental problem, because these gases play an important role in the atmospheric ozone destruction and global warming (Stepanov and Korpela, 1997). Furthermore, urban NO_x is regarded as one of the most important precursors to photochemical smog (Guicherit and Van den Hout, 1982, Princiotta, 1982). Smog products irritate eyes and throat, evoke asthmatic attacks, reduces visibility and damages plants and materials as well. Moreover, NO_x also contribute to acidification, because they dissolve in cloud and precipitation water to form the strong acids HNO_2 and HNO_3 (Hales, 1982, Stepanov and Korpela, 1997, Princiotta, 1982).

The presence of NO_x in ambient air is of great concern because of the toxicity of the individual compounds or the mixtures of nitrogen oxides. NO has cytotoxic properties and is implicated in the degeneration of neurons, associated with Parkinson's disease, dementia and stroke (Stepanov and Korpela, 1997). NO_2 irritates the respiratory tract (Posthumus, 1982).

Sources of NO_x

NO_x are naturally produced in the atmosphere due to lightning, volcanic activity and biological decay (Cole, 1993, Stepanov and Korpela, 1997). Naturally produced nitrogen oxides end up in the nitrogen cycle together with human-generated NO_x. For the last 20-30 years, the NO_x concentration in the atmosphere has increased due to human activities (Rasmussen and Khalil, 1986), mainly due to the combustion of fossil fuels (Fig. 1).

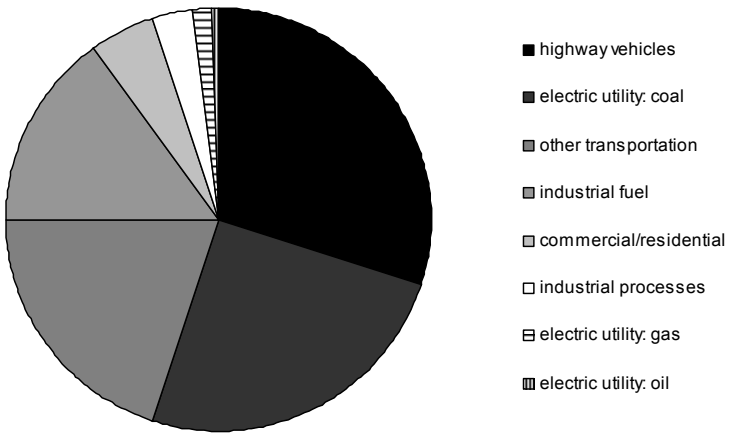

Figure 1

Relative contributions of anthropogenic NO_x emission sources (Schnelle and Brown, 2002).

The presence of nitrogen oxides in flue gases can be sub-devided in two groups: thermal NO_x and fuel NO_x (Jager, 2001; Kremer, 1982). Thermal NO_x are formed by the combustion reactions where molecular nitrogen reacts with $O^{\cdot}$ and $OH^{\cdot}$ radicals and molecular O_2 in high temperature regions (> 1800 K). The formation of this category of NO_x is highly influenced by the combustion temperature. Fuel NO_x is formed by oxidation of nitrogen compounds chemically bound in the fossil fuel. Formation of fuel NO_x takes place at lower temperatures (1000-1400 K) than thermal NO_x.

Table 1 shows typical concentrations in the flue gases from electric power generation plants as a function of the fuel utilised (Ramachandran *et al.*, 2000). Natural gas, for example, is a relatively clean fuel with respect to NO_x formation when compared with heavy fuel or coal. Natural gas does not contain any fuel nitrogen, i.e., nitrogen atoms bound in the hydrocarbon molecules, and, therefore, the only NO_x production route is via oxidation of the molecular nitrogen contained in the combustion air.

Table 1

Typical concentrations of emissions in flue gases from power generation plants using different types of fuel (Ramachandran *et al.*, 2000).

	Natural gas	**Fuel oil**	**Coal**
NO_x (ppm)	25-160	100-600	150-1000
SO_x (ppm	<0.5-20	200-2000	200-2000
CO_2 (%)	5-12	12-14	10-15
O_2 (%)	3-18	2-5	3-5
H_2O (%)	8-19	9-12	7-10
N_2	balance	balance	balance

Global NO_x emissions followed until recently an almost exponential increase over time, but over the last decade they seem to slow down. In Europe, the release of nitrogen oxides has even declined recently, but it still amounted to approximately 13 million tons in 2000 (European Environment Agency, 2003). The NO_x emission reduction targets for the European Union are based on a 55 % reduction of the total NO_x emission in 1990 by the year 2010 (Erisman *et al.*, 2003). In 2001, a reduction of 28 % compared to 1990 was achieved (Table 2). Approximately 24 million tons of NO was released to the atmosphere from US sources during 1998 (US EPA, 2000). Titles I and IV of the 1990 Clean Air Act Amendments regulate NO_x emissions from major stationary sources (Bradford *et al.*, 2002). The overall goal of these programs was to achieve NO_x reductions of 2 million tons per year below 1980 levels by the year 2000. However, the growing economies, especially in Southeast Asia, likely overwhelm any reductions in NO_x emissions made in Europe and North America (Fawler *et al.*, 1998). The rapid economic growth and the increasing consumption of fossil fuel there results in the emission of large amounts of NO_x into the atmosphere.

Table 2

NO_x emissions in the European Union by sector (ktonnes) (European Environmental Agency, 2003).

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	Change 1990-2001	% contribution to Change	Change 1990-2001 (%)
Energy Industries	4274	4019	3771	3433	3221	3191	3079	2858	2727	2612	2639	2640	-1634	-33.4%	-38%
Fugitive Emissions	40	34	34	35	35	34	36	35	34	42	38	35	-5	-0.1%	-12%
Industry (Energy)	2282	2253	2109	1882	1814	1787	1739	1717	1646	1615	1533	1548	-736	-15.0%	-32%
Industry (Processes)	287	246	208	179	182	157	158	158	160	158	152	148	-155	-2.8%	-48%
Other (Energy)	805	835	795	861	781	748	840	780	743	742	706	732	-44	-1.5%	-9%
Other (Non Energy)	0	0	0	1	1	1	0	0	0	0	0	0	0	0.0%	-42%
Road Transport	7481	7401	7371	7133	7001	6685	6533	6255	6078	5838	5570	5331	-2147	-44.0%	-29%
Other Transport	2022	1967	1910	1882	1873	1900	1989	2021	2031	1997	1894	1874	-204	-3.0%	-7%
Agriculture	144	142	137	129	129	134	146	148	147	143	153	146	-5	0.0%	2%
Waste	134	141	139	139	135	133	132	134	129	134	128	127	-16	-0.1%	-5%
Total	**17470**	**17039**	**16475**	**15675**	**15172**	**14771**	**14654**	**14107**	**13694**	**13280**	**12814**	**12581**	**-4947**	-	**-28%**

REMOVAL OF NITROGEN OXIDES

In principle, NO_x emissions due to the combustion of fossil fuels can be abated via two different strategies (Fig. 2): combustion modification (primary measures) and post-combustion techniques (end of pipe technologies). Usually post-combustion techniques are implemented in addition to primary measures when a high level of NO_x abatement is required.

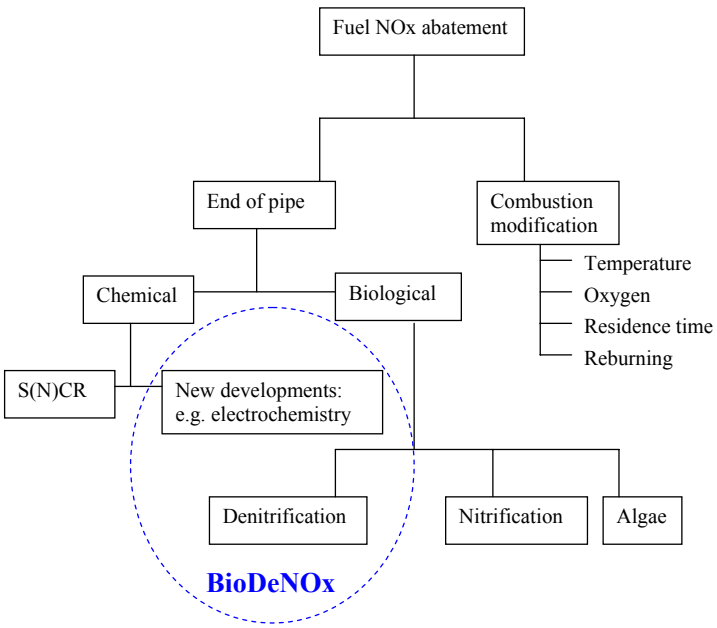

Figure 2

Overview of NO_x abatement principles.

Combustion modification

Combustion modification prevents or reduces the formation of NO_x during combustion. These modifications can give moderate (20-50 %) NO_x reductions at relatively low costs (Princiotta, 1982). The principles of combustion modifications are (Jager, 2001, Princiotta, 1982):

- decrease of the combustion temperature.
- decrease of the residence time in high temperature zones.
- decrease of the oxygen concentration in high temperature zones by lowering the excess air.
- Reburning/Infurnace NO_x reduction: here a second combustion zone is created by injecting fuel into the furnace above the main combustion zone. In this second combustion zone, hydrocarbon radicals from the secondary fuel reduce the NO_x to N_2.

Chemical NOx removal techniques

The most widely applied end of pipe technology for NO_x removal from flue gases is SCR, i.e. Selective Catalytic Reduction (Davis, 1992). SCR is based on chemical NO reduction by ammonia or ureum at high temperatures to form molecular nitrogen, according to (Bradford *et al.*, 2002):

$6NO + 4NH_3 \rightarrow 5N_2 + 6H_2O$

$2NO + 4NH_3 + 2O_2 \rightarrow 3N_2 + 6H_2O$

$6NO_2 + 8NH_3 \rightarrow 7N_2 + 12H_2O$

Ammonia is sprayed into the exhaust gas, which passes then through a catalyst where NO_x and NH_3 are converted into nitrogen and water at an optimum temperature between 300ºC and 400ºC. The catalyst is based on titanium oxide, vanadium oxide, zeolite, iron oxide, or activated carbon. The reactions are very efficient and therefore enable a very effective NH_3 injection control, based on feedback of the measured NO_x concentration in the flue gas. Efficiencies up to 90 percent can be reached with this technique, mainly depending on the catalyst activity (Jager, 2001). However, there are two significant problems associated with this process: a) the operating costs of the process are high due to catalyst deactivation and b) unreacted ammonia either forms ammonium sulphate that can clog equipment or ends up in the effluent gas (Lee, 2001). Both destinations of ammonia are undesirable in the SCR process.

Selective non-catalytic reduction (SNCR) is similar to SCR, but without the use of a catalyst. The temperature is higher than in SCR: 850ºC to 1100ºC. The point of injection is located between the combustion chamber and an economiser in the area of the superheater. Adequate mixing of chemicals and temperature control are essential for maximum NO_x reduction and minimum NH_3 slip (Jager, 2001). For practical SNCR systems, NO_x reduction efficiencies are only about 50 percent, but lower capital costs are necessary than for the SCR (Princiotta, 1982).

Electrochemical NO_x removal techniques

Kleifges *et al.* (1997) developed a novel electrochemical NO_x removal process, which is based on NO absorption into an aqueous Fe(II)EDTA^{2-} solution, followed by NO reduction using dithionite ($S_2O_4^{2-}$) as redox mediator. The latter is regenerated by cathodic reduction of SO_3^{2-} in an electrochemical cell connected to the absorption column by a liquid loop (Fig. 3).

The use of Fe(II)EDTA^{2-} for NO absorption is essential to achieve satisfactory NO removal efficiencies. In the absence of Fe(II)EDTA^{2-}, only 17 % of the NO was converted via $S_2O_4^{2-}$ oxidation. It was assumed that NO is reduced to N_2 via dithionite oxidation, but results of batch experiments showed that also ammonium and N_2O were formed as reaction products (Kleifges *et al.*, 1997):

$$Fe(II)EDTA\text{-}NO^{2-} + S_2O_4^{2-} + H_2O \rightarrow Fe(II)EDTA^{2-} + 2\ SO_3^{2-} + \tfrac{1}{2}\ N_2 + 2\ H^+$$

$$4\ Fe(II)EDTA\text{-}NO^{2-} + 4\ HSO_3^- \rightarrow 4\ Fe(II)EDTA^{2-} + 2\ HON(SO_3)_2^{2-} + N_2O$$

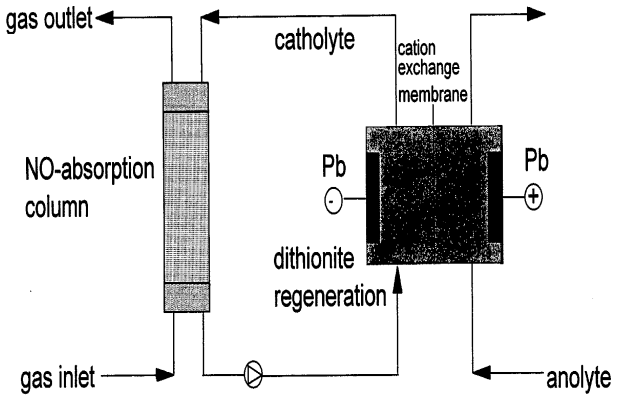

Figure 3

Schematic principle of electrochemical NO_x removal using Fe(II)EDTA^{2-} as absorbent and dithionite as redox mediator (Kleifges *et al.*, 1997).

Biological NO_x removal techniques

Biological post-treatment techniques offer advantages over chemical post-treatment techniques, especially when the concentration of the pollutant in the waste gas is low (Kirchner, 1989). An important advantage over other processes is that biological post-treatment techniques operate at low pressures and temperatures (Cesario, 1991). Basically, biological NO_x removal technologies can be classified into three different groups: (i) nitrification; (ii) denitrification and (iii) algae, as reviewed by Jin *et al.* (2005).

Nitrification

Davidova *et al.* (1997) were the first who demonstrated NO removal from gas streams via nitrification. However, a 90 % NO removal from a 100 ppm contaminated gas stream required long residence times

of around 13.7 minutes. Insufficient biomass growth for effective nitrification was considered as the cause of the rather inefficient NO removal. The above work was further extended using nitrite as a substrate to develop and enhance growth of the nitrifying biofilm in biotrickling filters (Hudepohl et al, 2000) (Fig. 4). The systems, operated at an empty bed retention time (EBRT) of 1 minute, exhibited a limited removal efficiency (10-15%), attributed to mass transfer limitation of the poorly water soluble NO from the gas to the liquid phase. The estimated residence time for 90% removal was 6 minutes (Davidova *et al.*, 1997). Further studies demonstrated that the high EBRT of 6 minutes was required due to mass-transfer limitations of the poorly soluble nitric oxide and oxygen (Nascimento et al, 1999; Nascimento *et al.*, 2000).

Thus, the key to economical biological treatment of NO is to reduce mass transfer limitations. For biotrickling filters, a maximisation of the surface-to-volume ratio can support the required biofilm surface without clogging of the pores.

NO removal using an aerobic biotrickling filter (Fig. 5) with a specific surface area of 120 $m^2.m^{-3}$ was investigated by Chou *et al.* (2000). Approximately 6 weeks were required to develop a biofilm for NO removal, using activated sludge as inoculum, while glucose, yeast powder, phosphate and $NaHCO_3$ were added as supplementary nutrients. At a gas residence time of two minutes, removal efficiencies of 80% were obtained, but it should be noted that the influent NO concentrations were relatively high (892-1237 ppmv NO). When glucose addition was ceased for two weeks, NO removal declined to 48%. This organic carbon deficiency resulted in the detachment of part of the biofilm from the packing surface. Also here, more than 90% of the eliminated NO was converted into nitrate, suggesting NO removal via nitrification. In other studies, soil was used as packing material for NO removing biofilters (Lukow and Diekmann, 1997; Yani *et al.*, 2000; Kim *et al.*, 2000). The NO removal efficiency was 60% on an average at gas residence times of 1 minute.

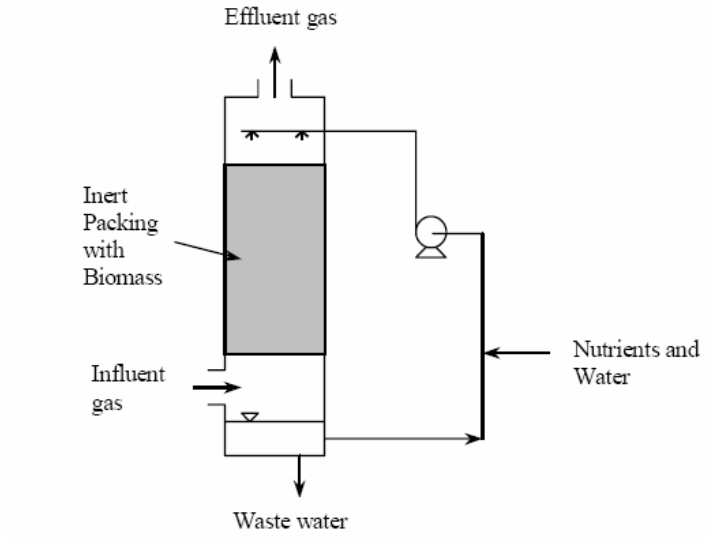

Figure 4

Schematic diagram of a biotrickling filter (Van Groenestijn and Hesselink, 1994).

To maximize the surface-to-volume ratio, the application of a Hollow Fiber Membrane Bioreactor (HFMB) might be promising. NO_x removal from waste gas using a HFMB configuration was evaluated by Min *et al.* (2002). The NO gas diffuses through the membrane pores into a nitrifying biofilm (Fig. 5), where it is oxidized to nitrate. The hollow fiber membranes serve as a support for the microbial populations and provide a large surface area for NO and oxygen mass transfer. Maximal NO removal rates of 27 $g.m^{-3}.d^{-1}$ were found for a overall gas residence time of 5 minutes, corresponding to a NO removal efficiency of 74 %.

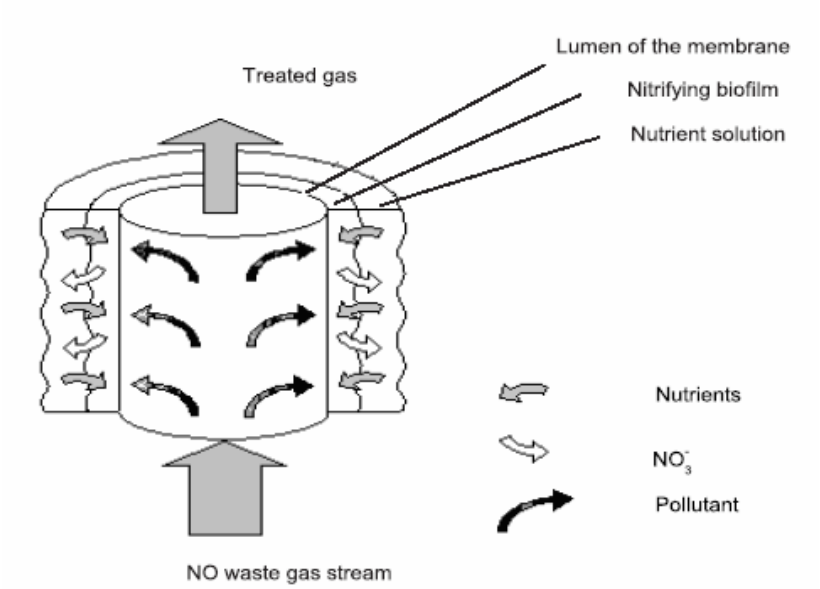

Figure 5
Principle of Hollow Fiber Membrane Bioreactor (Min *et al.*, 2002).

Denitrification

NO removal using heterotrophic denitrification has been observed in an aerobic toluene-treating biofilter (DePlessis *et al.*, 1998). When treating flue gas containing 17 % O_2, the development of a thick biofilm is a pre-requisite for creating anaerobic underlayers where denitrification can take place. Removal efficiencies of 75 % of 60 ppmv NO streams were observed at an EBRT of 6 minutes. Other researchers reported the occurrence of NO removal via denitrification in compost biofilters (Samdami *et al.*, 1993; Apel and Turick, 1993; Barnes *et al.*, 1995; Apel *et al.*, 1995; Lee *et al.*, 1999). High removal efficiencies (up to 90 %) were observed when treating a 500 ppmv NO gas stream at an EBRT of 1.3 minutes in the presence of a phosphate buffer containing either lactate or dextrose as electron donor and carbon source (Barnes *et al.*, 1995, Lee *et al.*, 2001; Klasson and Davison, 2001; Lacey *et al.*, 2002). NO removal efficiencies of over 85 % NO were observed, but only at EBRT of 70-80 s. Also here, the effect of oxygen on the NO removal efficiency by these biofilters was significant. In steady anaerobic operation, both biofilters showed NO removal efficiencies exceeding 50 %, but the NO removal dropped to 10 – 20 % when 2 % O_2 was present in the influent stream (Lee *et al.*, 1999). Heterotrophic NO reduction in the presence of succinate, yeast extract and heat/alkali pre-treated

municipal sewage sludge as carbon and energy source was investigated by Shanmugasundram *et al.* (1993) and Dasu *et al.* (1993).

The oxygen-sensitivity of denitrification is a major complicating factor in the scope of applying denitrifying bioreactors for NO_x removal from oxygen containing gas streams. Since certain fungi exhibit denitrification under aerobic conditions, adoption of NO reducing fungi may be an interesting option. NO removal from oxygen containing flue gas streams by means of fungal denitrification was evaluated in a biofilter using toluene as a sole carbon and energy source (Woertz *et al.*, 2001). The fungal bioreactor removed 93 % of the inlet 250 ppmv NO at an EBRT of 1 min, with an inlet NO loading rate of 17.2 $g.m^{-3}.h^{-1}$.

NO removal by *Thiobacillus denitrificans* via autotrophic denitrification using thiosulfate as electron donor was studied by Lee and Sublette (1990). They found up to 96 % removal of nitric oxide when treating a gas stream containing 5000 ppmv NO. Autotrophic denitrification of gas streams via the oxidation of reduced sulfur compounds in principle represents a very interesting option since it may enable to combine NO and SO_2 removal from flue gases. Therefore, studies were performed to check the feasibility to simultaneously remove SO_x and NO_x from cooled flue gas by contact with cultures of the sulfate-reducing bacterium *Desulfovibrio desulfuricans*, converting SO_2 (via aqueous HSO_3^-/HSO_4^-) to H_2S, and *Thiobacillus denitrificans*, transforming H_2S to SO_4^{2-} using NO as the electron acceptor. However, a simultaneous process combining the SO_x/NO_x removal was technically not feasible due to NO inhibition of SO_4^{2-} reduction by *D. desulfuricans* (Lee and Sublette, 1991).

Another system investigated is based on the pre-concentration of low concentrations of NO at a high volumetric flow onto activated carbon, followed by a thermal desorption of NO_x and biological denitrification treatment of the thermically desorbed gas (Chagnot *et al.*, 1998). The NO_x flow was denitrified by a pure culture of *Thiobaccillus denitrificans* in a trickling biofilter with a gas superficial velocity of 0.5 m/h. The NO_x inlet concentration was 8,000-16,000 ppm and the EBRT was 22 minutes.

NO removal by algae

Various studies demonstrate the capability of NO removal by algal cultures. A marine microalgae was found to simultaneously eliminate nitric oxide and carbon dioxide from a model flue gas (Yoshihara *et al.*, 1996). About 40 mg of NO and 3.5 g of CO_2 were eliminated per day in a 4 liter reactor column sparged with 300 ppm (v/v) NO and 15% (v/v) CO_2 in N_2 at a rate of 150 $mL.min^{-1}$. In order to understand the process of NO removal from flue gases by an algal culture more thoroughfully, a bioreactor system with the unicellular microalga *Dunaliella tertiolecta* was operated (Nagase *et al.*, 1997). They found that the presence of both algal cells and O_2 is important for the reactor system and it was hypothesized that the principle of NO removal is based on NO oxidation, followed by assimilation of that oxidized nitrogen by the algal cells. The results of the study indicate that the dissolution of nitric oxide in the aqueous phase is the rate-limiting step in this reactor system. This was confirmed in another study made by Nagase *et al.* (1998) where the gas-liquid contact area was increased by reducing the bubble size, thereby ensuring a higher NO dissolution rate. NO removal efficiencies up to

96 % were achieved with a counter-flow type airlift reactor aerated with small bubbles. The uptake pathway of nitric oxide by the algae *Dunaliella tertiolecta* was investigated in a bubble column type bioreactor (Nagase *et al.*, 2001). It was found that most of the NO permeated directly into the cells by diffusion; only a small part of the NO-nitrogen was oxidized to nitrite and nitrate. Nitric oxide taken up in the algal cells was then preferentially utilized as a nitrogen source for cell growth rather than nitrate.

Evaluation of NO removal techniques

Major drawbacks of the commonly applied chemical NO_x abatement techniques are the consumption of catalysts (SCR), high energy consumption because of high operation temperatures (SCR and SNCR), and the slip ammonia or ureum to the environment (SCR and SNCR). Since biological processes work at ambient temperatures, without chemical catalysts in the absence of ammonia or ureum, biological NO_x removal techniques principally are more sustainable than SCR and SNCR. However, the poor water solubility of NO implies that the mass transfer of NO from the gas into the water phase is relatively slow. Consequently, relatively long gas retention times (Table 3) and big reactor volumes are needed for NO_x removal in biofilters or biotrickling filters. This is a major (economic) drawback when biological NO_x removal has to be applied at voluminous gas streams, e.g. at power plants with gas flows exceeding 1.000.000 $Nm^3.h^{-1}$.

Table 3

Biological NO removal techniques: removal efficiencies (RE) and empty bed retention times (EBRT) required (after Jin *et al.*, 2005).

	NO conc. (ppmv)	EBRT (min)	Load ($gNO.m\text{-}3.h^{-1}$)	RE (%)	reference
Nitrification					
HFMB	100	5	1,48	69-75	Min *et al.* , 2002
celite	100	1	7,4	10-15	Nascimento *et al.* , 2000
slag	892-1237	2	2.75-22.4	80	Chou *et al.* , 2000
biosoil	1,6	1	0,12	60	Okuno *et al.* , 2000
Denitrification					
compost, heterotrophic	250	1	18	99	Samdani, 1993
compost, heterotrophic	500	1	37	90	Appel and Turick, 1993
compost, heterotrophic	500	1,3	28	95	Flanagan *et al.* , 2002
perlite, heterotrophic	500	1,18	31	94	Flanagan *et al.* , 2002
biofoam, heterotrophic	500	1,18	31	85,5	Flanagan *et al.* , 2002
silicate pellets, heterotrophic	60	3	0,75	75	du Plessis *et al.* , 1998
sulphur+marel, autotrophic	8978	22	4.15 $mmol.h^{-1}$	10	Chagnot *et al.* , 1998
silicate pellets, fungal	250	1	18,4	93	Woertz *et al.* , 2001
Microalgae	300	26,7	22.3 $mg.d^{-1}$	50-60	Nagase *et al.* , 2001

Use of aqueous Fe(II)EDTA^{2-} solutions for NO removal

The slow mass transfer of NO from the gas to the liquid phase can be overcome by the application of aqueous solutions of ferrous chelates, e.g. Fe(II)EDTA^{2-} (EDTA= ethylenediaminetetraacetic acid). The latter compound has the ability to absorb NO because NO binds selectively to the Fe centre of the Fe(II)EDTA^{2-} complex (Fig. 6) (Rocklin *et al.*, 1999).

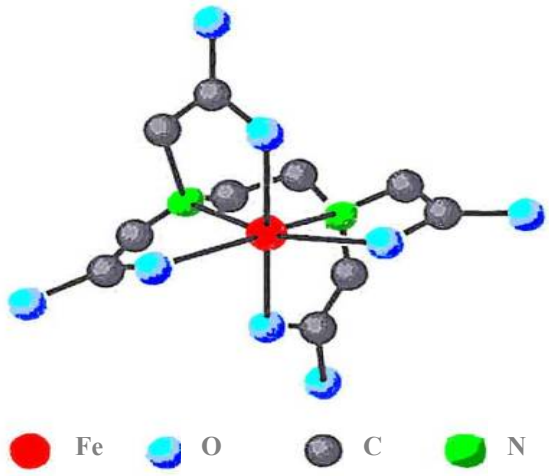

Figure 6
The Fe(II)EDTA)$^{2-}$ complex.

The absorption of NO into aqueous Fe(II)EDTA^{2-} solutions proceeds according to Wubs and Beenackers, (1993):

$$NO\ (g) \leftrightarrow NO\ (aq)$$

$$NO\ (aq) + Fe(II)EDTA^{2-} \leftrightarrow Fe(II)EDTA\text{-}NO^{2-}$$

The kinetics of NO absorption into aqueous Fe(II)EDTA^{2-} solutions have been investigated thoroughly in the last two decades. Several researchers reported that this reaction is first order in both NO and Fe(II)EDTA^{2-} (Demmink, 2000; Schneppensieper *et al.*, 2001). Recent studies have unequivocally proven that the reaction is reversible (Schneppensieper *et al.*, 2001). The reaction is intrinsically very fast and mass transfer limitation of both NO and the Fe(II)EDTA^{2-} complex plays an important role (Demmink, 2000). As a consequence, the reaction often takes place in the instantaneous regime of reactive gas absorption.

When aqueous Fe(II)EDTA^{2-} solutions are applied for NO removal from oxygen containing industrial flue gasses (O_2 content depends on fuel type, see Table 1), part of the Fe(II)EDTA^{2-} will be oxidized to Fe(III)EDTA^{-} according to:

$4\ Fe(II)EDTA^{2-} + O_2\ (aq) \rightarrow 4\ Fe(III)EDTA^{-} + 2\ H_2O$

The reaction of $Fe(II)EDTA^{2-}$ with oxygen has also been the subject of various studies and is known to be an irreversible reaction (Wubs and Beenackers, 1993; Zang and Van Eldik, 1990). The reaction is first order in oxygen, whereas the order in iron is a function of the iron chelate concentration (Wubs and Beenackers, 1993; Zang and Van Eldik, 1990). At low $Fe(II)EDTA^{2-}$ concentrations (< 10 mol/m^3), the reaction is first order in iron whereas it becomes second order at higher concentrations.

BIODENOX

The BioDeNOx process combines the principles of wet absorption of NO into an aqueous $Fe(II)EDTA^{2-}$ solution with biological regeneration of that scrubber liquor in a bioreactor (Buisman *et al.*, 1999) as schematically represented in Fig. 7.

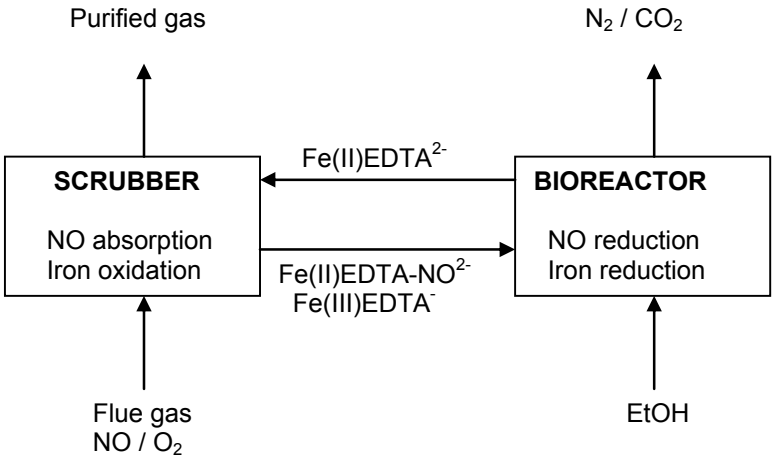

Figure 7

Schematic principle of the BioDeNOx concept.

BioDeNOx reactors normally operate under thermophilic conditions, at around 50-55 °C, which is the adiabatic temperature of scrubber liquors. When ethanol is used as electron donor, the denitrification reaction occurs according to the overall reaction (Buisman *et al.*, 1999):

$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 6\ Fe(II)EDTA^{2-} + 3\ N_2 + CO_2 + 3\ H_2O$

To regenerate the absorption liquor, the $Fe(III)EDTA^{-}$ that is formed via oxidation by oxygen has to be reduced back to $Fe(II)EDTA^{2-}$. Thus, besides NO reduction, reduction of EDTA chelated Fe(III) is a core reaction within the regeneration pathway of the BioDeNOx process (Buisman *et al.*, 1999):

$12\ Fe(III)EDTA^{-} + C_2H_5OH + 3\ H_2O \rightarrow 12Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^{+}$

NO reduction

Biological NO reduction is one of the sequential processes in microbial denitrification, i.e. the respiratory reduction of nitrate to dinitrogen gas. NO is reduced to dinitrogen (N_2) with nitrous oxide (N_2O) as intermediate:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Fig. 8 schematically depicts the production and consumption of nitric oxide during bacterial denitrification.

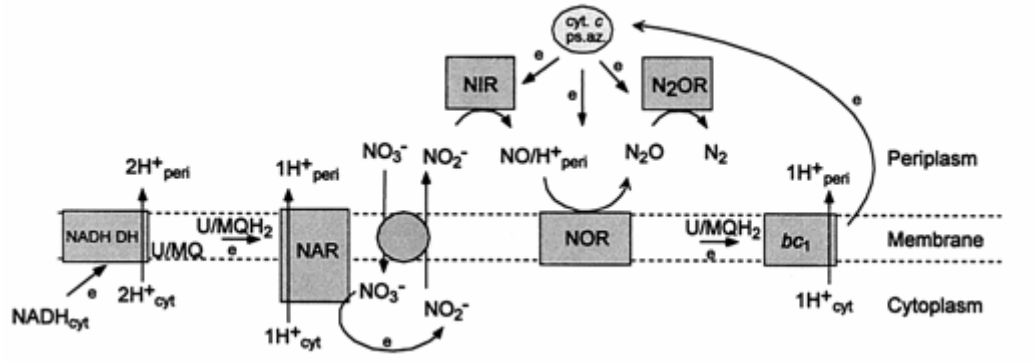

Figure 8

Production and consumption of nitric oxide in the denitrification pathway (Wasser *et al.*, 2002).

As reviewed by Zumft (1997), the formation and/or uptake of exogenous NO has been studied for a variety of bacteria, including *Pseudomonas fluorescens* (Firestone *et al.*, 1979), *P. stutzeri* (Frunzke and Zumft, 1986; Schäfer and Conrad, 1993), *P. aeruginosa* (Kalkowski and Conrad, 1991; Vosswinkel *et al.*, 1991) and *Flexibacter Canadensis* (Wu *et al.*, 1995). The amount of NO formed during denitrification depends on the organism and the culture conditions (Zumft, 1997). When a denitrifying culture of *P. stutzeri* is stripped by a carrier gas, it releases 50 to 75 % of the supplied nitrate as NO to the gas phase (Schäfer and Conrad, 1993; Zafiriou *et al.*, 1989). The concentration of extracellular NO during steady-state denitrification is in the nanomolar range. The value of 50 nM for *P. stutzeri* obtained by gas stripping (Zafiriou *et al.*, 1989; Goretski *et al.*, 1990) agrees well with 20 to 30 nM obtained by a direct sensitive NO measurement (Goretski *et al.*, 1990). Denitrifying *P. aeruginosa*, supplied with nitrate, accumulates nitrite transiently and develops a steady-state NO concentration of 1 to 2 nM in the liquid phase (Kalkowski and Conrad, 1991). Values ranging from 0.5 to 31 nM are representative of cell suspensions containing 0.1 mg of protein per ml of *P. stutzeri* JM300 and *Paracoccus denitrificans* within 40 min of reaction time. The steady-state NO concentrations are higher with nitrite as the substrate (7 to 31 nM) than with nitrate (0.5 to 7 nM) (Goretski *et al.*, 1990).

Also for denitrifiers, nitric oxide can be toxic. NO toxicity, manifested as a loss of cell division and viability, is expected to occur around 1 mM and above (Zumft, 1997). Because of its toxicity, it is generally assumed that exogenous NO will not support cell growth. NO-dependent bacterial growth has been found in strains of *Bacillus* that were isolated on nitrite by an anaerobic enrichment technique (Pichinoty, 1979). Bacterial cell mass approximately doubled within 5 h in the presence of 10 % NO, but the growth showed a long lag phase that could not be eliminated by subculturing. According to Zumft (1997), the successful approach for growing a bacterium on NO involves the presence of a low but continuous supply of NO and operation near the steady-state concentration of free NO during denitrification. For a proper investigation of bacterial growth on NO, the nonenzymatic conversion to N_2O and energy conservation via the latter substrate has to be excluded. Growth of an NO-evolving strain of *P. aeruginosa* was possible only when N_2O reduction was not inhibited by acetylene, which means that NO by itself does not sustain the bacterial energy metabolism (Vosswinkel *et al.*, 1991). Breakdown of organic carbon and increase of biomass was shown to depend on NO. With only 0.5 % NO in the feed gas, the cell mass of *Paracoccus denitrificans*, "*Pseudomonas denitrificans*," *Alcaligenes xylosoxidans* subsp. *denitrificans* and *Thiobacillus denitrificans* (all grown anaerobically for denitrification with nitrate), increased at the expense of oxidizing a complex carbon source, whereas the NO concentration fell to 0.01 - 0.02 % in the exhaust gas (Potter *et al.*, 1995).
Theoretically, growth on NO as the sole electron acceptor should be possible since NO reduction is coupled to proton translocation (Garber *et al.*, 1982; Shapleigh and Payne, 1985; Shapleigh and Payne, 1986; Vosswinkel *et al.*, 1991). The conversion of NO to N_2O has a redox potential, E^0 (pH 7), of 1177 mV and a free energy ($\Delta G°$) of -2306 kJ/mol. It is energetically comparable to the respiration of N_2O to N_2 [E^0(pH 7) 1352 mV; $\Delta G°$ -2340 kJ/mol]. Growth yield studies show that the overall reduction of nitrite to N_2O is energy conserving (Koike and Hattori, 1975). Because of the difficulty in growing cells with NO, no comparable studies are available. Electron transfer to NO reductase proceeds through cytochrome *bc*1 (Carr *et al.*, 1989; Hendriks *et al.*, 2002), and energy conservation is associated with a dehydrogenase and/or the cytochrome *bc*1 complex.
Nitric oxide reduction is not restricted to denitrifiers. Strains of *Rhodobactercapsulatus* transform NO to N_2O at a significant rate of 10 to 35 nmol.min^{-1}.mg protein^{-1} (Bell *et al.*, 1992). The methanotroph *Methylosinustrichosporium* (Krämer *et al.*, 1990), *Escherichia coli*, *P. putida*, *Pseudomonas viridiflava*, and the cyanobacterium *Synechococcus* (*Anacystis*) *nidulans* (Baumgärtner *et al.*, 1996) all consume NO under anoxic conditions without being able to denitrify, i.e. the full conversion of NO_3^- to N_2. Also several fungi (Shoun *et al.*, 1991; Shoun *et al.*, 1992) and yeasts (Tsuruta *et al.*, 1998) are capable to reduce NO to N_2O.

The bacterial reduction of NO to N_2O is catalyzed by the membrane bound Nitric Oxide Reductase (NOR):

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$$

Nitric oxide reductase is the last of the terminal oxidoreductases of denitrification whose molecular properties became known (Heiss *et al.*, 1989). At present, three different bacterial NO reductases have been characterized, as reviewed by Wasser *et al.* (2002). All three are integral membrane metalloenzymes containing a non-heme iron center. The best-studied NORs are the cytochrome *bc* complexes (cNOR) purified from gram-negative bacteria, e.g., *P. stutzeri*, *P. denitrificans*, and *Paracoccus halodentrificans* (Girsch and De Vries, 1997; Carr and Ferguson, 1990; Fujiwara and Fukumori, 1996; Heiss *et al.*, 1989; Hendriks *et al.*, 1998; Kastrau *et al.*, 1994). The purified cNORs consist of two subunits and use membrane or soluble c-type cytochromes or small blue copper proteins (azurin, pseudoazurin) as physiological electron donors (Berks *et al.*, 1995; Richardson *et al.*, 1999; Richardson, 2000). Another type of NOR, qNOR, uses ubihydroquinone or menahydroquinone as the electron donor. qNOR consists of one subunit and is not only present in soil and marine bacteria or in archaea but also in pathogenic microorganisms such as *Neisseria meningitides*, *Neisseria gonorrhea*, and *Corynebacterium diphteriae* which do not denitrify (Cramm *et al.*, 1997; Hendriks *et al.*, 2000). A third type of NOR, called qCu_ANOR, was isolated from the gram-positive bacterium *Bacillus azotoformans* (Pichinoty *et al.*, 1979) and uses menahydroquinone as an electron donor (Suharti *et al.*, 2001).

$\underline{N_2O}$ reduction

N_2O reduction to N_2 is the final conversion in the denitrification pathway:

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$$

Coupled to the oxidation of organic compounds, N_2O can serve as the sole electron acceptor for many denitrifying bacteria. Involvement of the cytochrome *bc1* complex in N_2O reduction was demonstrated for *P. denitrificans* (Boogerd *et al.*, 1980), *Rhodobacter sphaeroides* (Itoh *et al.*, 1989), and *Rhodobacter capsulatus* (Richardson *et al.*, 1989). The electron transfer through cytochrome *bc1* is accompanied by a proton transfer across the membrane. Nitrous oxide reduction is therefore energy conserving even though N_2O reductase is a periplasmic enzyme (Richardson *et al.*, 1991).

Just as the nitrate respiration is not obligatorily coupled to denitrification, also bacteria that respire N_2O without being denitrifiers (i.e. capable to reduce NO_3^- to N_2) are known (McEwan *et al.*, 1985; Yoshinari, 1980). *Vibrio succinogenes* was the first non-denitrifying strain shown to grow on N_2O (Yoshinari, 1980). The bacterium oxidizes formate by reducing nitrate to nitrite, nitrite to ammonia and N_2O to N_2. NO is not utilized by whole cells, and no N_2 is formed from nitrate reduction (Yoshinari *et al.*, 1980). Other examples that fall into this category are the nitrate-respiring but nondenitrifying *Campylobacter fetus* (Payne *et al.*, 1982). At high N_2O concentrations, reduction of N_2O at reasonable high rates (30 nmol per minute per mg of protein) has also been reported for *Escherichia coli* (Kaldorf *et al.*, 1993).

The key enzyme in N_2O reduction to N_2 by denitrifying bacteria is the periplasmic N_2O reductase (Zumft, 1997). It has been purified and characterized to different extents from heterotrophic, phototrophic, and chemolithotrophic gram-negative bacteria, which usually have a soluble enzyme with a functional location in the periplasm. Nitrous oxide reductase is a multicopper enzyme, which explains the requirement of Cu for N_2O respiration (Zumft, 1997). Cu deficient media lead to accumulation of N_2O during nitrate reduction and do not sustain cell growth on N_2O (Iwasaki *et al.*, 1980; Iwasaki and Terai, 1982; Matsubara and Zumft, 1982).

Fe(III) reduction

Ferric iron reduction has been an early form of respiration on Earth (Vargas *et al.*, 1998). Fe(III) can be the dominant electron acceptor for microbial respiration in many subsurface environments (Lovley *et al.*, 1995) and Fe(III)-reducing communities can be responsible for the majority of the organic matter oxidized in such environments (Lovley *et al.*, 1993). Until the 1990's, Fe(III) reduction in sedimentary environments was primarily considered as an abiotic process. As reviewed by Lovley (1991), this abiotic concept dates back to some of the earliest studies on microbial Fe(III) reduction. In these studies, it was concluded that Fe(III) reduction occurred when microorganisms generated chemical conditions which would promote the nonenzymatic conversion of Fe(III) to Fe(II) and that Fe(III) reduction was merely a result of changes in pH and/or redox potential. However, no direct evidence supporting this nonenzymatic Fe(III) reduction mechanism was provided (Lovley, 1991).

Dissimilatory Fe(III) reducing microorganisms

Dissimilatory Fe(III) reducing microorganisms can be separated into two major groups: (i) those that support growth by conserving energy from electron transfer to Fe(III) and (ii) those that do not conserve energy from that electron transfer (Lovley, 2000). The latter group consists of fermentative bacteria that are capable of reducing Fe(III) while fermenting sugars or amino acids and generating acetate, alcohols, H_2 and other fermentation products. However, less than 5% of the reducing equivalents from the metabolized substrates are transferred to Fe(III) in this process (Lovley 1991). Only a small percentage of Fe(III) reduction in sedimentary environments can be attributed to fermentative microorganisms. The majority of Fe(III) reduction in sediments is due to the oxidation of fermentation endproducts such as acetate by microorganisms that use Fe(III) as the terminal electron acceptor (Lovley 1993). *Geobacter metallireducens* was the first pure culture shown capable to completely oxidize acetate with Fe(III) as the electron acceptor (Lovley *et al.* 1993). This organism is a strict anaerobe, isolated from freshwater sediments, capable of oxidizing several organic acids and alcohols using Fe(III) as the electron acceptor (Lovley, 1993). A wide phylogenetic diversity of microorganisms that can grow via Fe(III) reduction have been recovered from subsurface environments (Fig. 9). The physiology and phylogeny of these organisms have been reviewed in detail by Lovley (2000).

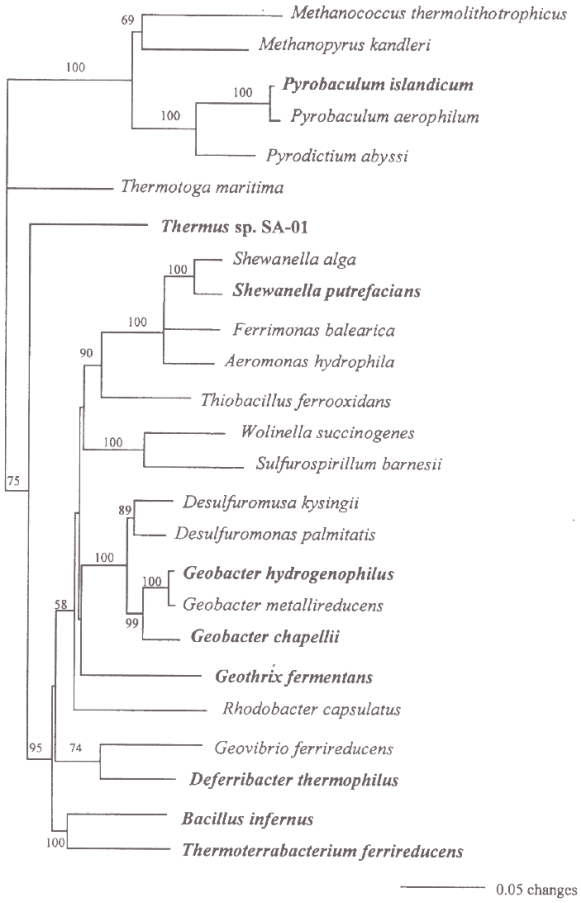

Figure 9

16S rRNA based phylogeny of Fe(III) reducing microorganisms. Organisms recovered from subsurface environments are in bold. Other Fe reducing microorganisms have been included to aid in defining the tree (Lovley, 2001).

Sulfate-reducing bacteria also reduce Fe(III) (Coleman *et al.*, 1993), although, similar to fermentatives, growth has not been demonstrated on this electron acceptor (Lovley *et al.*, 1993b). Moreover, the end product of sulfate reduction, sulfide, can nonenzymatically reduce Fe(III) and is often suggested to be the prime reductant for Fe(III), especially in marine sediments where there are large supplies of sulfate for dissimilatory sulfate reduction. However, such an abiotic reduction of Fe(III) by sulfide is not expected in the majority of sediments, in which Fe(III) is reduced prior to significant sulfate reduction prevails (Lovley *et al.*, 1991). As Fe(III) respiring microorganisms can outcompete sulfate-reducing microorganisms for important electron donors such as acetate and hydrogen, there is generally little sulfate reduction in the Fe(III) reduction zone of sediments. Fe(III) respiring microorganisms maintain the concentration of these electron donors too low for sulfate reduction (Lovley et al, 1993). Even in marine sediments, the inhibition of sulfate reduction has no effect on the Fe(III) reduction rates (Lovley *et al.*, 1993). Therefore, sulfide production from sulfate reduction is suggested to an unimportant mechanism for Fe(III) reduction in sediments. However, it is evident that there are close links between the microbial sulfur and iron cycles in anaerobic marine sediments (Lovley *et al.*, 1993).

Another possible mechanism for nonenzymatic Fe(III) reduction has arisen with the discovery that Fe(III) respiring microorganisms can reduce humic substances and other extracellular quinones (Lovley *et al.*, 1996; Lovley and Blunt-Harris, 1999). Once these extracellular quinones are reduced, they in turn can reduce Fe(III) oxides via a nonenzymatic reaction.

Mechanisms of biological Fe(III) reduction

Although iron is abundant in many sedimentary environments, Fe(III) has a solubility of approximately 10^{-9} M at neutral pH, based on calculations by Chipperfield and Ratledge (2000). Hence, the majority of Fe(III) in aquatic sediments is present as insoluble Fe(III) oxides. The mechanisms of microbial electron transport to insoluble acceptors are poorly understood. To respire on Fe(III), the microorganisms must be able to either (i) attach to the iron substrate and directly transfer electrons to it, or (ii) solubilize the iron and deliver it to the electron transport chain, or (iii) use available electron shuttling compounds or produce their own (Luu and Ramsy, 2003b). Given the phylogenetic diversity of Fe(III)-reducing microorganisms, it is likely that more than one strategy for Fe(III) reduction exists.

The mechanisms of Fe(III) reduction have been studied in most detail in *S. oneidensis* and *Geobacter sulfurreducens*. Although the terminal reductase has not yet been identified unequivocally in either organism, the involvement of *c*-type cytochromes in electron transport to Fe(III) is implicated in several studies (Gaspard *et al.*, 1998; Lloyd *et al.*, 2003; Magnuson *et al.*, 2000; Myers and Myers, 1993; Myers and Meyers, 1997). In some examples, activities have also been localised at the outer membrane or surface of the cell, consistent with a role in direct transfer of electrons to Fe(III) oxides that are highly insoluble at circumneutral pH (Gaspard *et al.*, 1998; DiChristina *et al.*, 2002). In addition to the proposed direct transfer of electrons to Fe(III) minerals, soluble 'electron shuttles' are also able to transfer electrons between metal-reducing prokaryotes and the mineral surface. According to this mechanism there is no requirement for direct contact between the micro-organism and the mineral. For example, humic acids and other extracellular quinones are utilised as electron acceptors by Fe(III)-reducing bacteria (Lovley *et al.*, 1996), and the reduced hydroquinone moieties are able to abiotically transfer electrons to Fe(III) minerals. Nevin and Lovley (2000) evaluated the potential of various substances to serve as electron shuttles between Fe(III)-reducing microorganisms and insoluble Fe(III) oxides in aquifer sediments in order to determine whether abiological mechanisms might play a role in the apparent microbial reduction of Fe(III) in subsurface sediments. They found that humics and other extracellular quinones can serve as electron shuttle to the Fe(III) oxides found in subsurface environments, suggesting that some Fe(III) reduction previously considered to be the result of direct enzymatic reduction, may instead result from abiotic reduction of Fe(III) by microbially reduced humics or other microbially generated hydroquinones (Nevin and Lovley, 2000). Very low concentrations of an electron shuttle, e.g. 100 nM of the humic acid analogue anthraquinone-2,6-disulfonate, can accelerate the reduction of Fe(III) oxides (Lloyd *et al.*, 1999). The principle of Fe(III) reduction via an electron shuttling compound is illustrated in Fig. 10.

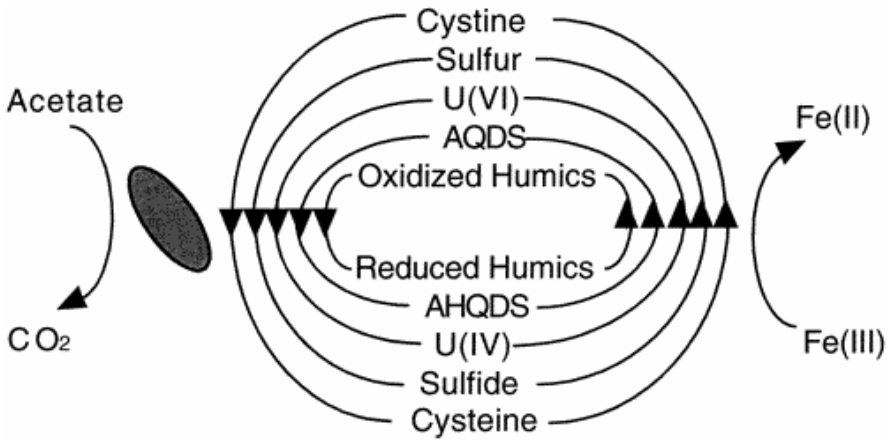

Figure 10

Model for various compounds serving as electron shuttles between Fe(III) reducing microorganisms and Fe(III) as the terminal electron acceptor (Nevin and Lovley, 2000).

The secretion of soluble electron shuttles by actively respiring Fe(III) reducers has also been proposed for both *S. oneidensis* and *G. sulfurreducens*, and remains hotly debated for the *Geobacter* species. Early studies suggested release of a small soluble *c*-type cytochrome by *G. sulfurreducens* (Seeliger *et al.*, 1998), but other studies suggest that this protein is not an effective electron shuttle (Lloyd *et al.*, 1999). Studies have also suggested that a small quinone-containing extracellular electron shuttle is released by *S. oneidensis*, which may also promote electron transfer to Fe(III) minerals (Newman and Kolter, 2000). Recently, it was shown that *G. metallireducens* synthesises pili and flagella when grown on insoluble Fe(III) minerals, but not soluble forms of the metals (Childers *et al.*, 2002). These results suggest that *Geobacter* species sense when soluble electron acceptors are depleted and synthesise the appropriate appendages that allow movement to Fe(III) minerals and subsequent attachment.

Biological reduction of Fe(III)EDTA$^-$

In contrast to the numerous investigations on the reduction of crystalline iron (e.g. poorly crystalline iron oxides), only limited information is available on the biological reduction of EDTA chelated Fe(III). An analogueous chelating agent, NTA, was shown to stimulate the reduction of a variety of Fe(III) forms by *Geobacter metallireducens* (Lovley *et al.*, 1996a; Lovley *et al.*, 1996b). It was suggested that NTA solubilization of insoluble Fe(III)-oxides is an important mechanism for stimulation of bacterial Fe(III) reduction. NTA also stimulated Fe(III) reduction in sediments from a petroleum contaminated aquifer (Lovley *et al.*, 1994). The authors presumed that this stimulation was a result of Fe(III) solubilization, and thereby, making Fe(III) more bio-available. However, based on results from incubations with a sediment derived culture, Luu and Ramsay (2003a) suggested that NTA improved Fe(III) reduction not by Fe(III) solubilization, but by extraction of electron shuttling humic substances from soil into the aqueous medium.

Haas and DiChristina (2002) investigated the effects of various ligands on dissimilatory Fe(III) reduction by *Shewanella putrefaciens*. They found a typical relation between the Fe(III) reduction rate

and the stability constants of the ferric chelates. In case of Fe(III)EDTA$^-$, no iron reduction occurred. In sediments, the presence of EDTA stimulated benzene oxidation coupled to Fe(III) reduction (Lovley *et al.*, 1996). Again, the observed stimulation was thought to be the result of the increased bio-availability of Fe(III) as an electron acceptor for iron reducing bacteria.. However, Fe(III)EDTA$^-$ at high concentrations (25 mM) is reported to be a rather poor e-acceptor for most known dissimilatory iron reducing bacteria (Finneran *et al.*, 2002).

SCOPE AND OUTLINE OF THIS THESIS

BioDeNOx may be a more sustainable and economically attractive concept compared to conventional NO_x removal techniques like SCR and biotrickling filters, since it does not need high temperatures and catalysts, while scrubber retention times can be limited due to the chemically enhanced NO absorption. In the scope of optimizing the BioDeNOx technology, the scrubber and bioreactor processes were investigated by a consortium of three research groups: University of Groningen (scrubber processes), Delft University (microbial ecology) and Wageningen University (bioreactor processes). In that scope, the objective of this thesis is to elucidate the mechanisms and kinetics of the key processes in BioDeNOx bioreactors, i.e. NO and Fe(III)EDTA$^-$ reduction.

Denitrification in aqueous Fe(II)EDTA^{2-} solutions is described in Chapter 2, whereas Chapter 3 deals with the NO reduction in that medium. The role of bio-catalysis was assessed, as well as the applicability of various inocula for NO reduction in aqueous Fe(II)EDTA^{2-} solutions. In Chapter 4, the feasibility of the BioDeNOx process was demonstrated using a continuous lab-scale reactor treating artificial flue gas. The influence of different operational parameters (NO and O_2 content of the flue gas, redox potential and substrate concentration in the bioreactor, role of inoculum) on the NO removal efficiency was determined. Chapter 5 describes the reduction of EDTA chelated Fe(III) in batch experiments and the mechanism and kinetics of this BioDeNOx key conversion were investigated in more detail in Chapter 6. The influence of the electron mediating compound, electron donor and $CaSO_3$ on the Fe(III)EDTA$^-$ reduction rate was determined. The mechanism and kinetics of NO reduction in BioDeNOx reactors are presented in Chapter 7, whereas the influence of sulfide on NO reduction is described in Chapter 8. Chapter 9 deals with investigations on the Fe(II)EDTA^{2-} regeneration capacity in a lab-scale BioDeNOx reactor, with special attention to biomass growth and EDTA degradation during continuous operation with an artificial flue gas. Finally, in Chapter 10 the results are summarized, discussed and related to the results obtained at the partner universities of Delft and Groningen, as well as to full scale BioDeNOx operation.

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CHAPTER 2

Denitrification in aqueous FeEDTA solutions

The biological reduction of nitric oxide (NO) in aqueous solutions of FeEDTA is an important key reaction within the BioDeNOx process. To explore the reduction of nitrogen oxide analogues, this chapter investigates the full denitrification pathway in aqueous FeEDTA solutions, i.e. the reduction of NO_3^-, NO_2^-, NO via N_2O to N_2 in this unusual medium. This was done in batch experiments at 30 °C with 25 mmol.L^{-1} FeEDTA solutions (pH 7.2 ±.0.2). Also Ca^{2+} (2 and 10 mmol.L^{-1}) and Mg^{2+} (2 mmol.L^{-1}) were added in excess to prevent free, uncomplexed EDTA. Nitrate reduction in aqueous solutions of Fe(III)EDTA is accompanied by the biological reduction of Fe(III) to Fe(II), for which ethanol, methanol and also acetate are suitable electron donors. Fe(II)$EDTA^{2-}$ can serve as electron donor for the biological reduction of nitrate to nitrite, with the concomitant oxidation of Fe(II)$EDTA^{2-}$ to Fe(III)$EDTA^-$. Moreover, Fe(II)$EDTA^{2-}$ can also serve as electron donor for the chemical reduction of nitrite to NO, with the concomitant formation of the nitrosyl-complex Fe(II)EDTA-NO^{2-}. The reduction of NO in Fe(II)EDTA was found to be catalyzed biologically and occurred about 3 times faster at 55 °C than NO reduction at 30 °C. This study showed that the nitrogen and iron cycles are strongly coupled and that FeEDTA has an electron mediating role during the subsequent reduction of nitrate, nitrite, nitric oxide and nitrous oxide to dinitrogen gas.

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INTRODUCTION

The emission of NO_x into the atmosphere is a major environmental problem: it contributes to the depletion of the ozone layer, global warming and the acidification of soils and water bodies. Moreover, it is one of the principal causes of smog formation. Approximately 24 million tons of NO_x were released to the atmosphere from US sources during 1998 (EPA, 2000). The Clean Air Act Amendments regulate NO_x emissions from major stationary sources like industrial flue gasses (Grano, 1995). One promising technique for the NO_x removal from industrial flue gasses is an integrated physico-chemical and biological process, called BioDeNOx (Buisman *et al.*, 1999). Basically the concept consists of two parts: 1) wet absorption of nitric oxide (NO) into an iron chelate solution and 2) regeneration of this iron chelate solution by a biological denitrification process (Fig.1).

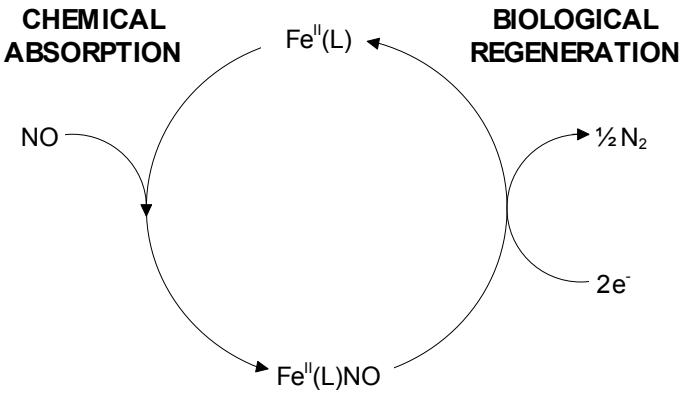

Figure 1

Schematic principle of BioDeNOx: 1) wet absorption of NO into a chelate solution and 2) regeneration of this chelate solution by a biological denitrification process.

When Fe(II)EDTA is used as chelating agent, absorption of NO takes place according to:

$$Fe(II)EDTA^{2-} + NO \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \quad (1)$$

The absorption liquor, e.g. the aqueous Fe(II)EDTA-NO solution, is biologically regenerated (denitrified) in a bioreactor, for which a proper electron donor needs to be supplied. In practical applications, the BioDeNOx scrubber liquor contains approximately 25 $mmol.L^{-1}$ FeEDTA, in which the iron can be present in both the reduced [$Fe(II)EDTA^{2-}$] and in the oxidized [$Fe(III)EDTA^{-}$] state [3]. When ethanol is used as electron donor, as is practiced in full scale installations (Biostar, personal communication), the recovery of Fe(II)EDTA from the nitrosyl-complex Fe(II)EDTA-NO proceeds according to reaction 2:

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 2\ CO_2 + 3\ N_2 + 3\ H_2O + 6\ Fe(II)EDTA^{2-} \quad (2)$$

However, under thermophilic conditions (55 °C) it was found that the absorbent itself, $Fe(II)EDTA^{2-}$, can also serve as electron donor for the biological reduction of NO to N_2 according to (Chapter 3):

$$2\ Fe(II)EDTA\text{-}NO^{2-} + 2\ Fe(II)EDTA^{2-} + 4H^{+} \rightarrow N_2 + 2\ H_2O + 4Fe(III)EDTA^{-} \qquad (3)$$

NO reduction to di-nitrogen gas (N_2) is part of the denitrification pathway (Ye *et al.*, 1994; Zumft, 1993), e.g. the biological reduction of nitrate (NO_3^-) to N_2. In order to get better insight in the nitrogen conversions in aqueous solutions of FeEDTA, the pathway of full denitrification in aqueous FeEDTA solutions was investigated in the present study. Using batch experiments (pH 7.2±0.2; 30 °C), the pathway of NO_3^-, nitrite (NO_2^-), and NO reduction was elucidated, as well as the influence of temperature elevation (55 °C) on NO reduction. The role of biocatalysis, electron donor (ethanol, methanol and acetate) and medium composition (the influence of EDTA-counter ions Ca^{2+} and Mg^{2+}) was investigated. The impact of uncomplexed EDTA on the biological denitrification process was determined as well.

MATERIALS AND METHODS

Inoculum

The inoculum used in this study originates from a full scale biological fluidised bed reactor (BFBR), located in Veendam (The Netherlands), treating surface water for oxygen and nitrate removal at a NO_3^- loading rate of ± 2 kg $NO_3^-.m^{-3}.d^{-1}$, with the use of methanol as carbon source and electron donor. The imposed temperature in the BFBR varied between 2 and 25 °C, i.e. the temperature of the surface water in winter and summer, respectively.

For the nitrate reduction experiments, the inoculum was pre-cultivated at 30 °C in basal medium (Van der Maas *et al.*, 2002) under denitrifying conditions for 1 week using methanol as electron donor. For the NO reduction experiments the sludge was pre-cultivated under denitrifying conditions in basal medium (Van der Maas *et al.*, 2002) at 55 °C for 2 days with ethanol as the electron donor.

Medium

The $Fe(III)EDTA^-$ medium contained (unless specified otherwise): Na_2H_2EDTA (Titriplex) 50 $mmol.L^{-1}$, $FeCl_3$ 50 $mmol.L^{-1}$, $MgSO_4.7H_2O$ 0.1 $g.L^{-1}$, $CaCl_2.2H_2O$ 0.01 $g.L^{-1}$, NH_4Cl 0.28 $g.L^{-1}$, KH_2PO_4 0.25 $g.L^{-1}$, $NaHCO_3$ 5,4 $g.L^{-1}$, yeast extract 0.1 $g.L^{-1}$ and trace elements solution (Visser *et al.*, 1993) 1 $ml.l^{-1}$. The medium was prepared by adding sequentially EDTA, $FeCl_3$, nutrients, and metals to demineralized water. Then the pH was adjusted to 7.2 by adding sodium hydroxide, and finally, $NaHCO_3$ (buffer) was added.

$Fe(II)EDTA^{2-}$ medium contained (unless specified otherwise): NaH_2EDTA (Titriplex) 20 $mmol.L^{-1}$, $FeCl_2.4H_2O$ 20 $mmol.L^{-1}$, $MgSO_4.7H_2O$ 0.1 $g.L^{-1}$, $CaCl_2.2H_2O$ 0.01 $g.L^{-1}$, NH_4Cl 0.28 $g.L^{-1}$, KH_2PO_4 0.25 $g.L^{-1}$, $NaHCO_3$ 5,4 $g.L^{-1}$ and trace elements solution 1 $mL.L^{-1}$. The medium was prepared by

adding subsequently EDTA, nutrients, and metals to demi water. To avoid oxidation of ferrous iron, oxygen was removed by flushing the solution with N_2 for 30 min before Fe^{2+} was added. Then the pH was adjusted to 7.2 by adding sodium hydroxide, and finally $NaHCO_3$ (buffer) was added. The pH of all media was 7.2 ± 0.2.

Denitrification experiments

Nitrate reduction experiments were performed in 2,5 litre stirred cell reactors, placed in a 30 °C climate room. The reactors were filled with 2 litre $Fe(III)EDTA^-$ medium and sludge to reach a concentration of ± 5 mg $VSS.L^{-1}$. The medium was flushed with nitrogen gas for approximately 2 minutes in order to remove oxygen. Nitrate was added as $NaNO_3$ at an initial concentration of ± 5 $mmol.L^{-1}$. Methanol or ethanol was supplied from a concentrated stock solution up to an initial concentration of ± 320 $mg.L^{-1}$, unless otherwise mentioned.

Nitrite and NO reduction experiments were conducted in 120 ml glass serum bottles filled up to 80 ml (40 ml headspace), placed in a waterbath at 30 °C. The bottles were filled with 80 ml of medium and with sludge up to a concentration of ± 5 or ± 20 mg $VSS.L^{-1}$ for, respectively, the nitrite and NO reduction experiments. Immediately after addition of medium and biomass, the vials were sealed and the headspace was replaced with Argon. Assays without biomass served as sterile controls. In these controls, the medium was autoclaved (two times for 20 minutes at 124 °C) before use. Nitrite (± 5 $mmol.L^{-1}$) and ethanol (± 2 $mmol.L^{-1}$) were added by syringe from concentrated stock solutions. The NO reduction experiments were initiated by generating the nitrosyl-complex at an initial concentration of 2 $mmol.L^{-1}$ $Fe(II)EDTA\text{-}NO^{2-}$. The nitrosyl-complex was formed by injection of pure NO gas by means of a gas syringe. NO reduction experiments were performed at both 30 and 55 °C.

Analyses and chemicals

The concentration of nitrate and nitrite was determined by HPLC with a Chrompack column, packing material Ionospher tmA (dim. 250 x 4.6) and UV detection at 205 nm (Spectroflow 783 UV absorbence detector). A phosphate buffer (K_2HPO_4 15 $g.L^{-1}$, pH adjusted to 3.0 by phosphoric acid) was used as eluent (flow: 1.5 $mL.min^{-1}$). Nitrogen and nitrous oxide (N_2O) were measured by Gas Chromatography (Hewlett Packard model 5890A), equipped with a Molsieve 5A Plot column (30 m x 0,53 mm, DF=15) and Thermal Conductivity Detection (TCD). The $Fe(II)EDTA\text{-}NO^{2-}$ concentration was monitored using UV-vis spectrofotometry (Perkin Elmer, Lambda 12) with 25 $mmol.L^{-1}$ $Fe(II)EDTA^{2-}$ medium as reference. The nitrosyl-complex shows typical intense absorption bands around 345 and 620 nm (Schneppensieper *et al.*, 2001). Samples for $Fe(II)EDTA\text{-}NO^{2-}$ determination were taken by syringe and injected in a quartz cuvet. This was performed under anaerobic conditions to avoid the formation of $Fe(III)EDTA^-$ by oxygen contact. Methanol, ethanol and acetate in the liquid phase were analyzed by gas chromatography (Cervantes *et al.*, 2000). Ferrous iron was determined colorimetrically according to Wang and Peverly (1998). Volatile suspended solids (VSS) were determined according to standard methods (APHA, 1985).

All chemicals used were of analytical grade, supplied by Merck (Darmstadt, Germany). Pure NO-gas *was supplied by Hoek Loos (Rotterdam, The Netherlands).*

Calculations and graphs

The reduction of nitric oxide was followed by monitoring the production of N_2O and N_2 in the headspace of the vials. Gas samples (100 μL) were taken with a gas syringe equipped with a locker, so that the pressure of the sample was equal to that in the headspace. The amount of N_2O and N_2 produced is calculated by the amount present in the headspace, corrected by the gas-liquid partitioning coefficient of N_2 and N_2O (Tchobanoglous *et al.*, 2002). Graphs show representative data of replicate experiments.

RESULTS

Medium composition

Fig. 2 presents the influence of the medium composition on the biological nitrate reduction with methanol. In case the medium contained both 50 mmol.L^{-1} EDTA and 50 mmol.L^{-1} Fe(III), denitrification started after a lag-phase of approximately 8 days. However, in case the medium contained 50 mmol.L^{-1} EDTA and only 48 mmol.L^{-1} Fe(III), e.g. 2 mmol.L^{-1} EDTA was uncomplexed, no activity was observed during the experiments (23 days incubation). When the medium contained a surplus of cations, i.e. the sum of cations was more than 50 mmol.L^{-1}, nitrate reduction started after a shorter lag-phase: addition of Ca^{2+}, Fe^{3+} and Mg^{2+} (2 mmol.L^{-1} of each cation) to the 50 mmol.L^{-1} Fe(III)$EDTA^-$ medium resulted in a lag-phase of only 5 days. In case the medium contained 50 mmol.L^{-1} Fe(III)$EDTA^-$ + 10 mmol.L^{-1} Ca^{2+}, the lag-phase was only 1 day (Fig. 2).

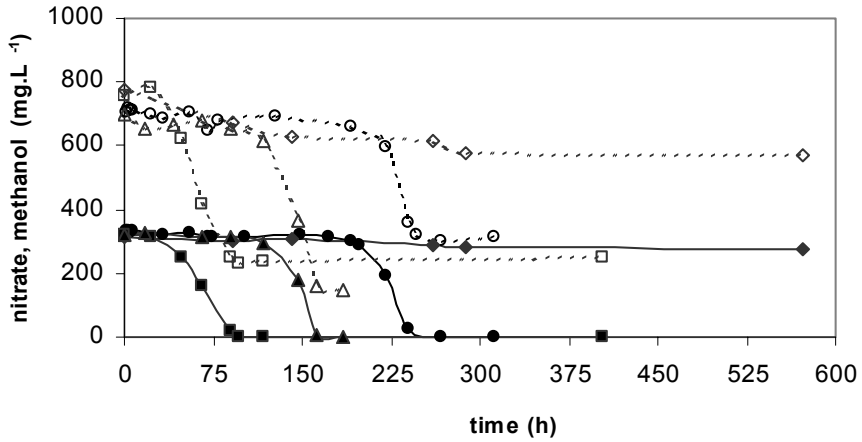

Figure 2

Depletion of methanol (closed line, closed marker) and nitrate (dotted line, open marker) in aqueous solutions of Fe(III)$EDTA^-$ at 30 °C. The medium with 50 mmol.L^{-1} EDTA contained also 48 mmol.L^{-1} Fe^{3+} (◇), 50 mmol.L^{-1} Fe^{3+} (○), 52 mmol.L^{-1} Fe^{3+} + 2 mmol.L^{-1} Ca^{2+} + 2 mmol.L^{-1} Mg^{2+} (△), 50 mmol.L^{-1} Fe^{3+} + 10 mmol.L^{-1} Ca^{2+} (□).

Nitrate reduction in aqueous FeEDTA solution with ethanol and methanol as e-donor

Fig. 3 shows the removal of nitrate in the inoculated Fe(III)EDTA$^-$ medium in the presence of either ethanol (A) and methanol (B) as electron donor. The consumption of both ethanol (initial concentration ± 350 mg.L^{-1}) and nitrate (± 300 mg.L^{-1}) started already after a lag-phase of ± 1 day. In case methanol (± 400 mg.L^{-1}) was added as the e-donor, the lag-phase of NO_3^- depletion was much longer, approximately 5 days. The consumption of e-donor coincided with an increase of Fe(II), indicating that a net iron reduction occurred. Contrary to methanol, with ethanol as the substrate, Fe-reduction was accompanied by an accumulation of acetate. After all the ethanol was consumed, the acetate was degraded completely (Fig. 3A). After 13 days, a fresh amount (± 350 mg.L^{-1}) of nitrate was added. Although ethanol or methanol was not available anymore as electron donor, still a fast depletion of nitrate proceeded at a nitrate reduction rate up to 0.5 mmol.L^{-1} NO_3^-.h^{-1} (Fig. 3A and 3B). Now, the denitrification process was accompanied by a decrease of the Fe(II) concentration, consequently oxidation of Fe(II) occurred.

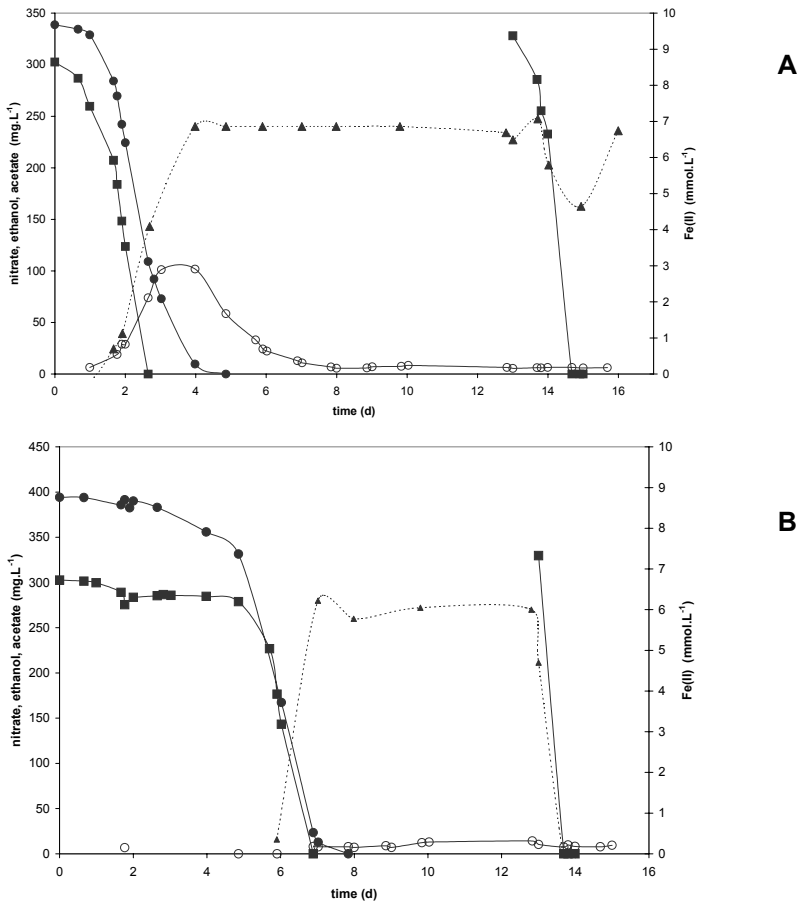

Figure 3

A: Depletion of nitrate (■) and production of Fe(II)EDTA^{2-} (▲, dotted line) and acetate (○) in Fe(III)EDTA$^-$ medium with ethanol (●) as e-donor at 30 °C. B: Depletion of nitrate (■) and production of Fe(II)EDTA^{2-} (▲, dotted line) and acetate (○) in Fe(III)EDTA$^-$ medium with methanol (●) as e-donor at 30 °C.

Fig. 4 shows the nitrate reduction in the inoculated Fe(II)EDTA^{2-} medium (40 mmol.L^{-1} Fe(II)EDTA^{2-} + 10 mmol.L^{-1} Ca^{2+}) in the presence of ethanol (initial concentration ± 320 mg.L^{-1}), and in the sterile control as well. In the biological assays, the total amount of nitrate (360 mg.L^{-1}) was consumed within 1 day at a nitrate consumption rate of about 30 mmol.L^{-1} NO_3^-.h^{-1}. Ethanol consumption started only after 1 day, i.e. after nearly all NO_3^- was consumed. During the denitrification process, the Fe-EDTA medium turned from colorless [Fe(II)EDTA^{2-}] via green [Fe(II)EDTA-NO^{2-}] to orange-brown [Fe(III)EDTA$^-$]. In contrast to the biological assays, the sterile controls did not show any nitrate depletion and no color change was observed.

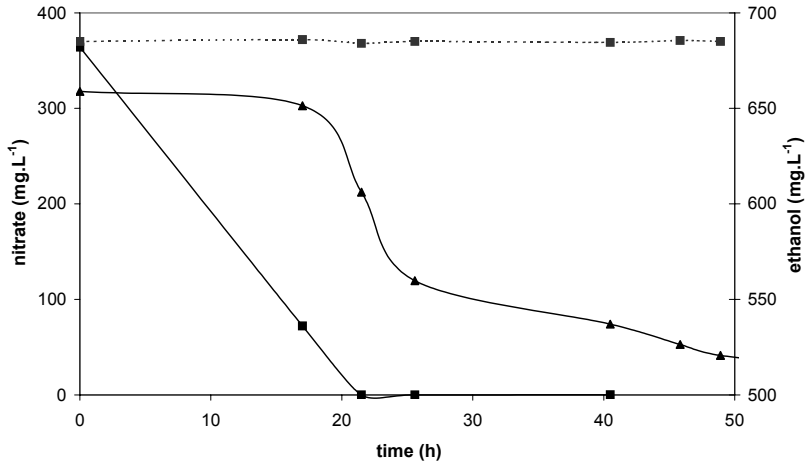

Figure 4

Depletion of nitrate (■) in Fe(II)EDTA^{2-} medium with ethanol (▲) in the bio-assays (closed line) as well as nitrate depletion in the sterile control (dotted line) at 30 °C.

Nitrite reduction in Fe(II)EDTA medium

In aqueous Fe(II)EDTA^{2-} medium, the nitrite depletion proceeded very rapidly (up to 1.5 mmol.L^{-1}.h^{-1}) during the initial stage of the experiment (t < 24 h), both in the inoculated vial and in the sterile control (Fig 5). During this period, the solution turned from colorless to green, indicating that the nitrosyl-complex was formed. Nitrite depletion proceeded only in the biological assays, while the NO_2^- concentration remained unchanged at 200 mg.L^{-1} in the chemical control (Fig. 5). The chemical control vial kept its green color during the experiments (125 h).

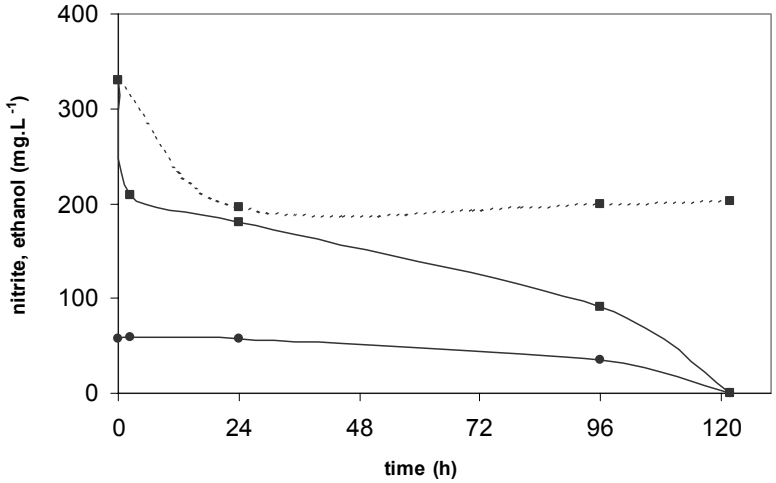

Figure 5

Depletion of nitrite (■) in Fe(II)EDTA^{2-} medium with ethanol (●) at 30 °C in the bio-assays (closed line) and in the sterile control (dotted line).

Nitric Oxide reduction in Fe(II)EDTA medium

Fig. 6A compares the reduction of NO in aqueous Fe(II)EDTA^{2-} medium in the biological assays in the presence and absence of ethanol. The initial Fe(II)EDTA-NO^{2-} concentration was ±1 mmol.L^{-1}. Merely in the inoculated vials depletion of the Fe(II)EDTA-NO^{2-} concentration occurred. Interestingly, Fig. 6A shows that the NO removal rate was independent of the presence of ethanol or acetate.

Fig. 6B compares the reduction of nitric oxide at 30 °C and 55 °C. NO reduction was followed by monitoring the gaseous N (N_2 + N_2O) in the headspace. The NO reduction rate amounted to approximately 12 μmol.L^{-1}.h^{-1} at 30 °C and approximately 35 μmol.L^{-1}.h^{-1} at 55 °C (Fig 6B). At both temperatures, the NO reduction rate was not affected by the availability of ethanol (Fig. 6B). In all assays, nitrous oxide (N_2O) was detected as an intermediate, although only as a small fraction (< 5 % of the total gaseous N amount). The sterile controls did not show any significant NO reduction (Fig 5A and 6B).

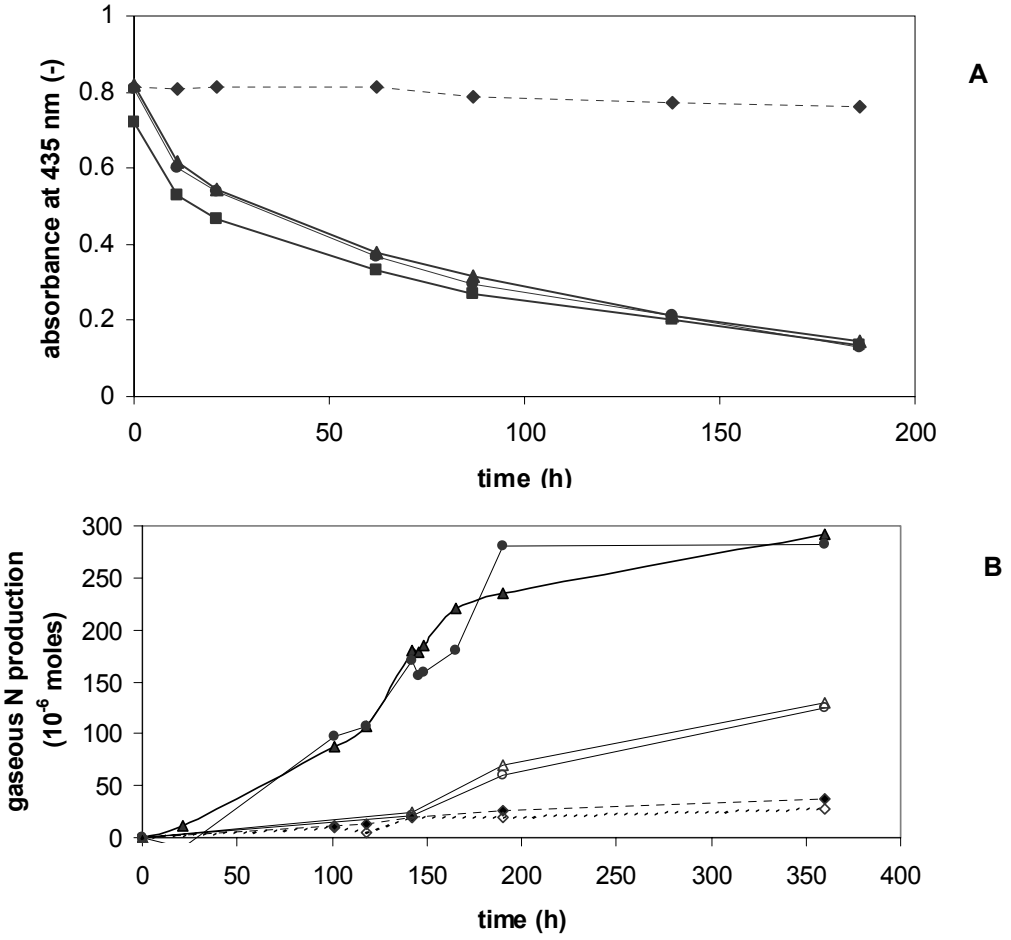

Figure 6

A: Depletion of the nitrosyl-complex Fe(II)EDTA-NO^{2-} (absorbance at 435 nm) in the presence of ethanol (●), acetate (▲) and in absence of any organic e-donor (■) in the bio-assays (closed line) and in the sterile control (dotted line). At t=0 the nitrosyl-complex (1 mmol.L^{-1}) was generated by injection of pure NO gas.

B: NO reduction in Fe(II)$EDTA^{2-}$ medium at 30 °C (open markers, closed lines) and 55 °C (closed markers, closed lines) monitored by the production of gaseous nitrogen (sum of N_2 and N_2O) in the headspace, in the presence (●) and in the absence (▲) of ethanol in the bio-assays and in the sterile controls (◆, dotted line). At t=0 the nitrosyl-complex (1 mmol.L^{-1}) was generated by injection of pure NO gas. This was repeated at t =100 h, t = 120 h and t = 160 h.

DISCUSSION

Reduction of nitrate and iron

This study shows that denitrification and Fe(III)$EDTA^-$ reduction occur simultaneously in Fe(III)$EDTA^-$ medium in the presence of ethanol (Fig 3A) or methanol (Fig. 3B). A net iron reduction, i.e. an increase of the Fe(II) concentration, however, only occurred when ethanol was available as electron donor. In the absence of ethanol, nitrate reduction was accompanied by a net iron oxidation (Fig. 3A and 3B) and apparently Fe(II)$EDTA^{2-}$ is then used as electron donor for nitrate reduction.

This nitrate reduction with Fe(II)EDTA^{2-} as electron donor was biologically catalyzed (Fig. 4). Microbial denitrification using Fe(II) as electron-donor has also been reported to occur in various natural environments (Straub *et al.*, 1996; Hauck *et al.*, 2001; Straub and Buchholz-Cleven, 1998; Straub *et al.*, 2001). The presence of nitrate dependent iron oxidation capacity in the sludge is also supported by the isolation of the bacterium *Paracoccus ferrooxidans* from the sludge used in the present study (Kumaraswamy *et al.*, 2005), as this bacterium is able to use Fe(II)EDTA^{2-} as e-donor for nitrate reduction (Kumaraswamy *et al.*, 2005).

The full denitrification of NO_3^- with Fe(II)EDTA as electron donor proceeds according to (overall reaction):

$$5\ Fe(II)EDTA^{2-} + NO_3^- + 6\ H^+ \leftrightarrow \tfrac{1}{2}\ N_2 + 5\ Fe(III)EDTA^- + 3\ H_2O \qquad (4)$$

The iron oxidation observed in this study (Fig. 3A and 3B) did, however, not proceed according to the stoichiometry of reaction 4. Since the stirred reactors were not completely air-tide, likely some uncontrolled oxidation of Fe(II)EDTA occurred due to micro-aerobic conditions locally prevailing in the stirred reactors.

Ethanol, acetate (Fig. 3A) and methanol (Fig. 3B) are suitable electron donors for the reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} (see also Chapter 6). However, the lag-phase with methanol (5 days) was substantial longer than with ethanol or acetate (1 day) as the electron donor. The same phenomenon was observed during iron reduction experiments in the absence of nitrate (Weelink, 2001). The difference in lag-phase is most probably related to the metabolic characteristic of Fe-reducers, as ethanol and acetate are reported to be suitable electron donors for dissimilatory Fe(III) reduction by various bacteria, while methanol is not (Lovley, 1991; Coates *et al.*, 1996; Kanso *et al.*, 2002).

Nitrite reduction

Fig. 5 shows that nitrite reduction was independent of the presence of biomass, which indicates that nitrite reduction by the oxidation of Fe(II)EDTA^{2-} is a chemical reaction. This is in agreement with Zang and Van Eldik (1990) in their studies dealing with the chemical equilibria of NO_2^- and NO in Fe(II)-ligand solutions. In this reaction, the green-colored nitrosyl-complex Fe(II)EDTA-NO is formed according to:

$$2\ Fe(II)EDTA^{2-} + NO_2^- + 2\ H^+ \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} + Fe(III)EDTA^- + H_2O \qquad (5)$$

The reduction of nitrite proceeds until a certain equilibrium is reached (reaction 5), which was at ± 200 mg.L^{-1} NO_2^- in our experiments (Fig. 5). Since the sterile control vial kept its green color, it is evident that the nitrosyl-complex Fe(II)EDTA-NO^{2-} is relatively stable under sterile conditions for prolonged periods of time (125h).

NO reduction

Depletion of the nitrosyl-complex (Fig. 6A) and gaseous nitrogen production (Fig. 6B) only occurred in the inoculated vials. This clearly shows that the reduction of NO to N_2 is biologically catalyzed. The NO reduction rates were found to be independent of the presence of ethanol or acetate (Fig. 5A and B), suggesting that Fe(II) served as electron donor for NO reduction to N_2 with N_2O as an intermediate, i.e. according to reactions 6 and 7:

$$2\ Fe(II)EDTA\text{-}NO^{2-} + 2H^+ \rightarrow N_2O + H_2O + 2\ Fe(III)EDTA^- \quad (6)$$

$$N_2O + 2\ Fe(II)EDTA^{2-} + 2\ H^+ \rightarrow N_2 + H_2O + 2\ Fe(III)EDTA^- \quad (7)$$

The occurrence of NO reduction with Fe(II) as electron donor has recently been reported for several inocula under thermophilic conditions (55 °C) (Chapter 3). Compared to the specific NO reduction rate reported in that study (1.6 mmol.gVSS^{-1}.day^{-1} for the original Veendam inoculum), the value found in the present study (42 mmol.gVSS^{-1}.day^{-1}) was distinctly higher. The higher specific denitrifying activity observed in this study can be attributed to the fact that the sludge had been pre-cultivated under nitrate reducing thermophilic conditions for 2 days.

Independent of the presence of ethanol, the NO reduction rate at 55 °C (± 35 μmol.L^{-1}.h^{-1}) was about 3 times higher than at 30 °C (± 12 μmol.L^{-1}.h^{-1}). Besides the lower denitrifying activity of the biomass, the NO reduction rate at 30 °C may have been limited by the lower availability of free NO (aq). The latter compound, and not its complexed form Fe(II)EDTA-NO^{2-}, might be the electron acceptor in the denitrification pathway (Chapter 3). Since at 55 °C the nitrosyl-complex Fe(II)EDTA-NO is less stable than at 30 °C (Demmink *et al.*, 1997), more free NO (aq) (which is always in equilibrium with the nitrosyl-complex according to reaction 2), is available at elevated temperatures. To determine if free or chelated NO is used as electron acceptor in the NO reduction pathway, further research is required using different ligands to create nitrosyl-complexes with different stabilities, and thus different free NO concentrations.

FeEDTA as electron mediator in the denitrification pathway

Figure 7 depicts schematically the pathway of denitrification in aqueous FeEDTA solutions. Nitrate reduction to nitrite is biologically catalyzed using Fe(II) as electron donor. This study does, however, not allow to exclude that ethanol or methanol can also serve as electron donor for this first reduction step. Subsequently nitrite is chemically reduced to nitric oxide with Fe(II)EDTA^{2-} as electron donor, which leads to the formation of the nitrosyl-complex Fe(II)EDTA-NO^{2-} and Fe(III)EDTA$^-$. Nitric oxide, i.e. presumably free NO (aq) in equilibrium with the nitrosyl-complex, is then finally reduced biologically to N_2 with N_2O as an intermediate. During that reaction Fe(II)EDTA^{2-} is oxidized to Fe(III)EDTA$^-$.

This study does not allow to assign whether $Fe(II)EDTA^{2-}$ is exclusively used as electron donor for denitrification in case also an organic electron donor (ethanol or acetate) is available. This warrants future research to determine if organic or inorganic electron donors are preferred for NO_3^- and NO reduction in aqueous FeEDTA solution.

Ethanol, methanol or acetate can be used as electron donor for the dissimilatory reduction of $Fe(III)EDTA^-$ (Fig. 3). This study further demonstrates that, under nitrate- and nitrite-reducing conditions with ethanol or methanol as electron donor, FeEDTA serves both as electron donor [$Fe(II)EDTA^{2-}$] and electron acceptor [$Fe(III)EDTA^-$]. Such a mediating role of iron in the electron transport during denitrification has also been suggested for nitrite reduction by *Escherichia coli* (Brons *et al.*, 1991) and for denitrification processes in groundwater (Ludvigsen *et al.*, 1998; Caldwell *et al.*, 1999), paddy soil (Klueber and Conrad, 1998) and thermophilic wastewater treatment (Percheron *et al.*, 1998).

Medium composition

Fig. 2 clearly demonstrates that the presence of uncomplexed EDTA needs to be avoided in media used for biological denitrification. Free, uncomplexed EDTA, inhibits the denitrification process (Fig. 2), which can be attributed to free EDTA toxicity. Indeed, free EDTA has been reported to increase the permeability of the outer membrane of gram negative bacteria, leading to cell decay (Asbell and Eagon, 1966; Leive, 1968; Temple *et al.*, 1992). Addition of an excess of the cations Fe^{3+}, Ca^{2+} and Mg^{2+} in the medium is an elegant way to overcome this free EDTA toxicity. This is in agreement with Ayres *et al.* (1998), who found that addition of divalent cations substantially reduces EDTA induced lysozym lysis. Cation addition also leads to a substantial shorter lag-phase (Fig. 2), which likely can be attributed to the absence of free EDTA or to the stimulating role that calcium can play on bacterial activity (Herbaud *et al.*, 1998).

CONCLUSION

This study shows that in aqueous $Fe(III)EDTA^-$ solutions, denitrification is accompanied by biological iron reduction. Ethanol, acetate and methanol were found to be suitable electron donors for the reduction of $Fe(III)EDTA^-$ to $Fe(II)EDTA^{2-}$. The latter compound can serve as electron donor for the biological nitrate reduction to nitrite, for the chemical reduction of nitrite to nitric oxide and also for the biological reduction of NO reduction to N_2 with nitrous oxide as an intermediate. It is concluded that the pathway of full denitrification in aqueous FeEDTA solutions involves both biological and chemical reduction steps with FeEDTA as an electron mediating compound. Besides that, this paper demonstrates that free, uncomplexed EDTA strongly inhibits the biological denitrification activity. Thus, an excess of divalent cations is essential to guarantee complete denitrification in aqueous FeEDTA solutions.

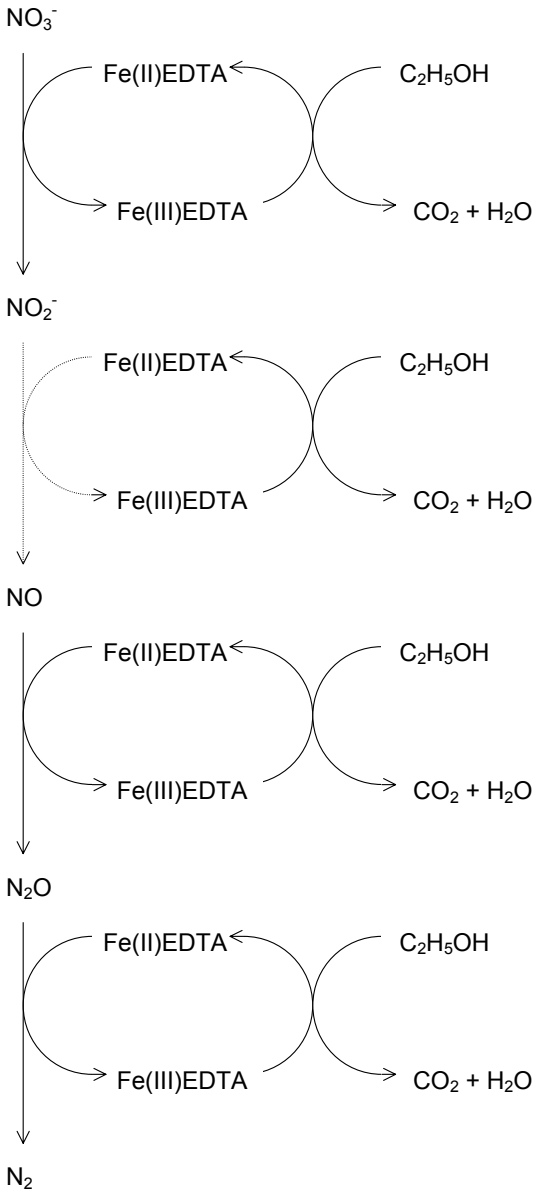

Figure 7

Denitrification pathway in aqueous $Fe(II)EDTA^{2-}$ solutions in the presence of ethanol. The reduction of NO_3^- to N_2 involves both biological (closed lines) and chemical (dotted lines) processes.

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CHAPTER 3

Biological reduction of nitric oxide in aqueous $FeEDTA^{2-}$ solutions

The reduction of nitric oxide in aqueous solutions of $Fe(II)EDTA^{2-}$ (20-25 mM, pH 7.2 ± 0.2) was investigated in batch experiments at 55 °C. Reduction of NO to N_2 was found to be biologically catalyzed with nitrous oxide (N_2O) as an intermediate. Various sludges from full-scale denitrifying and anaerobic reactors were capable to catalyze NO reduction under thermophilic conditions. The NO reduction rate was not affected by the presence of ethanol or acetate. EDTA-chelated Fe(II) was found to be a suitable electron donor for the biological reduction of nitric oxide to N_2, with the concomitant formation of $Fe(III)EDTA^-$. In the presence of ethanol, EDTA-chelated Fe(III) was reduced to $Fe(II)EDTA^{2-}$. This study strongly indicates that redox cycling of FeEDTA plays an important role in the biological denitrification process within the BioDeNOx concept.

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INTRODUCTION

The emission of nitrogen oxides (NO_x), consisting of ±95% nitric oxide (NO) and ±5% nitrogen dioxide (NO_2), by industrial flue gases causes serious environmental problems, e.g., acid rain and depletion of the ozone layer (Harding *et al.*, 1996; Flanagan *et al.*, 2002). To date, mainly chemical processes such as selective catalytic reduction (SCR) are used for NO_x emission abatement. However, all of these processes require high temperatures (>300 °C) and expensive catalysts (Davis *et al.*, 1992). Therefore, biological NO_x removal techniques using denitrification may represent promising alternatives to the conventional SCR techniques, because denitrification occurs at ambient temperatures using cheap microbial inocula (e.g., soil or activated sludges). However, biological techniques such as trickling filtration may suffer from low treatment efficiencies at economic retention times (Chou and Lin, 2000; Du Plessis *et al.*, 1998). When water is used as scrubbing liquor, the transfer of NO from the gas to the liquid phase is limited by the low solubility of NO in water. Fortunately, the low solubility of NO can be overcome by the use of an aqueous solution of $Fe(II)EDTA^{2-}$, which has the ability to form stable complexes with NO and therefore provides high absorption efficiencies for gaseous NO (Demmink *et al.*, 1997). In an instantaneous reaction, the nitrosyl complex is formed according to reactions 1 and 2 (Demmink *et al.*, 1997):

$$NO\ (g) \leftrightarrow NO\ (aq) \qquad (1)$$

$$NO\ (aq) + Fe(II)EDTA^{2-} \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \qquad (2)$$

Aqueous $Fe(II)EDTA^{2-}$ solutions have already been adopted in biological flue gas treatment for NO_x removal, e.g., in the so-called BioDeNOx process. This process combines the principles of wet absorption of NO (in a ±25 mM Fe(II)EDTA solution) with biological reduction of NO in a bioreactor (Buisman *et al.*, 1999). In this process, the biological reduction of NO to dinitrogen gas (N_2) takes place under thermophilic conditions, at around 50-55 °C, which is the adiabatic temperature of scrubber liquors. When ethanol is used as electron donor, as is applied in full-scale BioDeNOx plants (Biostar, personal communication, the denitrification reaction occurs according to the overall reaction 3 (Buisman *et al.*, 1999):

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 2\ CO_2 + 3\ N_2 + 3\ H_2O + 6\ Fe(II)EDTA^{2-} \qquad (3)$$

It should be noted here that, supplementary to organic compounds such as ethanol, also ferrous iron (Fe^{2+}) can serve as electron donor for denitrification (Straub *et al.*, 2001). Iron-dependent denitrification has been observed in groundwater treatment (Devlin *et al.*, 2000) and activated sludge systems (Nielsen and Nielsen, 1998). At neutral pH, the redox potential for NO reduction (NO/N_2O = +1180 mV and N_2O/N_2 = +1350 mV) is much more positive than the redox potential for iron reduction ($Fe(III)EDTA^{-}/Fe(II)EDTA^{2-}$ = +96 mV) (Straub *et al.*, 2001). This implies that the absorbent

(Fe(II)EDTA^{2-}) might also play a role in the biological regeneration (denitrification) pathway, as presented by the overall reaction 3.

The biological reduction of NO in aqueous Fe(II)EDTA^{2-} solutions has to the best of our knowledge not been described in the literature so far. In this study, the pathway of NO reduction in aqueous Fe(II)EDTA^{2-} solutions was investigated by using batch experiments at neutral pH under thermophilic (55 °C) conditions. The role of biocatalysis was assessed, as well as the applicability of various inocula for NO reduction in aqueous Fe(II)EDTA^{2-} solutions. Special attention was paid to the electron donors involved in the NO reduction pathway, as well as to inhibiting and rate-limiting factors.

MATERIALS AND METHODS

Inoculum

The inoculum used for most experiments described in this study was taken from a full-scale fluidized bed denitrification reactor (Veendam, The Netherlands), which treats surface water for oxygen and nitrate removal at a NO_3^- loading rate of ±2 kg NO_3^- m^{-3} d^{-1} using methanol as carbon source and electron donor. The imposed temperature conditions in the reactor varied between 2 and 25 °C, i.e., the temperature of the surface water in winter and summer, respectively.

To screen NO reducing capacities of various inocula, three other seed sludges were also tested: "Emmen", "Nedalco", and "Eerbeek". The Emmen sludge originated from a biological wastewater treatment plant, operating under aerobic and anoxic (denitrifying) conditions at 10-20 °C (Living Machine, Emmen Zoo, The Netherlands). Nedalco sludge comprises a completely anaerobic, methanogenic granular sludge, taken from a full-scale expanded granular sludge bed (EGSB) reactor treating distillery wastewater at 30 °C. The Eerbeek sludge was anaerobic methanogenic granular sludge as well, cultivated in a full-scale upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater at 30 °C.

Medium

The standard Fe(II)EDTA^{2-} medium contained (unless specified otherwise): NaH_2EDTA (Titriplex) 25 mM, $FeCl_2 \cdot 4H_2O$ 30 mM, $MgSO_4 \cdot 7H_2O$ 5 mM, $CaCl_2 \cdot 2H_2O$ 5 mM, NH_4Cl 0.28 g/L, KH_2PO_4 0.25 g/L, $NaHCO_3$ 5.4 g/L, and trace elements solution 1 mL/L (Visser *et al.*, 1999). The medium was prepared by adding subsequently EDTA, nutrients, and metals to demi water. To avoid oxidation of ferrous iron, oxygen was removed by flushing the solution with N_2 for 30 min before Fe^{2+} was added. Then the pH was adjusted to 7.2 by adding sodium hydroxide, and finally $NaHCO_3$ (buffer) was added.

NO Reduction Experiments

NO reduction experiments with various sludges were carried out in 120-mL glass serum vials with a liquid volume of 80 mL (40-mL headspace) at 55 °C in duplicate. The bottles were filled with 80 mL of medium and inoculum to reach a concentration of ca. 1.5 g VSS L^{-1}. Before use, the inocula were washed with 25 mM Fe(II)EDTA^{2-} medium.

Immediately after addition of the sludge and medium, the vials were sealed and the headspace was replaced with Ar/CO_2 (v/v 80/20). Assays without biomass served as sterile controls, and here the medium was autoclaved before use (two times for 20 min at 124 °C). The vials were preincubated at 55 °C overnight for temperature equilibration. This procedure was found to result in the formation of acetate (ca. 1 mM) in the inoculated vials, which was most probably formed as a result of cell lysis upon exposing the biomass to the $Fe(II)EDTA^{2-}$ solution at the elevated temperature. Ethanol was added to part of the vials up to an initial concentration of 1 mM by syringe from a concentrated stock solution.

During the experiments to screen various inocula on their NO reduction capacity, besides ethanol, also acetate, molasses, and hydrogen were added to part of the vials as external electron donor. Acetate and molasses (initial concentration 1 mM and 0.1% v/v, respectively) were added by syringe from a concentrated stock solution. In case of hydrogen, the headspace was replaced by H_2/CO_2 (v/v 80/20) at a pressure of 1.6 bar.

The bioconversion process in the experiments was initiated by generating the nitrosyl complex at an initial concentration of 2.5 mM $Fe(II)EDTA\text{-}NO^{2-}$, by adding 2.5 mM nitrite (NO_2^-) from a concentrated stock solution (equal to 200 μmol N per vial). In an aqueous $Fe(II)EDTA^{2-}$ medium, 2.5 mM nitrite forms 2.5 mM $Fe(II)\text{-}EDTA\text{-}NO^{2-}$ and 2.5 mM $Fe(III)EDTA^-$ (Zang and Van Eldik, 1990).

Analyses and Chemicals

Nitrite was analyzed by HPLC with a Chrompack column, packing material Ionospher tmA, and UV detection at 205 nm (Spectroflow 783 UV absorbance detector). A phosphate buffer (K_2HPO_4 15 g/L, pH adjusted to 3.0 by phosphoric acid) was used as eluent (flow 1.5 mL/min). Nitrogen and nitrous oxide (N_2O) were measured by gas chromatography (Hewlett-Packard model 5890A), using a Molsieve 5A Plot column (30 m × 0.53 mm, DF = 15) and thermal conductivity detection (TCD). Ethanol and volatile fatty acids (VFA) in the liquid phase were analyzed by gas chromatography according to Cervantes *et al.* (2000). Ferrous iron was determined colorimetrically using the 1,10-phenantroline method according to Akzo Nobel (1996). Volatile suspended solids (VSS) were determined according to standard methods (APHA, 1985).

All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany).

Calculations

Reduction of NO was monitored by the production of N_2O and N_2 in the headspace of the vials. Gas samples (100 μL) were taken with a gas syringe equipped with a locker, so that the pressure of the sample was equal to that in the headspace. The amount of N_2O and N_2 produced was calculated by the amount present in the headspace, corrected by the gas-liquid partitioning coefficient of N_2 and N_2O at 55 °C (Tchobanoglous *et al.*, 2002).

RESULTS

Reduction of Nitrite and Nitric oxide

Figure 1 shows the reduction of NO_2^- and NO in aqueous Fe(II)EDTA^{2-} medium in the presence of ethanol and acetate. The sterile controls showed a fast NO_2^- removal during the first 2 h, but then the nitrite concentration stabilized at ca. 0.3 mM (even after 2 days nitrite still was present). The sterile controls did show no significant NO reduction. In the inoculated vials, on the contrary, NO reduction, monitored by N_2O production, started after ca. 2 h and the nitrite was completely removed within 4 h. This means that at t = 2 h, practically the total amount of nitrogen oxides was present as the nitrosyl complex Fe(II)EDTA-NO^{2-}. In the bioassays, within 1 day ca. 200 μmol of NO was reduced to gaseous nitrogen compounds (N_2O + N_2). The reduction rate of NO amounted to 1.6 mmol N gVSS^{-1} d^{-1}. The nitrogen balance (Table 1) clearly shows that the nitrosyl complex Fe(II)EDTA-NO^{2-} is relatively stable under sterile conditions. In the bioassays, consumption of ethanol (initial concentration 1 mM) started only after a lag phase of ca. 1 day (Figure 1). The acetate concentration remained rather constant at ±1 mM throughout the incubation (Figure 1).

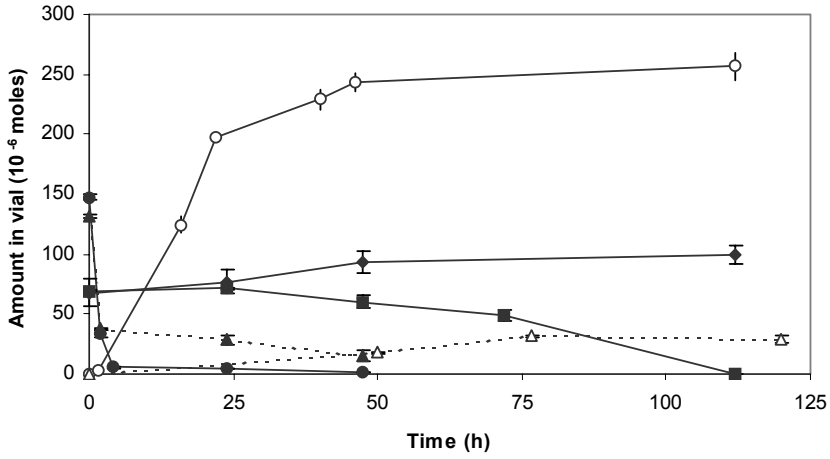

Figure 1

Reduction of nitrite and production of total gaseous nitrogen (sum of N_2 + N_2O) in batch incubations at 55 °C in the presence of both ethanol (■) and acetate (◆). Nitrite (●) and total gaseous nitrogen (○) in bioassays (closed line) and sterile controls (dotted line). At t = 0 the nitrosyl complex was generated by injection of 2.5 mM nitrite (= 200 μmol N).

Effect of Ethanol on NO Reduction

The effect of ethanol on the production of gaseous nitrogen compounds (N_2O + N_2) was investigated in the standard medium, containing 5 mM sulfate (Figure 2) and in a sulfate-free medium (Figure 3). Interestingly, NO reduction to N_2O was found to be independent of the availability of ethanol. Although ethanol (when present) was consumed (Figure 1), the NO reduction rate was similar to that in the absence of ethanol (Table 2). Nitrous oxide (N_2O) accumulated in the headspace, and its

reduction to N_2 started only after a lag phase of between 1 and 4 days (Figures 2 and 3). The lag phase of N_2O reduction varied strongly between the duplicates, resulting in rather high standard deviations (Fig. 2).

After 7 days, the nitrosyl complex was generated again by injection of 2.5 mM nitrite (Figure 2). The same phenomenon manifested, i.e., the reduction of NO to N_2O started after a very short (less than 2 h) lag phase, followed by reduction of N_2O to N_2 after 1-2 days (Figure 2). Although the lag phase of N_2O reduction varied significantly over the vials, the presence of ethanol (similar to NO reduction) did not significantly stimulate the N_2O reduction rate (varied between <0.2 and 1.0 mM N $gVSS^{-1}$ d^{-1}).

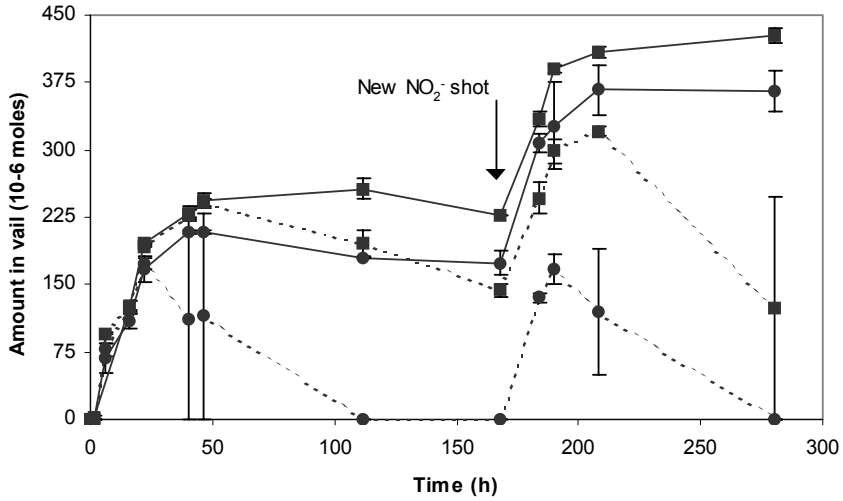

Figure 2

Production of N_2O (dotted line) and total gaseous nitrogen (sum of N_2 + N_2O, closed line) in batch incubations at 55 °C in sulfate-containing medium in the presence (■) and absence (●) of ethanol. At t = 0 and t = 167 h, the nitrosyl complex was generated by injection of 2.5 mM nitrite (= 200 μmol N).

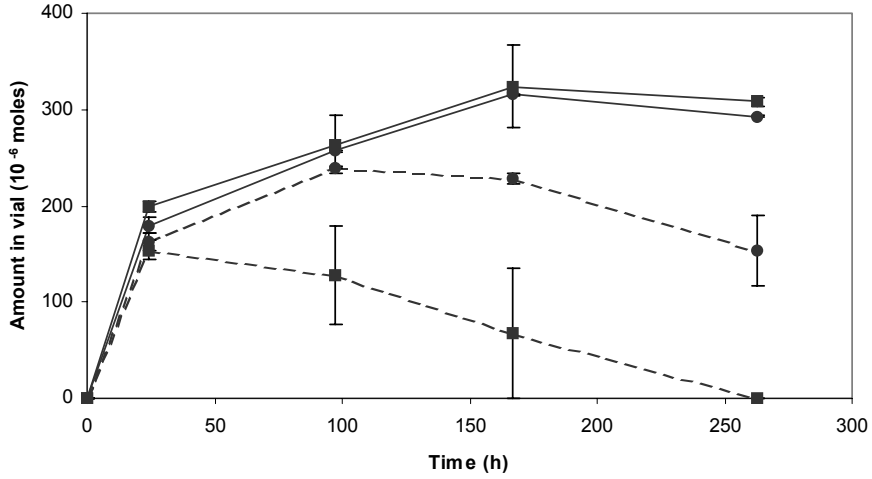

Figure 3

Production of N_2O (dotted line) and total gaseous nitrogen (sum of N_2 + N_2O, closed line) in batch incubations at 55 ºC in sulfate-free medium in the presence (■) and absence (●) of ethanol. At t = 0 the nitrosyl complex was generated by injection of 4 mM nitrite (= 320 μmol N).

Table 1

Effect of Inoculation and Ethanol on Nitrogen Balance: Total Gaseous N Produced at t = 263 h after Nitrite Reduction in Batch Incubations at 55 ºC[a]

Experimental condition	NO_2^--N reduced (μmoles)	Total gaseous N produced (μmoles)	NO-N as nitrosyl-complex (μmoles)
Biological, ethanol present	320	313 ± 5	7 ± 5
Biological, ethanol absent	320	315 ± 2	5 ± 2
Sterile control	160	11 ± 1	149 ± 1

[a] At t = 0 h, the nitrosyl complex was generated by injection of 320 μmol (biological assays) or 160 μmol (sterile controls) of nitrite. From these data, the final theoretical nitrosyl concentration Fe(II)EDTA-NO is calculated.

Screening Inocula

Table 2 shows that NO reduction in aqueous Fe(II)EDTA^{2-} solution also proceeded with the denitrifying sludge (Emmen) and the anaerobic granular sludge Nedalco. In contrast, the anaerobic Eerbeek sludge was not able to reduce NO. In all cases that NO reduction proceeded, N_2O was found as an intermediate and the presence of an external electron donor, i.e., ethanol, acetate, hydrogen, or molasses, did not influence the NO reduction rates (Table 2).

Table 2

NO Reduction Rates (mmol N $gVSS^{-1}$ d^{-1}) of Various Inocula during NO Reduction in Aqueous $Fe(II)EDTA^{2-}$ Solutions at 55 °C with Different External Electron Donors[a]

	External electon donor available				
Inoculum	**None**	**Acetate**	**Ethanol**	**Molasses**	**Hydrogen**
Veendam	1.6	1.6	1.6	n.a.	n.a.
Eerbeek	0.0	0.0	0.0	n.a.	n.a.
Nedalco	0.8	n.a.	n.a.	0.8	0.8
Emmen	0.3	n.a.	n.a.	0.3	0.3
Sterile control	0.0	0.0	0.0	n.a.	0.0

[a] Initial concentrations: ethanol or acetate 1 mM, molasses 0.1 % v/v. Hydrogen was added in the headspace as H_2/CO_2 (v/v 80/20) at a pressure of 1.6 bar. na = not analyzed.

Iron Oxidation and Reduction

Table 3 presents the amount of Fe(II) in the vials before and after reduction of 200 µmol of nitrite in the presence and absence of ethanol. In both cases, 80% of the NO_2^--N was converted to N_2O, while the remaining 20% was fully reduced to N_2 (data not shown). In the absence of ethanol, the Fe(II) concentration decreased during the experiment, i.e., net iron oxidation occurred. However, in the presence of ethanol contrarily a net iron reduction took place. These results were confirmed by visual observations: in the absence of ethanol, a color change was observed from colorless [$Fe(II)EDTA^{2-}$] via green/brown [$Fe(II)EDTA\text{-}NO^{2-}$] to orange/brown [$Fe(III)EDTA^-$], indicating iron oxidation (Figure 4). In contrast, in the presence of ethanol, the vials ended up colorless, indicating that all iron was present as $Fe(II)EDTA^{2-}$. The sterile control vials kept their green color, indicating that the nitrosyl complex $Fe(II)EDTA\text{-}NO^{2-}$ was relatively stable within the time frame of the performed batch experiments (11 days).

Table 3

Effect of Ethanol on Amount of Fe(II) in Biological Vials, Prior to and after Reduction of 200 µmol of NO_2^- to 160 µmol of N_2O-N and 40 µmol of N_2-N at 55 °C.

Assay	**Fe(II) before denitrification (µmoles)**	**Fe(II) after denitrification (µmoles)**	**Fe oxidation / reduction**
Presence of ethanol	2062 ± 24	2102 ± 10	± 40 µmol reduced
Absence of ethanol	2101 ± 42	1855 ± 94	± 250 µmol oxidized

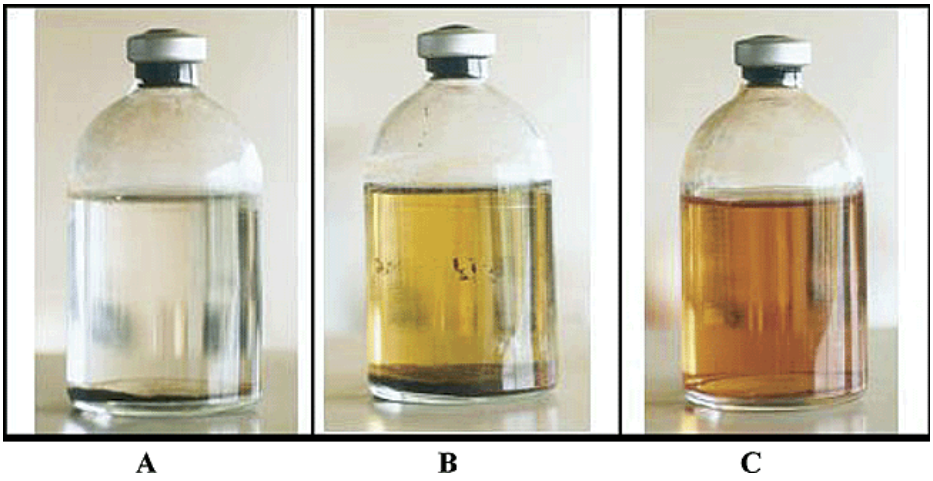

Figure 4

Color change during nitrite reduction (200 µmol) at 55 °C in the biological assays: from colorless [Fe(II)EDTA^{2-}] (A, t = 0) via green [Fe(II)EDTA-NO^{2-}] (B, t = 2 h) to orange-brown [Fe(III)EDTA^{-}] (C, t = 120 h).

DISCUSSION

Reduction of Nitrogen Oxides

The fast depletion of nitrite in the sterile controls (Figure 1) reveals that reduction of NO_2^- in aqueous Fe(II)EDTA^{2-} solutions is a chemical process, as reported by Zang and Van Eldik (1990). After addition of NO_2^- to the Fe(II)EDTA^{2-} solution, the nitrosyl complex Fe(II)EDTA-NO^{2-} is formed according to reaction 4:

$$2\ \text{Fe(II)EDTA}^{2-} + \text{NO}_2^- + 2\ \text{H}^+ \leftrightarrow \text{Fe(II)EDTA-NO}^{2-} + \text{Fe(III)EDTA}^- + \text{H}_2\text{O} \qquad (4)$$

On the basis of the fast nitrite depletion in both the sterile and the biological vials during the initial stage, it is plausible that also in the biological vials reduction of NO_2^- to NO was a chemical process. The subsequent reaction, i.e., the reduction of NO bound in the nitrosyl complex Fe(II)EDTA-NO^{2-}, was clearly biologically catalyzed, because a significant NO reduction (2.4 mmol L^{-1} d^{-1}) proceeded only in the biological assays (Figure 1; Table 2). A chemical NO reduction to N_2 occurred as well (Table 1) but at a much lower rate, ±0.1 mmol L^{-1} d^{-1} (Figure 1). Chemical NO reduction in aqueous Fe(II)EDTA^{2-} solutions at lower temperature (23 °C) and lower Fe(II)EDTA concentration (1 mM) was reported before by Schneppensieper *et al.* (2001a). They observed a slow and irreversible redox process accompanied by the dissociation of the nitrosyl complex to Fe(III)EDTA$^-$ and N_2O (Scheppensieper *et al.*, 2001a).

The biological NO reduction resulted in a complete nitrite depletion in the bioassays in less than 4 h, whereas in the chemical controls nitrite still could be detected after 2 days (Figure 1). Since the chemical nitrite reduction in aqueous Fe(II)EDTA^{2-} proceeds according to an equilibrium reaction

(reaction 4), it is evident that lowering the nitrosyl concentration by bioconversion stimulates the chemical nitrite reduction.

NO reduction was found to be catalyzed by three out of the four inocula tested (Table 2). Since NO is an intermediate in denitrification, NO reduction can be expected in the denitrifying sludges, e.g., Veendam and Emmen. However, these sludges originated from mesophilic and psychophilic reactors, while the NO reduction experiments were performed at thermophilic temperatures (55 ºC). Apparently, thermophilic denitrifying bacteria were present in the sludges used, or the NO reduction was catalyzed by mesophilic or psychophilic species, still active under nonoptimal temperatures. Although the Nedalco sludge was not cultivated under denitrifying conditions, this seed sludge also catalyzed NO reduction. Apparently, in contrast to the other anaerobic inoculum, Eerbeek, a NO-reducing bacterial population was present in the Nedalco sludge. Denitrifying populations can be present in methanogenic (Percheron *et al.*, 1999) and sulfidogenic (Lens *et al.*, 2000) mesophilic sludges and have been utilized to integrate nitrogen removal in methanogenic wastewater treatment (Percheron *et al.*, 1999).

The maximal NO reduction rate was observed in the initial stage (Figures 1-3), which might indicate that not the nitrosyl complex Fe(II)EDTA-NO^{2-} but rather free NO(aq) was involved as an electron acceptor in the denitrification pathway. As free NO(aq) is always in equilibrium with the nitrosyl complex Fe(II)EDTA-NO^{2-} (reaction 2) (Demmink *et al.*, 1997), based on the value of the stability constant of the nitrosyl complex Fe(II)EDTA-NO^{2-}, $K_{NO} = 6 \times 10^5$ M^{-1} at 55 °C (Schneppensieper *et al.*, 2001b), only a very low concentration of free NO(aq) can be expected. In our experiments, the concentration of free NO(aq) was estimated to be $\pm$ 1 $\times$ 10^{-7} M at t = 2 h, e.g., at the maximum concentration of the nitrosyl complex (±2 mM) and free NO(aq). In case the Monod constant for NO reduction (K_m) would be 4 $\times$ 10^{-7} M for NO (Zafiriou *et al.*, 1989), it is clear that the concentration of free NO(aq) always will be far below that K_m value. This strongly suggests that free NO(aq) was the reactant in the NO reduction.

All assays demonstrate that N_2O is an intermediate in the NO reduction pathway (Figures 2 and 3). Since the reduction of N_2O to N_2 (redox potential N_2O/N_2 = +1350 mV) energetically is more favorable than the formation of N_2O from NO (redox potential NO/N_2O = +1180 mV) (Straub *et al.*, 2001), the accumulation of N_2O cannot be explained on that basis. However, the accumulation of N_2O, well-known in other denitrifying systems as activated sludge systems (Von Schulthess *et al.*, 1995) and soil (Flessa *et al.*, 2002), can be caused by factors such as a low C/N ratio (Itokawa *et al.*, 2001), a high oxygen concentration (Otte *et al.*, 1996), or inhibition of nitrous oxide reductase (N_2OR) (Knowles, 1982). In this study, nitrous oxide accumulated in the presence of ethanol and acetate at a high C/N ratio in the absence of oxygen. Thus, N_2OR inhibition is the most likely reason for the observed N_2O accumulation. Indeed, sulfide (S^{2-}) can inhibit N_2O reduction at extremely low concentrations (ca. 1 μM) (Sörensen *et al.*, 1987). Since the standard medium contained 5 mM sulfate, low concentrations of S^{2-} might have been present in the vials under reducing conditions, especially when ethanol, an excellent electron donor for biological sulfate reduction under thermophilic conditions (Widdel and Hanssen, 1991), was available. Nevertheless, in the absence of sulfate in the medium (Figure 3), the rate of N_2O reduction appeared to be lower (≤0.2 mM N $gVSS^{-1}$ d^{-1}) than in the presence of 5 mM

sulfate (Figure 2). Besides S^{2-} inhibition, also extracellular NO(aq) can inhibit N_2O reduction. Von Schulthess *et al.* (1995) showed that addition of NO gas (NO(aq) ≤0.8 mg L^{-1}) during denitrification in batch experiments can cause N_2O accumulation. However, since the free NO(aq) concentration was low in this study (see above), it is unlikely that the inhibition of N_2O reduction was caused by extracellular NO.

Production of Acetate

Although not supplied to the medium, acetate was always present in the vials, likely due to lysis of the biomass resulting from the temperature shift of the original reactor temperature (psychrophilic or mesophilic) to thermophilic conditions. Elevation of temperature to thermophilic conditions has been reported to promote cell lysis (Rocher *et al.*, 1999), and this can be accompanied by the production of acetate (Mason *et al.*, 1986). Although acetate production could also be caused by EDTA degradation (Nörtemann, 1999), it is very unlikely that this process plays a major role in this study since similar amounts of acetate were produced in inoculated vials without EDTA in the medium (data not shown).

Electron Donor for NO Reduction

For all inocula tested in this study, the NO reduction rate was apparently independent of the availability of an external electron donor, i.e., ethanol, acetate, hydrogen, or molasses (Figure 2; Table 2). The observed net iron oxidation in the absence of ethanol (Table 3) shows that Fe(II)EDTA^{2-} can serve as electron donor for the biological reduction of NO to N_2O. In that reaction, oxidation of iron takes place according to reaction 5. Scheppensieper *et al.* (2001a) proposed that the same reaction occurs during the chemical NO reduction in aqueous Fe(II)EDTA-NO^{2-} solutions:

$$2\ \text{Fe(II)EDTA-NO}^{2-} + 2\text{H}^{+} \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} + 2\ \text{Fe(III)EDTA}^{-} \qquad (5)$$

Similarly as found for NO reduction, the presence of ethanol did not affect the rate of N_2O reduction to N_2 (Figures 2 and 3). This indicates that electrons provided by Fe(II) also served N_2O reduction, according to reaction 6:

$$\text{N}_2\text{O} + 2\ \text{Fe(II)EDTA}^{2-} + 2\ \text{H}^{+} \rightarrow \text{N}_2 + \text{H}_2\text{O} + 2\ \text{Fe(III)EDTA}^{-} \qquad (6)$$

On the basis of the partial reactions 4-6, the overall reaction of the total reduction of nitrite in aqueous Fe(II)EDTA^{2-} proceeds according to reaction 7. When nitrite is only reduced to nitrous oxide, the reaction proceeds according to reaction 8:

$$3\ \text{Fe(II)EDTA}^{2-} + \text{NO}_2^{-} + 4\ \text{H}^{+} \rightarrow 0.5\ \text{N}_2 + 3\ \text{Fe(III)EDTA}^{-} + 2\ \text{H}_2\text{O} \qquad (7)$$

$$2\ \text{Fe(II)EDTA}^{2-} + \text{NO}_2^{-} + 3\ \text{H}^{+} \rightarrow 0.5\ \text{N}_2 + 2\ \text{Fe(III)EDTA}^{-} + 1.5\ \text{H}_2\text{O} \qquad (8)$$

Table 2 reveals that the reduction of nitrite to N_2O and N_2 coincides with the oxidation of Fe(II) to Fe(III) in the absence of ethanol. According to reactions 7 and 8, reduction of 200 µmol of nitrite to 160 µmol of N_2O-N and 40 µmol of N_2-N is theoretically coupled to the oxidation of 480 µmol of Fe(II). Although the observed iron oxidation amounts to only about 55% of that value (Table 3), which likely can be attributed to analytical limitations to fully quantify the fast redox Fe conversions, this study shows clearly that Fe(II) is a suitable electron donor for NO reduction. This is in agreement with Straub *et al.* (1996), who showed that chemolithotrophic bacteria can use Fe(II) as electron donor for the full reduction of nitrate to N_2.

At neutral pH, the redox potential of NO reduction (NO/N_2O = +1180 mV and N_2O/N_2 = +1350 mV) is much more positive than that of iron ($Fe(III)EDTA^-/Fe(II)EDTA^{2-}$ = +96 mV). Therefore, $Fe(II)EDTA^-$ is in principle a favorable electron donor for the reduction of both NO and N_2O, even though the oxidation of ethanol to carbon dioxide (CO_2) is energetically more favorable than the oxidation of $Fe(II)EDTA^{2-}$ to $Fe(III)EDTA^-$ (Straub *et al.*, 2001; Thaurer *et al.*, 1997) The independency of the NO reduction rate on the presence of ethanol or acetate (Figure 2) suggests that NO reduction is exclusively catalyzed by chemolithotrophic bacteria using Fe(II) as the electron donor. Another explanation for the independency of ethanol might be that not the electron donor, but, e.g., the transport of free NO(aq) (or the nitrosyl complex) from the bulk liquid to the bacterial cell limits the NO reduction rate. The latter can, however, not be the sole factor determining the NO reduction rate, as experiments performed under the same conditions with thermophilic denitrifying sludge showed much higher NO reduction rates (Chapter 2).

Iron Reduction

In the presence of ethanol, the Fe(III) produced during NO_2^- (reaction 4) and NO reduction (reaction 5 and 6) was reduced by biological iron reduction (Table 3). Thus, the oxidation and reduction of EDTA chelated iron play a key role during the reduction of NO in the presence of ethanol (Figure 5) as, e.g., in BioDeNOx reactors.

It is well-known that various bacteria can use ethanol as electron donor for dissimilatory Fe(III) reduction (Lovley, 1991; Lovley *et al.*, 1993; Kanso *et al.*, 2002). On the other hand it is questionable whether Fe(III), when bound to strong chelating agents such as EDTA, is still bioavailable. According to Lovley *et al.* (1996) soluble chelated iron, e.g., $Fe(III)EDTA^-$, is well available for microbial reduction. In contrast, Haas and DiChristina (2002) concluded that Fe(III) is not available for biological reduction by *Schewanella putrefaciens* when bound to EDTA, because of the high stability of this ligand. This warrants further research on Fe(III) reduction in ligand (EDTA)-metal systems as adopted in BioDeNOx reactors.

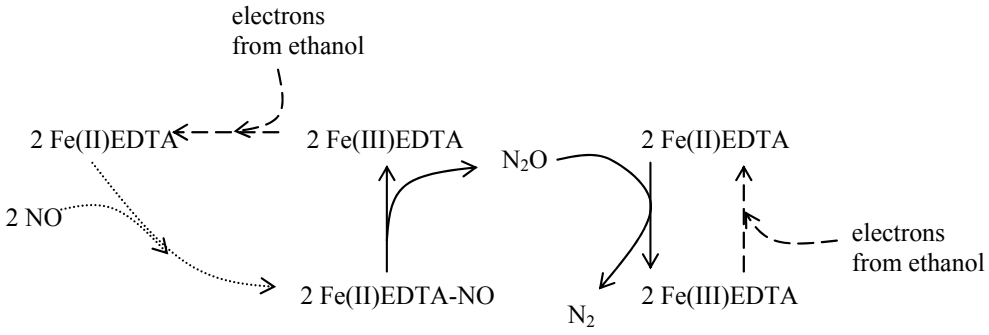

Figure 5

Redox cycling of the metal ligand FeEDTA during the reduction of NO in the presence of ethanol. The roles of FeEDTA: Fe(II)EDTA^{2-} as absorbent for NO (reaction 2) (·····), Fe(II)EDTA^{2-} as electron donor for denitrification (reactions 5 and 6) (———), and Fe(III)EDTA^{-} as electron acceptor for Fe reduction (- - - - - -).

CONCLUSION

This study showed that reduction of NO in aqueous Fe(II)EDTA^{2-} solutions is biologically catalyzed. Various inocula are capable to catalyze NO reduction under thermophilic conditions. NO reduction to N_2 occurs with N_2O as an intermediate. The NO reduction rate was not affected by the presence of ethanol or acetate under the experimental conditions applied. EDTA-chelated Fe(II) is a suitable electron donor for the biological reduction of nitric oxide; in that reaction Fe(III)EDTA^{-} is formed. In the presence of ethanol, the EDTA-chelated Fe(III) is reduced to Fe(II)EDTA^{2-}. Redox cycling of FeEDTA plays a key role in the biological NO reduction, as adopted in the BioDeNOx concept.

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CHAPTER 4

Demonstration of the BioDeNOx process

The study described in this chapter demonstrates the technical feasibility of this BioDeNOx concept in a bench-scale installation with a continuous flue gas flow of 650 l/h (70 – 500 ppm NO; 0.8 - 3.3% O_2). Stable NO removal with an efficiency of at least 70 % was obtained in case the artificial flue gas contained 300 ppm NO and 1 % O_2 when the bioreactor was inoculated with a denitrifying sludge. An increase of the O_2 concentration of only 0.3 % resulted in a rapid elevation of the redox potential (ORP) in the bioreactor, accompanied by a drastic decline of the NO removal efficiency. This was not due to a limitation or inhibition of the NO reduction, but to a limited biological iron reduction capacity. The latter leads to a depletion of the absorption capacity of NO of the scrubber liquor, and thus to a poor NO removal efficiency. Bio-augmentation of the reactor mixed liquor with an anaerobic granular sludge with a high Fe(III) reduction capacity succesfully improved the bioreactor efficiency and enabled to treat a flue gas containing at least 3.3% O_2 and 500 ppm NO with an NO removal efficiency of over 80%. The ORP in the bioreactor was found to be a proper signal for the control of the ethanol supply, needed as electron donor for the biological regeneration process. The NO removal efficiency as well as the $Fe(III)EDTA^-$ reduction rate were found to decline at ORP values higher than –140 mV (pH 7.0). For stable BioDeNOx operation, the supply of electron donor (ethanol) can be used to control the ORP below that critical value.

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INTRODUCTION

The emission of nitrogen oxides (NO_x), consisting of ± 95 % nitric oxide (NO) and ± 5 % nitrogen dioxide (NO_2), to the atmosphere causes serious environmental problems, e.g. acid rain and depletion of the ozone layer (Grano, 1995). To date, mainly chemical processes like selective catalytic reduction (SCR) are used for NO_x emission abatement. However, all these processes require high temperatures (> 300 °C) and expensive catalysts (Davis, 1992). Therefore, biological NO_x removal techniques using denitrification may represent promising alternatives for the conventional SCR techniques, because denitrification occurs at ambient temperatures with the use of cheap microbial inocula (e.g. soil or activated sludges). When water is used as scrubbing liquor, the transfer of NO from the gas to the liquid phase is limited by the low solubility of NO in water. Biological techniques like trickling-bed filtration may consequently suffer from low treatment efficiencies at economic retention times (Du Plessis, 1998). The low solubility of NO can be overcome by the use of an aqueous solution of $Fe(II)EDTA^{2-}$, which has the ability to form stable complexes with NO, and therefore provides high absorption efficiencies for gaseous NO (Demmink et al, 1997). In an instantaneous reaction, the nitrosyl-complex is formed according to the reactions 1 and 2:

$$NO\ (g) \leftrightarrow NO\ (aq) \qquad (1)$$

$$NO\ (aq) + Fe(II)EDTA^{2-} \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \qquad (2)$$

The so called BioDeNOx process combines the principles of wet absorption of NO (in a ± 25 mM $Fe(II)EDTA^{2-}$ solution) with biological reduction of NO in a bioreactor (Buisman *et al.*, 1999). In this process, the biological reduction of NO to di-nitrogen gas (N_2) takes place under thermophilic conditions, at around 50-55 °C, which is the adiabatic temperature of scrubber liquors. When ethanol is used as electron donor, the denitrification reaction occurs according to the overall reaction 3 (Buisman *et al.*, 1999):

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 6\ Fe(II)EDTA^{2-} + 3\ N_2 + 2\ CO_2 + 3\ H_2O \qquad (3)$$

Since industrial flue gasses generally contain 2-8 % oxygen, part of the $Fe(II)EDTA^{2-}$ is oxidized during the scrubbing process to $Fe(III)EDTA^{-}$ according to:

$$4\ Fe(II)EDTA^{2-} + O_2 + 4\ H^+ \rightarrow 4Fe(III)EDTA^{-} + 2\ H_2O \qquad (4)$$

To regenerate the absorption liquor, the $Fe(III)EDTA^{-}$ that is formed by reaction 4 has to be reduced back to $Fe(II)EDTA^{2-}$ according to:

$$12\ Fe(III)EDTA^{-} + C_2H_5OH\ + 3\ H_2O \rightarrow 12\ Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^+ \qquad (5)$$

Thus, besides NO reduction, reduction of EDTA chelated Fe(III) is a core reaction within the regeneration pathway of the BioDeNOx process (Fig. 1).

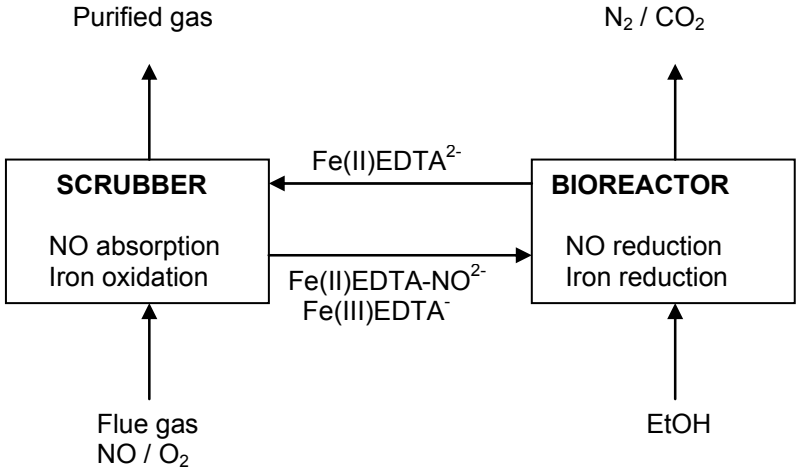


Figure 1
Schematic principle of the BioDeNOx concept

The aim of the present work was to demonstrate the BioDeNOx process in a bench scale installation. The influence of different operational parameters (NO and O_2 content of the flue gas, redox potential and substrate concentration in the bioreactor, role of inoculum) on the NO removal efficiency was determined. The applicability of the redox potential as a steering parameter for the supply of the electron donor ethanol was also investigated. Moreover, the reactor capacity for the biological regeneration of Fe(II)EDTA^{2-} was estimated.

MATERIALS AND METHODS

Reactor configuration

Figure 2 represents schematically the reactor configuration used in this study, which basically consisted of a scrubber that was mounted on top of a bioreactor. A perforated sheet was fixed between the scrubber and the bioreactor to prevent the occurrence of short-circuit streams. The scrubber was configured as a bubble column (height = 0.15 m) with a wet volume of 1.8 dm^3, in which the artificial flue gas entered via a single tube with a diameter of 6 mm. The effluent gas (atmospheric pressure) was used to make up influent flue gas by supplying pure NO and air. Nitrogen gas was supplied to compensate the amount of sampling gas needed for continuous NO_x monitoring (Fig. 2).

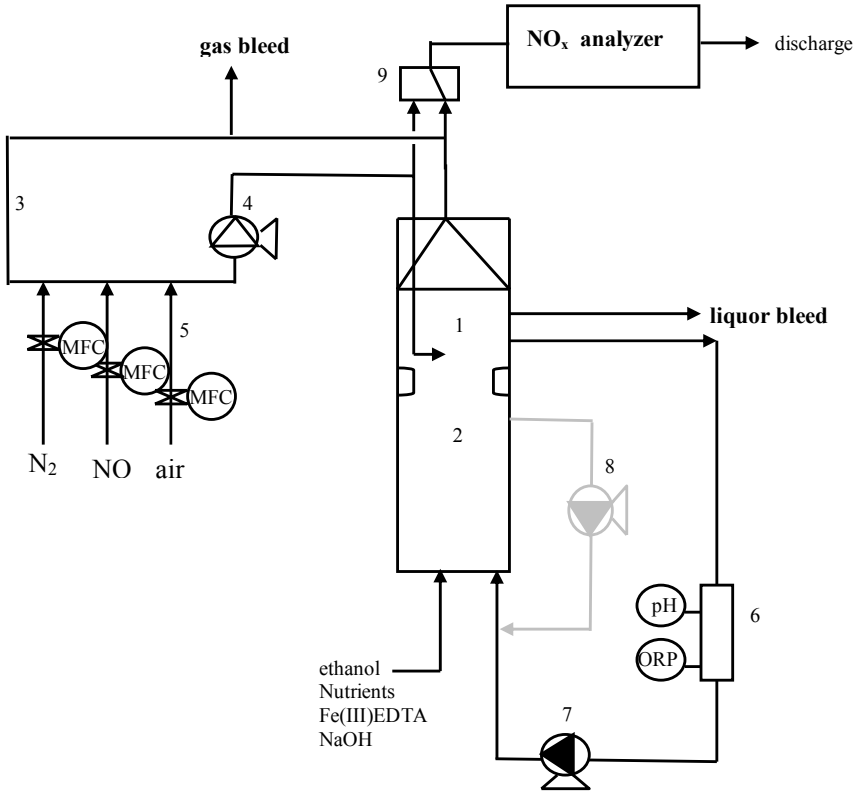


Figure 2

Configuration of the lab-scale bioreactor used in this study. Flue gas is entering the scrubber (1), which is situated on top of the bioreactor (2). The effluent gas is recycled (3) by a gas pump (4) while NO, N_2 and air is supplied continuously by mass flow controllers (5). The scrubber liquor passes a cell with an ORP and pH electrode (6), after which it is pumped (7) to the bioreactor (2). Bioreactor mixing is provided by a recirculation pump (8). The NO_x concentration of the flue gas is monitored continuously, using a selector (9) for in- and effluent sampling.

The bioreactor consisted of a 5.6 dm^3 cylindrical glass reactor. At the bottom of the bioreactor, inlet points were present for the addition of HCl, ethanol and basal medium with nutrients. The scrubber liquid was continuously withdrawn from the scrubber and pumped into the bottom of the bioreactor at a continuous flow rate of 15 l h^{-1}, corresponding to a superficial upflow velocity of 3 m/h. In this way, the reactor liquor was continuously recycled with a hydraulic retention time (HRT) of 22.4 minutes. The pH was automatically controlled by HCl addition at an upper limit of 7.6. Ethanol was supplied both manually and automatically controlled by the ORP signal.

Protein measurements over the reactor height indicated that the reactor content was poorly mixed. Therefore, a second recirculation flow was applied to provide extra mixing of the bioreactor liquor and to increase the superficial upflow velocity to 18 m/h. The temperature of the bioreactor was controlled at 55° C by means of a temperature controlled water jacket. The temperature of the flue gas was not controlled, but it may be assumed that it was slightly below the bioreactor temperature (55 ºC), since the wet scrubber volume determined the major part of the total gas volume.

The redox potential (ORP) and pH of the bioreactor were monitored continuously (Sentix electrodes, WTW, Germany). The measured ORP of the system $Fe(III)EDTA^-/Fe(II)EDTA^{2-}$ depends on the pH (Kolthoff *et al.*, 1952). Under the experimental conditions applied, the ORP increased ± 100 mV per pH unit decrease. ORP values presented in this study are corrected for pH = 7.0 (ORP_c) unless specified otherwise.

Gas and medium composition

The artificial flue gas was composed by combining pure N_2 gas, pure NO gas and pressured air in adjustable amounts with mass flow controllers (Brooks, The Netherlands). The gas was continuously recycled over the scrubber part of the installation (Fig. 2) with a compressor (KNF, Germany). Both the influent and effluent gas were analyzed for NO concentrations using an automatically operating gas selector (WUR, The Netherlands). Gas analysis required a continuous sample flow of 100 ml/min. Therefore, N_2 gas was supplied to the flue gas to prevent underpressure.

The medium used for all experiments contained 25 mM Na_2H_2EDTA (titriplex, Merck), 30 mM $FeCl_3$, 5 mM $MgSO_4$, 5 mM $CaCl_2$ and macro-nutrients (see Chapter 3). The excess of di- and trivalent cations over EDTA was applied to prevent cell lysis (Ayres *et al.*, 1998).

Sources of biomass

Two types of inocula were used: a denitrifying and a methanogenic sludge. 'Veendam' sludge originated from a methanol fed denitrifying fluidized bed reactor, treating surface water (Veendam, The Netherlands). The sludge was pre-cultivated at 55 °C under denitrifying conditions using ethanol as electron donor. Veendam sludge has a high NO removal capacity (Chapter 3). The methanogenic 'Eerbeek' granular sludge originated from a full scale UASB reactor treating pulp and paper mill wastewater (Eerbeek, The Netherlands). Eerbeek sludge has a high EDTA chelated Fe(III) reducing activity (Chapter 5), but no NO reducing activity (Chapter 3).

Start-up bioreactor

The loading of the bioreactor was divided in three periods (Table 1). For the start-up of the BioDeNOx installation, the reactor was filled with medium, which was heated overnight to 55° C. The reactor was inoculated with 250 ml of pre-cultured 'Veendam' sludge to obtain an initial VSS concentration of ca. 0.05 g/l. Ethanol was supplied manually as the sole electron donor. To stimulate the reduction of $Fe(III)EDTA^-$ to $Fe(II)EDTA^{2-}$, initially the flue gas contained no oxygen or NO, but only di-nitrogen (N_2) gas. After all iron was reduced (redox potential < -250 mV), the reactor was loaded with flue gas containing NO and O_2.

Table 1

Flue gas flow and composition during different experimental periods.

period	start-up	continuous operation	after bio-augmentation
Days	0-115	115-196	196-209
Flue gas flow ($l.h^{-1}$) [a]	± 500 [b]	± 550 [c]	± 650 [c]
NO concentration (ppm)	0-170	60-300	60-540
O_2 concentration (%)	0.3-1.3	0.8-1.7	0.9-3.2

[a] Atmospheric pressure, temperature ± 50 ºC
[b] flow based on the gas pump capacity
[c] calculated by means of NO mass balance: ([NO]infl. – [NO]effl.) / NO supplied by MFC

Experimental design

After the start-up period, the reactor was continuously loaded with artificial flue gas containing NO (70 – 300 ppm). The influence of O_2 was determined by a gradual increase of the O_2 concentration, starting with a concentration of 0.8 %. Ethanol was supplied automatically based on the redox potential inside the reactor. Dosing started when the ORP was higher than –180 mV and it switched off at an ORP below -220 mV. Per day, ca. 250 ml fresh medium was added to the reactor. To increase the Fe-reduction capacity, 185 ml of the iron reducing 'Eerbeek' sludge (ca. 24 g VSS) was added to the reactor mixed liquor on day 81 of the continuous operation period. In this final period, the O_2 concentration in the flue gas was stepwise increased up to 3.3 %.

Activity tests

Batch experiments were performed to examine the NO reduction activity of the reactor liquor. These were performed at 55 °C in duplicate using 120 ml serum bottles, as described in Chapter 3. The NO reduction activity of the reactor biomass was determined using reactor liquor containing 3.5 mM ethanol, when the NO load on the bioreactor amounted 11.5 mmol l^{-1} d^{-1} (day 54 of the continuous operation period).

Analyses

The NO concentrations in the in- and effluent gas of the bench-scale installation was measured continuously by a chemolumisence NO analyzer (Beckman model 951). The H_2, O_2, CH_4 and N_2O content of the flue gas as well as the headspace of the batch flasks was analyzed using gas chromatography (Hewlett Packard model 5890A), equipped with a Molsieve 5A Plot column (30 m x 0.53 mm, DF=15) and Thermal Conductivity Detection (TCD). H_2, O_2, N_2, CO_2 and CH_4 were separated at 70° C, N_2O at 150° C. Fe(II)EDTA^{2-} was determined colorimetrically using 1,10-phenantroline (Akzo Nobel, 1996). Ethanol and acetate were determined by gas chromatography (Cervantes *et al.*, 2000).

The biomass content of the reactor liquor was determined via the protein concentration. Reactor samples were centrifuged (10 min, 10.000 rpm) and the supernatant was discarded. The pellet was

dissolved in 1 N NaOH and placed in boiling water for 15 min. After boiling, the sample was neutralised with 1 N HCl and the protein concentration was determined (Bradford, 1976).

Load calculation

The bioreactor load, i.e. the amount of Fe(III)EDTA$^-$ and Fe(II)EDTA-NO^{2-} supplied to the bioreactor, was calculated using mass balances for O_2 and NO. The load of the electron acceptors (O_2, Fe(III)EDTA$^-$ and NO) and the electron donor (ethanol) is expressed as *molar electron equivalents (meq)*: 1 mol ethanol, O_2, NO and Fe(III)EDTA$^-$ corresponds to 12, 4, 2 and 1 meq, respectively.

RESULTS

Iron reduction

To start up the BioDeNOx process, the aqueous Fe(III)EDTA$^-$ solution has to be reduced to Fe(II)EDTA^{2-}. The reduction of EDTA chelated Fe(III) started ± 2 days after the inoculation with Veendam sludge and ethanol supply (Fig 3A). This was accompanied by ethanol consumption and acetate production, as well as a decrease of the redox potential (Fig 3B). After all ethanol was consumed, the acetate concentration depleted as well (Fig. 3A), indicating that also acetate served as electron donor for iron-reduction. The relation between the Fe(II) concentration and the ORP_c (measured ORP but corrected for pH=7) appeared to be well correlated to the Nernst equation (Fig. 3C). The standard redox potential (i.e. the ORP where the concentration of Fe(II)EDTA^{2-} is equal to Fe(III)EDTA$^-$) under the experimental conditions, showed to be around –140 mV versus Ag/AgCl (Fig. 3C).

Redox potential and NO removal efficiency during start up

When the reactor was initially loaded with flue gas (500 l/h) containing 300 ppm NO and 0.8 % O_2 , the ORP_c increased from –300 mV to –150 mV within 1 day, followed by a slow increase to –50 mV within a week. This indicated that the iron reduction capacity of the Veendam inoculum was rather poor. The increase of the redox potential was accompanied by a continuous decrease of the NO removal efficiency. Fig. 4 shows that the NO removal efficiency from the gas phase strongly dropped in case the ORP_c of the bioreactor exceeds -140 mV (Fig. 4).

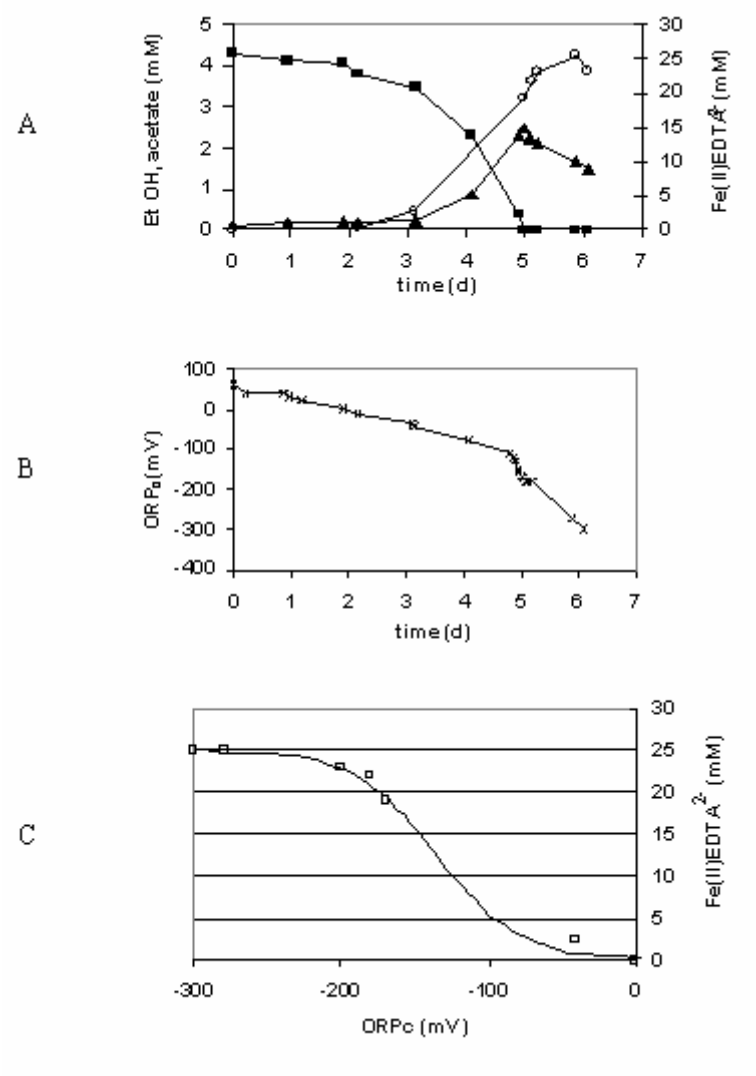


Figure 3

Reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} during the start-up of the BioDeNOx reactor. Fe(III)EDTA$^-$ reduction (A) was accompanied by a decrease of the redox potential (ORPc) (B). The Fe(II)EDTA^{2-} concentration (marks) is well correlated to the ORP_c according to the Nernst equation (line) (C).

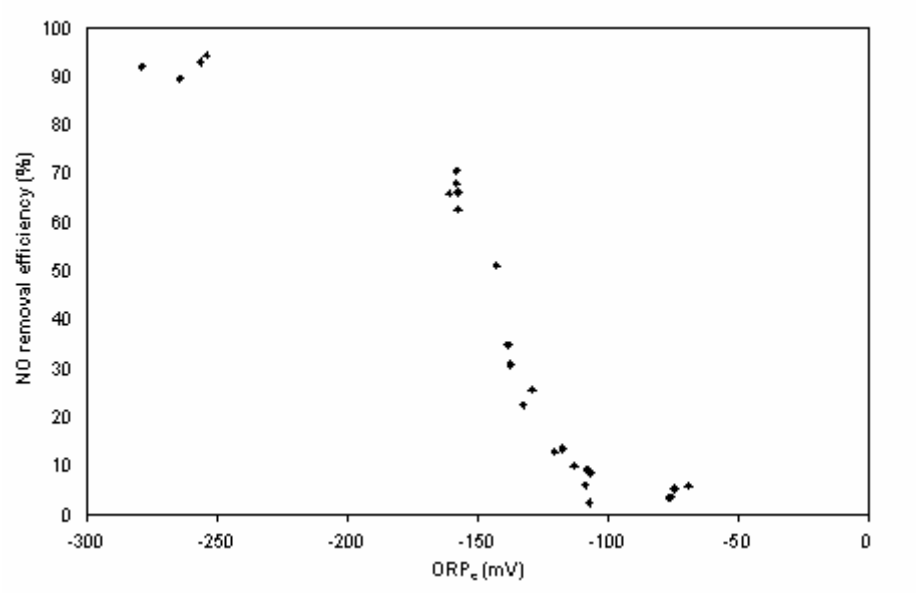


Figure 4

Relation between the ORP_c of the bioreactor and NO removal efficiency from the gas phase.

Continuous operation

The NO removal efficiency from the gas-phase amounted to 40 – 70 %, depending on both the NO influent concentration and the redox potential in the bioreactor (Fig. 5). The NO removal efficiency dropped when the redox potential exceeded –140 mV (Fig. 5A). An increase of the redox potential, i.e. a limiting iron reduction capacity, occurred when the ethanol concentration in the bioreactor was lower than 0.8 mM. As long as enough ethanol was present, the ORP decreased with a simultaneous increase of the NO removal efficiency (Fig. 5A).

After 10 days of continuous operation the NO concentration in the influent gas was stepwise increased to around 300 ppm, while the O_2 concentration remained constant around 0.9%. The increased NO concentration in the flue gas did not affect the ORP_c of the bioreactor. Under these conditions, average NO removal efficiencies of 70% were achieved (Fig. 5A). The load during that period corresponded to 0.023 meq l^{-1} d^{-1} for NO (16 mg N l^{-1} d^{-1}) and 0.047 meq l^{-1} d^{-1} for O_2 (0.38 g O_2 l^{-1} d^{-1}) (Fig. 5B).

After 2 weeks of continuous operation (t = 129 d), ethanol was automatically supplied when the ORP_c exceeded –140 mV. The supply was stopped when the ORP_c dropped below -190 mV. Fig. 5B shows that the intermitted supply of ethanol was successful in keeping the ORP_c at the desired level and to get a stable NO removal efficiency. No acetate accumulated during the whole period of continuous operation (Fig. 5A).

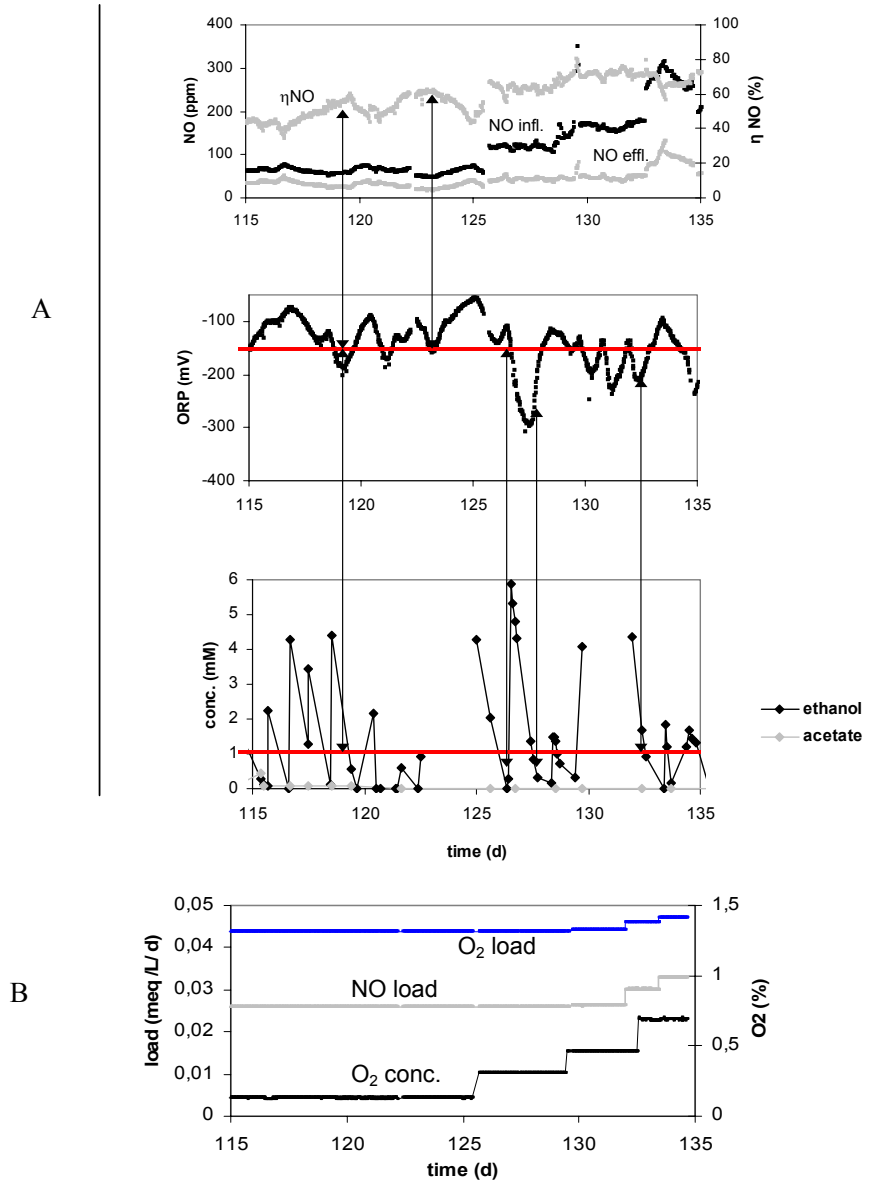


Figure 5

NO removal during continuous operation: the removal efficiency dropped when the ORP exceeded –140 mV, which was the cace at low ethanol concentrations (A). The load of the bioreactor was stepwise increased to 0.023 and 0.027 meq.l^{-1}.day^{-1} for NO and O_2 respectively (B). The flue gas volumetric flow rate was held constant at 550 $l.h^{-1}$.

Increase of oxygen concentration and bio-augmentation

After 37 days of continuous operation (t = 152 d), the O_2 concentration in the flue gas was increased from 0.9 to 1.2 % (Fig. 6A). This resulted in an immediate increase of the ORP from –340 to –100 mV (Fig. 6A) and a simultaneous drop of the NO removal efficiency from 75 to 40% (Fig. 6B). This procedure, e.g. increasing the O_2 concentration to over 1 %, was repeated twice, both resulting in the same phenomenon: a fast increase of the ORP and with a simultaneous decrease of the NO removal

efficiency (data not shown). Apparently, the Fe-reduction capacity of the bioreactor was insufficient to cope with higher oxygen concentrations. To increase the Fe-reduction capacity, 185 ml of anaerobic granular 'Eerbeek' sludge (ca. 24 g VSS) was added to the reactor as an additional inoculum. During a period of 8 days, the NO and O_2 concentrations in the flue gas were then gradually increased (Fig. 7). At a gas flow rate of 650 l/h and an O_2 concentration of 3.3%, the ORP_c remained below –140 mV (Fig. 7C) and the NO removal efficiency remained between 70 and 80 % (Fig. 7A). After the bio-augmentation with the Eerbeek sludge, the bioreactor performance was stable, while the load amounted 0.13 meq l^{-1} d^{-1} for O_2 (130 mmol Fe(III)$EDTA^-$ l^{-1} d^{-1}) and 0.019 meq l^{-1} d^{-1} for NO (9.5 mmol NO l^{-1} d^{-1}) (Fig. 7B). Before the Eerbeek sludge was added, the regeneration capacity was limited at app. 50 % of that load (Table 2). Figure 8 shows that the consumption rate of the electron donor (ethanol) corresponded to the maximal loading of the bioreactor.

Table 2

Flue gas characteristics and bioreactor load before and after bio-augmentation.

	before bio-augmentation		**after bio-augmentation**	
	influent concentration	**bioreactor load (meq l^{-1} d^{-1})**	**influent concentration**	**bioreactor load (meq l^{-1} d^{-1})**
NO	300 ppm	0.023	330 ppm	0.019
O_2	1.0 %	0.047	3.3 %	0.13
Total	-	0.070	-	0.14
Flow ($l.h^{-1}$) [a]	± 550		± 650	

[a] Atmospheric pressure, temperature ± 50 ºC

Biomass characteristics

From the increase of the protein concentration (Fig. 9A) and the ethanol consumption rate (Fig. 8), a biomass yield of 0.04 g protein per meq ethanol can be estimated. The great spreading of the protein concentrations in the early stage is most probably due to an insufficient mixing of the reactor liquor during the start-up period. The cells were homogeneous in morphology. Cells occurred single or in pairs and no flocks were present. Individual cells were rod-shaped, 2-8 µm long and 0.8 µm wide, with rounded ends (Fig. 9B).

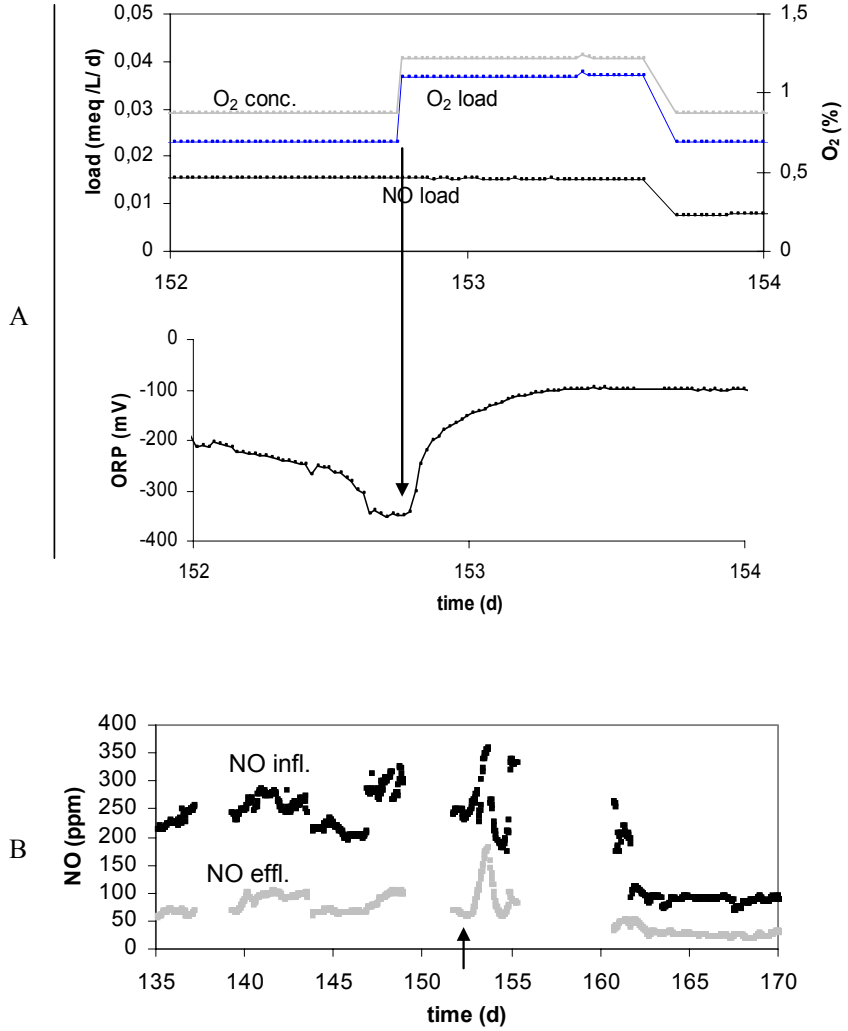


Figure 6

Effect of the increase of the O_2 concentration and O_2 load (day 152) on the ORP_c (A) and the NO removal efficiency (B) of the BioDeNOx reactor. The flue gas volumetric flow rate was held constant at 550 $l.h^{-1}$.

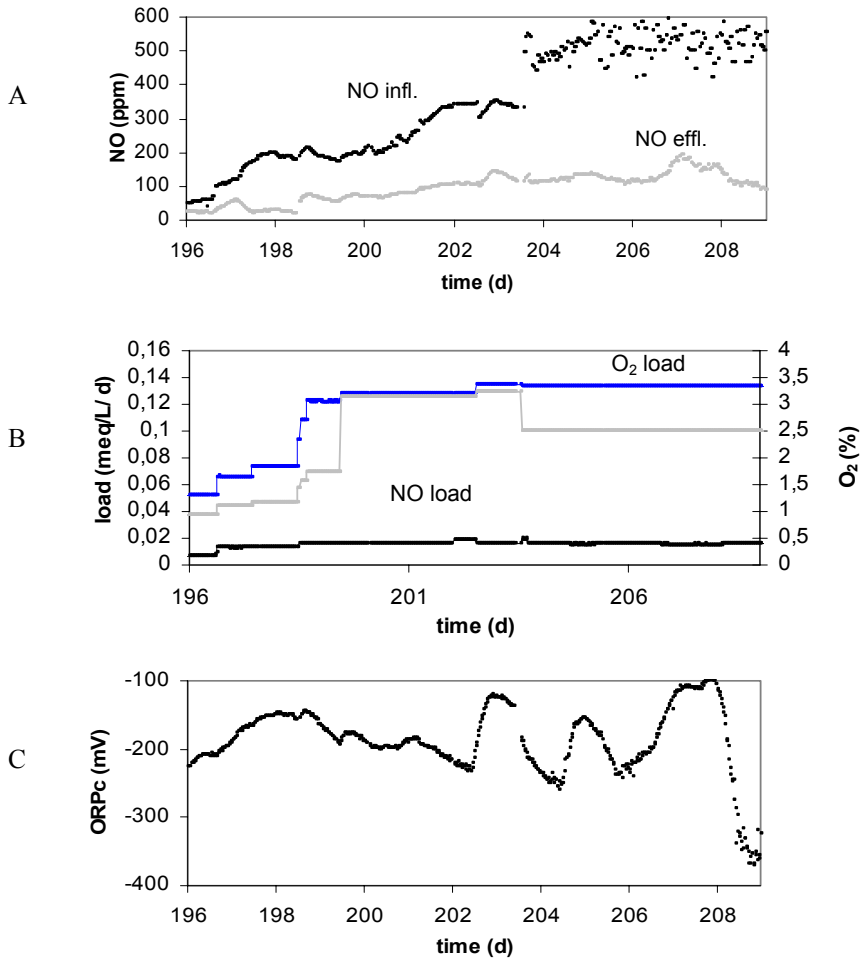


Figure 7

NO removal after bio-augmentation of the BioDeNOx reactor with Eerbeek sludge. The NO removal efficiency (A) was not effected by the stepwise increase of the O_2 concentration and O_2 load (B) and the ORP_c remained below –140 mV (C). The flue gas volumetric flow rate was held constant at 650 $l.h^{-1}$.

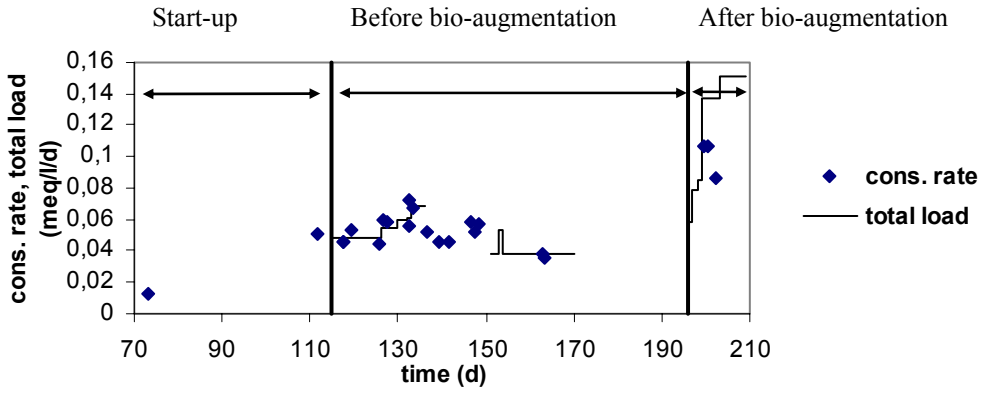
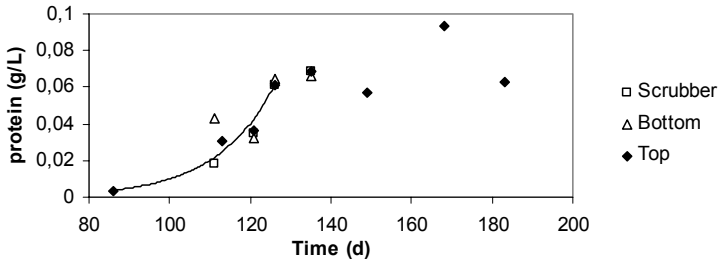


Figure 8

Maximum ethanol consumption rate and total load (sum of NO and O_2 load) on the bioreactor before and after bio-augmentation with Eerbeek sludge.

A



B

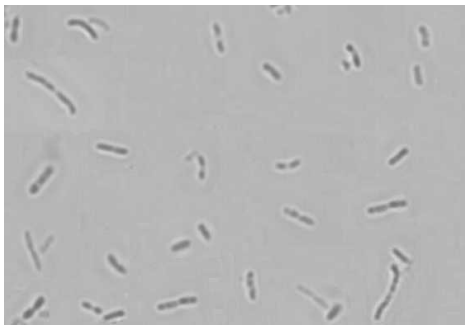


Figure 9

Biomass characteristics of the BioDeNOx reactor liquor: evolution of the protein concentration with time (A) and bacterial morphology (phase-contrast microscopy) of the reactor mixed liquor 6 days after bio-augmentation with Eerbeek sludge (B).

NO reduction in batch mode

Figure 10 shows the reduction of NO to gaseous nitrogen compounds in batch experiments. Within several hours, N_2O accumulated in the headspace. At the time all NO was reduced, also the N_2O was converted to N_2. The rate of NO conversion to N_2 amounted to at least 0.67 mM h^{-1}, corresponding to 0.032 mmeq l^{-1} d^{-1}.

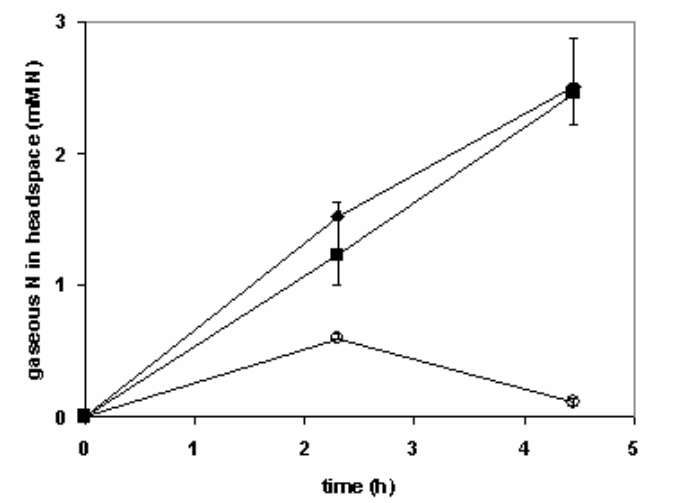


Figure 10

Production of N_2O (○), N_2 (■) and total gaseous nitrogen (sum of N_2 and N_2O) (◆) during the reduction of NO in batch experiments using BioDeNOx reactor sludge sampled after 54 days of continuous operation.

DISCUSSION

NO removal efficiency and redox potential

This study showed that a stable NO removal with an efficiency of more than 80 % from the gas phase can be obtained by the BioDeNOx concept, when treating a flue gas containing 500 ppm NO and 3.3 % O_2 (Fig. 7). A bioreactor load up to 0.023 meq.l^{-1}.d^{-1} for NO and 0.13 meq.l^{-1}.d^{-1} for Fe(III)$EDTA^-$ was completely regenerated in case the redox potential of the bioreactor was maintained below -140 mV (corrected for pH 7). For stable operation of the BioDeNOx reactor, a high Fe(II)EDTA concentration, e.g. a low redox potential (< -140 mV) is needed (Fig. 4). When the ORP_c exceeds that value, the NO removal efficiency strongly declined (Fig. 5). This critical ORP_c value corresponds with the redox potential where 50 % of the of the aqueous FeEDTA is in the oxidized state (Fig. 3C), as well as with the E^0 values of the Fe(II)$EDTA^{2-}$/Fe(III)$EDTA^-$ redox system that are reported in literature (Kolthoff *et al.*, 1952). To keep the redox potential low, the NO and iron reduction (reaction 3 and 5) rate should be equal to or higher than the NO and Fe(III) loading rates, respectively (reactions 2 and 4).

Note that the NO removal efficiency from the gas phase was limited by the absorption kinetics of the scrubber. Since the flue gas was supply bubbled through the solution via a single open tube, suboptimal mass transfer probably contributed to the limited NO removal from the gas phase. The NO and iron reduction efficiency of the bioreactor was 100 %, i.e. the total NO and Fe(III)$EDTA^-$ load of the bioreactor was reduced to N_2 and Fe(II)$EDTA^{2-}$, respectively. The latter can be derived from the fact that the ORP_c is maintained constant below -140 mV. An incomplete regeneration would lead to accumulation of Fe(III)$EDTA^-$ and/or Fe(II)EDTA-NO^{2-} in the bioreactor, resulting in an increasing redox potential. The NO removal efficiency from the gas phase primary depends on the kinetics of NO absorption to the scrubber liquor, i.e. the aqueous FeEDTA solution. The NO absorption rate follows first order kinetics for both NO and Fe(II)$EDTA^{2-}$ concentration (Demmink *et al.*, 1997). This explains why the NO removal efficiency from the gas phase strongly depends on the oxidation state of the aqueous FeEDTA solution, and thus on its redox potential (Fig. 4). Furthermore, the observation that the removal efficiency from the gas phase increased at higher NO influent concentrations (Fig. 5A) can be explained by the first order absorption kinetics. Note that the NO absorption rate is not directly affected by the presence of oxygen (Gambardella *et al.*, 2005), but indirectly due to a lower Fe(II)$EDTA^{2-}$ concentration, i.e. a higher redox potential.

Depletion of ethanol below the critical value of 0.8 mM caused an insufficient Fe(II)$EDTA^{2-}$ regeneration rate, resulting in an increasing redox potential (Fig. 5A). However, timely supply of ethanol lowered the redox potential again (Fig. 5A). Redox potentials below -300 mV indicate an overdosing of ethanol: while all FeEDTA is in the reduced state, ethanol and or acetate will be used for other biological processes like sulfate reduction and methanogenesis (Chapter 5). As this represents an inefficient ethanol consumption, a proper control of the electron donor supply on the BioDeNOx bioreactor is important.

Regeneration capacity – NO reduction

As mentioned above, the regeneration capacity of the bioreactor is determined by the volumetric rate of two processes: reduction of NO to N_2 and reduction of $Fe(III)EDTA^-$ to $Fe(II)EDTA^{2-}$. As reported in the Chapters 2 and 3, the reduction of NO in aqueous $Fe(II)EDTA^{2-}$ solutions is catalyzed by a biological conversion in which Fe(II)EDTA is most likely used as the electron donor, according to:

$$2\ Fe(II)EDTA\text{-}NO^{2-} + 2\ Fe(II)EDTA^{2-} + 4\ H+ \rightarrow 2\ H_2O + N_2 + 4\ Fe(III)EDTA^-$$

The volumetric NO reduction rate was higher than the maximal NO load applied to the bioreactor (Table 2). This means that NO reduction was not limiting the regeneration capacity of the bioreactor. The reduction of NO to N_2 in aqueous $Fe(II)EDTA^{2-}$ solutions was shown to be a two step reaction with N_2O as an intermediate (Chapter 3). Contrary to the batch experiments (Fig. 10), no accumulation of N_2O was detected during the continuous reactor experiments (data not shown).

Some bacterial species can indeed use ferrous iron as electron donor for denitrification (Straub *et al.* 2001), which might be the mechanism of NO reduction in aqueous $Fe(II)EDTA^{2-}$ solutions. Alternatively, the classical denitrification pathway is followed. Since NO reduction to N_2 is involved in the denitrification pathway (Zumft, 1993) and the Veendam inoculum used in this study originates from a denitrifying plant, it is likely that the two bacterial enzymes, namely NO reductase (NOR) as well as N_2O reductase (N_2OR) are present in the sludge and thus are involved in the BioDeNOx regeneration process. As $Fe(II)EDTA^{2-}$ is reported to be an excellent electron donor for Cytochrome c reduction (Hodges *et al.*, 1974), which is an electron carrier for both NOR and N_2OR (Wasser *et al.*, 2002), the chelated ferrous iron might enhance the denitrification rates by chemical regeneration of the electron carriers.

Regeneration capacity – $Fe(III)EDTA^-$ reduction

In contrast to NO reduction, the reduction of EDTA chelated Fe(III) was the limiting process for the regeneration of scrubber liquor. A slight increase of the O_2 concentration in the flue gas from 0.9 to 1.2 % resulted in an increasing OPR and a decreasing NO removal efficiency (Fig. 6). Other studies showed that original Veendam inoculum used in this study has indeed a rather low potential for the reduction of EDTA chelated Fe(III) (Chapter 5). The maximal O_2 load that could be applied amounted to only 0.047 meq $l^{-1}.d^{-1}$, which was already reached when the flue gas contained only 1.0 % O_2. Bio-augmentation of the Veendam sludge with Eerbeek sludge resulted in a strong increase of the Fe-reduction capacity. Also when the flue gas contained 3.3 % O_2, the ORP_c of the bioreactor remained below the critical value of – 140 mV (Fig. 7). In that case, the $Fe(III)EDTA^-$ load of the bioreactor (0.13 meq.$l^{-1}.d^{-1}$) was nearly 3 times as high as the maximal load that could be applied in case the reactor was inoculated with Veendam sludge (0.047 meq.$l^{-1}.d^{-1}$).

The principle of Fe(III) reduction when bound to strong chelating agents like EDTA is still a matter of debate. Chelating agents like NTA and EDTA might stimulate dissimilatory iron reduction because they keep Fe(III) soluble, and therefore they promote the bio-availability under neutral pH conditions (Lovley *et al.*, 1990). In contrast, others concluded that EDTA chelated Fe(III) was not bio-available

for *Shewanella putrefaciens* (Haas and DiChristina., 2002). They presumed that the strong binding properties of EDTA inhibited the bacterial uptake of Fe(III). However, the set-up of that study (Haas and DiChristina, 2002) suggests the presence of high concentrations of free EDTA, e.g. not complexed to iron or any other transition metal. This is important to notice, since free EDTA can strongly inhibit bacterial activity (Leive, 1968).

Regeneration capacity – biotic versus chemical processes

The electron flow within the regeneration pathway involves most likely a sequence of redox processes (Table 3). First of all, ethanol is partly oxidized to acetate (Fig. 3), probably coupled to hydrogen production. The latter compound may be the electron donor for sulfate or elemental sulfur reducing bacteria, which convert endogeneous sulfur sources to sulfide. This reductant is known to react very fast with Fe(III)EDTA$^-$ with the concomitant production of elemental sulfur and Fe(II)EDTA^{2-} (Dalrymple *et al.*, 1989). Fe(II)EDTA^{2-} is most likely involved in enzymatic NO reduction, with the concomitant formation of Fe(III)EDTA$^-$ (Chapter 3). The proposed regeneration pathway is schematically presented in Fig. 11. The proposed pathway implicates that both biological and chemical conversions are involved in the BioDeNOx regeneration process. This could explain the low biomass yield measured by the protein concentration (Fig. 9A). Also the electron balance (Fig. 8) suggests that the biomass yield is low. To reduce the NO and Fe(III)EDTA$^-$ load, practically all ethanol has to be used as electron donor, so nearly no carbon is left for cell synthesis.

Further developments

This study showed that for the successful application of BioDeNOx, it is crucial that the redox potential in the bioreactor is kept low (< -140 mV versus Ag/AgCl). Therefore, it is important to optimize the iron reduction process, as well as to minimize the influence of oxygen in the flue gas on the iron oxidation rate. Minimization of the iron oxidation rate can possibly be achieved by the application lower FeEDTA concentrations or by replacing EDTA by another ligand that is less sensitive for oxidation by oxygen, e.g. methyliminodiacetic acid (MIDA) or ethylenediamine-*N,N'*-diacetic acid (EDDA). However, a strong correlation was shown between the NO absorption properties of a ligand and its sensitivity for oxidation by oxygen (Schneppensieper *et al.*, 2001). Although that research was performed at lower a pH (pH = 5), replacing the ligand might lead to lower NO removal rates.

Batch experiments on the kinetics of Fe(III) reduction indicated that the presence of reduced sulfur compounds increases the Fe-reduction rate (Chapter 5). Further research is required to demonstrate that higher volumetric iron reduction rates can be obtained when reduced sulfur compounds are supplied to enhance the electron transfer to Fe(III)EDTA$^-$. Alternatively, ethanol might be replaced by carbohydrates like glucose or molasses, since these substrates showed higher volumetric Fe(III)EDTA$^-$ reduction rates (Chapters 5 and 6).

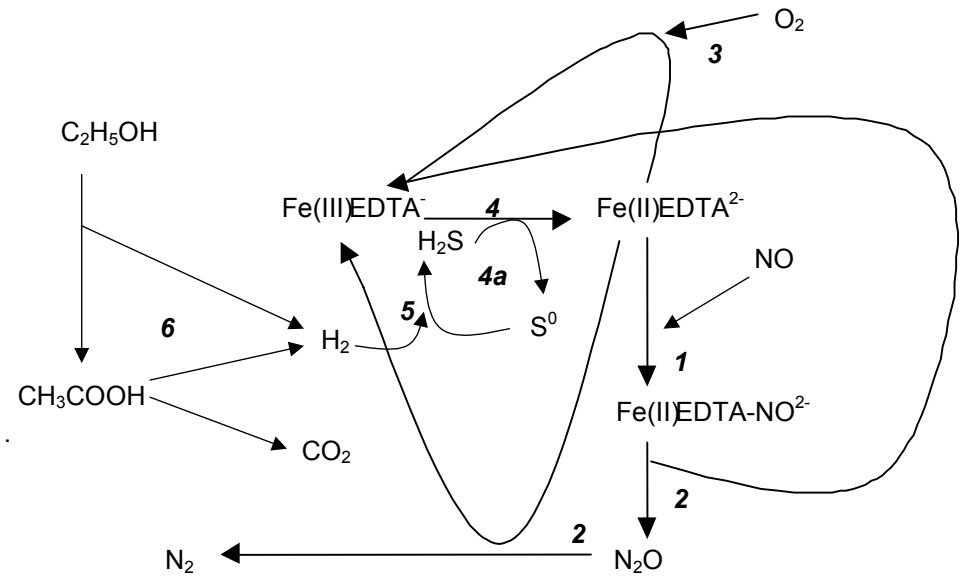


Figure 11

Proposed pathway of the BioDeNOx regeneration process. 1. Absorption of NO to aqueous Fe(II)EDTA^{2-} . 2. Denitrification of the sorbed NO with Fe(II)EDTA^{2-} as electron donor. 3. Oxidation of chelated Fe(II) by the oxygen present in the flue gas. 4. Reduction of Fe(III)EDTA^{-} with sulfide / elemental sulfur as electron shuttle (4a). 5. Biological sulfur reduction with electrons provided by the syntrophic converion of ethanol (6).

Table 3

Possible redox reactions involved in the BioDeNOx regeneration process.

Ethanol oxidation (biological)
$C_2H_5OH + H_2O \rightarrow CH_3COO^- + 2\ H_2 + H^+$
$CH_3COO^- + 2\ H_2O + H^+ \rightarrow 4\ H_2 + 2\ CO_2$
Sulfur reduction (biological)
$S^0 + H_2 \rightarrow H_2S$
$4\ S^0 + CH_3COO^- + 2\ H_2O + H^+ \rightarrow 4\ H_2S + 2\ CO_2$
$6\ S^0 + C_2H_5OH + 3\ H_2O \rightarrow 6\ H_2S + 2\ CO_2$
Iron reduction (chemical)
2 Fe(III)EDTA$^- + H_2S \rightarrow S^0 + 2$ Fe(II)EDTA$^{2-} + 2\ H^+$
NO reduction (biological)
NO + Fe(II)EDTA$^{2-} + H^+ \rightarrow$ Fe(III)EDTA$^- + ½\ N_2O + H_2O$
$N_2O + 2$ Fe(II)EDTA$^{2-} + 2\ H^+ \rightarrow H_2O + N_2 + 2$ Fe(III)EDTA$^-$

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CHAPTER 5

Enzymatic versus nonenzymatic conversions during the reduction of ferric EDTA

Reduction of EDTA-chelated Fe(III) is one of the core processes in the BioDeNOx process. The capacity of *Escherichia coli*, three mixed cultures from full scale methanogenic granular sludge reactors, one denitrifying sludge, and a BioDeNOx sludge to reduce Fe(III)EDTA$^-$ (25 mM) was determined at 37 and 55 °C using batch experiments. All inocula were capable to reduce Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-}. Addition of catalytic amounts of sulfide to the mixed cultures greatly accelerated Fe(III)EDTA$^-$ reduction, indicating that biological Fe(III)EDTA$^-$ reduction is not a direct, enzymatic conversion but an indirect reduction with involvement of an electron-mediating compound, presumably polysulfides. It is suggested that not thermophilic dissimilatory iron-reducing bacteria but reducers of elemental sulfur or polysulfides are primarily involved in the reduction of EDTA-chelated Fe(III) in BioDeNOx reactors.

A modified version of this chapter is published as:

Van der Maas P, Peng S, Klapwijk B and Lens P (2005). Enzymatic versus Nonenzymatic Conversions during the reduction of EDTA-chelated Fe(III) in BioDeNOx Reactors. *Envion. Sci. Technol.*, 39, 2616-2623.

INTRODUCTION

Reduction of EDTA-chelated Fe(III) is one of the core processes in the BioDeNOx process, a chemically enhanced technique for biological NO_x removal from industrial flue gases (Buisman *et al.*, 1999). In the BioDeNO$_x$ concept reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} takes place in a bioreactor under thermophilic (±55 ºC) conditions and near neutral pH (pH 7.0-7.4). As only Fe(II)EDTA^{2-} and not Fe(III)EDTA$^-$ can absorb NO, the NO removal capacity of a BioDeNOx installation strongly depends on the iron reduction capacity of the bioreactor (Chapter 4). Thus, optimization of Fe(III)EDTA$^-$ reduction is essential in the development of BioDeNOx technology.

Iron reduction by dissimilatory iron-reducing bacteria, a special group of microorganisms able to conserve energy to support growth from electron transport to Fe(III), is widely documented in the literature, as reviewed by Lovley (1991 and 1997) and Lloyd (2003). However, most investigations have focused on the reduction of crystalline iron (e.g., poorly crystalline iron oxides) using pure cultures (Lovley *et al.*, 1993; Dobbin *et al.*, 1999) or mixed cultures from natural environments such as soil and sediments (Tuccillo *et al.*, 1999) or activated sludge (Nielsen *et al.*, 1997). Very limited information is available on biological reduction of EDTA-chelated Fe(III). Lovley *et al.* (1996a and 1996b) suggested that chelation to NTA or EDTA enhances the microbial Fe(III) reduction rate because then Fe(III) stays soluble under neutral pH, thus making Fe(III) more accessible to the microbial cell. However, Fe(III)EDTA$^-$ in high concentrations (25 mM), typical concentrations for the BioDeNOx concept (Buisman *et al.*, 1999), is reported to be a rather poor electron acceptor for most known dissimilatory iron-reducing bacteria (Finneran *et al.*, 2002).

A variety of electron donors can reduce Fe(III)EDTA$^-$ chemically, e.g., sulfide (Wubs and Beenackers, 1994), dithionite (Kleifges *et al.*, 1997), ascorbic acid (Mauricio *et al.*, 2003) or thionine (Tanaka *et al.*, 1983). This raises the question of whether in the BioDeNOx process the reduction of Fe(III)EDTA$^-$ is a direct biological conversion by iron-reducing bacteria or an indirect chemical reduction with involvement of an extracellular electron-mediating compound that transfers electrons from (nondissimilatory iron reducing) bacteria to the EDTA-chelated Fe(III), as illustrated by Figure 1.

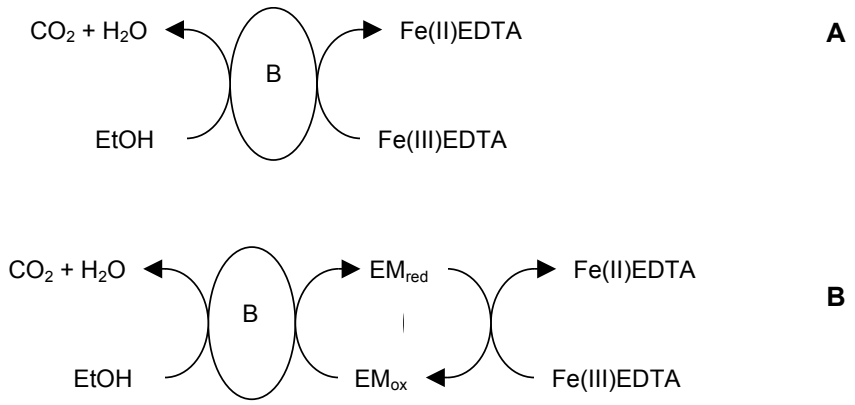

Figure 1

Schematic representation of direct (A) versus indirect (B) reduction of Fe(III)EDTA$^-$. B = bacterium/bacterial consortium. EM = electron mediator.

The goal of the experiments described in this chapter was to illuminate the reaction mechanism of EDTA-chelated Fe(III) reduction under BioDeNOx conditions using batch experiments. *Escherichia coli*, known to be incapable of reducing crystalline Fe(III) (Lovley *et al.*, 1991), and mixed cultures from a laboratory BioDeNOx reactor as well as full-scale anaerobic and denitrifying reactors were screened for their capacity to reduce 25 mM Fe(III)EDTA$^-$ at 37 and 55 ºC. This Fe(III)EDTA$^-$ concentration is representative for practical applications of BioDeNOx (Buisman *et al.*, 1999). The potential of the sulfide/elemental sulfur redox couple to act as an electron-mediating compound during Fe(III)EDTA$^-$ reduction was also investigated.

MATERIALS AND METHODS

Medium

The standard Fe(III)EDTA$^-$ medium contained (unless specified otherwise) NaH_2EDTA (Titriplex; 25 mmol/L), $FeCl_3$ (30 mmol/L), $MgSO_4 \cdot 7H_2O$ (5 mmol/L), $CaCl_2 \cdot 2H_2O$ (5 mmol/L), NH_4Cl (0.28 g/L), KH_2PO_4 (0.25 g/L), $NaHCO_3$ (5.4 g/L), and trace elements solution 1 mL·L (Visser *et al.*, 1993). The medium was prepared by adding sequentially EDTA, $FeCl_3$, nutrients, and metals to demineralized water. Then the pH was adjusted to 7.2 by adding sodium hydroxide, and finally, $NaHCO_3$ (buffer) was added. Medium without sulfate was prepared as the standard medium, but $MgSO_4 \cdot 7H_2O$ was replaced by $MgCl_2 \cdot 2H_2O$. Medium without EDTA was prepared as the standard medium but without EDTA addition. In that case brown iron precipitates were formed during pH neutralization (crystalline ferric iron). All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany).

Sources of Biomass

E. coli NCTC 9002 was kindly provided by the Microbiology Department of Wageningen University. The strain was precultivated for 2 days at 37 ºC in LB medium (Rainer *et al.*, 1990). 'Nedalco' sludge is comprised of an anaerobic, methanogenic granular sludge taken from a full-scale expanded granular sludge bed (EGSB) reactor treating alcohol distillery wastewater at 30 °C (Bergen op Zoom, The Netherlands). 'Eerbeek' and 'Hoogezand' sludge are anaerobic methanogenic granular sludges as well, cultivated in full-scale upflow anaerobic sludge bed (UASB) reactors treating paper mill wastewater at 30 °C (located in Eerbeek and Hoogezand, The Netherlands, respectively). 'Veendam sluge' was taken from a full-scale fluidized bed denitrification reactor treating surface water under psychrophilic conditions (Veendam, The Netherlands) (Chapter 3). BioDeNOx sludge was taken from a 6-L lab-scale BioDeNOx reactor after 2 months of operation. The reactor, originally inoculated with Veendam sludge, treated artificial flue gas at a NO and O_2 loading rate of ca. 0.7 and 6 $mmol \cdot L^{-1} \cdot h^{-1}$, respectively.

Before use the Eerbeek, Nedalco, and Hoogezand granules were crushed in standard medium (1/3 m/m) with a handheld blender (Braun, 160 W) for 5 min. The Veendam inoculum (biofilm on sand) and BioDeNOx sludge were used without pretreatment.

Determination of the Fe(III)EDTA$^-$ Reduction Rate.

To investigate the Fe reduction rate of various sludges, batch experiments were performed in duplicate at 55 °C using 300-mL double-jacket glass reactors. The reactors were equipped with an pH and ORP electrode (WTW Sentix, Germany) for online pH and redox potential (ORP) measurement. The reactors were filled with 200 mL of standard Fe(III)EDTA$^-$ medium, and then the headspace was replaced with Ar/CO_2 (80/20 v/v). The reactors were subsequently inoculated by injection of the BioDeNOx sludge or the crushed granules (Nedalco, Eerbeek, Hoogezand) or by addition of the biomass on sand (Veendam). In the latter case the biomass was added before headspace replacement. Unless otherwise specified, the initial VSS concentration was ± 0.07 g/L. Finally, ethanol (10 mM) and Na_2S (0.1-5 mM) were added from concentrated stock solutions by syringe as potential electron donor and potential electron mediator, respectively. The reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} was monitored online by means of the ORP. As illustrated in Figure 2, the relation between the ORP and Fe(II)EDTA^{2-} concentration is well correlated with the Nernst equation according to:

$$[Fe(II)EDTA^{2-}] = [FeEDTA_{tot}] / (1 +(\exp((ORP - E_0)/RT/nF)))$$

where

$[FeEDTA_{tot}] = [Fe(II)EDTA^{2-}] + [Fe(III)EDTA^-] = 25$ mM

ORP = redox potential versus Ag/AgCl under experimental conditions, and E_0 = standard redox potential versus Ag/AgCl under experimental conditions = -140 mV (Schwarzenbach and Heller, 1951).

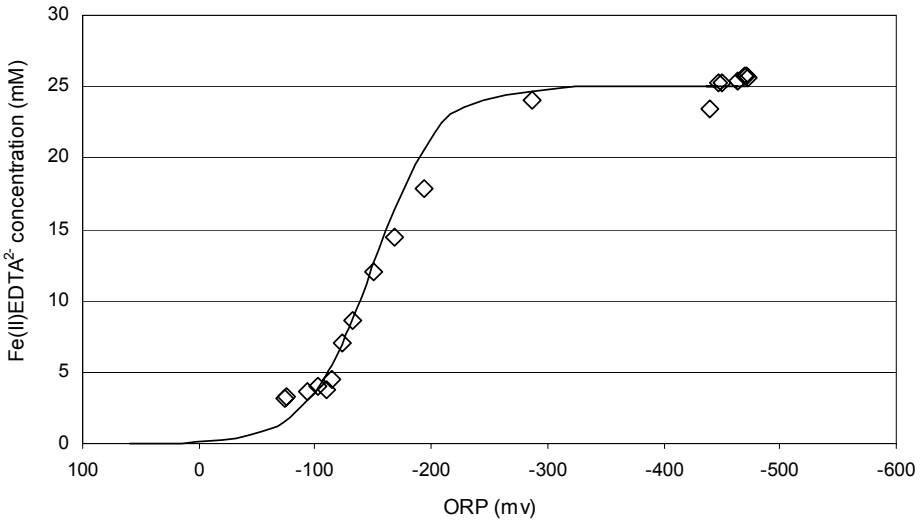

Figure 2

Relation between ORP (mV versus Ag/AgCl) and Fe(II)EDTA^{2-} concentration according to analytical data (◇) and predicted by the Nernst equation (line).

Iron Reduction by E. coli and Eerbeek Sludge at 37 °C

Iron reduction by *E. coli* was investigated using batch experiments at 37 °C. Serum bottles (120 mL) were filled with 80 mL of medium and sealed with thick butyl-rubber stoppers (Rubber BV, The Netherlands). The headspace (40 mL) was replaced with Ar/CO_2 (80/20 v/v), and glucose (10 mM) was added from a stock solution by means of a syringe. After sterilization (autoclaving 2 times for 20 minutes at 124 °C) the vials were stored at 37 °C overnight for temperature equilibration. Bottles were inoculated by injection of 1 mL of the precultivated *E. coli* suspension and incubated at 37 °C. As a reference, similar experiments were conducted using 1 mL of crushed Eerbeek sludge as inoculum. Reduction of EDTA-chelated Fe(III) was monitored by measuring the Fe(II) concentration. Also, the headspace was analyzed for hydrogen and methane formation. Assays without biomass served as chemical control.

Analyses

Ethanol and volatile fatty acids (VFA) in the liquid phase were analyzed by gas chromatography according to Cervantes *et al.* (2000). Ferrous iron was determined colori metrically using the 1,10-phenantroline method according to Akzo Nobel (1996). Volatile suspended solids (VSS) were determined according to standard methods (APHA, 1985). The protein concentration was determined according to Bradford (1976). Sulfate was measured by ion chromatography (IC DX-600, Dionex Corp., Salt Lake City, UT). The hydrogen and methane concentrations in the gas phase were determined by gas chromatography according to Weijma *et al.* (2000).

RESULTS

Iron Reduction by E. coli and Eerbeek Sludge at 37 ºC

Figure 3A shows that *E. coli*, reported to be incapable of reducing poorly crystalline Fe(III) oxides, was capable of reducing EDTA-chelated Fe(III) at 37 ºC during the oxidation of glucose. In the case where EDTA was absent, no substantial Fe(III) reduction occurred (Figure 3A). Glucose conversion by *E. coli* was accompanied by H_2 production (Table 1). Also, in the case where the vials were inoculated with Eerbeek sludge, it is clear that the presence of EDTA stimulated the reduction of Fe(III) to Fe(II) (Figure 3B). Note that injection of crushed Eerbeek sludge at $t = 0$ resulted in an immediate production of $Fe(II)EDTA^{2-}$ (Figure 3B). In contrast to *E. coli*, the Eerbeek inoculum was capable of reducing crystalline Fe(III) but Fe reduction stopped when a concentration of 5 mM Fe(II) was reached (Figure 3B). Besides iron reduction, glucose conver sion by the Eerbeek sludge yielded H_2 and CH_4 (Table 1). Fe reduction was negligible when the bottles were not inoculated (Figure 3B) or (in the experiments using *E. coli*) in the absence of glucose (Figure 3A).

Table 1

Final H_2 and CH_4 Concentration Detected in the Headspace of Various Assays

Assay	H_2 (%)	CH_4 (%)
Fe(III)EDTA$^-$ medium		
E-coli, 37 °C, glucose	1.2 ± 0.3	not detectable
Eerbeek, 37 °C, glucose	3.1 ± 1.0 *	7.5 ± 0.2
Eerbeek, 55 °C, ethanol	1.0 ± 0.1 *	4.7 ± 1.4
Crystalline Fe medium		
E-coli, 37 °C, glucose	1.2 ± 0.2	not detectable
Eerbeek, 37 °C, glucose	1.4 ± 0.1	1.0 ± 0.4

[a] Not final but maximal concentration of H_2 during the experiment.

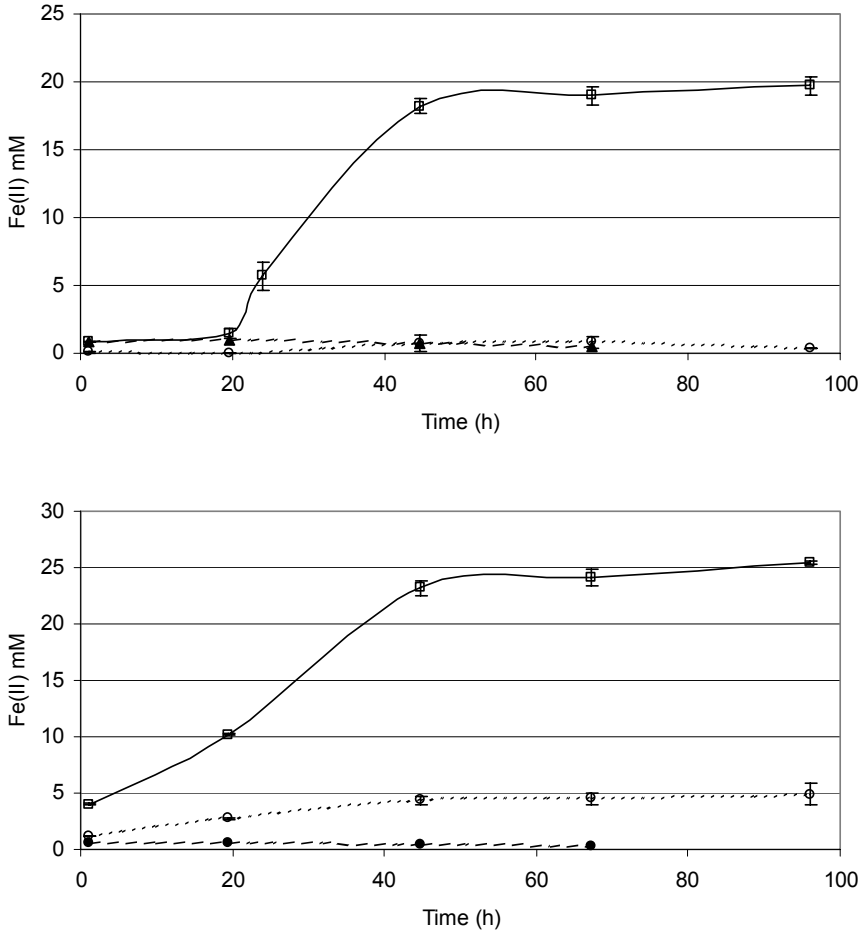

Figure 3

Production of Fe(II)EDTA^{2-} (□) by *E. coli* NCTC 9002 (A) and Eerbeek sludge (B) at 37 °C (pH 7.2 ± 0.2) with glucose as electron donor. No or only limited Fe(II) production occurred in the absence of EDTA (○) or in the absence of glucose (▲) or biomass (●).

Iron Reduction at 55 °C

Figure 4 shows Fe(III)EDTA$^-$ reduction by Eerbeek sludge with ethanol as the electron donor at 55 °C. Upon termination of the experiment, H_2 and CH_4 accumulated in the headspace of the test bottles (Table 1). Also, the Veendam, Hoogezand, and Nedalco inocula were capable of reducing Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} at 55 °C at high conversion rates with ethanol as electron donor (Table 2). Fe(III)EDTA$^-$ reduction was accompanied by ethanol consumption and acetate production. After iron reduction was complete, the sulfate concentration decreased (Figure 4). Figure 5 shows that the presence of 5 mM sulfate did not influence the iron reduction rate. In the case where the medium contained 25 mM $FeCl_3$ and 30 mM EDTA^{4-}, thus 5 mM EDTA^{4-} was uncomplexed, Fe reduction was completely inhibited (Figure 5).

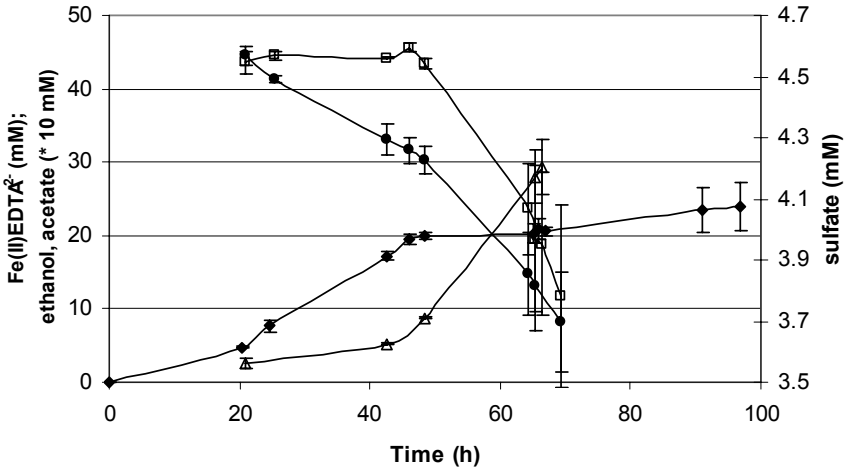

Figure 4

Production of Fe(II)EDTA^{2-} (◆) by Eerbeek sludge with ethanol at 55 °C. Ethanol (●) consumption was accompanied by production of acetate (△). Reduction of sulfate (□) occurred after iron reduction was practically finished. Ethanol was injected at t = 19 hours.

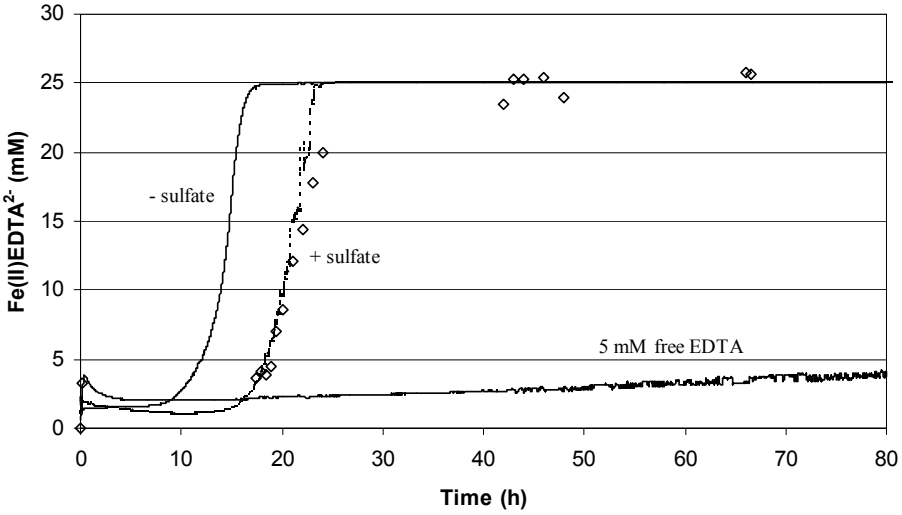

Figure 5

$Fe(II)EDTA^{2-}$ production by Eerbeek sludge at 55 °C with ethanol in the presence and absence of 5 mM sulfate. $Fe(III)EDTA^{-}$ reduction was strongly inhibited in case the medium contained 30 mM EDTA and 25 mM Fe(III). The $Fe(II)EDTA^{-}$ concentration was monitored by the ORP using the Nernst equation (lines) and checked by $Fe(II)EDTA^{-}$ measurements (◇) in medium with sulfate.

Influence of Sulfide on Fe Reduction Rate

Addition of low concentrations of sulfide (0.1 mM) resulted in higher $Fe(III)EDTA^{-}$ reduction rates. Figure 6 shows that an increasing sulfide concentration resulted in an increasing reduction rate for both Eerbeek and Nedalco sludges. Addition of Na_2S to the bottles (t = 0) resulted in an immediate formation of $Fe(II)EDTA^{2-}$ (Figure 6). The highest $Fe(III)EDTA^{-}$ reduction rate was observed when 1 mM sulfide (Eerbeek, 8.3 $mmol \cdot L^{-1} \cdot h^{-1}$) or 2 mM sulfide (Nedalco, 6.7 $mmol \cdot L^{-1} \cdot h^{-1}$) was added to the assays. Addition of 1 mM sulfide also accelerated the Fe reduction by the Veendam and the Hoogezand inocula with 97% and 220%, respectively (Table 2).

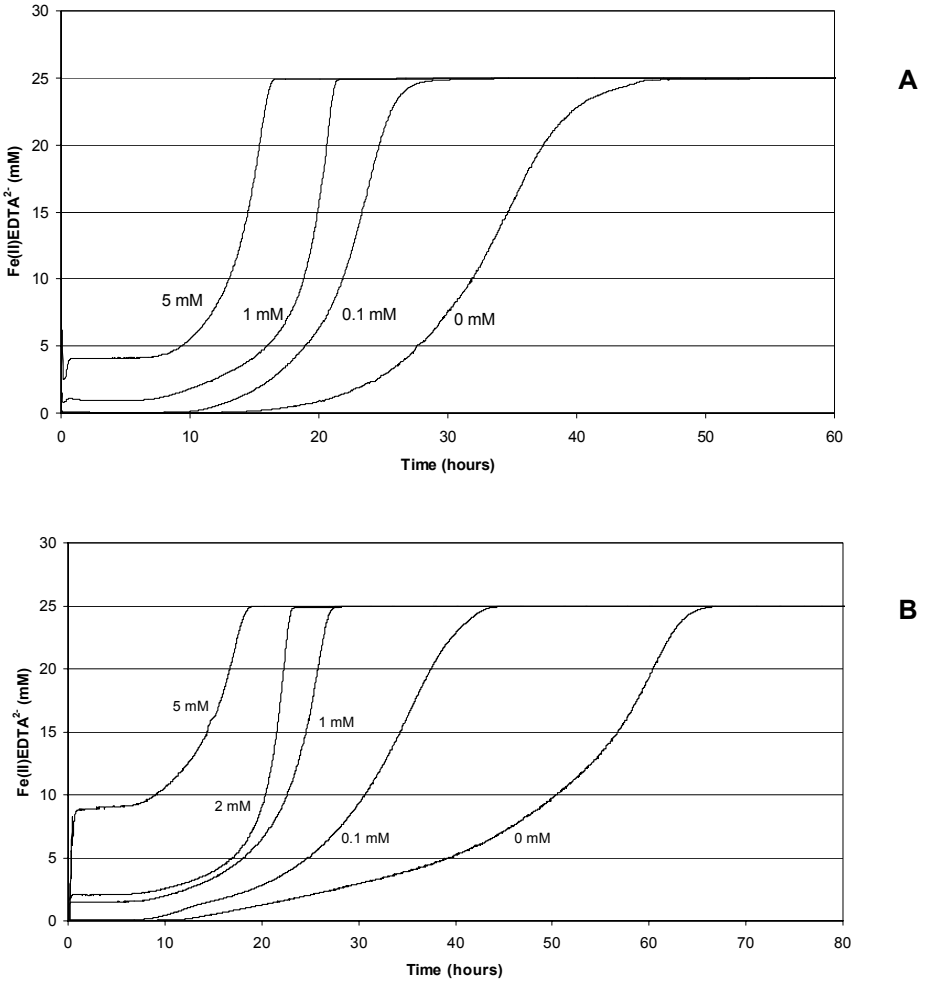

Figure 6

Influence of the addition of various amounts Na_2S on Fe(III)EDTA$^-$ reduction, monitored by ORP/Nernst, by Eerbeek (A) and Nedalco (B) with ethanol as the electron donor at 55 °C (pH 7.0 ±.0.2). Note that part of the aqueous Fe(III)EDTA$^-$ medium is chemically reduced upon injection of sulfide at t = 0.

Figure 7 shows the influence of sulfide on Fe(III)EDTA$^-$ reduction in vials inoculated with BioDeNOx sludge. Addition of low concentrations (0.2-1.0 mmol·L^{-1}) of sulfide resulted in high Fe(III)EDTA$^-$ reduction rates of ±6 mmol·L^{-1}·h^{-1}, compared to only ±0.2 mmol·L^{-1}·h^{-1} when no external sulfide was added. Addition of 0.2 mmol·L^{-1} Na_2S to this control assay at t = 70 h induced a sharp increase in the Fe(III)EDTA$^-$ reduction rate (Figure 7). Also, addition of 0.5 mM elemental sulfur powder (S^0) accelerated the Fe(III)EDTA$^-$ reduction rate (Figure 8), although the lag phase (±40 h) was a bit longer than when 0.5 mM sulfide was added (±30 h lag phase).

Table 2

Effect of 1 mM Na_2S Addition on the Fe(III)EDTA$^-$ Reduction Rate (mmol $Fe \cdot L^{-1} \cdot h^{-1}$) of Various Inocula at 55 ºC (pH 7.2 ± 0.2) with Ethanol as Electron Donor[a].

Inoculum	Without NaS addition	Addition of 1 mM NaS
Eerbeek	2.1 ± 0.3	7.4 ± 0.4
Nedalco	1.4 ± 0.2	4.1 ± 0.2
Veendam	2.9	5.7
Hoogezand	1.5	4.8
BioDeNOx	0.2	6.0

[a] Initial biomass concentration: ±1.5 mg of protein/L (BioDeNOx) or ±0.07 g of VSS/L (rest).

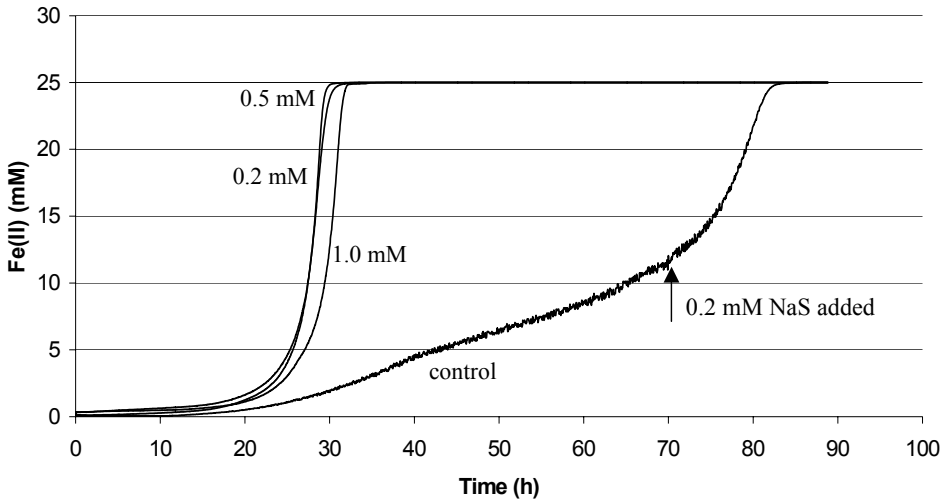

Figure 7

Influence of the addition of various amounts of sulfide on Fe(III)EDTA$^-$ reduction by BioDeNOx sludge with ethanol as electron donor at 55 °C (pH 7.2 ± 0.2). At t = 0 h the vials were inoculated by injection of 4 mL of BioDeNOx reactor liquor into 200 mL of Fe(III)EDTA$^-$ medium (initial protein concentration ±1.5 mg/L). At t = 70 h 0.2 mM Na_2S was added to the 'control' (containing no sulfide).

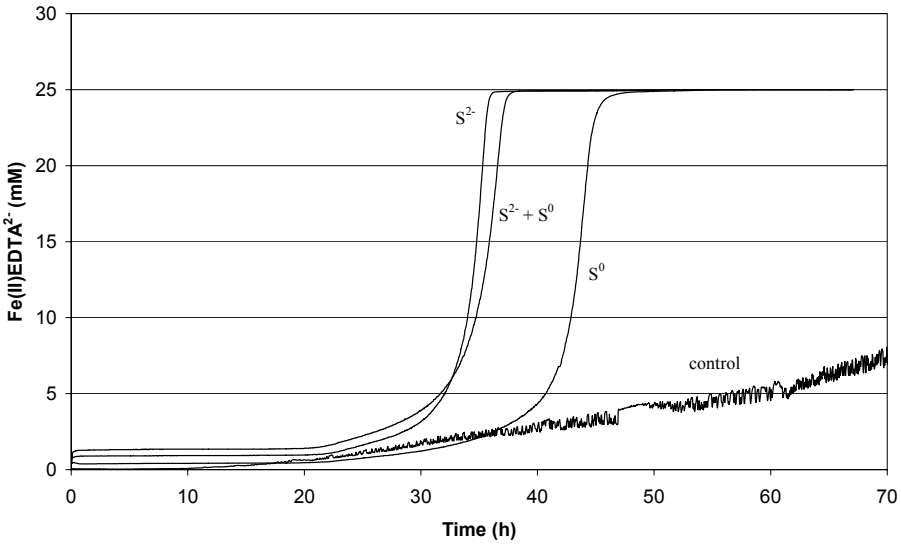

Figure 8

Influence of the addition of sulfide (0.5 mM) and elemental sulfur (0.5 mM) on Fe(III)EDTA$^-$ reduction by BioDeNOx sludge with ethanol at 55 °C (pH 7.2 ± 0.2). At t = 0 h the vials were inoculated by injection of 2 mL of BioDeNOx reactor liquor into 200 mL of Fe(III)EDTA$^-$ medium (initial protein concentration ±1 mg/L).

DISCUSSION

Principle of Fe(III)EDTA$^-$ Reduction

This study strongly indicates that Fe(III)EDTA$^-$ reduction is not a direct, enzymatic conversion by dissimilatory iron-reducing bacteria but an indirect reduction with involvement of an electron shuttle. When chelated to EDTA, Fe(III) stays soluble at neutral pH. In this form iron is very reactive and changes readily between the divalent and trivalent state (Straub *et al.*, 2001). Therefore, complexed iron species often react quite unspecifically with any kind of electron-releasing or -accepting system (Straub *et al.*, 2001). Indeed, injection of crushed Eerbeek inoculum resulted in an immediate Fe reduction (Figures 3 and 5). This indicates a chemical reduction of Fe(III)EDTA$^-$ by an electron donor, e.g., sulfide, present in the injected crushed Eerbeek sludge. Polarographic research demonstrated that the Fe(III)EDTA$^-$/Fe(II)EDTA^{2-} system is redox reversible in the pH range 4-9 (Kolthoff and Auerbach, 1952). This means that Fe(III)EDTA$^-$ can be chemically reduced by compounds with standard redox potentials below + 96 mV versus NHE, such as sulfide ($E^{0'}$ S^0/S^{2-} = -270 mV), thionine (E^0 = +56 mV), riboflavin (E^0 = -208 mV), or AHQDS (E^0 AQDS/AHQDS = -184 mV).

Electron Shuttling by Sulfide

The observation that addition of small amounts of sulfide at least doubled the Fe reduction rate of mixed cultures of Eerbeek, Nedalco, Veendam, and Hoogezand (Figure 6, Table 2) as well as the BioDeNOx sludges (Figure 7) indicates that sulfide acted as an electron-shuttling compound. Chemical

sulfide oxidation by Fe(III)EDTA$^-$ is extensively reported in the literature, as reviewed by Wubs *et al.* (1994). Sulfide chemically reduces Fe(III)EDTA$^-$ very fast according to:

$$S^{2-} + 2\ Fe(III)EDTA^- \rightarrow S^0 + 2\ Fe(II)EDTA^{2-} \quad (1)$$

This chemical Fe(III)EDTA$^-$ reduction by sulfide (eq 1) is shown in Figure 6, where Fe(II)EDTA^{2-} is immediately produced upon injection of sulfide in Fe(III)EDTA$^-$ medium. Elemental sulfur is readily converted to polysulfides in aqueous solutions of sulfide (eq 2):

$$S^{2-} + n\ S^0 \rightarrow S_n^{2-} \quad (2)$$

Since sulfides are a product of the reduction of elemental sulfur as well as sulfate reduction, polysulfides can be an intermediate of sulfur respiration in general (Schauder and Muller, 1993). Since the lag phase of Fe(III)EDTA$^-$ reduction was longer when only elemental sulfur powder was added (Figure 8), it is more likely that polysulfides are involved in the electron transfer than elemental sulfur. Indeed, many bacteria and archaea can reduce elemental sulfur or polysulfides to H_2S, as reviewed by Hedderich *et al.* (1999). The sulfide produced can reduce Fe(III)EDTA$^-$ again according to Eq 1. In that way, Fe(III)EDTA$^-$ reduction is continued via the continuous (biological) reduction and (chemical) oxidation of polysulfides. A lag phase of about 10-40 h (Figures 6-8) is needed to induce this $S^{2-}/S^0/S_n^{2-}$ cycle.

According to Nevin and Lovley (2002), electron shuttling by sulfide/elemental sulfur did not appear to be important in stimulating crystalline Fe(III) reduction under the conditions found in subsurface sediments, i.e., in the case of iron oxide particles. In contrast, sulfide/elemental sulfur has been proposed to catalyze the reduction of chromium (Smillie *et al.*, 1981), technetium (Lloyd *et al.*, 1998), and molybdenum (Tucker *et al.*, 1997). Moreover, this electron shuttle is involved in the biological decolorization of azo dyes (Van der Zee *et al.*, 2003).

It should be noted that the influence of external sulfide on the Fe(III)EDTA$^-$ reduction rate was only substantial when the assays were inoculated with a low sludge concentration (data not shown). In the case of high sludge concentrations, the endogeneous sulfide concentration was high enough to catalyze the Fe reduction, thus masking the stimulating effect of externally added sulfide. Anaerobic granular sludge itself contains a certain amount of sulfide, e.g., the Eerbeek inoculum contains ± 42 mg of sulfur per gram of VSS (Osuna *et al.*, 2004).

Fe(III) Reduction by Eerbeek Sludge.

Both at 37 °C with glucose (Figure 3B) and at 55 °C with ethanol (Figures 4 and 5) Fe(III)EDTA$^-$ was rapidly reduced by the Eerbeek sludge. In the latter case (Figure 4) ± 83% of the electron equivalents of ethanol were coupled to Fe(III)EDTA$^-$ reduction. The rest (17%) was partly oxidized to acetate according to:

$$15\ Fe(III)EDTA^- + 1.5\ C_2H_5OH + 3.7\ H_2O \rightarrow$$

$$15\ Fe(II)EDTA^{2-} + 0.4\ CH_3COOH + 15\ H^+ + 2.2\ CO_2 \qquad (3)$$

Note that equation 3 only represents the overall reaction, i.e., without expressing the proposed involvement of $S^{2-}/S^0/S_n^{2-}$ as electron shuttle. Figure 4 and eq 3 show that Fe(III)EDTA$^-$ was the final electron acceptor in the Eerbeek assays. Also, sulfate was used as electron acceptor but only after Fe(III)EDTA$^-$ was completely reduced (Figure 4). This sequence indicates that the oxidized electron-shuttling compound, i.e., polysulfide, is the preferred electron acceptor instead of sulfate. Various sulfate-reducing bacteria, e.g., *Desulfobacter* sp. and *Desulfovibrio* sp., are reported to be able to reduce Fe(III) complexed with NTA, but none of these sulfate reducers have been shown to grow with Fe(III)NTA$^-$ as the sole electron acceptor (Lovley *et al.*, 1993). In this study the presence of sulfate did not influence the Fe(III)EDTA$^-$ reduction rate (Figure 5). Also, in this case endogenous sulfide, present in the inoculum, most likely catalyzed the Fe(III)EDTA$^-$ reduc tion. In the absence of sulfide, biological sulfate reduction may be a source of biogenic sulfide, thus stimulating Fe(III)EDTA$^-$ reduction.

Fe(III) Reduction by E. coli

Although *E. coli* is not a Fe-respiring bacterium (Lovley *et al.*, 1991), the strain rapidly reduced Fe(III)EDTA$^-$ coupled to the oxidation of glucose (Figure 3A). These results are in accordance with Tanaka *et al.* (1983), who demonstrated that *E. coli* can be used in fuel cell applications when FeEDTA was applied as the electron-mediating compound. Coves and Fontecave (1993) reported that *E. coli* contains a powerful flavin reductase for the reduction of free flavins, FMN, FAD, or riboflavin by NADH or NADPH. Reduced flavins, in turn, transfer their electrons to the ferric complexes (Coveds and Fontecave, 1993). Since the *E. coli* strain used in this study was precultivated in LB medium (Rayner *et al.*, 1990) containing tryptone (including riboflavin), it is suggested that riboflavin acted as an electron-shuttling compound during the reduction of EDTA-chelated Fe(III) by *E. coli*.

Methanogenic granular sludges contain many microorganisms which grow via fermentation. Apart from fermenta tive metabolism, these microorganisms can use Fe(III) as a minor electron acceptor. In that case less than 5% of the reducing equivalents are typically transferred to Fe(III), while most of the electron equivalents in the fermentable substrates are still recovered in organic fermentation products and hydrogen (Lovley and Phillips, 1988). Lovley *et al.* (1991) demonstrated that a low redox potential (up to -600 mV versus NHE), created by glucose fermentation by *E. coli*, did not induce the reduction of crystalline Fe(III), as confirmed by this study (Figure 3A).

Bioavailability of Fe(III)EDTA

In nature ferric iron is predominantly present as iron oxides, which are barely soluble at neutral pH. Therefore, dissimilatory iron-reducing bacteria use different strategies to transfer electrons to insoluble Fe(III): (1) secretion of Fe(III) chelators (siderophores), (2) iron respiration via direct contact to the insoluble iron crystals, or (3) reduction of insoluble iron via an electron shuttle (see literature reviews

by Schröder *et al.* (2003) and Lovley (2000)). When chelated to EDTA, the solubility problem does not exist.

Haas and DiChristina (2002) investigated the effects of various ligands on dissimilatory Fe(III) reduction by *Shewanella putrefaciens*. They found a typical relation between the Fe(III) reduction rate and the stability constants of the ferric chelates. In the case of Fe(III)EDTA$^-$, no Fe reduction occurred. The authors proposed that the sorption of Fe(III) by functional groups on the cell surface is restricted by the presence of strong chelating agents such as EDTA (Finneran *et al.*, 2002). However, to the best of our understanding, the Fe:EDTA ratio used in that study was 5:50 (mM:mM), indicating that 45 mM EDTA was uncomplexed. Much lower amounts of free EDTA concentration already inhibit the Fe(III)EDTA$^-$ reduction (Figure 5). Also, other studies have shown that the presence of free EDTA strongly inhibits microbial activity (Leive, 1968; Ayres *et al.*, 1998; Chapter 3).

NTA addition has been shown to stimulate Fe(III) reduction in sediments from a petroleum-contaminated aquifer (Lovley *et al.*, 1996). As NTA also stimulated the reduction of a variety of Fe(III) forms by *Geobacter metallireducens*, the authors suggested that NTA solubilization of insoluble Fe(III) oxide is an important mechanism for stimulation of bacterial Fe(III) reduction. Moreover, chelation of Fe(III) to EDTA stimulated benzene oxidation coupled to Fe(III) reduction (Lovley and Woodward, 1996). Again, the observed stimulation was thought to be a result of the increased bioavailability of Fe(III) as an electron acceptor for iron-reducing bacteria. The studies of Lovley (Lovely *et al.*, 1996; Lovley and Woodward, 1996) do, however, not allow one to conclude that EDTA stimulates the bioavailability of Fe(III) to dissimilatory iron-reducing bacteria. This should be further investigated using an experimental setup in which the possible involvement of an electron-shuttling compound in the reduction of Fe(III)EDTA$^-$, i.e., indirect, chemical Fe reduction, can be excluded.

Relevance for BioDeNOx.

This study strongly indicates that Fe(III)EDTA$^-$ reduction is not a direct biological conversion but an indirect reduction with involvement of a sulfur compound as an extracellular electron shuttle. However, relatively slow, direct enzymatic reduction of Fe(III)EDTA$^-$ cannot be excluded. Since NO_x-contaminated flue gases generally also contain some SO_2 (Philip and Deshusses, 2003), reduced sulfur compounds are expected to be available in the mixed reactor liquor of BioDeNOx reactors under practical conditions. This implicates that not dissimilatory iron-reducing bacteria but reducers of elemental sulfur or polysulfides play a key role in the reduction of EDTA-chelated Fe(III) within the BioDeNOx concept.

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CHAPTER 6

Fe(III)EDTA$^-$ reduction in BioDeNOx reactors: kinetics and mechanism

High rate biological Fe(III)EDTA$^-$ reduction is a factor of crucial importance for a succesful application of BioDeNOx, a novel technique for NO_x removal from flue gasses. This chapter investigates the kinetics and mechanism of biological Fe(III)EDTA$^-$ reduction by unadapted anaerobic methanogenic sludge and BioDeNOx reactor mixed liquor using batch experiments (21 mM Fe(III)EDTA$^-$, 55 ºC, pH 7.2 ± 0.2). The influence of different electron donors, electron mediating compounds and $CaSO_3$ on the Fe(III)EDTA$^-$ reduction rate was determined. The type of electron donor affected the Fe(III)EDTA$^-$ reduction rate, the highest rate (13.9 mM.h^{-1}) was observed with glucose, followed by ethanol, acetate and hydrogen. Relatively slow Fe(III)EDTA$^-$ reduction (4.1 mM.h^{-1}) occurred with methanol as the electron donor. Fe(III)EDTA$^-$ reduction was accelerated by the addition of small amounts (0.5 mM) of sulfide, cysteine or elemental sulfur, from which an unknown electron mediating compound, presumably polysulfides, is formed. Using ethanol as electron donor, the specific Fe(III)EDTA$^-$ reduction rate showed a linear relation with the amount of sulfide supplied and amounted to 1.0 mmol.mg$_{prot}^{-1}$.h^{-1} in case 1 mM sulfide was added to the 21 mM Fe(III)EDTA$^-$ medium. The electron mediators AQDS, AQS and riboflavin did not affect the Fe(III)EDTA$^-$ reduction, wheras thionine inhibited Fe(III)EDTA$^-$ reduction at the concentration investigated (0.5 mM). Also $CaSO_3$ (0.5-100 mM) inhibited Fe(III)EDTA$^-$ reduction, possibly because SO_3^{2-} scavenged the electron mediating compound.

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INTRODUCTION

Reduction of EDTA chelated Fe(III) is one of the core reactions in BioDeNOx, a novel technique for NO_x removal from industrial flue gases (Buisman *et al.*, 1999; Chapter 4). An aqueous $Fe(II)EDTA^{2-}$ solution (20-25 mM) is used to enhance the removal of nitric oxide (NO) from the gas phase in a wet scrubbing process according to (Demmink *et al.*, 1997):

$$Fe(II)EDTA^{2-}\ (aq) + NO\ (aq) \leftrightarrow Fe(II)EDTA\text{-}NO^{2-}\ (aq) \quad (1)$$

Since industrial flue gases generally contain 2 – 8 % oxygen, part of the $Fe(II)EDTA^{2-}$ is oxidized to $Fe(III)EDTA^-$ during the gas scrubbing according to (Wubs and Beenackers, 1993):

$$4\ Fe(II)EDTA^{2-} + O_2 + 4\ H^+ \rightarrow 4\ Fe(III)EDTA^- + 2\ H_2O \quad (2)$$

In the bioreactor, the scrubber liquor is regenerated by means of two key conversions: reduction of $Fe(III)EDTA^-$ to $Fe(II)EDTA^{2-}$ and reduction of NO to di-nitrogen gas (N_2). When ethanol is used as electron donor, as it is done in practice (Biostar, personal communication), reduction of EDTA chelated ferric iron proceeds according to the overall reaction (Buisman *et al.*, 1999):

$$12\ Fe(III)EDTA^- + C_2H_5OH + 3\ H_2O \rightarrow 12Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^+ \quad (3)$$

The experiments reported in Chapter 5 showed that the addition of low amounts (0.2-1.0 mM) of sulfide strongly increased the $Fe(III)EDTA^-$ reduction rate. Therefore, an indirect $Fe(III)EDTA^-$ reduction mechanism was proposed, with the involvement of a sulfur compound acting as an electron shuttle (Fig. 1 of Chapter 5). When treating gas streams which contain elevated oxygen concentrations, EDTA chelated Fe(III) reduction can become the limiting process for the regeneration of the scrubber liquor (Chapter 4). Therefore, optimization of the $Fe(III)EDTA^-$ reduction rate is an important factor for the succesful application of the BioDeNOx technology.

The applicability of the BioDeNOx concept for flue gas treatment will increase when NO_x removal can be combined with desulfurization, as the process will become more cost effective. One opportunity to implement this may be the retrofitting of limestone-gypsum plants, which eliminate SO_2 from flue gases via $CaSO_3$ precipitation, with the BioDeNOx technology. In that case, the reactor mixed liquor will also contain high $CaSO_3$ concentrations, up to 0.8 M (Biostar, personal communication), which might interfere with the biological regeneration of the BioDeNOx scrubber liquor.

In this chapter the kinetics and mechanism of $Fe(III)EDTA^-$ reduction in BioDeNOx reactors using batch experiments (25 mM $Fe(III)EDTA^-$, 55 ºC, pH 7.2 ± 0.2). The influence of the electron mediating compound, electron donor and $CaSO_3$ on the $Fe(III)EDTA^-$ reduction rate was determined.

MATERIAL AND METHODS

Sources of biomass

Samples were taken from the mixed liquor of a continuously operated lab-scale BioDeNOx reactor (55 ºC, pH 7.2 ± 0.2, 15 – 20 mM Fe(II)EDTA^{2-}), treating an artifical flue gas containing 60-190 ppm NO and 3.9 % O_2 (Chapter 6). The protein content of this sludge amounted to 628 mg.l^{-1}. Also anaerobic Eerbeek sludge (5), applied as inoculum for the BioDeNOx reactor (Chapter 4) was used in tests that investigated the effect of the electron donor and calcium sulfite on the Fe(III)EDTA$^-$ reduction rate. Before use, the Eerbeek granules were mixed with the standard medium (1/3 w/w) and crushed with a kitchen blender for 5 minutes. To assess the effect of crushing, one experiment was performed with intact granules.

Medium

The standard Fe(III)EDTA$^-$ medium contained (unless specified otherwise): Na_2H_2EDTA (Titriplex) 25 mM, $FeCl_3$ 30 mM, $MgSO_4.7H_2O$ 5 mM, $CaCl_2.2H_2O$ 5 mM, NH_4Cl 0.28 g/l, KH_2PO_4 0.25 g/l, $NaHCO_3$ 5.4 g/l and trace elements solution 1 ml.l^{-1} (Visser *et al.*, 1993). The medium was prepared by adding subsequently EDTA, $FeCl_3$, nutrients and metals to demiwater, after which the pH was adjusted to 7.2 by adding sodium hydroxide and finally $NaHCO_3$ (buffer) was added. After preparation, the medium contained 21 mM Fe(III)EDTA$^-$, indicating that part of the EDTA^{4-} was complexed with Ca^{2+} and/or Mg^{2+}. All chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany).

Fe(III)EDTA$^-$ reduction experiments

Fe(III)EDTA$^-$ reduction rates were determined in batch experiments as described in Chapter 5. The assays were conducted at 55 ºC using 300 ml double jacket glass reactors, shaken at 150 rpm and equipped with a redox potential (ORP) and a pH electrode (WTW Sentix, Germany) for online pH and ORP measurement. The reactors were filled with 200 ml standard Fe(III)EDTA$^-$ medium and the headspace was replaced with Ar/CO_2 (80/20 v/v). The reactors were then inoculated by injection of 2 ml BioDeNOx mixed reactor liquor, corresponding to a protein concentration of 6.3 mg.l^{-1}, or with 70 mg VSS.l^{-1} crushed Eerbeek sludge, unless specified otherwise.

The electron donor (ethanol, methanol, acetate or glucose) was added to the reactors from a concentrated stock solution to an initial concentration of 5 mM, unless specified otherwise. In case of hydrogen, the headspace was replaced by H_2/CO_2 gas (80/20 v/v) to an initial pressure of 1.6 bar. Electron mediating compounds (Na_2S, riboflavin, vitamin B_{12}, cysteine, AQS, AQDS or thionine) were added from concentrated stock solutions to an initial concentration of 0.5 mM, unless specified otherwise. Elemental sulfur (0.5 mM) was added as powder prior to capping the vials and headspace replacement.

The reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} was monitored online by means of the redox potential (ORP) as described in Chapter 5. The Fe(III)EDTA$^-$ reduction rate was determined by the slope of the Fe(II)EDTA$^-$ production curve, e.g. d[Fe(II)EDTA^{2-}]/dt. All experiments were performed at least in duplicate. The data shown are averages, standard deviations were less than 10 % of the averages.

Analyses

$Fe(II)EDTA^{2-}$ was determined colorimetrically using 1,10-phenantroline (Akzo Nobel, 1996). The hydrogen and methane concentration in the gas phase was determined by gas chromatography according to Weijma *et al.* (2000). Ethanol and acetate were determined by gas chromatography (Cervantes *et al.*, 2000). For biomass protein determination, samples were centrifuged (10 min, 10.000 rpm) and the supernatant was discarded. The pellet was dissolved in 1 N NaOH and placed in boiling water for 15 min. After boiling, the sample was neutralised with 1 N HCl and the protein concentration was determined according to Bradford (1976). Volatile suspended solids (VSS) were determined according to standard methods (APHA, 1985).

RESULTS

Screening of electron mediating compounds

Fig. 1 shows the effect of the addition of electron mediating compounds (0.5 mM) on the $Fe(III)EDTA^-$ reduction rate in batch reactors inoculated with BioDeNOx reactor mixed liquor (6.3 mg protein.l^{-1}) in the presence of 5 mM ethanol as electron donor. In the absence of any externally added electron mediating compound, the $Fe(III)EDTA^-$ reduction rate amounted to 0.9 mM.h^{-1}. Addition of sulfide or elemental sulfur induced a substantial acceleration of the $Fe(III)EDTA^-$ reduction rate to, respectively, 8.3 and 6.7 mM.h^{-1}. Also L-cystein (5.3 mM.h^{-1}) and vitamin B_{12} (3.0 mM.h^{-1}) stimulated $Fe(III)EDTA^-$ reduction (Fig. 2). In contrast, thionin inhibited $Fe(III)EDTA^-$ reduction, while AQS, AQDS and riboflavin did not have a significant effect at the concentration investigated. No $Fe(III)EDTA^-$ reduction occurred in the absence of biomass (Fig. 1).

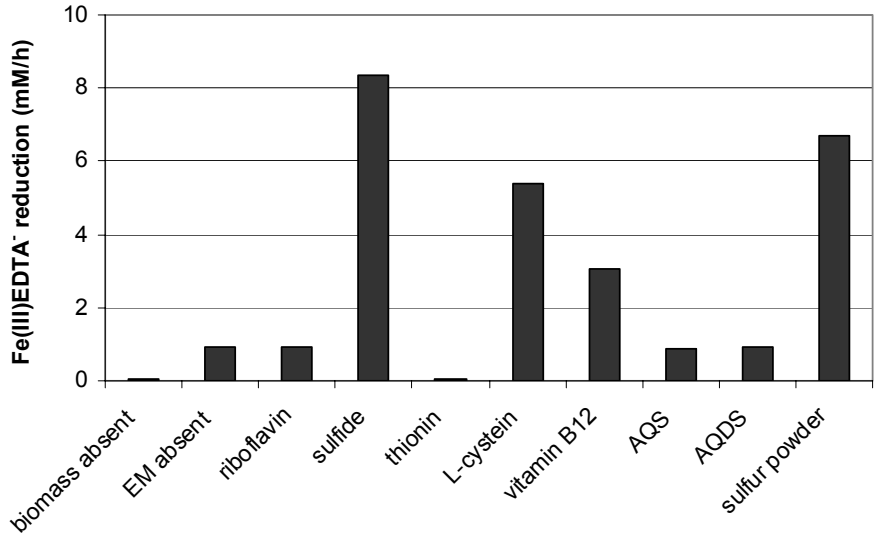


Figure 1

Effect of various electron mediating compounds, supplied in a concentration of 0.5 mM, on the $Fe(III)EDTA^-$ reduction rate with ethanol as electron donor (initial concentration 5 mM). Assays were inoculated with 1 % (v/v) BioDeNOx mixed reactor liquor.

Effect of sufide concentration

Injection of minor amounts of sulfide (0.25 – 1.0 mM) stimulated the Fe(III)EDTA$^-$ reduction by the BioDeNOx reactor mixed liquor (6.3 mg protein.l^{-1}) with 400-900 % in the presence of 5 mM ethanol as electron donor (Fig. 2A and 3C). Addition of only 0.25 mM sulfide already accelerated the reduction rate of the BioDeNOx sludge by a factor of 4 compared to the control, to which no sulfide was added. Injection of more sulfide in the concentration range 0.25 – 1.0 mM resulted in a linear increase of the Fe(III)EDTA$^-$ reduction rate up to 10.2 mM.h^{-1} in case 1.0 mM sulfide was supplied (Fig. 3B). Assuming that the biomass concentration was 10 $mg_{prot}.l^{-1}$ at the time the Fe(III)EDTA$^-$ reduction rate was maximal (during the batch experiments, the protein concentration increased from 6.3 to 12.2 $mg_{prot}.l^{-1}$), the specific Fe(III)EDTA$^-$ redution rate was 1.0 $mmol.mg_{prot}^{-1}.h^{-1}$ in case 1 mM sulfide was supplied to the assays.

The volumetric Fe(III)EDTA$^-$ reduction rate in Fig. 2C can be expressed as:

$$- d[Fe(III)EDTA^-]/dt = k_1 * [S^{2-} \text{ injected}] \quad \text{(Eq. 1)}$$

where k_1 has a constant value of 9.2 h^{-1} (Fig. 2C).

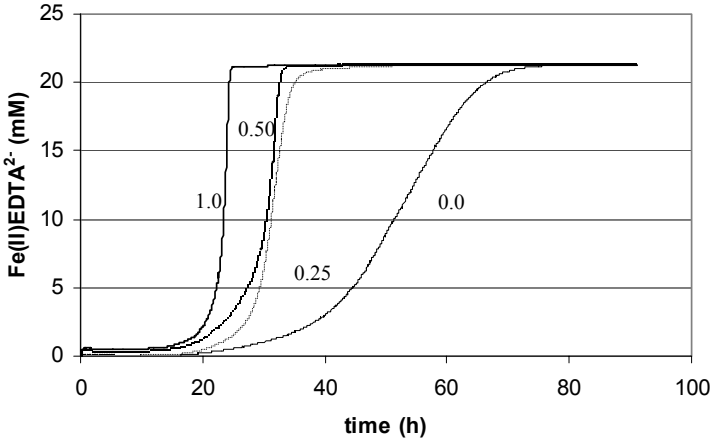
Following the lag-phase, the Fe(III)EDTA$^-$ reduction rate increased exponentially until a maximum rate was observed (Fig. 2A). Fig. 2B shows that not only the maximal Fe(III)EDTA$^-$ reduction rate, but also the exponential acceleration increased at increasing amounts of sulfide injected. The volumetric Fe(III)EDTA$^-$ reduction rate (r) increased exponentially according to:

$$r(t) = r(0) \, . \, e^{A.t} \quad \text{(Eq. 2)}$$

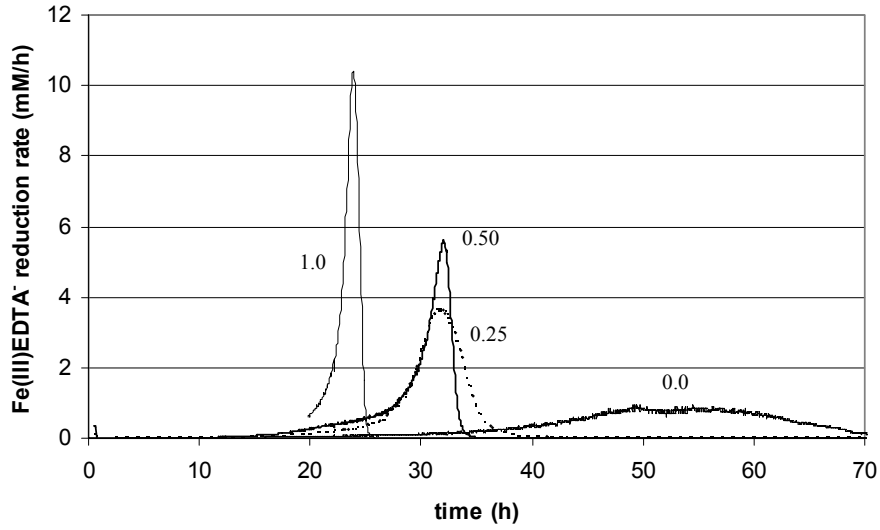
where r(0) is the volumetric reduction rate at the start of the exponential phase and A is the acceleration constant. By means of fitting the exponential phase, parameter A was estimated to be 0.52, 0.27, 0.25 and 0.10 h^{-1} in case, respectively, 1.0, 0.5, 0.25 and 0 mM sulfide was added to the assays.

Effect of electron donor

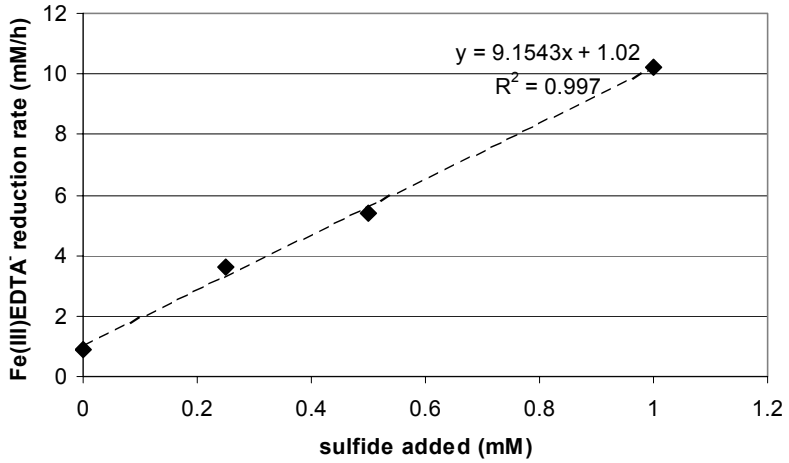
Screening tests conducted with a number of different electron donors show that the BioDeNOx reactor mixed liquor (with 0.5 mM sulfide added prior to Fe(III)EDTA$^-$ reduction) were well capable to use these electron donors for Fe(III)EDTA$^-$ reduction (Fig. 3). Glucose gave the highest Fe(III)EDTA$^-$ reduction rate (13.9 mM.h^{-1}), followed by ethanol (8.2 mM.h^{-1}), hydrogen and acetate (both 5.1 mM.h^{-1}). Methanol supported a relatively slow Fe(III)EDTA$^-$ reduction rate (4.1 mM.h^{-1}). Also Eerbeek sludge (with no sulfide added) showed the highest Fe(III)EDTA$^-$ reduction rate with glucose as the electron donor (7.2 mM.h^{-1}), followed by ethanol (6.2 mM.h^{-1}) and hydrogen (3.0 mM.h^{-1}). Also here, methanol gave the lowest rate (0.9 mM/h) (Fig. 3).



A



B



C

Figure 2

Effect of the amount of sulfide added on the Fe(III)EDTA$^-$ reduction rate by BioDeNOx mixed reactor liquor (inoculum 1 % v/v) with ethanol as electron donor (initial concentration 5 mM) at 55 ºC and pH 7.2 (± 0.2). Evolution of the Fe(II)EDTA^{2-} concentration with time (A), Fe(III)EDTA$^-$ reduction rate versus time (B) and Fe(III)EDTA$^-$ reduction rate as a function of the amount of sulfide suplied (C).

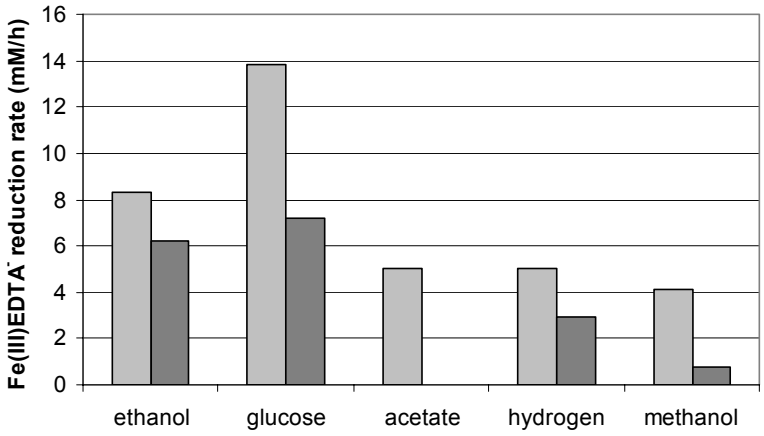


Figure 3

Effect of electron donor on the Fe(III)EDTA$^-$ reduction rate by BioDeNOx mixed reactor liquor (inoculum 1 % v/v, left bars) and crushed Eerbeek sludge (inoculum 70 mg VSS.l^{-1}, right bars) at 55 ºC and pH 7.2 (± 0.2). The initial concentration of electron donor was 5 mM or 1.6 bar (hydrogen).

Effect of crushing and calcium sulfite on Fe(III)EDTA$^-$ reduction by Eerbeek granules

Crushing of Eerbeek granular sludge stimulated Fe(III)EDTA$^-$ reduction (Fig. 4): the Fe(III)EDTA$^-$ reduction rate with ethanol as electron donor increased from 3 mM.h^{-1} to 11 mM.h^{-1} at an inoculum concentration of 0.63 g VSS.l^{-1}. Addition of calcium sulfite resulted in lower Fe(III)EDTA$^-$ reduction rates by Eerbeek sludge (with no external sulfide) in the presence of 5 mM ethanol as electron donor (Fig. 5). Addition of only 0.5 mM $CaSO_3$ already inhibited the Fe(III)EDTA$^-$ reduction rate to approximately 30 % of the reference value, i.e. without sulfite addition. The Fe(III)EDTA$^-$ reduction was completely inhibited in the presence of 100 mM $CaSO_3$ (Fig. 5).

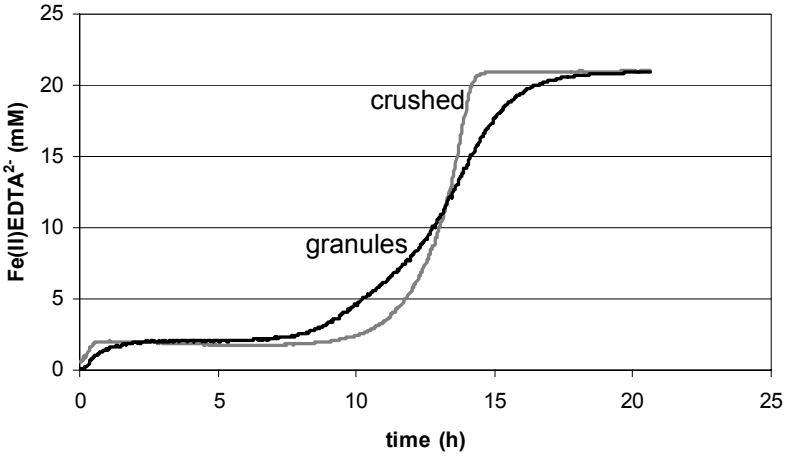


Figure 4

Effect of crushing Eerbeek granular sludge on the Fe(III)EDTA$^-$ reduction rate at 55 ºC and pH 7.2 (± 0.2) with ethanol as electron donor (initial concentration 5 mM).

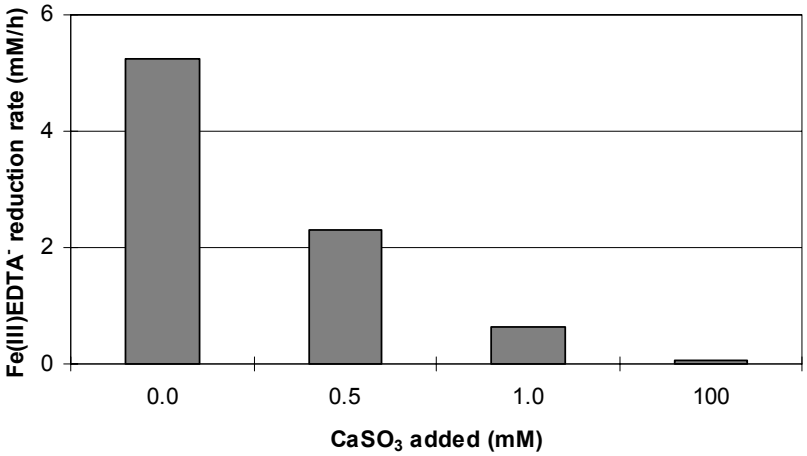


Figure 5

Effect of calcium sulfite on the Fe(III)EDTA$^-$ reduction rate by crushed Eerbeek sludge (inoculum 0.63 g VSS.l^{-1}) with ethanol as electron donor (initial concentration 5 mM). No external sulfide was supplied.

DISCUSSION

Catalytic effects of reduced sulfur species on Fe(III)EDTA$^-$ reduction

This study shows that Fe(III)EDTA$^-$ reduction by sludges originating from a BioDeNOx reactor and a methanogenic UASB reactor can be significantly accelerated by the addition of low amounts (0.5 mM) of sulfide, cysteine and elemental sulfur (Fig. 1). This suggests that Fe(III)EDTA$^-$ reduction is accelerated by a sulfur species, formed out of these compounds and facilitating an electron shuttle between the microbes and Fe(III)EDTA$^-$.

Although determination of the chemical structure of this electron mediating compound was beyond the scope of this study, the linear relation between the amount of sulfide added to the bio-assays and the the specific Fe(III)EDTA$^-$ reduction rate (Fig. 2) strongly suggests that polysulfides are the electron mediating compound. Injection of sulfide into an aqueous Fe(III)EDTA$^-$ solution leads to the formation of elemental sulfur and Fe(II)EDTA^{2-} according to reaction 4 (McManus and Martell, 1997, Wubs and Beenackers, 1994). As a result of that reaction, monosulfides polymerize to polysulfides, S_n^{2-}, with n = 2 – 7 (reaction 5, overall reaction 6) (Clarke *et al.*, 1994):

$$2\ \text{Fe(III)EDTA}^- + S^{2-} \rightarrow 2\ \text{Fe(II)EDTA} + S^0\ (s) \qquad (4)$$

$$S^0 + n\ S^{2-} \rightarrow S_{n+1}^{2-} \qquad (5)$$

$$2n\ \text{Fe(III)EDTA}^- + (n+1)\ S^{2-} \rightarrow S_{n+1}^{2-} + 2n\ \text{Fe(II)EDTA}^{2-} \qquad (6)$$

Polysulfides may also be responsible for the observed acceleration of Fe(III)EDTA$^-$ reduction upon S^0 or cysteine supply (Fig. 1), as they can be formed via sulfide formation upon reduction of S^0 (Hedderich *et al.*, 1999) and cysteine (Morra and Warren, 1991). However, can also form an electron

shuttle with its oxidized form, i.e. cystine, as described by Doong and Schink (2002) in the scope of reduction of poorly crystalline Fe(III) oxides by *Geobacter sulfurreducens*.

In the presence of anaerobic granular sludge as well as BioDeNOx reactor sludge, the oxidized polysulfides are likely reduced continuously via microbial polysulfide reduction (Hedderich *et al.*, 1999). Hence, Fe(III)EDTA$^-$ reduction in BioDeNOx reactors is a result of both biological reduction (reaction 7) and chemical oxidation (reaction 8) of (poly)sulfide:

$$S_{n+1}^{2-} + 2\ e^- \rightarrow S_n^{2-} + S^{2-} \quad (7)$$

$$S_n^{2-} + 2\ Fe(III)EDTA^- + S^{2-} \rightarrow S_{n+1}^{2-} + 2\ Fe(II)EDTA^{2-} \quad \text{(overall)} \quad (8)$$

Acceleration of Fe(III)EDTA$^-$ reduction by *Escherichia coli* via the application of an electron mediating compound is reported by Tanaka *et al.* (1983), who found a linear, i.e. first order, relation between the Fe(III)EDTA$^-$ reduction rate and the amount of thionine (5 – 200 µM) added as electron mediator. Surprisingly, in the present study thionine (0.5 mM) inhibited Fe(III)EDTA$^-$ reduction with both seed materials tested (Fig. 1). Possibly, the higher concentration used here (0.5 mM) was toxic to the microbial populations present in the bio-assays.

High rate Fe(III)EDTA$^-$ reduction by dissimilatory iron reducers, e.g. *Geobacter sp., Shewanella sp.* or *Geothrix sp.* without the involvement of an electron mediating compound, has, to the best of our knowledge, not been reported so far. Kumaraswamy *et al.* (2005) reported the presence of the dissimilatory iron reducer *Deferribacter thermophilus* in BioDeNOx reactors, but batch assays showed that the reduction of 5 mM Fe(III)EDTA$^-$ took 7 days (initial protein concentration 10 mg.l^{-1}) (Kumaraswamy, personal communication), indicating a very low specific Fe(III)EDTA$^-$ reduction rate. Indeed, in contrast to other ferric iron species, e.g. poorly crystalline iron oxides (Coates *et al.*, 1995; Lovley and Phillips, 1988; Straub *et al.*, 1998), Fe(III)citrate (Lonergan *et al.*, 1996) or Fe(III)NTA (Lovley *et al.*, 1995), Fe(III)EDTA$^-$ is reported to be a poor electron acceptor for reduction by dissimilatory iron reducing bateria (Finneran *et al.*, 2002). Although Fe(III)EDTA$^-$ is a suitable terminal electron acceptor for assimilatory ferric iron reductases (Schröder *et al.*, 2003), nearly all the enzymes characterized so far showed flavin reductase activity and need a flavin as co-factor. For example, Vadas *et al.* (1999) reported a high Fe(III)EDTA$^-$ reductase activity in the soluble protein fraction of *Archaeoglobus fulgidus*, but this reductase required flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as a catalytic intermediate for Fe(III)EDTA$^-$ reduction. Thus, also literature indicates that high rate microbial Fe(III)EDTA$^-$ reduction can only be achieved in the presence of electron shuttling compounds.

In contrast to Fe(III)EDTA$^-$, elemental sulfur and polysulfides are suitable electron acceptors for many bacteria and archaea (Hedderich *et al.*, 1999). It is difficult to distinguish between elemental sulfur and polysulfides as electron acceptor and polysulfides may even be an obligatory intermediate of sulfur respiration (Schauder and Müller, 1993). Interestingly, Stetter and Gaag (1983) reported high rate

elemental sulfur reduction by a broad range of methanogenic archeae. In the presence of elemental sulfur, most of the methanogens investigated even strongly favoured H_2S formation over methanogenesis with hydrogen as electron donor (Stetter and Gaag, 1983). The Eerbeek sludge contains a methanogenic population (Santegoeds *et al.*, 1999), as is also the case with the BioDeNOx mixed reactor liquor. The latter is evidenced by methanogenic activity following complete $Fe(III)EDTA^-$ reduction to $Fe(II)EDTA^{2-}$ (data not shown). Therefore, methanogens might play a key role in $Fe(III)EDTA^-$ reduction in BioDeNOx reactors by using polysulfide or elemental sulfur as electron acceptor and thus regenerating the electron shuttle. Further research using molecular tools as denaturating gradient gel electroforesis (DGGE) and fluorescent in situ hybridization (FISH) (Kumaraswamy *et al.*, 2004*)* or DNA chips (Junca and Pieper, 2003) is needed to identify and characterize the microbial population involved in the high rate reduction of EDTA chelated Fe(III).

Rate of $Fe(III)EDTA^-$ reduction

Chemical $Fe(III)EDTA^-$ reduction via sulfide oxidation (reaction 4) is extensively reported in the literature, as reviewd by Demmink (2000). It is widely applied as the key reaction in the LO-CAT gas desulfurization process (McManus and Kin, 1986). The rate is first order in both the $Fe(III)EDTA^-$ and sulfide concentration according to equation 3 (Demmink and Beenackers, 1998):

$$d[S^{2-}]/dt = - d[Fe(III)EDTA^-]/dt = K_2 * [Fe(III)EDTA^-]*[S^{2-}] \quad \text{(Eq. 3)}$$

where K_2 is a first order rate constant ($l.mol^{-1}.s^{-1}$).

In this study, the overall $Fe(III)EDTA^-$ reduction rate was limited by the biological generation of monosulfide via polysulfide reduction (reaction 7), since the $Fe(III)EDTA^-$ reduction rate was linearly related to the amount of sulfide injected, i.e. the polysulfide concentration (Fig. 2C). The volumetric ($mmol.l^{-1}.h^{-1}$) and the specific ($mmol.g_{prot}^{-1}.h^{-1}$) reduction rate of the oxidized electron mediator (EMox, polysulfide) can be expressed by, respectively, equation 4 and 5:

$$d[Fe(III)EDTA^-]/dt = d[EMox]/dt = k_1 * [EMox]_0 \quad \text{(Eq. 4)}$$

$$R_{spec.} = k_1 * [EMox]_0 / [biomass] \quad \text{(Eq. 5)}$$

where $[EMox]_0$ is the amount of electron mediator formed upon the injection of sulfide into the aqueous $Fe(III)EDTA^-$ solution and k_1 is a first order rate constant (h^{-1}).

Note that theoretically, according to equation 5, the specific $Fe(III)EDTA^-$ reduction rate is inversely proportional with the biomass concentration in the presence of a constant concentration of the electron mediating compound. This relation was indeed confirmed by data obtained during BioDeNOx reactor activity tests (see Chapter 9), and it explains why the catalysing effect of low amounts (0.25 – 1 mM) of sulfide was only observed at low (< 1 $gVSS.l^{-1}$) biomass concentrations. At higher biomass concentrations, the catalytic effect of these small amounts of sulfide was not observed (data not

shown), likely because in that case the amount of electron mediator available per gram protein was too low to induce the acceleration.

During Fe(III)EDTA$^-$ reduction, a steady state is reached when the sulfide oxidation rate (eq. 3) is equal to the Fe(III)EDTA$^-$ reduction rate (eq. 4), thus when:

$$k_1 * [EMox]_0 = k_2 * [EMred] * [Fe(III)EDTA^-] \quad \text{(Eq. 6)}$$

During the steady state, the rate of (chemical) Fe(III)EDTA$^-$ reduction is directly determined by and equal to the rate of electron mediator (polysulfide) reduction. Therefore, Fig. 2B can be considered as microbial growth curves where the growth rate is limited by the substrate, i.e. electron mediator concentration. Hence, the acceleration constant (factor A in eq. 2) equals the observed growth rate ($\mu_{obs.}$).

Equation 6 can be rewritten as:

$$[EMox] / [EMred] = (k_2 / k_1) * [Fe(III)EDTA^-] \quad \text{(Eq. 7)}$$

With a determined first order rate constant k_1 of 9 h^{-1} (Fig. 2C) and an assumed second order rate constant k_2 of 275 $l.mmol^{-1}.s^{-1}$ (Demmink *et al.*, 1998), it is evident that the electron mediator is predominantly in the oxidized state during the biological reduction of aqueous Fe(III)EDTA$^-$ (20-25 mM). Only in case of very low Fe(III)EDTA$^-$ concentrations, i.e. (nearly) all Fe(III)EDTA$^-$ has been converted to Fe(II)EDTA^{2-}, accumulation of sulfide may be expected since in that case polysulfide reduction (reaction 7) is not balanced anymore by (poly)sulfide oxidation (reaction 8). Besides, previous research showed the occurrence of sulfate reduction in completely reduced FeEDTA media (Chapter 5) which also generates sulfide accumulation. This situation is unwanted for BioDeNOx applications, since sulfide is a toxicant for denitrification and inhibits the second step of NO reduction, i.e. N_2O reduction to N_2, already at very low (< 0.1 mg/l) concentrations (Chapter 8). Moreover, accumulation of sulfide may induce FeS formation (reactions 9 and 10), meaning that the reactive sulfur species has changed to a non or slowly reactive form:

$$S_n^{2-} + (2n-2)\, e^- \rightarrow n\, S^{2-} \quad (9)$$

$$2\, Fe^{2+} + S^{2-} \rightarrow 2\, FeS\ (s) \quad (10)$$

Electron donor

In addition to the (poly)sulfide concentration, the Fe(III)EDTA$^-$ reduction rate is also clearly affected by the type of electron donor (Fig. 3). Hydrogen may be an intermediate during the thermophilic oxidation of ethanol (Bryant *et al.*, 1977), glucose (Ianotti *et al.*, 1973) and methanol (Paulo *et al.*, 2003) and thus be involved in the (indirect) reduction of Fe(III)EDTA$^-$. This contrasts, however, with the only moderate Fe(III)EDTA$^-$ reduction rates obtained with hydrogen as electron donor (Fig. 3). It

should, however, be noted that the experimental set-up used in this study did not allow conditions of hydrogen overpressure in the headspace. Hence, the hydrogen consumption might have been mass transfer limited during the experiments. Further research is needed to illuminate the possible role of hydrogen in the Fe(III)EDTA$^-$ reduction pathway and to determine if BioDeNOx reactors can be operated with hydrogen as electron donor.

Inhibition by calcium sulfite

The strong inhibition of Fe(III)EDTA$^-$ reduction by calcium sulfite (Fig. 5) may be due to a direct toxic effect of sulfite on the bacterial population. For instance, 0.9 mM sodium sulfite completely inhibited methanogenic activity of *Methanobacterium fornicicum* (Balderston and Payne, 1976). It should be noted that the solubility of calcium sulfite in water is poor, i.e. 0.05 mM at 291 K (Weast, 1984), but in the present study, its solubility may be higher due to the the presence of EDTA^{4-}. Apart from a direct sulfite toxicity, an indirect mechanism may be responsible for the strong inhibition of Fe(III)EDTA$^-$ reduction by $CaSO_3$, namely depletion of the electron mediating compound, i.e. elemental sulfur and/or polysulfide. Indeed, since no sulfide was added to these assays, only low concentrations of sulfur and/or polysulfide (± 0.9 mM maximally), formed out of the endogenous sulfur stock present in the Eerbeek inoculum (which contains ± 42 mg total S/g VSS (Osuna *et al.*, 2004)) were present in the medium. Addition of sulfite leads to the formation of thiosulfate (McManus and Martell, 1997) according to:

$$S^0 + SO_3^{2-} \rightarrow S_2O_3^{2-} \quad (11)$$

$$S_n^{2-} + SO_3^{2-} \rightarrow S_2O_3^{2-} + S_{n-1}^{2-} \quad (12)$$

$$S^{2-} + SO_3^{2-} + 2\ Fe(III)EDTA^- \rightarrow S_2O_3^{2-} + 2\ Fe(II)EDTA^{2-} \quad (13)$$

This mechanism would imply that sulfite is only inhibiting Fe(III)EDTA$^-$ reduction at very low electron mediator (sulfur and/or polysulfide) concentrations, i.e. in the absence of externally supplied sulfide, elemental sulfur or cysteine. Interestingly, sulfite can be reduced to S^{2-} by sulfate or sulfite reducing bacteria (Hedderich *et al.*, 1999) meaning that, in principle, sulfite can be a source of the electron mediating compound. However, this is only the case at low redox potentials, since sulfate/sulfite reduction only proceeds in completely reduced FeEDTA media, e.g. after all Fe(III)EDTA$^-$ is reduced to Fe(II)EDTA^{2-} (Chapter 5). Further research is required to develop appropriate measures to overcome calcium sulfite inhibition in BioDeNOx applications combined with the calcium-gypsum desulfurization process, e.g. via a continous supply of sulfide controlled by a selective sulfide electrode (Müller and Stierli, 1999).

Relevance for BioDeNOx

The volumetric Fe(III)EDTA$^-$ reduction rate of BioDeNOx reactors limits the Fe(III)EDTA$^-$ load, and therefore, the load of O_2 containing flue gas that can be treated (Chapter 4). Therefore, it is of crucial importance for the successful application of the BioDeNOx technology that Fe(III)EDTA$^-$ reduction

proceeds at a high rate. This study demonstrated that the Fe(III)EDTA$^-$ reduction rate by BioDeNOx mixed reactor liquor is limited by the concentration of an electron mediating compound, presumably polysulfide. This means that high Fe(III)EDTA$^-$ reduction rates can be obtained when the electron mediator is maintained in the reactor mixed liquor. To avoid polysulfide depletion via FeS formation (as discussed above), Fe(III)EDTA$^-$ should always be present in the BioDeNOx mixed reactor liquor, i.e. a fraction (10-20 %) of the FeEDTA liquor in BioDeNOx reactors should be in the oxidized state. This can be achieved by operating BioDeNOx reactors at a redox potential between -180 and -200 mV versus Ag/AgCl (pH 7.2 ± 0.2).

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CHAPTER 7

Nitric oxide reduction in BioDeNOx reactors: kinetics and mechanism

Biological reduction of nitric oxide (NO) to di-nitrogen (N_2) in aqueous Fe(II)EDTA^{2-} solutions is a key reaction in BioDeNOx, a novel process for NO_x removal from flue gases. In this chapter, the mechanism and kinetics of the first step of NO reduction, i.e. the conversion of NO to N_2O, was investigated in batch experiments using various types of inocula: denitrifying sludge, anaerobic methanogenic granular sludge and mixed liquor from a BioDeNOx reactor. The experiments were performed in Fe(II)EDTA^{2-} medium (5-25 mM) under BioDeNOx reactor conditions (55 ºC, pH 7.2 ± 0.2) with ethanol as external electron donor. The specific NO (to N_2O) reduction rate depended on the NO (aq) and Fe(II)EDTA^{2-} concentration as well as the temperature. The highest NO reduction rates (± 0.37 nmol.s^{-1}.mg$_{prot}^{-1}$) were found for the BioDeNOx reactor mixed liquor with an estimated K_m value below 10 nM for NO. The experimental results, complemented with kinetic and thermodynamic considerations, confirm that Fe(II)EDTA^{2-} is the primary electron donor for NO reduction, i.e. the BioDeNOx reactor medium (the redox system Fe(II)EDTA^{2-}/Fe(III)EDTA^{-}) interferes with the NO reduction electron transfer chain.

A modified version of this chapter is submitted for publication in Environmental Science and Technology.

INTRODUCTION

The principle of BioDeNOx, a novel process for NO_x removal from industrial flue gases, is based on wet absorption of NO into an aqueous $Fe(II)EDTA^{2-}$ solution (20-25 mM), followed by the biological regeneration of the $Fe(II)EDTA^{2-}$ scrubber liquor in a bioreactor (Buisman *et al.*, 1999). In that reactor, two main conversions take place to regenerate $Fe(II)EDTA^{2-}$: a) reduction of $Fe(II)EDTA^{2-}$ complexed nitric oxide (NO) to di-nitrogen gas (N_2) and b) reduction of $Fe(III)EDTA^-$ to $Fe(II)EDTA^{2-}$.
When ethanol is supplied as electron donor, as is the normal practice in full-scale BioDeNOx applications (Biostar, personal communication), these conversions are described by the overall reactions 1 and 2:

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 6\ Fe(II)EDTA^{2-} + 3\ N_2 + CO_2 + 3\ H_2O \quad (1)$$

$$12\ Fe(III)EDTA^- + C_2H_5OH + 3\ H_2O \rightarrow 12\ Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^+ \quad (2)$$

Chapters 2 and 3 demonstrated that $Fe(II)EDTA^{2-}$ can also be used as electron donor for biological reduction of NO to N_2 with nitrous oxide (N_2O) as intermediate, as described by reactions 3 and 4:

$$2\ Fe(II)EDTA\text{-}NO^{2-} + 2H^+ \rightarrow N_2O + H_2O + 2\ Fe(III)EDTA^- \quad (3)$$

$$N_2O + 2\ Fe(II)EDTA^{2-} + 2\ H^+ \rightarrow N_2 + H_2O + 2\ Fe(III)EDTA^- \quad (4)$$

In the presence of ethanol, the $Fe(III)EDTA^-$ produced by reactions 3 and 4 is reduced back to $Fe(II)EDTA^{2-}$ according to overall reaction 2. Therefore, the $Fe(III)EDTA^-$/$Fe(II)EDTA^{2-}$ system acts as an electron shuttle during NO reduction to N_2 in BioDeNOx reactors (Chapter 3).
This chapter explores the mechanism and kinetics of the first step in NO reduction, i.e. NO conversion to N_2O, under BioDeNOx reactor conditions. The sequential step, i.e. N_2O reduction to N_2, was inhibited by low sulfide concentrations (3-5 mg/l), which are formed endogeneously in the batch assays (Chapter 8). The influence of several factors, i.e. source and amount of inoculum, NO and $Fe(II)EDTA^{2-}$ concentration and temperature, on the NO reduction rate was investigated in batch experiments (pH 7.2 ± 0.2). Based on the experimental results as well as kinetic and thermodynamic considerations, the electron transfer chain of NO reduction under BioDeNOx reactor conditions is discussed.

MATERIAL AND METHODS

Sources of biomass

Three types of inocula were used in the experiments: denitrifying sludge "Veendam" (see Chapter 2), anaerobic methanogenic granular sludge "Eerbeek" (see Chapter 5) and BioDeNOx reactor mixed liquor. Veendam and Eerbeek sludge were stored at 4 °C before use. The BioDeNOx sludge was directly taken from a laboratory scale (6 liter) reactor treating artificial flue gas (60 - 190 ppm NO,

3.9% O_2) at 55 ºC (pH 7.2 ± 0.2) with a load of 0.65 mmol NO $l^{-1}.h^{-1}$ and 8 mmol Fe(III)EDTA$^-$ $l^{-1}.h^{-1}$ (Chapter 9). The mixed reactor liquor contained ± 13 mM Fe(II)EDTA^{2-} and had a redox potential of about -250 mV versus Ag/AgCl. Assays with autoclaved biomass and medium (twice for 20 min at 124 ^{0}C, after iron reduction) served as sterile controls.

Previous experiments showed that the "Veendam" sludge has good NO reduction properties, whereas the "Eerbeek" inoculum is not able to reduce NO (Chapter 3). On the other hand, Eerbeek sludge reduces Fe(III)EDTA$^-$ at high specific rates (Chapter 5). Before use, the Eerbeek granules were crushed by means of a kitchen blender as described in Chapter 5. The other inocula were used without further treatment.

Medium

The standard medium used in this study contained 25 mM Na_2H_2EDTA (Titriplex), 30 mM $FeCl_3$, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and micro-nutrients as described in Chapter 3. The pH was adjusted to 7.2 (± 0.2) by adding sodium hydroxide from a concentrated stock solution. To assess the influence of the Fe(II)EDTA^{2-} concentration on NO reduction, assays were conducted at varying EDTA^{4-} concentrations in the medium (5, 15 and 25 mM).

NO reduction activity tests

NO reduction experiments were conducted at 55 and 30 ºC in 120 ml serum bottles filled with 80 ml medium and various amounts of inoculum. NO reduction in aqueous Fe(II)EDTA^{2-} solutions were prepared by chemical reduction of NO_2^- in the presence of Fe(II)EDTA^{2-} (Schneppensieper *et al.*, 2001; Chapters 2 and 3). The latter was generated from a Fe(III)EDTA$^-$ solution, which needed to be reduced to Fe(II)EDTA^{2-}. As the Veendam sludge had a relatively poor Fe(III)EDTA$^-$ reduction capacity,, the Fe(III)EDTA$^-$ was reduced either biologically by anaerobic Eerbeek sludge (0.6 g VSS.l^{-1}) or chemically by addition of Fe^0.

Unless specified otherwise, 0.6 gVSS/l anaerobic Eerbeek sludge was injected in the vials containing various amounts of denitrifying Veendam sludge (0, 0.65, 1.3, 1.95 and 2.6 gVSS/l). After addition of medium and biomass, the vials were sealed and the headspace was replaced by Ar/CO_2. Next, ethanol (5 mM) was added to the bottles as external electron donor by syringe from a concentrated stock solution. The bottles were incubated in the dark at 55 or 30 ºC in a shaking (60 rpm) waterbath (Salm en Kipp, The Netherlands). Under these conditions, the Fe(III)EDTA$^-$ medium was biologically reduced to Fe(II)EDTA^{2-} within 24 hours (see Chapter 5). The BioDeNOx mixed reactor liquor was already in the reduced state when sampled from the reactor. After sampling, these vials were immediately flushed with Ar/CO_2 to prevent oxidation by air. In the completely reduced Fe(II)EDTA^{2-} medium, low concentrations (3-5 mg/l) of endogeneously formed sulfide were present in all cases. These sulfide concentrations inhibit N_2O reduction to N_2, but not NO reduction to N_2O (Chapter 8). In the absence of ethanol (Fig. 5), the aqueous Fe(II)EDTA^{2-} medium was obtained via chemical Fe(III)EDTA$^-$ reduction using Fe^0 (15 mM) as reducing agent (see Chapter 8).

After complete reduction of the medium (generally obtained within 24 hours incubation), the nitrosyl-complex Fe(II)EDTA-NO^{2-} was generated by the injection of 2 mM nitrite from a concentrated stock solution to the aqueous Fe(II)$EDTA^{2-}$ medium (Schneppensieper *et al.*, 2001; Chapter 3). The NO reduction rate was determined by monitoring the N_2O and N_2 concentration in the headspace of the activity test vials as described in Chapter 3. The solubility of these gases (Lide, 2001) was taken into account to calculate the total amount of gaseous nitrogen produced. All experiments were conducted in duplicate, graphs show average values of the data. Standard deviations were less than 10 % of the average.

Analytical methods

Nitrogen and nitrous oxide were measured by Gas Chromatography as described in Chapter 3. Nitrite was determined by Ion Chromatography (Dionex, IonPac AS17) as described in Chapter 8. Nitrite samples were measured immediately to minimize nitrite removal by chemical reduction with Fe(II)$EDTA^{2-}$ in the period between sampling and analysis. Ethanol and acetate were analyzed by liquid Gas Chromatography as described in Chapter 3. Ferrous ion was determined colorimetrically using the 1,10-phenantroline assay according to Akzo Nobel (1996). Volatile suspended solids (VSS) were determined according to Standard Methods (APHA, 1985) Protein concentrations were determined according to Bradford (1976) using Bovine Serum Albumin (BSA) as standard.

Calculations

The nitrosyl concentration was estimated from the nitrite and N_2O concentration according to:

$$Fe(II)EDTA\text{-}NO^{2-}_{(t)} = NO_2^-{}_{(0)} - NO_2^-{}_{(t)} - N_2O_{(t)} \quad \text{(Eq. 1)}$$

The NO (aq) concentration was calculated from the nitrosyl and Fe(II)$EDTA^{2-}$ concentration according to: $[NO\ (aq)] = [Fe(II)EDTA\text{-}NO^{2-}] / ([Fe(II)EDTA^{2-}].K)$ (Eq. 2)

where K is the constant for the equilibrium between the nitrosyl complex and NO (aq):

$$NO\ (aq) + Fe(II)EDTA^{2-} \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \quad (5)$$

The equilibrium constant K is temperature dependent (Demmink *et al.*, 1997). Fig. 1 presents the theoretical relation between the nitrosyl and NO (aq) concentration at 303, 313 and 328 K.

The first order rate constant k_1 and the substrate affinity constant K_m for NO reduction were determined by plotting the NO reduction rate against the NO (aq) concentration. k_1 was determined by the slope of the curve, while K_m was calculated as the NO (aq) concentration where the NO reduction rate $v = ½\ v_{max}$.

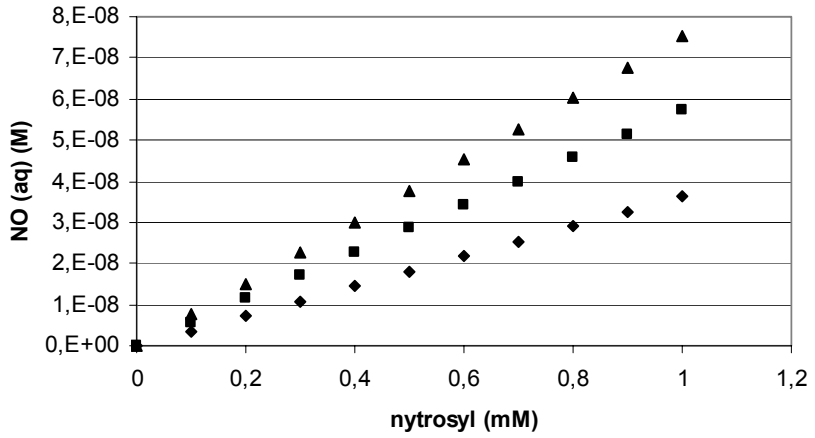

Figure 1

Theoretical relation between the Fe(II)EDTA-NO^{2-} and NO (aq) concentration in aqueous Fe(II)$EDTA^{2-}$ solutions (25 mM) at 303(◆), 313 (■) and 328 K (▲).

RESULTS

NO reduction to N_2O by various inocula

Fig. 2A and 2B show the evolution of the N_2O production upon the injection of 2 mM nitrite into the aqueous Fe(II)$EDTA^{2-}$ medium, inoculated with a mixture of denitrifying Veendam sludge (1.3 gVSS/l) and crushed anaerobic Eerbeek granules (0.6 gVSS/l). Nitrite reduction to NO, i.e. formation of the nitrosyl-complex Fe(II)EDTA-NO^{2-}, was followed by NO reduction to N_2O. The lag-phase, e.g. the time between the injection of nitrite and the start of N_2O production, amounted to ± 2 hours (Fig. 2A). No N_2 production, i.e. N_2O reduction was observed within the timescale of the experiment (98 hours). Re-plotting the data from Fig. 2B shows that the NO reduction rate depended on the NO concentration according to a non-linear, probably sigmoidal, relation (Fig 2C). From Fig. 2C, the K_m value for NO reduction by Veendam sludge cannot be determined exactly, but the data indicate that the K_m value was higher than 30 nM.

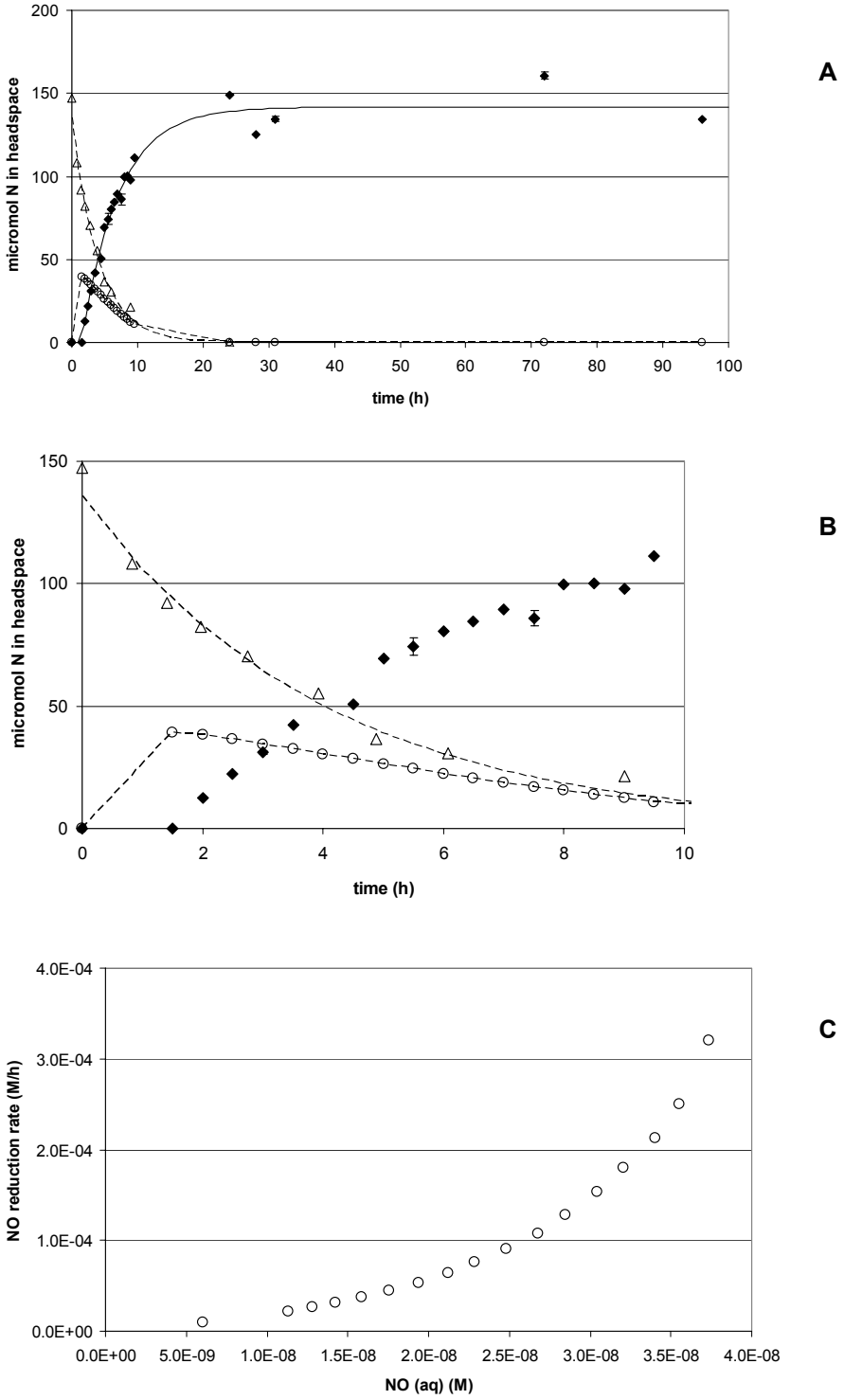

Figure 2

NO_2^- depletion (△) and N_2O production (◆) in assays inoculated with a mixture of anaerobic Eerbeek sludgde (0.6 g VSS/l) and Veendam denitrifying sludge (1.3 g VSS/l) in the first hours (B) and the total timescale of the experiment (A). The nitrosyl-complex Fe(II)EDTA-NO (○), was generated by the injection of 2 mM nitrite into the $Fe(II)EDTA^{2-}$ medium at t = 0h. Prior to nitrosyl-generation, $Fe(II)EDTA^{2-}$ was produced via biological Fe(III)EDTA reduction with ethanol as electron donor (initial concentration 5 mM). The NO reduction rate, calculated from N_2O accumulation in the headspace versus the theoretical NO (aq) concentration (C).

The volumetric NO reduction rate observed in bio-assays inoculated with the denitrifying Veendam sludge was linearly related with the initial biomass concentration (Fig. 3A). A comparable, linear relation was observed by the BioDeNOx reactor mixed liquor (Fig. 3B). Assays with protein concentrations of, respectively, 530, 180 and 100 mg.l^{-1} showed NO reduction rates of 0.67, 0.25 and 0.14 mM.h^{-1}, corresponding to a specific reduction rate of 1.2 µmol.mg_{prot}^{-1}.h^{-1} or 0.34 nmol.mg_{prot}^{-1}.s^{-1}.

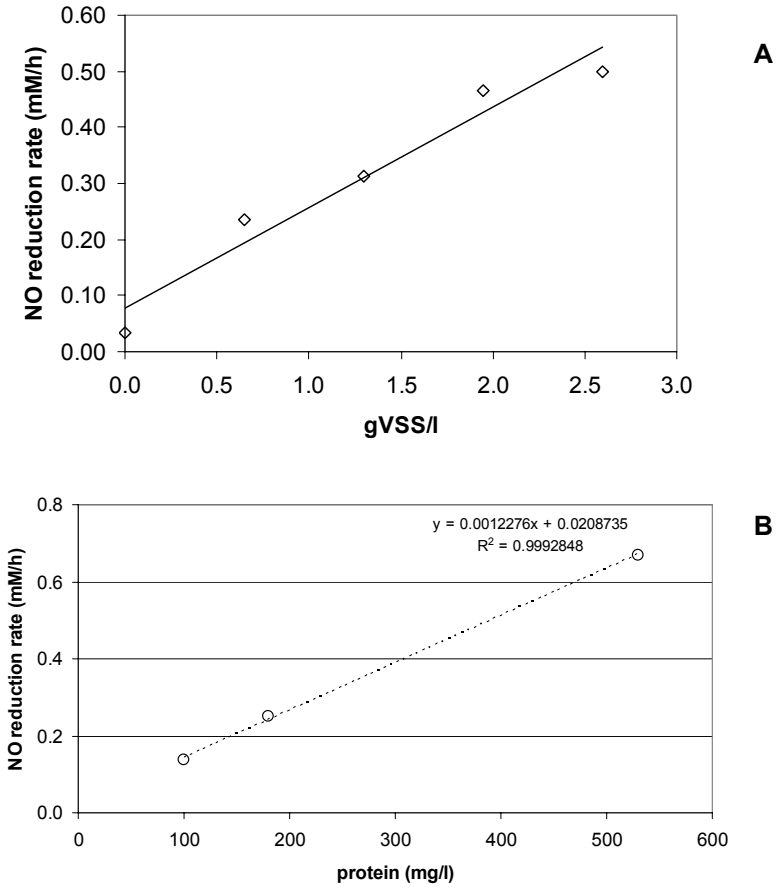

Figure 3

NO reduction rates, monitored observed at various concentrations of denitrifying Veendam sludge and a fixed amount (0.6 g VSS/l) of anaerobic Eerbeek sludge (A) and various protein concentrations of the BioDeNOx mixed reactor liquor (B). Ethanol was available as external electron donor (initial concentration 5 mM).

During activity test using BioDeNOx reactor mixed liquor the NO reduction rate stayed constant until at least 80 % of the spiked NO_2^- (equal to 1.6 mM, Fig. 4A) was converted into N_2O, i.e. when the nitrosyl-concentration dropped below 0.2 mM. Taking into account the equilibrium constant for the system $Fe(II)EDTA^{2-}$/NO/$Fe(II)EDTA\text{-}NO^{2-}$, a K_m-value for NO reduction by BioDeNOx slude of lower than 10 nM can be estimated (Fig. 4B). The curve in Fig. 4B is fitted using a K_m-value of 3 nM.

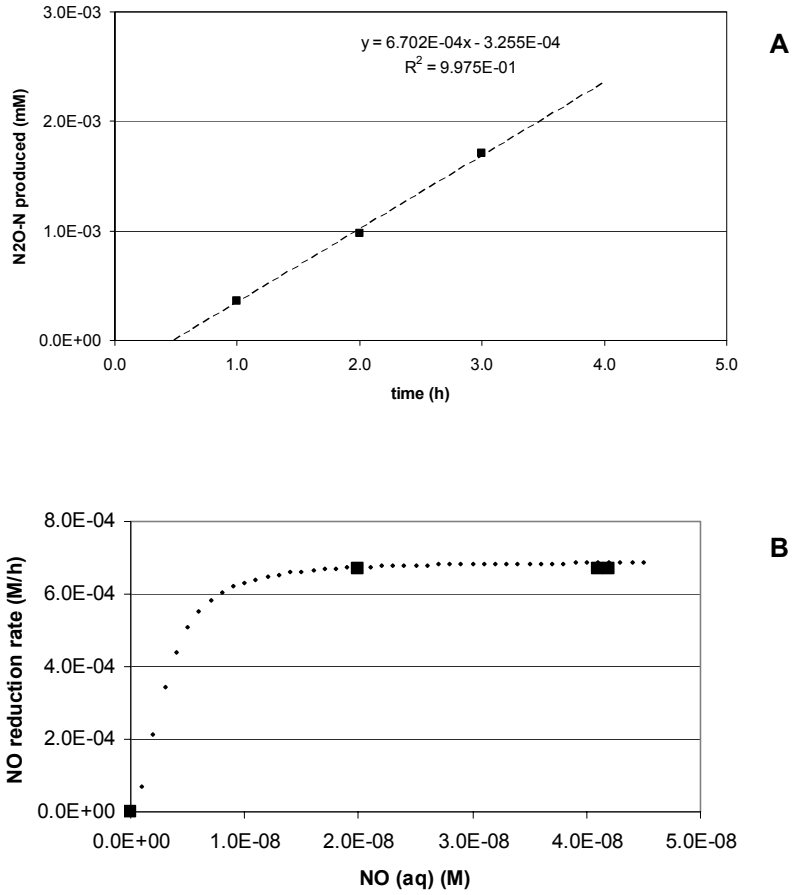

Figure 4

NO reduction, monitored by N_2O accumulation versus time, in a BioDeNOx mixed liquor (530 mg protein per liter) (A). The nitrosyl-complex Fe(II)EDTA-NO was generated by the injection of 2 mM nitrite into the $Fe(II)EDTA^{2-}$ medium at t = 0h. Ethanol (5 mM) was available as electron donor. Plotting the NO reduction rate versus the NO (aq) concentration (B) indicates that the K_m-value is below 10 nM (3 nM in fit curve).

Effect of ethanol and $Fe(II)EDTA^{2-}$ concentration

Fig. 5 shows that NO reduction in aqueous $Fe(II)EDTA^{2-}$ medium, inoculated with a mixture of anaerobic Eerbeek and denitrifying Veendam inoculum, was independent of the presence of ethanol. Interestingly, the NO reduction rate was affected by the $Fe(II)EDTA^{2-}$ concentration: higher NO reduction rates were found at higher $Fe(II)EDTA^{2-}$ concentrations (Fig. 6A). However, in media containing 60 mM Na_2H_2EDTA, i.e. when part of the $EDTA^{4-}$ was uncomplexed, no NO reduction was observed (data not shown). Only 60 % of the $Fe(II)EDTA^{2-}$ complexed NO was recovered as N_2O in case the medium contained 5 mM or 15 mM $Fe(II)EDTA^{2-}$ (Fig. 6B). Visual observations confirmed that the depletion of the nitrosyl complex remained uncompleted in case of 5 and 15 mM $Fe(II)EDTA^{2-}$, since these assays kept their green color, typical for the nitrosyl complex. In case of 25 mM $Fe(II)EDTA^{2-}$, all nitrogen, originally supplied as nitrite, was recovered as gaseous N (Fig. 6B and 6C).

A second nitrite injection (t = 164h) showed the same pattern: approximately 60 % of the $Fe(II)EDTA^{2-}$ complexed NO was recovered at lower $Fe(II)EDTA^{2-}$ concentrations, whereas all nitrogen was recovered as N_2O in case the medium contained 25 mM $Fe(II)EDTA^{2-}$ (Fig. 6B). The relation between the $Fe(II)EDTA^{2-}$ concentration and the NO reduction rate appeared to be first order, with rate constants (k_1) of 438, 1419 and 4038 h^{-1} for the assays containing 5, 10 and 25 mM $Fe(II)EDTA^{2-}$, respectively (Fig. 6A). K_m-values for NO reduction were estimated to be, respectively, >30 nM, >120 nM and >200 nM.

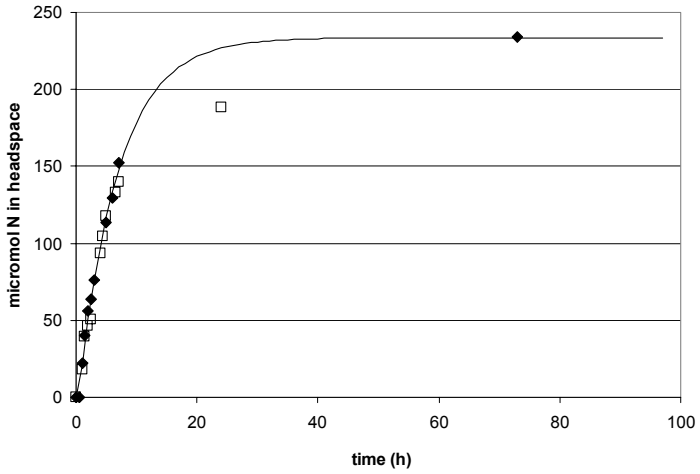

Figure 5

N_2O accumulation in assay inoculated with a mixture of anaerobic Eerbeek sludge (0.6 g VSS/l) and denitrifying Veendam sludge (1.3 mgVSS/l). in presence (◆) and absence (□) of 5 mM ethanol. Prior to the generation of the nitrosyl-complex by 2 mM nitrite injection at t = 0 hours, the original $Fe(III)EDTA^-$ medium was reduced chemically, using 15 mM Fe^0 as reducing agent.

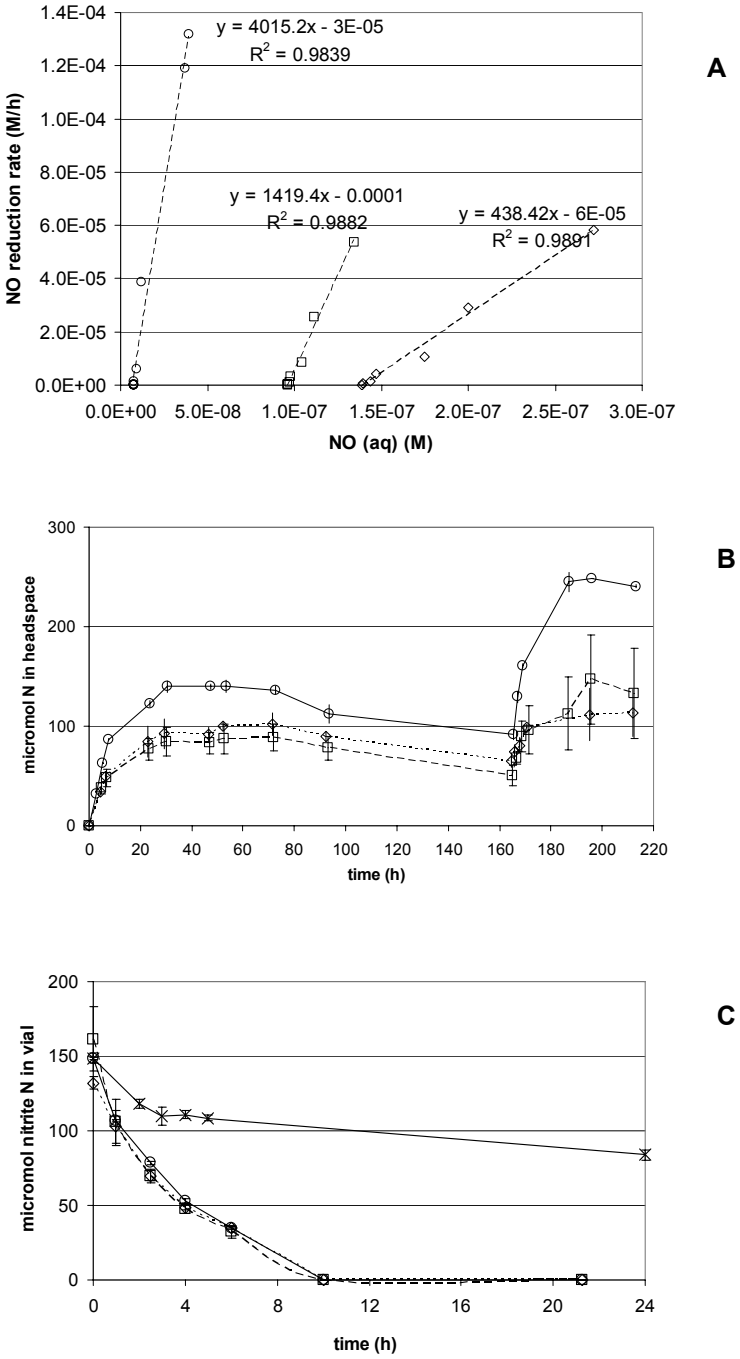

Figure 6

NO reduction rate versus NO (aq) concentration (A) in assays inoculated with a mixture of anaerobic Eerbeek sludge (0.6 g VSS/l) and denitrifying Veendam sludge (1.3 mgVSS/l), containing 5 mM (◇), 15 mM (□) and 25 mM (○)Fe(II)EDTA^{2-}. Ethanol was available as external electron donor (initial concentration 5 mM). The NO reduction rate was calculated from the N_2O accumulation in the headspace (B). The N_2O production was accompanied by simultaneous nitrite depletion in the bio-assays, as well as in the sterile control containing 25 mM Fe(II)EDTA^{2-} (×) (C).

Effect of temperature

Fig. 7 shows the production of N_2O by the denitrifying Veendam inoculum at 30 and 55 °C. Before the generation of the nitrosyl complex Fe(II)EDTA-NO^{2-}, the assays were incubated for 2 days at these temperatures for Fe(III)$EDTA^{2-}$ reduction. The N_2O accumulation patterns suggest zero-order kinetics at 30 ºC, while pseudo first order at 55 ºC (Fig. 1-3). The observed volumetric NO reduction rate is ± 12 times higher at 55 ºC: 0.25 $mM.h^{-1}$ versus only 0.02 $mM.h^{-1}$ at 30 ºC. Moreover, the NO reduction at 30 ºC was incomplete within the timeframe of the experiment, i.e. only 20 % of the nitrite injected (35 µg N per vial) was recovered as gaseous nitrogen (Fig. 7). The vials at 30 ºC kept their green color, indicating that part of the NO stayed in solution as the nitrosyl-complex Fe(II)EDTA-NO^{2-}.

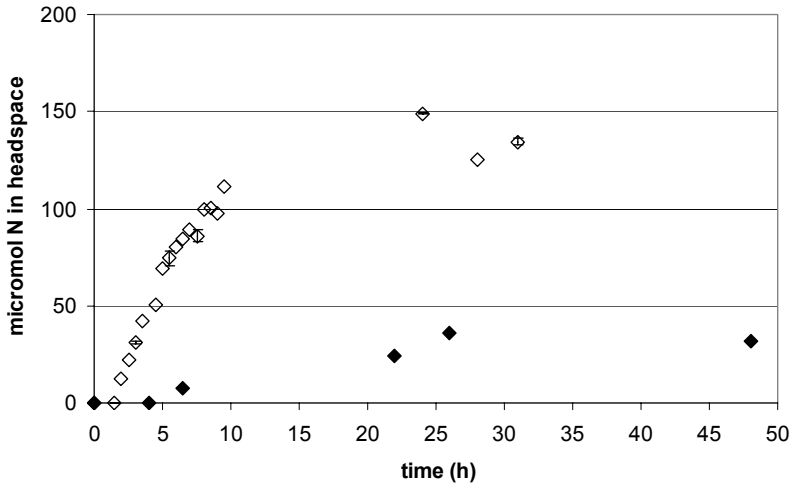

Figure 7

N_2O production by denitrifying Veendam sludge (1.3 mgVSS/l) at 30 (◆) and 55 ºC (◇). Ethanol was present as electron donor (initial concentration 5 mM).

DISCUSSION

Role of Fe(II)$EDTA^{2-}$ in NO reduction

This study shows that the specific rate of biological NO reduction to N_2O in aqueous Fe(II)$EDTA^{2-}$ media is independent of the presence of ethanol (Fig. 5), but depends on the Fe(II)$EDTA^{2-}$ concentration (Fig. 6). This strongly suggests that in BioDeNOx reactors, i.e. in aqueous Fe(II)$EDTA^{2-}$ solutions, the chelated Fe(II) is the direct electron donor for NO reduction, and not ethanol. This is, at first sight, somewhat surprising, since ethanol is known to be an excellent electron donor for denitrification (Constantin and Fick, 1997). However, the redox properties of FeEDTA and bacterial Nitric Oxide Reductase (NOR), the denitrification enzyme that catalyzes NO reduction to N_2O, allow that Fe(II)$EDTA^{2-}$ can be directly involved in NOR reduction, with the concomitant formation of N_2O. Fig. 8 schematically depicts the possible roles of Fe(II)$EDTA^{2-}$ as electron donor in NO reduction. Thus, Fe(II)$EDTA^{2-}$ not only enhances the NO transfer from the gas into the liquid phase, the reason

why it is applied in BioDeNOx reactors, but also plays a key role in the biological denitrification process.

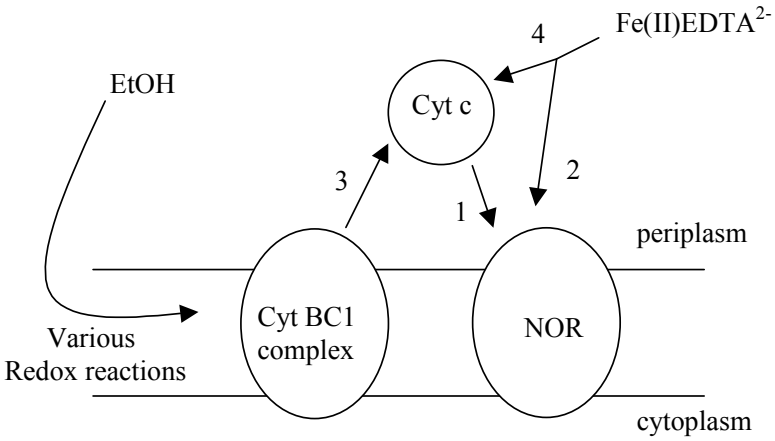

Figure 8

Possible electron transfer pathways for NOR reduction

1. NOR reduction by reduced Cytochrome c
2. Direct NOR reduction by $Fe(II)EDTA^{2-}$
3. The conventional electron transfer chain: Cytochrome c reduction by the Cyt *bc*1 complex
4. Cytochrome c reduction by $Fe(II)EDTA^{2-}$

$Fe(II)EDTA^{2-}$ - NOR interactions

The $Fe(II)EDTA^{2-}$/$Fe(III)EDTA^{-}$ system is redox reversible and has a midpoint potential of + 96 mV (Kolthoff and Auerbach, 1952). This implies that it easily accepts electrons from a system with a lower midpoint potential and that it donates electrons to any redox sensitive system with a higher standard redox potential (Straub *et al.*, 2001). Based on the molecular weight (360 Da), both $Fe(II)EDA^{2-}$ and $Fe(III)EDTA^{-}$ may penetrate into the periplasmic space, where these compounds can be involved in various biochemical redox reactions (Fig. 8). Bacterial NOR is a membrane bound enzyme that receives both its electron donor and acceptor (NO) from the periplasmic side (Berks *et al.*, 1995; Hendriks *et al.*, 2002). The midpoint potential of bacterial NO reductases ranges between +280 and +320 mV (Wasser *et al.*, 2002). This means that, from thermodynamic point of view, $Fe(II)EDTA^{2-}$ is a suitable electron donor for bacterial NOR reduction (reaction 6). Direct NOR reduction by $Fe(II)EDTA^{2-}$ (reaction 6) has, to the best of our knowledge, not been reported in literature. However, $Fe(II)EDTA^{2-}$ is an excellent electron donor for Cytochrome c reduction (reaction 7), as reported by Hodges *et al.* (1974):

$$NOR_{ox} + 2\ Fe(II)EDTA^{2-} \leftrightarrow NOR_{red} + 2\ Fe(III)EDTA^{-} \quad (6)$$

$$Cytc_{ox} + Fe(II)EDTA^{2-} \leftrightarrow Cytc_{red} + Fe(III)EDTA^{-} \quad (7)$$

Cytochrome c is a physiological electron donor of cNOR, the most abundant NOR type reported so far (Hendriks *et al.*, 2002; Wasser *et al.*, 2002). NOR reduction by Cytochrome c, and the associated NO reduction to N_2O by reduced bacterial NOR, proceeds subsequently according to reactions 8 and 9:

$$NOR_{ox} + 2\ Cytc_{red} \leftrightarrow NOR_{red} + 2\ Cyt_{ox} \quad (8)$$

$$NOR_{red} + 2\ NO \leftrightarrow NOR_{ox} + N_2O \quad (9)$$

In case of NOR reduction by $Fe(II)EDTA^{2-}$, whether direct or indirect via Cytochrome c, both the electron acceptor (NO) and donor ($Fe(II)EDTA^{2-}$) are coming from the periplasmic side. Therefore, the proposed electron transfer chain does not generate a proton motive force (PMF). Energy production and cell growth can, however, still be maintained by a PMF generated via a-specific $Fe(III)EDTA^{-}$ reduction (Rosen and Klebanoff, 1981) by an enzyme-system that translocates protons. This alternative electron acceptor, $Fe(III)EDTA^{-}$, is produced by NO reduction (reactions 3 and 4) and by oxidation of $Fe(II)EDTA^{2-}$ with O_2 present in the flue gas (Chapter 4). Note that the present data do not exclude that NO reduction occurs via the more conventional electron supply chain, i.e. via the Cytochrome *bc*1 complex and prior redox couples, following ethanol oxidation (Fig. 8, pathway 3). Quantification of the electron flow of the latter pathway in redox buffered media as $Fe(II)EDTA^{2-}$ requires further research, e.g. by using antimycin for the specific inhibition of the Cytochrome *bc*1 complex (Slater, 1974).

Kinetics of NO reduction

The positive relation between the $Fe(II)EDTA^{2-}$ concentration and the NO reduction rate (Fig. 6) supports the hypothesis that the $Fe(II)EDTA^{2-}$ concentration affects the fraction of NOR that is in the reduced state, whether via direct NOR reduction (reaction 6) or indirectly via Cytochrome c reduction (reaction 7). It should be noted that, similar to the normal denitrification pathway, where NO is an intermediate, presumably also in $Fe(II)EDTA^{2-}$ media, uncharged, free NO (aq) is the electron accepting compound instead of NO bound in the charged nitrosyl-complex.

The apparent logarithmic relation between the bulk $Fe(II)EDTA^{2-}$ concentration and the NO reduction rate (Fig. 6B) suggests diffusion limitation of $Fe(II)EDTA^{2-}$ and $Fe(III)EDTA^{-}$ between the periplasmic and extracellular environments. Literature data support that the electron donor concentration influences the enzyme reduction rate. Using horseheart Cytochrome c, Hodges *et al.* (1974) found first order kinetics for both the $Fe(II)EDTA^{2-}$ and protein concentration according to:

$$d[Cyt\ c_{ox}] / dt = K * [Fe(II)EDTA^{2-}] * [Cyt\ c_{ox}] \quad (Eq.\ 3)$$

where the 2nd order rate constant K amounts $7.34 * 10^4$ $mol.l^{-1}.s^{-1}$ at 328 K (Hodges *et al.*, 1974), i.e. the operating temperature of BioDeNOx reactors (Buisman *et al.*, 1999) and the temperature applied in the present study. Also Koutny and Kucera (1999) found that the concentration of the redox mediator concentration (i.e. the electron donor for NOR reduction), in their case *N*,*N*,*N*′,*N*′-tetramethyl-*p*-

phenylenediamine (TMPD), influenced the NO reduction rate. Therefore, they modified the mechanistic model of the nitric oxide reductase of *Paracoccus denitrificans*, as proposed by Girsch and De Vries (1997). The latter authors assumed that NOR reduction was much faster than NO reduction, i.e. NOR oxidation, and therefore did not take NOR reduction and reductant concentration into account in their original model (Girsch and De Vries, 1997). They assumed that, the NO reduction rate depends on the turnover constant (k_{cat}) and the concentration of the reduced NOR enzyme that is complexed with 2 NO molecules according to:

$$d[NO]/dt = k_{cat} * [NOR_{red}(NO)_2] \quad \text{(Eq. 4)}$$

When the NO reduction rate is not limited by the NO concentration (Fig. 4B), i.e. the NO concentration is much higher than the K_m value, the volumetric NO reduction rate depended both on the denitrifying biomass, i.e. NOR, concentration (Fig. 3B and 4A) and the $Fe(II)EDTA^{2-}$ concentration (Fig. 6B). The latter is proposed to determine the fraction of NOR that is reduced (see above). Assuming that the NOR concentration of the inoculum, expressed as mol NOR per gram of total protein, is constant within the timescale of the NO reduction rate experiments (several hours), the volumetric NO reduction rate can be expressed as:

$$R_{vol.\ apparent} = [NOR] * \text{fraction } NOR_{red} * k_{cat} \quad \text{(Eq. 5)}$$

At low NO concentrations, i.e. in the range of the K_m, the NO concentration affects the NO reduction rate (Fig. 2B, Fig. 6A). This corresponds with the kinetic model of Girsch and De Vries (1997), since the NO complexed NOR concentration (which is rate determining according to equation 4) depends on the (low) NO concentration. Since two NO molecules are involved in NOR oxidation, a sigmoidal relation may be expected (Girsch and De Vries, 1997). Fig. 2B fits with such a relation, in contrast to Fig. 6A, which appears to be a first order relation. However, Fig. 6A shows only a limited range of (low) NO concentrations, which cannot be extrapolated to higher NO concentrations.

The estimated K_m values for NO (<10 nM to >200 nM) are in agreement with values reported in the literature (Table 1). By definition, K_m values are directly related to the maximum NO reduction rate, which was only reached in the BioDeNOx reactor mixed liquor assays (Fig. 4B), and not for the Veendam sludge (Fig. 2B). At first sight, the maximum specific NO reduction rate of the BioDeNOx sludge (0.34 nmol NO.mg $prot^{-1}.s^{-1}$) seems low compared to literature data, which vary from 2.3 to 183 nmol $NO.mg^{-1}.s^{-1}$ (Table 1). It should be kept in mind, however, that the latter values were obtained using the membrane fraction of pure cultures of denitrifying bacteria, e.g. *Paracoccus* denitrificans. It may be expected that membrane fractions contain (± 3 times) higher NOR concentration (Zumft and Frunzke, 1982) than whole cells, which were used for protein determination in this study. Moreover, it may be assumed that in the BioDeNOx mixed liquor, only a limited part of the protein concentration consisted of denitrifying, i.e. NO reducing, biomass, since NO reduction covers only 10-15 % of the electron flow, while the rest of the electron flow covers $Fe(III)EDTA^-$ reduction (Chapters 4 and 9). Finally, the Bradford method used in this study often gives higher values for protein concentration

when BSA is used as standard (Goetz *et al.*, 2004), compared to Lowry, which is the method used for protein estimation in the reference studies (Table 1).

Table 1

Specific NO reduction rates and K_m values in literature

NO reduction rate ($nmol.s^{-1}.mg_{prot}$)	**K_m value (nM)**	**Culture**	**Reference**
8 (membrane fraction)	27	*Paracoccus denitrificans*	Koutny and Kucera, 1999
10 (membrane fraction)	0.55	*Paracoccus denitrificans*	Girsch and De Vries, 1997
n.d.	250	*Paracoccus denitrificans*	Fujiwara and Fukumori, 1996
183 (pure NOR)	< 10.000	*Paracoccus denitrificans*	Carr and Ferguson, 1990
70 (pure NOR)	n.d	*Achromobacter cycloclastes*	Jones and Hollocher, 1993
2.3 (membrane fraction)	60.000	*Pseudomonas stutzeri*	Heiss *et al.*, 1989
n.d.	> 200	Veendam (mixed culture)	This study
0.34 (total protein)	< 10	BioDeNOx (mixed culture)	This study

n.d. = not determined

Effect of temperature

NO reduction in aqueous Fe(II)EDTA^{2-} at 30 ºC proceeded slowly compared to 55 °C (Fig. 7), despite that the Veendam inoculum originated from a denitrification reactor operating at low temperatures (5-25 ºC). From enzymatic point of view, this might be caused by a substantially faster NOR reduction, directly or indirectly via Cytochrome C, at elevated temperatures (Schichman *et al.*, 1996). Another factor that might cause the lower NOR reduction rate at 30 °C is the approximately 2 times lower free NO (aq) concentration at that temperature (Fig. 1), as a result of a lower equilibrium constant for reaction 3 (Demmink *et al.*, 1997).

NO reduction in continuous BioDeNOx reactors

In BioDeNOx reactors, the volumetric NO reduction rate should be equal to or higher than the volumetric NO-load, i.e. the amount of NO that is absorbed from the flue gas into the scrubber liquor. The rate observed in this study, 0.67 $mM.h^{-1}$, was high enough for a stable operation of a lab-scale BioDeNOx reactor treating 1.5 $m^3.h^{-1}$ artificial flue gas containing 155 ppm NO (and 3.5-3.9 % O_2) (Chapter 9). This study shows that the NO reduction capacity in BioDeNOx reactors at 55 ºC is limited by the denitrifying biomass, more specifically the NOR concentration and/or low free NO (aq) concentrations, i.e. below the K_m value. Besides, the NO reduction rate might be limited by low Fe(II)EDTA^{2-} concentrations, which may occur under full scale conditions because of EDTA degradation (Chapter 9) or poor Fe(III)EDTA^{-} reduction capacities (Chapter 4). Increasing the Fe(II)EDTA^{2-} concentration will, however, probably not result in higher NO reduction rates, since in

that case lower free NO (aq) concentrations are expected (Eq. 2). Consequently, the volumetric NO reduction capacity of BioDeNOx reactors can only be elevated by reactor operation at higher denitrifying biomass concentrations. This can be achieved by biomass retention, e.g. via the application of ultrafiltration on the reactor bleed stream or by introduction of floc or granule formation.

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CHAPTER 8

Effect of sulfur compounds on NO reduction in aqueous Fe(II)EDTA^{2-} solutions

Biological reduction of nitric oxide (NO) in aqueous solutions of EDTA chelated Fe(II) is one of the main steps in the BioDeNOx process, a novel bioprocess for the removal of nitrogen oxides (NO_x) from polluted gas streams. Since NO_x contaminated gases usually also contain sulfurous pollutants, the possible interferences of these sulfur compounds with the BioDeNOx process need to be identified. Therefore, the effect of the sulfur compounds Na_2SO_4, Na_2SO_3 and H_2S on the biological NO reduction in aqueous solutions of Fe(II)EDTA^{2-} (25 mM, pH 7.2, 55 $^{\circ}$C) was studied in batch experiments. Sulfate and sulfite were found not to affect the reduction rate of Fe(II)EDTA^{2-} complexed NO under the conditions tested. Sulfide, either dosed externally or formed out of endogenous sulfur sources or the supplied sulfate or sulfite during the batch incubation, influences the production and consumption of the intermediate nitrous oxide (N_2O) during Fe(II)EDTA^{2-} bound NO reduction. At low concentrations (0.2 gVSS/l) of denitrifying sludge, 0.2 mM free sulfide completely inhibited the nitrosyl-complex reduction. At higher biomass concentrations (1.3 to 2.3 gVSS/l), sulfide (from 15 μM to 0.8 mM) induced an incomplete NO denitrification with N_2O accumulation. The NO reduction rates to N_2O were enhanced by the presence of anaerobic (non denitrifying) sludge, presumably because it kept FeEDTA in the reduced state.

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INTRODUCTION

The BioDeNOx process is an integrated physicochemical and biological technique for NO_x removal from flue gases. The principle is based on wet absorption of nitric oxide (NO) into an aqueous $Fe(II)EDTA^{2-}$ solution, followed by biological regeneration of the absorbent liquor in a bioreactor. Nitric oxide forms a stable nitrosyl complex with EDTA chelated ferrous iron (Demmink *et al.*, 1997) according to the following reaction:

$$Fe(II)EDTA^{2-} + NO \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \quad (1)$$

To regenerate $Fe(II)EDTA^{2-}$, biological NO reduction to N_2 takes place in the bioreactor under thermophilic conditions (50-55 $^{\circ}$C) using ethanol as an electron donor according to the overall reaction:

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 6\ Fe(II)EDTA^{2-} + 3\ N_2 + CO_2 + 3\ H_2O \quad (2)$$

NO reduction to N_2 is biologically catalysed with nitrous oxide (N_2O) as an intermediate (Chapter 3). Various inoculum types originating from anaerobic as well as aerobic wastewater treatment plants were shown to be able to reduce NO to N_2 under thermophilic conditions (55 $^{\circ}$C). Moreover, it was found that the absorbent itself, $Fe(II)EDTA^{2-}$ can also serve as electron donor for biological reduction of nitric oxide, with the concomitant formation of $Fe(III)EDTA^{-}$ (Chapter 3):

$$2\ Fe(II)EDTA\text{-}NO^{2-} + 2\ Fe(II)EDTA^{2-} + 4\ H^{+} \rightarrow N_2 + 2\ H_2O + 4\ Fe(III)EDTA^{-} \quad (3)$$

To maintain continuous regeneration of $Fe(II)EDTA^{2-}$, besides reduction of $Fe(II)EDTA\text{-}NO^{2-}$, also $Fe(III)EDTA^{-}$ (formed according equation (3) and through oxidation of $Fe(II)EDTA^{2-}$ by oxygen in the flue gas) must be reduced back to $Fe(II)EDTA^{2-}$. In the presence of ethanol, $Fe(III)EDTA^{-}$ is reduced to $Fe(II)EDTA^{2-}$ according to the overall reaction:

$$12\ Fe(III)EDTA^{-} + C_2H_5OH + 3\ H_2O \rightarrow 12\ Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^{+} \quad (4)$$

In practice, the effect of sulfur compounds on the BioDeNOx process needs to be taken into account, as NO containing flue gasses mostly also contain sulfurous pollutants as e.g. sulfur dioxide (SO_2). The latter dissolves as sulfite (SO_3^{2-}) or sulfate (SO_4^{2-}) in the scrubber liquor, which can be reduced to H_2S in the bioreactor. Also when retrofitting the limestone-gypsum process by integrating BioDeNOx for NO removal in this chemical SO_2 removal process (Lagas, 2000), the bioreactor mixed liquor will contain elevated concentrations of sulfur compounds, as the limestone-gypsum process typically works with high (up to 800 mM) $CaSO_3$ concentrations (Biostar, personal communication).

The role of sulfide in the BioDeNOx process is somewhat controversial (Figure 1). On the one hand, sulfide is known to inhibit bacterial activity (Koster *et al.*, 1986; Hilton and Oleskiewicz, 1988; Percheron *et al.*, 1998) and inhibition by sulfide of the NO and N_2O reduction have been reported extensively in the literature (Sörensen *et al.*, 1980; Tam and Knowles, 1979) (Figure 1, line b). Sulfide was found responsible for accumulation of these intermediate gases during denitrification at expense of the final product N_2. On the other hand, sulfide stimulates the reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} (Figure 1, line a) because of its role in an electron shuttling mechanism (Chapter 6). Since Fe(II)EDTA^{2-} is an electron donor in the Fe(II)EDTA-NO^{2-} reduction via N_2O and N_2, sulfide may consequentially stimulate the NO reduction in Fe(II)EDTA^{2-} solutions by keeping Fe(II)EDTA^{2-} in the reduced state and thus providing optimal conditions for NO reduction (Figure 1, line c).
This chapter investigates the effect of sulfide on the biological NO reduction in aqueous Fe(II)EDTA^{2-} solutions and discern between its potential inhibiting and stimulating effect. The effect of the sulfur compounds sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}), whose reduction may result in the generation of sulfide, was also investigated using batch experiments.

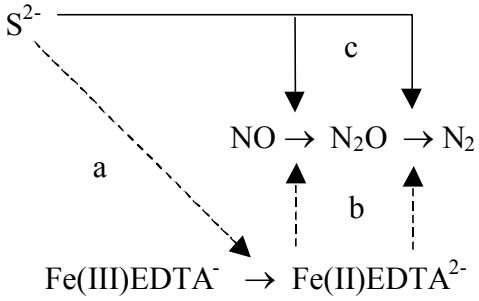

Figure 1
The possible stimulating and inhibiting roles of sulfide on NO reduction: sulfide enhances the reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} (line a) and indirectly may enhance the NO reduction which use Fe(II)EDTA^{2-} as electron donor (line b); sulfide may inhibit the reduction of NO to N_2 via the intermediate N_2O (line c).

MATERIALS AND METHODS

Medium

The composition and preparation of the Fe(III)EDTA$^-$ medium used in this study is described in Chapter 3. Experiments were performed in medium both with and without 5 mM sulfate. In latter case, $MgSO_4.7H_2O$ was replaced by $MgCl_2.H_2O$. The effect of trace metals on NO reduction was investigated by increasing the trace metal content of the Fe(III)EDTA$^-$ medium. This was done by the addition of 5 ml trace metal stock solution (Visser *et al.*, 1993) per liter medium, instead of 1 ml//l.

Inoculum

Denitrifying "Veendam" sludge originated from a denitrifying fluidized bed reactor (Veendam, the Netherlands), treating nitrate contaminated surface water (See Chapter 3).The sludge was collected

from the top of the reactor either before or after being separated from the sand used as biomass carrier. In sludge sampled before the biomass removal, i.e. still containing the sand, anaerobic conditions established fast during storage and consequently sulfide was generated from endogenous sulfur sources as evidenced by blackening of the sludge. In some assays, the denitrifying "Veendam" inoculum was pre-cultivated for one day with nitrate (5 mM) and ethanol (10 mM) at 55 °C in FeEDTA free medium as described elsewhere (Van der Maas *et al.*, 2002).

Anaerobic methanogenic granular "Eerbeek" sludge originated from a full scale upflow anaerobic sludge bed (UASB) reactor in Eerbeek (the Netherlands) treating paper mill wastewater (See Chapter 5) BioDeNOx sludge was taken from a lab scale BioDeNOx reactor that treated an artificial NO contaminated flue gas (Chapter 9).

Activity test procedure

NO reduction experiments were conducted at 55 ^{0}C in duplicate in 120 ml serum bottles filled with 80 ml oxidized Fe(III)EDTA$^-$ containing medium and various amount of denitrifying sludge (0.2, 1.3 or 2.3 gVSS/l). The vials were then sealed and the headspace replaced by Ar/CO_2. Bottles were fed with ethanol as external electron donor by syringe from a concentrated stock solution to a final concentration of 5 mM.

Biological reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} using ethanol as electron donor ethanol was performed in order to obtain the desired reduced state of iron before starting NO reduction experiments. Since denitrifying sludge has a relatively low iron reducing capacity and to avoid long time of incubation, the Fe(III)EDTA$^-$ reduction was enhanced either biologically by anaerobic sludge (0.6 or 1.2 gVSS/l) injected by syringe after headspace replacement to the vials containing the denitrifying inoculum (1.3 gVSS/l) or chemically by adding a reducing agent to the medium and denitrifying biomass containing vials (1.3 gVSS/l). Both Fe^0 and Ti(III)-citrate were used as reducing agents for Fe(III)EDTA$^-$ reduction. Fe^0 was added at an initial concentration of 13 mM, slightly in excess with respect to the stoichiometric value (12.5 mM) needed to completely reduce 25 mM Fe(III)EDTA$^-$. Ti(III)-citrate, 15 mM, was injected from a concentrated Ti(III)-citrate solution (Zehnder and Wuhrmann, 1976) after replacement of the headspace. After complete reduction of Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} in the medium, NO reduction experiments were initiated by generating the nitrosyl complex by adding 2 mM nitrite (= 180 µmol in vial) from a concentrated $NaNO_2$ stock solution. In this way, nitrite is reduced chemically to form the nitrosyl complex with Fe(II)EDTA^{2-} (Zang and Van Eldik, 1990). The nitrite stock solution was made fresh in all assays and its concentration measured. Prior to nitrite injection, Fe(II)EDTA^{2-}, ethanol and acetate concentrations were measured. Based on the ethanol residue, ethanol was added from a concentrated stock solution to the vials to reach an initial concentration of 5 mM. The sulfide concentration was also measured after iron reduction just before NO_2^- addition.

Activity tests with BioDeNOx sludge were done in serum bottles filled with 80 ml reduced BioDeNOx liquor, pre-incubated at 55 °C for one night before adding 1 mM nitrite (90 µmol in vial).

No ethanol addition was needed since the reactor liquor already contained sufficiently high ethanol concentrations (± 10 mM). The protein concentration of the BioDeNOx reactor liquor was 0.2 g/l.

Experimental design

To determine the influence of sulfide on NO reduction by denitrifying sludge, 1 mM Na_2S was injected from a concentrated stock solution to the vials containing denitrifying inoculum at two different concentrations, 0.2 and 1.3 gVSS/l; sulfide was injected after iron reduction just before NO_2^- injection. The effect of sulfide was also tested indirectly: different inoculum sludge concentrations were used, in which sulfide was produced from endogenous sulfur sources during the pre-incubation of the vials for Fe(III)EDTA$^-$ reduction and that consequently corresponded to different initial sulfide concentrations. Both "Veendam", when collected together with the sand (see above) and stored for several weeks in the fridge, and the anaerobic granular sludge "Eerbeek" led to the generation of a certain amount of sulfide. Activity tests were carried out at 1.3 and 2.3 gVSS/l of denitrifying sludge and at 1.3 gVSS/l of denitrifying sludge mixed with 0.6 or 1.2 gVSS/l of anaerobic sludge (the latter used with the purpose of enhancing the Fe(III)EDTA$^-$ reduction).

The influence of sulfate was investigated by performing experiments with mixed inocula (denitrifying "Veendam" and anaerobic "Eerbeek") in sulfate free and sulfate containing (5 mM) medium. To investigate the effect of sulfite on NO reduction by denitrifying sludge, sodium sulfite (1 mM) was added from concentrated stock solutions after complete iron reduction.

Analytical methods

Analytical methods for quantifying N_2 and N_2O in the headspace, ethanol, acetate and ferrous ion in the liquid phase have been described in Chapter 3. Nitrate, nitrite and sulfate concentrations were determined by Ion Chromatography (Dionex, IonPac AS17) with a 4x250 mm P/N 055682 anion exchange column. The eluent was made using an EG40 Eluent Generator configured with a KOH-cartridge (Dionex P/N 053921) and deionized water as the carrier. Sulfide was determined colorimetrically using a cuvette-test (LYW 653, Lange) and a XION 500 spectrophotometer. The Protein concentration was determined according to Bradford (1976). Volatile suspended solids (VSS) were measured according to the Standard Methods of the APHA (1985).

Determination of the NO reduction rate

NO reduction was monitored by following N_2O and N_2 production in the headspace. The amount of gases present in the headspace was corrected for N_2O and N_2 dissolved in the aqueous phase (Lide, 2001). Data are the means of the duplicate and are reported as micromoles per vial. Bars in the graphs, which show representative data of replicate experiments, indicate the deviation standard between duplicates. First order rate constants of the NO reduction were determined by fitting the N-total data (i.e. sum of the measured N_2O and N_2) with an exponential curve (equation 5):

$$A(t) = A_0(1 - e^{-k \cdot t}) \qquad (5)$$

Where:

A(t): N-gas total amount in vial at time t (mol);

A_0: amount of N (nitrite) injected at time 0 (mol);

k: first order rate constant (d^{-1}).

Specific NO reduction rates were calculated from the first order rate constant devided the amount of VSS in vial and the nitrogen added.

RESULTS

Influence of sulfide on NO reduction by denitrifying sludge

Figure 2 shows the effect of sulfide (1mM) on the NO reduction by the denitrifying sludge in sulfate-free Fe(II)EDTA^{2-} medium. When using 0.2 g VSS/l of freshly collected denitrifying sludge (i.e. stored in the fridge for only a few days) and in the absence of externally supplied sulfide, the nitrosyl-complex generated at t = 0 was reduced completely to dinitrogen gas within almost 20 hours without any apparent lag phase (Figure 2A). Nitrous oxide could not be detected as intermediate. In contrast, at an initial concentration of 1 mM sulfide, NO reduction by 0.2 g VSS/l denitrifying sludge was completely inhibited (Figure 2B). The nitrosyl-complex was formed from the chemical reaction of nitrite with Fe(II)EDTA^{2-} but only traces of nitrous oxide and dinitrogen gas were detected within 150 hours incubation.

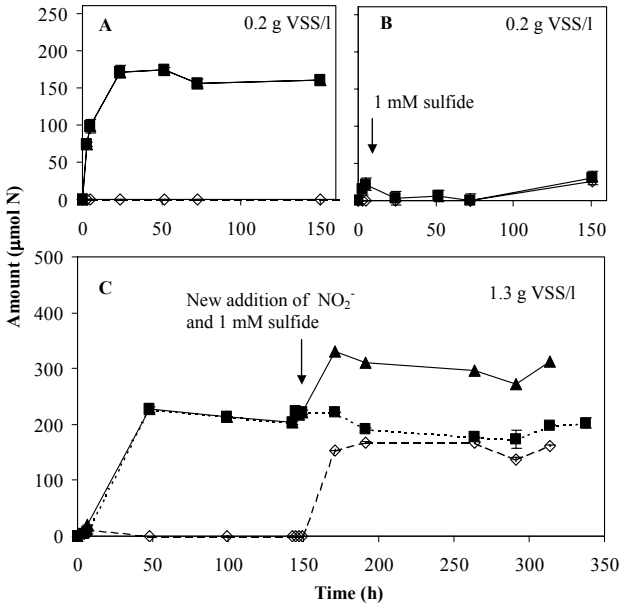

Figure 2

Effect of sulfide on NO reduction by denitrifying sludge in Fe(II)EDTA^{2-} medium. Production of N_2O (◇) and N_2 (■) and total gaseous nitrogen (▲), sum of N_2O and N_2, at 0.2 gVSS/l without (A) and with (B)1 mM sulfide injected. (C) NO reduction at an inoculum concentration of 1.3 gVSS/l. The nitrosyl-complex was generated twice (t = 0 and t = 149 hours); 1mM sulfide was added together with the second NO_2^- addition t = 149 hours).

Complete reduction of the Fe(II)EDTA^{2-} complexed NO to N_2 was also observed when using a higher inoculum concentration (1.3 g VSS/l) and without sulfide addition (Figure 2C). After a second injection of NO_2^- together with 1 mM sulfide, the nitrosyl-complex was completely converted to N_2O, which accumulated in the headspace at the expense of N_2. An average concentration of 0.22 (± 0.02) mM free sulfide was measured in the cases of sulfide addition (Table 2), so less than the concentration expected (1mM), probably due to the interaction S^{2-} with medium compounds, e.g. precipitation of metal sulfides.

Figure 3 shows NO reduction in sulfate-free Fe(II)EDTA^{2-} medium using denitrifying sludge alone, stored in the fridge for several weeks, at two different concentrations: 1.3 (Figure 3A) and 2.3 gVSS/l (Figure 3B). Although no external sulfide was added in these assays and the medium was sulfate free, a soluble sulfide concentration of 15 μM and 0.25 mM was measured prior to nitrite injection, in the vial containing 1.3 and 2.3 gVSS/l, respectively. This sulfide was probably generated from reduction of endogenous sulfur sources present in the sludge during storage. Therefore, increasing denitrifying sludge concentrations corresponded indirectly with increasing sulfide concentrations. After a short lag phase of less than 2 hours upon generation of the nitrosyl-complex at t = 0, NO reduction to N_2O occurred in the vials containing 1.3 gVSS/l sludge (Figure 3A). After 50 hours, N_2 production could also be detected. N_2O accumulation was transient: it was produced first and subsequently totally converted to N_2 within 7 days. In case of a higher sludge concentration, i.e. 2.3 g VSS/l (Figure 3B), Fe(II)EDTA^{2-} complexed NO, generated at t = 0, was reduced to N_2O at a specific rate similar as detected at lower biomass concentration (Table 2), but N_2O accumulated longer. The N_2O started to be reduced to N_2 after 100 hours of incubation and at a slower rate. Complete disappearance of N_2O and conversion to dinitrogen gas required 388 hours, instead of 120 hours in the vails with 1.3 g VSS/l.

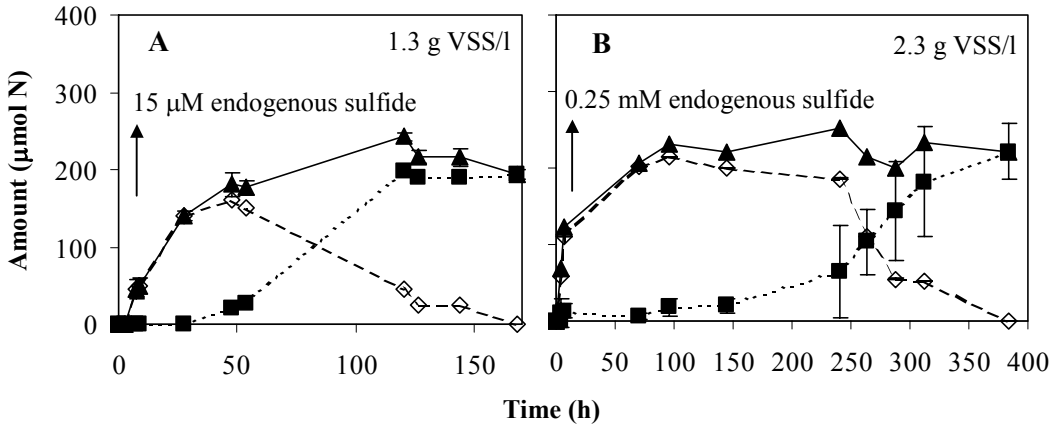

Figure 3

Production of N_2O (◇) and N_2 (■) and total gaseous nitrogen (▲), sum of N_2O and N_2, by denitrifying sludge at 1.3 gVSS/l (A) and 2.3 gVSS/l (B). Increasing endogenous sulfide concentrations corresponded indirectly with the increasing biomass amount: 15 μM and 0.25 mM, respectively in (A) and (B).

Table 2

Specific NO reduction rates and sulfide concentrations in the conditions tested. The rates values (except the first one) are referred to the first step reduction of NO to N_2O since N_2 was either not formed during the batch or formed only after total conversion of the N-added to N_2O. *a* Endogeneously produced. *b* Concentration after 1 mM S^{2-} injected. *c* mmol $N.g_{prot}.d^{-1}$. *d* $g_{prot}.l^{-1}$.

	Conditions	Sludge concentration (g VSS/l)			S^{2-} (mM)	NO reduction rate (mmol N/g VSS_{den} $.d^{-1}$)
		Denitrifying	Anaerobic	BioDeNOx		
Figure 2A	Iron reduction with Fe^0	0.2	0	/	0	32.44
Figure 2B	Iron reduction with Fe^0; 1 mM S^{2-} added	0.2	0	/	0.22±0.02[b]	0
Figure 2C	Biological iron reduction; 1 mM S^{2-} added	1.3	0	/	nd	2.4
Figure 3A	Biological iron reduction	1.3	0	/	0.015[a]	2.7
Figure 3B	Biological iron reduction	2.3	0	/	0.25[a]	2.55
Figure 4B	Biological iron reduction	1.3	0	/	0.015[a]	2.52
		1.3	0.6	/	0.22±0.11[a]	8.20
		1.3	1.2	/	0.88±0.03[a]	11.98
Figure 5	Biological iron reduction; increased trace metals	1.3	0	/	0.013[a]	6.11
Figure 6	Biological iron reduction	0	0	0.2[d]	0.1[a]	33.6[c]
Figure 7A	Biological iron reduction;	1.3	0.6	/		
	SO_4^{2-}-containing medium				0.16[a]	5.14
	SO_4^{2-}-free medium				0.15[a]	5.37
Figure 7B	Iron reduction with Ti(III)	1.3	0	/	nd	6.78
Figure 7B	Iron reduction with Ti(III); 1 mM SO_3^{-} added	1.3	0	/	nd	7.04

NO reduction by mixed inocula: influence of anaerobic biomass

With the addition of anaerobic sludge to the 1.3 gVSS/l denitrifying sludge, the nitrosyl complex was reduced to N_2O, but no appreciable N_2 production was observed in 150 hours. All added nitrogen (187 µmol N per vial) was converted to N_2O and accumulated in the headspace at the expense of N_2, whereas N_2O accumulation by solely denitrifying sludge at that biomass concentration was transient in this period (Figure 4A).

Despite that the medium was sulfate free, rather high sulfide concentrations (from 0.16 to 0.88 mM) were detected in the case of mixed inocula (Table 2). Reduction of oxidized sulfur compounds endogenously present in the anaerobic sludge can lead to the production of up to 3 mM sulfide (Lens *et al.*, 2003). Based on the elemental composition of this sludge (Osunna *et al.*, 1994), 3.57 mg S is present in the vials (Table 3). Therefore, the addition of increasing amounts of anaerobic sludge corresponded indirectly with the addition of increasing amounts of sulfur compounds (from which sulfide was generated) and trace metals.

Interestingly, even if the anaerobic "Eerbeek" sludge is not able to reduce NO (Chapter 3), which is confirmed in control assays with anaerobic sludge alone (Figure 4A), the NO reduction rate (to N_2O since N_2 was not formed) by mixed inocula was higher than by the denitrifying inoculum alone. Figure 4B shows profiles obtained after fitting the experimental data with an exponential curve (equation 5). Table 2 shows that the corresponding NO reduction rate to N_2O, calculated considering

only the VSS of the denitrifying sludge, increased with increasing concentrations of anaerobic sludge present.

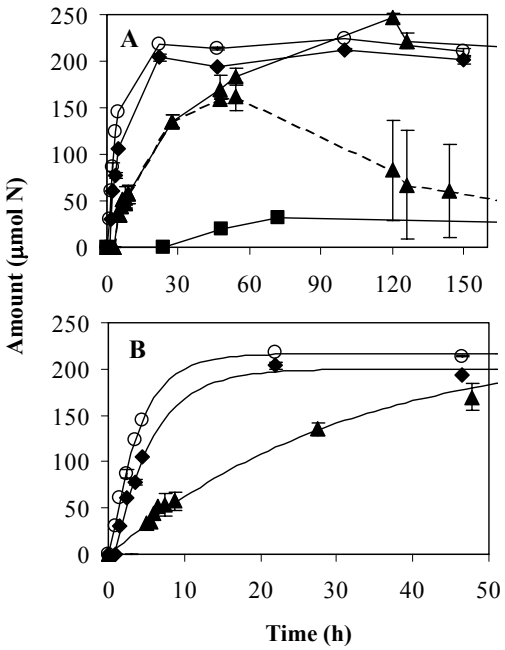

Figure 4

Effect of anaerobic sludge on NO reduction in Fe(II)EDTA^{2-} medium. A: production of N_2O (dotted line) and total gaseous nitrogen (closed line) with fixed denitrifying sludge (1.3 gVSS/l) and different anaerobic sludge concentrations: 0 g VSS/l (▲), 0.6 g VSS/l (◆), 1.2 g VSS/l (○) and with only the anaerobic sludge as a control (■). In case of mixed inocula, the total gaseous nitrogen coincides with N_2O because N_2 was not formed in 150 hours. B: Exponential curves fitting experimental data to determine the k values (equation 5).

Table 3

Total metal amounts introduced into the vials with anaerobic sludge (0.6 g VSS/l) and with trace metal solution (when the concentration was increased 5 times).

	mg (in vials) Anaerobic sludge	mg (in vials) Trace metal solution
Cobalt	0.005	0.226
Nickel	0.003	0.015
Copper	0.010	0.006
Zinc	0.016	0.011
Manganese	0.023	0.076
Iron	3.804	0.253
Sulfur	3.566	

Effect of trace metals

To determine whether essential trace metals present in the anaerobic sludge were stimulating the NO reduction rate of the denitrifying sludge (Figure 4), the effect of trace metals on NO reduction by solely denitrifying sludge was investigated. Indeed, an increase in the total trace metal content led to a subtantial higher NO reduction rate (Figure 5 and Table 2).

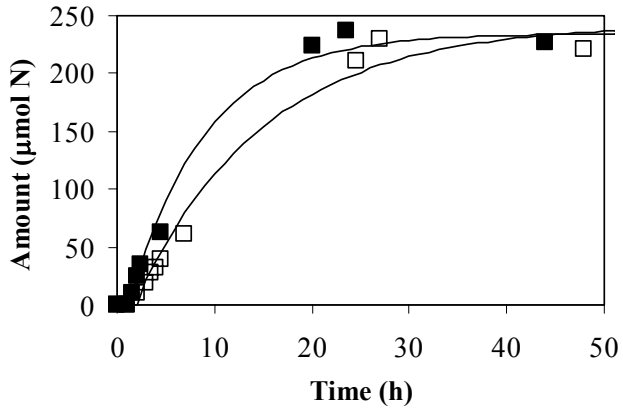

Figure 5

Influence of trace metals on NO reduction by denitrifying sludge. Total gaseous nitrogen (which coincided with nitrous oxide) with standard trace metal concentration (■) and 5 times increased concentration (□). Lines represent exponentially fitted curves.

NO reduction by BioDeNOx sludge

Upon the generation of the nitrosyl complex Fe(II)EDTA-NO^{2-} at t = 0 hours, Figure 6 shows that the BioDeNOx reactor liquor produced both N_2O and N_2 without a lag-phase. The specific NO reduction rate was higher than that of the denitrifying Veendam sludge (Table 2). After 24 hours, 80 µmol NO was converted to N-gas, 50 µmol as nitrous oxide and 30 µmol as nitrogen gas (Figure 6). These concentrations stayed constant during the rest of the experiment. The Fe(II)$EDTA^{2-}$ at the time of nitrite injection was 15 mM; the sulfide concentration at time zero was 0.1 mM.

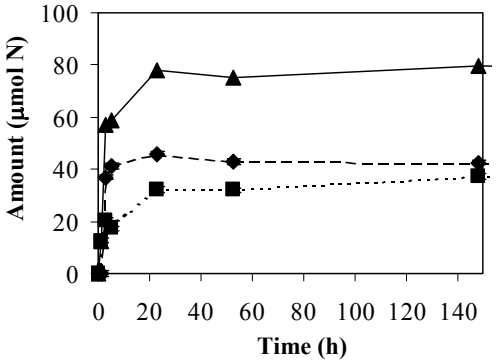

Figure 6

Production of N_2O (◆), N_2 (■) and total gaseous nitrogen (▲), sum of N_2O and N_2, out of Fe(II)$EDTA^{2-}$ complexed NO by BioDeNOx sludge. The nitrosyl complex Fe(II)EDTA-NO^{2-} was generated at t = 0 hours.

Influence of sulfate and sulfite

Figure 7A shows that the NO reduction rate by mixed inocula (denitrifying and anaerobic sludge) are similar in sulfate free medium and sulfate (5 mM) containing medium. N_2 production could not be detected in either case during the 98 hours experiment. Table 2 shows that comparable sulfide concentrations were present in both cases.

Figure 7B shows that the NO reduction rate to N_2O by denitrifying sludge was not influenced by 1 mM Na_2SO_3. After 24 hours, all nitrogen supplied was converted to N_2O without any N_2 production. In the presence of 1 mM sulfite, the N_2O concentration remained unchanged (Figure 7B). In the control without sulfite, the N_2O concentration was zero after 10 days, and only N_2 was measured as the end product, even if the nitrogen balance could not be completely closed (almost 100 µmol N was measured).

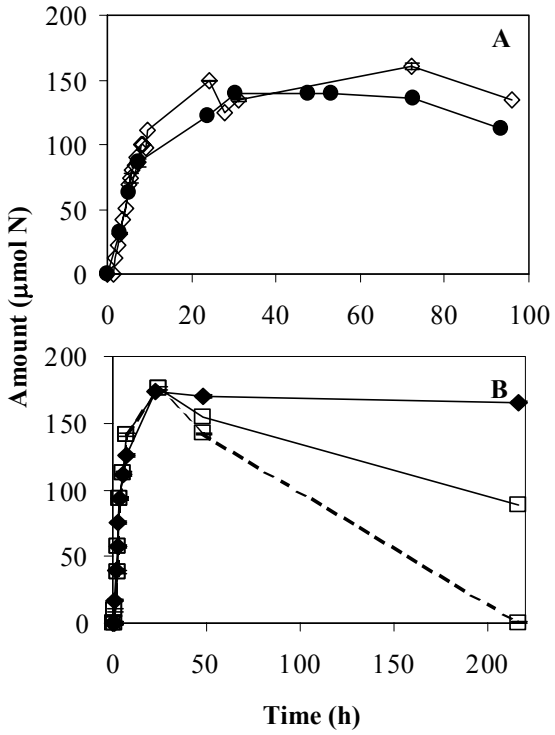

Figure 7

Effect of sulfate (A) and sulfite (B) on NO reduction in Fe(II)EDTA^{2-} medium. A: total gaseous nitrogen (closed line) in SO_4^{2-}-free medium (◇) and in SO_4^{2-}-containing (●) medium; total gaseous nitrogen coincide with N_2O because N_2 was not formed. B: Effect of sulfite on NO reduction: production of N_2O (dotted line) and total gaseous nitrogen (closed line) in the control without sulfite (□) and with 1 mM $NaSO_3$ (◆).

DISCUSSION

Influence of sulfide on NO reduction

This study showed that sulfide (at concentrations from 15 µM to 0.8 mM) inhibits the reduction of the Fe(II)EDTA^{2-} bound NO to N_2 at 55 °C and pH 7.2 (Figures 2, 3, 4). Sulfide inhibits both steps of the nitrosyl-complex reduction, depending on the specific amount of sulfide per g VSS of denitrifying

inoculum, with N_2O conversion to N_2 being the most vulnerable step. Indeed, 0.2 mM free sulfide completely inhibited the nitrosyl-complex reduction by 0.2 g VSS/l (Figure 2B), whereas at higher inoculum concentrations (1.3 and 2.3 g VSS/l) there was no major effect of sulfide at the level of NO reduction but the reduction of the $Fe(II)EDTA^{2-}$ bound NO showed a significant accumulation of nitrous oxide instead of dinitrogen gas, thus only the second step was inhibited (Figure 2C, 3, 4). Nitrous oxide formation was transient or it accumulated longer depending on the initial sulfide concentration (Table 2). This is in agreement with what is reported in the literature. Hydrogen sulfide has been associated with the release of nitrous oxide in natural environments as soil and marine sediments (Sörensen, 1978; Firestone *et al.*, 1980) and in activated sludge system (Schönharting *et al.*, 1998; Hanaki *et al.*, 1992). Sörensen *et al.* (1980) showed that only 0.3 mM S^{2-} causes a partial inhibition of NO reduction and a strong inhibition of N_2O reduction by *Pseudomonas fluorescens*. Tam and Knowles (1979) reported a decreasing N_2O reduction rate in soil by the addition of only 8 to 64 μmol sulfide g^{-1} soil and a complete inhibition of N_2O reduction by cell suspensions of *Pseudomonas aeruginosa* in the presence of 8 mM sulfide.

Nitrous oxide is an obligatory intermediate in the pathway of dissimilatory denitrification (Ye *et al.*, 1994; Zumft, 1997). Several factors can lower the rate of the reduction steps and terminate denitrification at the level of N_2O instead of dinitrogen gas, including a low C/N ratio (Itokawa *et al.*, 2001), high oxygen concentration (Otte *et al.*, 1996) and the lack or the inhibition of nitrous oxide reductase (N_2OR). In this study, nitrous oxide accumulated in the presence of ethanol and acetate (high C/N ratio) and the absence of oxygen. All the denitrification enzymes were present and active in the denitrifying sludge investigated, as indicated by the full reduction of nitrate to N_2 via nitrite and nitrous oxide or directly to N_2 (data not showed) during pre-cultivation at 55 °C of the denitrifying sludge in FeEDTA free medium with nitrate as electron acceptor and ethanol as electron donor. Thus, the inhibition of the N_2OR by sulfide is likely the reason for the observed N_2O accumulation. Beside sulfide, also NO is an inhibitor of N_2O reduction at concentrations below 20 μM in the liquid phase (Frunzke and Zumft, 1986). However, as reported in Chapter 7, only very low concentrations of NO ($NO(aq) \leq 0.1$ μM) can be expected to be present in the activity test vials in this study.

The observed stronger inhibitory effect by sulfide on the reduction of N_2O to N_2 suggests that nitrous oxide reductase (N_2OR) is much more sensitive to sulfide than the nitric oxide reductase (NOR). Indeed, Sorensen *et al.* (1987) reported that N_2OR is very sensitive to the presence of sulfide, even at very low concentrations (< 1 μM). Consequently, sulfide could constitute a bigger problem for the accumulation of nitrous oxide during $Fe(II)EDTA^{2-}$ bound NO reduction than for the inhibition of NO reduction. In a denitrification process as BioDeNOx, nitrous oxide accumulation is unwanted, because it is a potential greenhouse gas and it is involved in the chemical destruction of the ozone layer (Badr and Probert, 1993). Nitrous oxide reductase is a metalloenzyme (Zumft, 1997) and in particular copper has been individuated as a functional part of N_2O reductase in denitrifying bacteria (Matsubara *et al.*, 1982; Iwasaka *et al.*, 1981). Sulfide can readily react with copper to form copper sulfide precipitates, resulting in a lower copper concentration available for N_2OR activity, or denaturation of the N_2OR enzyme. Further studies are needed to determine the specific action of sulfide on the N_2OR,

which can lead to ways to overcome the N_2O accumulation during heterotrophic denitrification in sulfide containing wastewaters.

Effect of anaerobic sludge on NO reduction

Figure 4 shows that the rate of the NO reduction to N_2O by denitrifying sludge increased with increasing concentrations of anaerobic sludge, despite that elevated sulfide concentrations, which led to N_2O accumulation at expense of N_2, were generated. The accelerating effect of the anaerobic sludge might be due to the presence of some trace metals in the anaerobic inoculum that were limiting the denitrification capacity. Indeed, experiments with the denitrifying sludge with elevated trace metal concentrations showed that some metal in the trace metal solution used was limiting (Figure 5). Table 3 compares the total metal amounts introduced to the vials via the anaerobic sludge with those via the trace metal solution (supplied at a 5 times increased concentration). The amount of metals present in the anaerobic sludge is not much greater than their amount present in solution, except for iron. However, this element can be excluded to be a limiting element since the medium is an iron-based medium. It must also be considered that the values reported are the total amounts, measured after digestion of sludge with strong acids, so not all metals are readily available for microbial metabolism (Table 3). Since the anaerobic sludge contained an endogenous sulfur stock leading to more elevated sulfide concentrations (Table 2), also a potential sulfide scavenging by the trace metals or the sludge cannot explain the higher NO reduction rates.

The more likely explanation for the accelerated NO reduction rate to N_2O is the higher iron reducing capacity of the anaerobic sludge. Fe(II)EDTA^{2-} is a suitable electron donor for NO reduction, and Chapter 7 showed that the NO reduction rate of the denitrifying Veendam biomass depended on the Fe(II)EDTA^{2-} concentration in the medium. When using mixed inocula, the Fe(II)EDTA^{2-} concentration is kept high (and the redox potential low): Fe(III)EDTA^{-} screening experiments (Van de Sandt, 2002) showed that "Eerbeek" sludge has a high EDTA-chelated iron reduction capacity (30 mmol Fe gVSS^{-1} h^{-1}), whereas the chelated iron reduction capacity of "Veendam" sludge is relatively low (1.4 mmol Fe gVSS^{-1} h^{-1}). Thus, the increasing NO reduction rates to N_2O by mixed inocula observed at increasing amounts of anaerobic sludge is likely due to the higher FeEDTA reducing capacity creating low redox potentials.

Higher rates of NO reduction to N_2O were also found when iron reduction was accelerated using the reducing agents Fe^0 and Ti(III) in vials incubated with solely the denitrifying sludge (Table 2). The reducing agents had the same effect as "Eerbeek" sludge on keeping the Fe(II)EDTA^{2-} concentration high, thus providing a low redox potential.

Influence of sulfate and sulfite

When sulfate is present, dissimilatory sulfate reducing bacteria (SRB) use this oxyanion as a terminal electron acceptor for the degradation of organic compounds (like ethanol) under anaerobic conditions, which results in the generation of sulfide (Oude Elferink *et al.*, 1994). In addition to sulfate reduction, also reduction of sulfite to sulfide is common among SRB. Moreover, also non-sulfate reducing bacteria, as e.g. *Clostridia* and *Escherichia coli*, can reduce SO_3^- to sulfide (Robles *et al.*, 2000). On

the other hand, beside sulfide also sulfite has been shown to be inhibitory to denitrifying bacteria, i.e. *Thiobacillus denitrifican*s (Lee and Sublette, 1991) as well as to methanogens (Balderston and Payne, 1976).

Figure 7A demonstrated that the presence of 5 mM sulfate in the medium did not influence the NO reduction rate by the mixed inocula. In that case, the endogenous sulfide present in the inoculum was probably high enough to mask the effect of sulfide produced from biological reduction of the supplied sulfate (Table 2). In case sulfate reduction would occur, sulfide can be expected to have the same inhibitory effect on the NO reduction to N_2, causing nitrous oxide accumulation or complete inhibition of NO conversion, depending on the amount of sulfide formed and biomass present. Figure 7B shows that sulfite at the concentrations tested did not influence the NO reduction rate by mixed inocula. Since the solubility of calcium sulfite is very low (43 mg/l), the SO_3^{2-} concentration tested in this study (1 mM = 80 mg/l) is already higher than the maximum concentration in solution in case of 80 mM $CaSO_3$, that is the typical $CaSO_3$ concentration in the limestone-gypsum process (Lagas, 2000). Thus sulfite ions, eventually present from dissolved sulfur dioxide or because of the $CaSO_3$ formed during chemical removal of sulfur dioxide, does not represent a problem for the NO reduction in the BiodeNOx process. As in the case of sulfate, if sulfite is reduced to sulfide, the latter is expected to cause nitrous oxide accumulation or complete inhibition of the denitrification pathway. The total conversion of the $Fe(II)EDTA^{2-}$ bound NO to N_2 in the control without sulfite (Figure 6B) after almost 200 hours compared to the accumulation of nitrous oxide in vials with sulfite present at that time suggests that sulfide (not determined) was formed by sulfite reduction causing a higher sulfide concentration and longer N_2O accumulation.

CONCLUSIONS

Sulfide, either dosed or formed during the batch incubation from endogenous sulfur sources, influences the production and consumption of the intermediate nitrous oxide during the reduction of $Fe(II)EDTA^{2-}$ complexed NO. Already 0.2 mM free sulfide completely inhibited the NO reduction by 0.2 gVSS/l denitrifying biomass, whereas at higher (from 1.3 to 2.3 gVSS/l) biomass concentrations sulfide (from 15 μM to 0.8 mM) exerted an inhibition of the NO reduction at the level of N_2O reduction since N_2O accumulated at expense of N_2. NO reduction rates to N_2O were enhanced by anaerobic sludge, presumably because it kept FeEDTA in the reduced state. Sulfate and sulfite do not affect the reduction rate of $Fe(II)EDTA^{2-}$ complexed NO under the conditions tested. However, sulfide formed from the reduction of these compounds under anaerobic conditions will lead to incomplete NO reduction.

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CHAPTER 9

Fe(II)EDTA^{2-} regeneration, biomass growth and EDTA degradation in BioDeNOx reactors

The technical and economical feasibility of the BioDeNOx concept is strongly determined by high rate biological regeneration of the aqueous Fe(II)EDTA^{2-} scrubber liquor and by EDTA degradation. This chapter deals with the Fe(II)EDTA^{2-} regeneration capacity and EDTA degradation in a lab-scale BioDeNOx reactor (10-20 mM Fe(II)EDTA^{2-}, pH 7.2 ± 0.2, 55 ºC), treating an artificial flue gas (1.5 $m^3.h^{-1}$) containing 60 – 155 ppm NO and 3.5-3.9 % O_2. The results obtained show a contradiction between the optimal redox state of the aqueous FeEDTA solution for NO absorption and the biological regeneration. A low redox potential (below – 150 mV vs Ag/AgCl) is needed to obtain a maximal NO removal efficiency from the gas phase via Fe(II)EDTA^{2-} absorption. Fe(III)EDTA^{-} reduction was found to be too slow to keep all FeEDTA in the reduced state. Stimulation of Fe(III)EDTA^{-} reduction via periodical sulfide additions (2 mM spikes twice a week for the conditions applied in this study) were found to be necessary to regenerate the Fe(II)EDTA^{2-} scrubber liquor and to achieve stable operation at redox potentials below –150 mV (pH 7.2±0.2). However, redox potentials of below –200 mV should be avoided since sulfide accumulation is unwanted because it is toxic for NO reduction. Very low values for biomass growth rate and yield, respectively, 0.043 d^{-1} and 0.009 mg protein per mg ethanol, were observed. This might be due to substrate limitations, i.e. the electron acceptors NO and presumably polysulfide, or to physiological stress conditions induced by the EDTA rich medium or by radicals formed in the scrubber upon the oxidation of Fe(II)EDTA^{2-} by oxygen present in the flue gas. Radicals possibly also induce EDTA degradation, which occurs at a substantial rate: 2.1 (± 0.1) $mM.d^{-1}$ under the conditions investigated.

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INTRODUCTION

Industrial flue gases are a major source of NO_x emission to the atmosphere (Schnelle and Brown, 2002). At present selective catalytic reduction (SCR) is the most common technology applied for NO_x abatement. High energy consumption and costs, as well as ammonia emissions are, however, major drawbacks of the SCR technology. Biological NO_x removal techniques may become attractive alternatives, but the poor solubility of NO in water results into relatively high residence times of at least 1 minute (Jin *et al.*, 2005), and, therefore, large reactor volumes. To enhance the NO transfer from the gas to the liquid phase, aqueous solutions of ferrous chelates, e.g. $Fe(II)EDTA^{2-}$, can be applied as scrubber liquor, because ferrous EDTA reactively absorbs NO according to:

$$NO\ (aq) + Fe(II)EDTA^{2-} \leftrightarrow Fe(II)EDTA\text{-}NO^{2-} \quad (1)$$

The BioDeNOx process (Buisman *et al.*, 1999) utilises this principle of wet absorption of NO in an aqueous $Fe(II)EDTA^{2-}$ solution and combines it with biological reduction of the sorbed NO according to the overall reactions:

$$6\ Fe(II)EDTA\text{-}NO^{2-} + C_2H_5OH \rightarrow 6\ Fe(II)EDTA^{2-} + 3\ N_2 + 2\ CO_2 + 3\ H_2O \quad (2)$$

Since flue gases generally contain oxygen, part of the $Fe(II)EDTA^{2-}$ is oxidized to $Fe(III)EDTA^-$ (reaction 3). Therefore, reduction of EDTA chelated Fe(III) (overall reaction 4) is, besides NO reduction, a core reaction of the biological regeneration pathway in the BioDeNOx process.

$$4\ Fe(II)EDTA^{2-} + O_2 + 4\ H^+ \rightarrow 4Fe(III)EDTA^- + 2\ H_2O \quad (3)$$

$$12\ Fe(III)EDTA^- + C_2H_5OH\ + 3\ H_2O \rightarrow 12\ Fe(II)EDTA^{2-} + 2\ CO_2 + 12\ H^+ \quad (4)$$

The results of chapter 7 showed that the volumetric NO reduction rate in aqueous $Fe(II)EDTA^{2-}$ solutions is determined by the NO and $Fe(II)EDTA^{2-}$ concentration and by the amount of denitrifying biomass. However, not the NO reduction but $Fe(III)EDTA^-$ reduction was found to be the process rate limiting factor of BioDeNOx reactors treating flue gas with more than 1 % O_2 oxygen (Chapter 4). The volumetric $Fe(III)EDTA^-$ reduction rate can be enhanced by the presence of low concentrations of an electron mediating compound (EM), presumably polysulfides, that are formed upon addition of sodium sulfide to the aqueous $Fe(III)EDTA^-$ solution (Chapter 6).

Except a high rate biological regeneration of the aqueous $Fe(II)EDTA^{2-}$ scrubber liquor, pilot scale trials using real flue gases indicated that the technical and economical feasibility of the BioDeNOx concept is strongly determined by EDTA degradation (Biostar, personal communication). Both chemical (Witschel *et al.*, 2001) and biological (Nörtemann, 1999) degradation of EDTA is well documented, but it is unknown to what extend these processes contribute to EDTA degradation in BioDeNOx reactors.

The present study investigates the effect of sulfide addition and biomass concentration on the Fe(II)EDTA^{2-} regeneration capacity of BioDeNOx reactors, i.e. the volumetric NO and Fe(III)EDTA^{-} reduction rate. The investigations were conducted using a continuous operating laboratory scale BioDeNOx reactor (pH 7.2 ± 0.2, 55 ºC), treating an artificial flue gas (1.5 $m^3.h^{-1}$) containing 60 – 155 ppm NO and 3.5-3.9 % O_2. Furthermore, the biomass growth and EDTA degradation during BioDeNOx operation was determined.

MATERIAL AND METHODS

Reactor configuration

The reactor configuration used in this study is schematically presented in Fig. 1. The bioreactor consisted of a 5.5 dm^3 cylindrical double jacket glass column. Scrubbing of the artificial flue gas occurred in a simple bubble column (wet volume 0.5 liter), mounted on top of the bioreactor (Fig. 1). The artificial flue gas was composed of pure N_2 gas, pure NO gas and pressured air in adjusted amounts with mass flow controllers (Brooks, Veenendaal, The Netherlands). The gas was continuously recirculated over the gas scrubbing column with a compressor (KNF, Freiburg, Germany) at a fixed flow of 1.5 $m^3.h^{-1}$, resulting in a gas residence time of 1.2 seconds. The NO concentrations in both the influent and effluent gas were analyzed using an automatically operating gas selector (Wageningen University, The Netherlands).

The scrubber liquor was continuously recirculated over the bioreactor with a flow of 17 $l.h^{-1}$, corresponding to a bioreactor residence time of 20 minutes. A second recirculation flow was applied to provide extra mixing of the bioreactor liquor and to increase the superficial liquid upflow velocity to 18 m/h. The temperature of the bioreactor was controlled at 55° C by means of a temperature controlled water jacket. The redox potential (ORP) and pH of the bioreactor were monitored continuously (WTW Sentix electrodes, Weilheim, Germany). The pH was controlled at 7.2 (± 0.2) by means of automatic HCl or NaOH addition. Ethanol was supplied as electron donor both manually and automatically. In the latter case, the ORP signal was used as steering parameter: the ethanol supply switched on when the ORP became higher than -175 mV. Ethanol supply stopped when the ORP of the bioreactor liquor dropped below -180 mV versus Ag/AgCl.

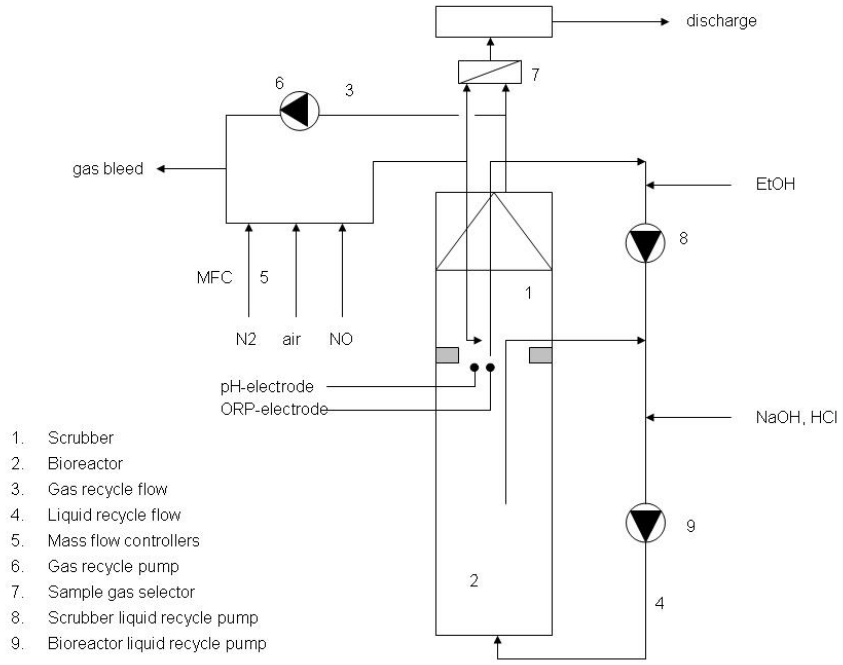

Figure 1

Schematic flow diagram of the continuous BioDeNOx reactor used in this study.

Medium composition

The medium used for all experiments contained 25 mM Na_2H_2EDTA (titriplex, Merck), 30 mM $FeCl_3$, 5 mM $MgSO_4$, 5 mM $CaCl_2$ and nutrients as described in Chapter 3. The excess of di- and trivalent cations over Na_2H_2EDTA was applied to prevent cell lysis (Ayres, 1998).

Sources of biomass

The bioreactor was inoculated with two sources of biomass: denitrifying Veendam sludge and methanogenic Eerbeek sludge. The denitrifying 'Veendam' sludge originated from a methanol fed denitrifying fluidized bed reactor treating surface water (Veendam, The Netherlands). Veendam sludge has a high NO removal capacity (Chapter 3). The methanogenic 'Eerbeek' granular sludge originated from a full scale UASB reactor treating pulp and paper mill wastewater (Eerbeek, The Netherlands). Eerbeek sludge has a high Fe(III)EDTA$^-$ reducing activity (Chapter 5), but no NO reducing activity (Chapter 3). Before use, the Eerbeek granules were mixed with the Fe(III)EDTA$^-$ medium (1/4 w/w) and crushed for 3 minutes with a kitchen blender.

Start up of the BioDeNOx reactor

The BioDeNOx reactor was started up with 5.4 liter of Fe(III)EDTA$^-$ medium inoculated with 600 ml Veendam sludge up to an initial VSS concentration of 60 mg/l (equal to 30 mg/l protein). To enhance the Fe(III)EDTA$^-$ reduction capacity, 2 mM Na_2S was added to the reactor mixed liquor from a concentrated stock solution. In aqueous Fe(III)EDTA$^-$ solutions, these sulfide additions generate polysulfides according to (Clarke *et al.*, 1994):

$$2n\ Fe(III)EDTA^- + (n+1)\ S^{2-} \rightarrow S_{n+1}^{2-} + 2n\ Fe(II)EDTA^{2-} \qquad (5)$$

The reactor mixed liquor was pre-cultivated for 79 days by means of discontinuous NO (100 ppm) and O_2 (3%) supply to the flue gas (1.5 $m^3.h^{-1}$). The NO and O_2 loads were switched off when the ORP of the bioreactor liquor became higher than -150 mV, meaning that the FeEDTA was partly (± 40%) in the oxidized state and that the NO and/or Fe(III)EDTA$^-$ reduction capacity was lower than the NO and O_2 load of the bioreactor (see Chapter 4).

Continuous operation and experimental design

After 79 days of discontinuous operation, 3 liter of reactor liquor was replaced with 2.8 liter of fresh Fe(III)EDTA$^-$ medium and 0.2 liter of Eerbeek sludge (± 20 g VSS). From then onwards, the reactor was continuously operated with 1.5 $m^3.h^{-1}$ flue gas containing NO (55-190 ppm) and O_2 (1.0-3.9 %). The NO and Fe(III)EDTA$^-$ load were gradually increased by increasing the NO and O_2 concentration of the flue gas, respectively. This load elevation was continued until the regeneration capacity, i.e. the volumetric NO and/or Fe(III)EDTA$^-$ reduction rate, became limiting. In that case, the ORP of the bioreactor exceeded the value of -150 mV at pH 7.2 (±0.2), which is equal to an ORP of -140 mV versus Ag/AgCl when corrected for pH 7.0 (Chapter 4). To supply nutrients and EDTA (to compensate degradation), 300 ml fresh Fe(III)EDTA$^-$ medium was added daily to the reactor with the simultaneous withdrawal of 300 ml reactor liquor, resulting a hydraulic retention time (HRT) of 20 days.

During the continuous operation period, the scrubber performance was characterized by determination of the relation between the ORP of the reactor mixed liquor and the NO removal efficiency from the gas phase. To investigate the effect of bio-augmentation on the NO reduction capacity, ± 5 g VSS Veendam sludge was added to the reactor at day 98. At the start of the continuous regeneration regime (day 80), the Fe(III)EDTA$^-$ reduction capacity was enhanced by the addition of 0.2 liter Eerbeek sludge to the bioreactor. To investigate the effect of sulfide supply on the Fe(III)EDTA$^-$ reduction rate, Na_2S was added in shots of 2 mM at days 93, 98 and 111. The regeneration capacity, biomass growth and EDTA degradation were quantified as described below.

Quantification of the bioreactor regeneration capacity

To quantify the overall regeneration capacity of the bioreactor, the flue gas flow was switched off periodically, i.e. the NO and Fe(III)EDTA$^-$ load to the bioreactor were set at zero. The volumetric Fe(II)EDTA^{2-} production rate was measured by monitoring the redox potential of the reactor mixed liquor, because it is a proper indicator of the Fe(II)EDTA^{2-} concentration (Kolthoff and Auerbach, 1952; Chapter 5).

The specific NO and Fe(III)EDTA$^-$ reduction rate of the reactor mixed liquor was determined in batch experiments at 55 °C in at least duplicate. The NO reduction rate was quantified by measuring the N_2O and N_2 production as described in Chapter 3. The Fe(III)EDTA$^-$ reduction rate was measured by means of the redox potential as described in Chapter 5.

Quantification of biomass growth and EDTA degradation

Biomass growth and EDTA degradation were quantified in daily samples by measuring the protein and EDTA concentration of the reactor mixed liquor, respectively. The ethanol consumption was quantified on the basis of a mass balance using the amount of ethanol supplied to the bioreactor and the ethanol concentration of the reactor mixed liquor. To investigate EDTA degradation in the absence of oxygen, bioreactor mixed liquor was incubated in 120 ml serum flasks in the dark at both 20 and 55 ºC.

Analyses and chemicals

The NO concentrations in the in- and effluent gas of the bench-scale installation was measured continuously by a chemolumisence NO analyzer (Beckman model 951). H_2, O_2, CH_4 in the gas phase were determined by gas chromatography as described by Weijma *et al.* (2000). The N_2 and N_2O concentration were also determined by gas chromatography as described in Chapter 3. The Fe(II)EDTA^{2-} concentration was determined colorimetrically using 1,10-phenantroline (Akzo Nobel, 1996). Ethanol and acetate were determined by gas chromatography as described by Cervantes *et al.* (2000). EDTA was determined by High Performance Liquid Chromatography (HPLC) with UV spectrofotometric detection at 354 nm. The HPLC was equipped with a Vydac 302IC4.6 column (pressure 50 bar, 20°C) and a phosphate buffer (KH_2PO_4 13.6 g/l, pH 3) was used as eluent (flow rate 1.5 ml/min).

The biomass content of the reactor liquor was quantified via its protein concentration. Reactor samples were centrifuged (10 min, 10.000 rpm) and the supernatant was discharged. The pellet was dissolved in 1 N NaOH and placed in boiling water for 15 min. After boiling, the sample was neutralized with 1 N HCl and the protein concentration was determined according to Bradford (1976).

Load calculations

The bioreactor load, i.e. the amount of Fe(III)EDTA$^-$ and Fe(II)EDTA-NO^{2-} supplied to the bioreactor, was calculated using mass balances for O_2 and NO as described in Chapter 4). The load of the electron acceptors (O_2, Fe(III)EDTA$^-$ and NO) and the electron donor (ethanol) is expressed as molar electron

equivalents (meq): 1 mol of ethanol, O_2, NO and Fe(III)EDTA$^-$ corresponds to 12, 4, 2 and 1 meq, respectively.

RESULTS

Effect of redox potential on NO_x removal from the gas phase

The NO_x removal efficiency from the gas phase showed to be greatly influenced by the redox potential of the bioreactor mixed liquor. Fig. 2 shows that the redox potential should be maintained below -150 mV vs Ag/AgCl for a maximal NO removal efficiency. Under these conditions, the NO concentration in the flue gas decreased from 155 to 93 ppm, meaning that 39 % of the gaseous NO was sorbed into the aqueous Fe(II)EDTA^{2-} solution. At redox potentials exceeding -150 mV, the relative scrubber performance and thus the NO removal efficiency strongly declined (Fig. 2). It should be noted that the results of Fig. 2 relate to the NO removal capacity of the scrubber, and not to the bio-regeneration capacity (see Discussion).

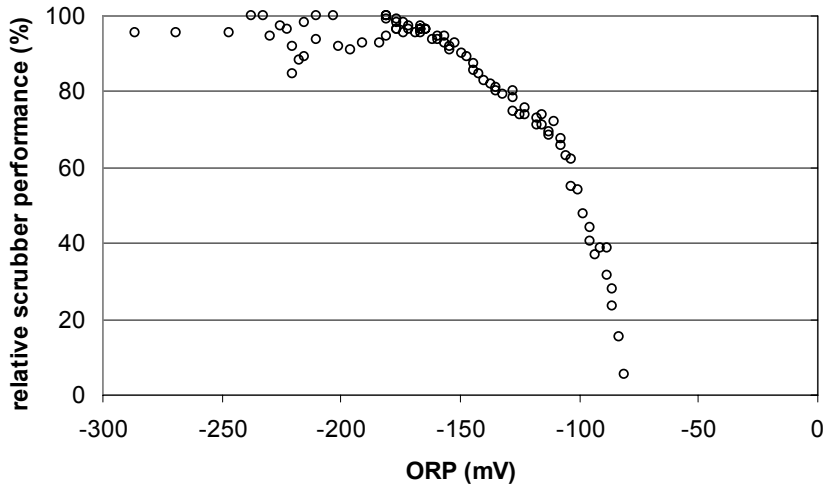

Figure 2

Relation between the redox potential (ORP) of the bioreactor mixed liquor and the relative scrubber performance for NO removal.

Quantification of the bioreactor loading rate

The redox potential of the bioreactor mixed liquor, i.e. the oxidation state of the bioreactor mixed liquor, depended on two factors: (1) the bioreactor loading rate, i.e. the amount of Fe(III)EDTA$^-$ as well as NO that enters the bioreactor via the scrubber, and (2) the regeneration capacity of the bioreactor, i.e. the volumetric Fe(III)EDTA$^-$ and NO reduction rate. Fig. 3 and Table 1, respectively, show the relation between the O_2 and NO content of the flue gas and the calculated bioreactor load based on mass balances. During the continuous operation with 1.5 $m^3.h^{-1}$ flue gas containing 3.5-3.9 %

O_2 and 155 ppm NO, the NO and O_2 removal from the gas phase amounted to, respectively, 65 and 405 mmol.d^{-1}. The NO removal corresponds to an electron acceptor flow of 0.043 meq.1^{-1}.d^{-1}, whereas the O_2 removal corresponds to a Fe(III)EDTA$^-$ loading rate of 11.3 mM.h^{-1} or 0.27 meq.l^{-1}.d^{-1}, based on the stoichiometric 1:4 ratio for Fe(II)EDTA^{2-} oxidation by oxygen (reaction 3). The volumetric ethanol consumption rate was fairly constant at 1.0 mM.h^{-1}.

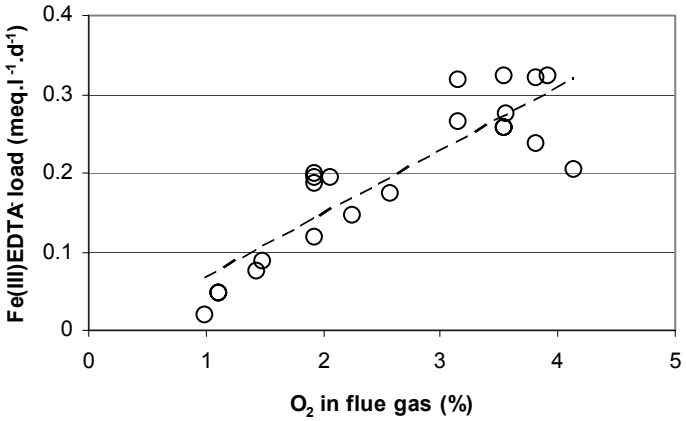

Figure 3

Relation between O_2 concentration of flue gas and the Fe(III)EDTA$^-$ loading rate of the bioreactor.

Table 1

Amount of NO absorbed andthe NO loading rate of the bioreactor in relation to the NO concentration of the flue gas.

NO concentration (ppm)	NO absorbed (mmol.h^{-1})	NO loading rate (meq.l^{-1}.d^{-1})
56	1,17	0.028
100	2.45	0.039
164	2.71	0.043

Effect of sulfide additions on the Fe(III)EDTA$^-$ reduction capacity

In order to maintain the redox potential below -150 mV versus Ag/AgCl, i.e. to avoid Fe(III)EDTA$^-$ overloading, the Fe(III)EDTA$^-$ load of the bioreactor was controlled by the air supply, e.g. the O_2 concentration in the flue gas. The ethanol concentration in the bioreactor mixed liquor was kept above 5 mM, i.e. the electron donor was available in excess. Fig. 4 shows that increasing the Fe(III)EDTA$^-$ load from 0.12 to 0.27 meq.l^{-1}.d^{-1} at day 108 resulted in an elevation of the redox potential from -480 to -120 mV vs Ag/AgCl, i.e. an increase of the Fe(III)EDTA$^-$ concentration, but the redox potential subsequently dropped to -480 mV within 1 day.

Repetition of this load increase did, however, not always lead to such a successful recovery of a low redox potential. Increase of the Fe(III)EDTA$^-$ load from 11 to 16 mM.h^{-1}, e.g. on day 110 resulted in an

ORP elevation to above -80 mV (Fig. 4), indicating that the Fe(III)EDTA^{-} reduction capacity decreased in time. Fig. 4 shows that a supply of 2 mM sulfide addition on day 111 induced an elevated Fe(III)EDTA^{-} reduction capacity, i.e. the redox potential dropped rapidly to –400 mV (pH 7.2±0.2). These low redox potentials (below -200 mV) were maintained for approximately 4 days, after which the redox potential increased again (results not shown), indicating that the sulfide additions were only effective for limited periods of time.

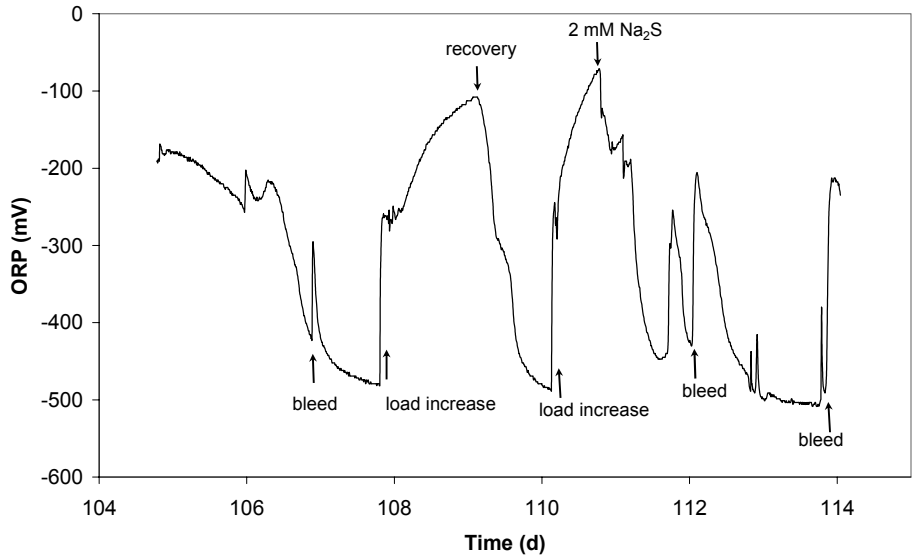

Figure 4

Effect of Fe(III)EDTA^{-} load increase (day 108 and 110) and sulfide addition (day 111) on the redox potential (ORP) of the bioreactor mixed liquor (pH 7.2±0.2). The Fe(III)EDTA^{-} load was increased due to elevation of the O_2 concentration of the flue gas.

The volumetric Fe(III)EDTA^{-} reduction rate in the reactor mixed liquor at day 116, i.e. when the total FeEDTA concentration was 15 mM and 5 days after the final sulfide shot, amounted 3.3 mM.h^{-1} (Fig 5), corresponding to a specific reduction rate of 0.06 mmol.mg prot.$^{-1}$.h^{-1}. Batch experiments showed, however, that the specific Fe(III)EDTA^{-} reduction rate depended on the biomass (protein) concentration in case a fixed amount of sulfide (0.5 mM) was added to the assays and that the specific Fe(III)EDTA^{-} reduction rate is linearly related to the amount of sulfide added per gram of protein (Table 2).

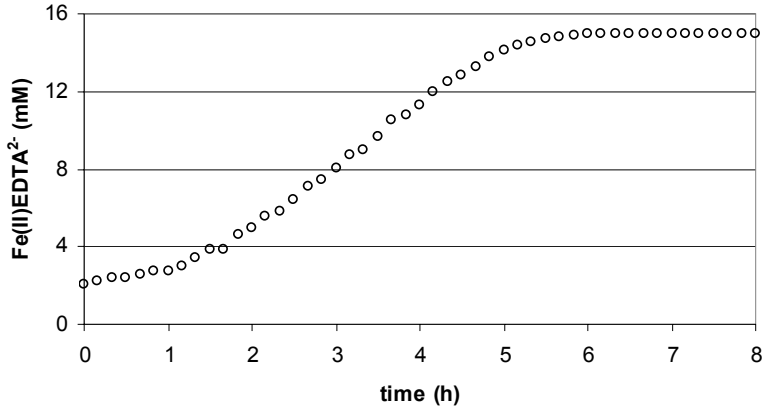

Figure 5

Fe(III)EDTA$^-$ reduction observed in the continuous reactor at day 115 (no Fe(III)EDTA$^-$ load).

Table 2

Effect of the protein concentration and the relative amount of sulfide added on the specific Fe(III)EDTA$^-$ reduction rate.

Initial protein concentration (mg.l^{-1})	Specific amount S^{2-} added (mmol.mg protein^{-1})	Specific Fe(III)EDTA$^-$ reduction rate (mmol.mg prot.h^{-1})
6.3	0.080	1.33
18.8	0.027	0.53
62.8	0.008	0.25

Effect of bio-augmentation on NO reduction capacity

Table 3 shows the volumetric NO reduction rate observed at various protein concentrations. From these data, a specific NO reduction rate of 1.2 µmol. $mg_{prot}^{-1}.h^{-1}$ can be extracted. This means that, with a NO flue gas concentration of 155 ppm, i.e. an NO loading rate of 0.45 $mM.h^{-1}$, complete Fe(II)EDTA^{2-} regeneration from Fe(II)EDTA-NO^{2-} was only possible when the reactor mixed liquor had a sufficiently high denitrification capacity, i.e. when the reactor mixed liquor contained protein concentrations higher than 350 mg/l. Bio-augmentation of the reactor with denitrifying Veendam sludge on day 98 to a biomass (protein) concentration higher than 350 mg/l (Fig. 6A) indeed induced an ORP drop of the reactor mixed liquor to below -200 mV (results not shown), indicating that the total NO load of the bioreactor was reduced.

Biomass growth and yield

Fig. 6A shows that the protein concentration of the BioDeNOx reactor amounted circa 180 mg.l^{-1} during the continuous operation period (starting at day 80), in which the reactor was operated at an HRT of 20 days. High protein concentrations were detected only after bio-augmentation with methanogenic Eerbeek sludge (day 80) and denitrifying Veendam sludge (day 98), but these

concentrations stabilized again around the average value of 150 mg.l^{-1} within 2 weeks (Fig. 6A). When the data of Fig. 7A are corrected for dilution due to medium supply to create a bleed stream, the protein concentration follows an exponential curve with time (Fig. 6B), from which an observed growth rate (μ_{obs}) of 0.043 d^{-1} can be estimated. The observed biomass yield (Y_{obs}) amounted only 0.009 mg protein per mg ethanol, based on the average protein increase (45 mg.d^{-1}) and the average ethanol consumption rate (0.8 mM.h^{-1}) during the continuous operation.

Table 3

Volumetric NO reduction rates of the BioDeNOx reactor mixed liquor at various protein concentrations.

Day of operation	Protein concentration (mg.l^{-1})	NO reduction rate (mM.h^{-1})
105	540	0.64
128	100	0.14
175	180	0.25

EDTA degradation

Fig. 7A shows the course of the EDTA concentration of the bioreactor mixed liquor during the continuous operation with 1.5 m^3.h^{-1} flue gas containing 3.5-3.9 % O_2 and 55-155 ppm NO. When corrected for the EDTA that was supplied by daily addition of fresh medium, a degradation rate of 2.1 (± 0.1) mM.d^{-1} was observed. Taking the O_2 load of 0.27 meq.l^{-1}.d^{-1} into account (Fig. 3), the relative EDTA degradation amounted 0.9 % (mol EDTA degraded per mol Fe(II)EDTA^{2-} oxidized). No EDTA degradation was observed in anaerobic batch incubations of the reactor liquor in the dark, both at 20 and 55 ºC, during 35 days (Fig. 7B).

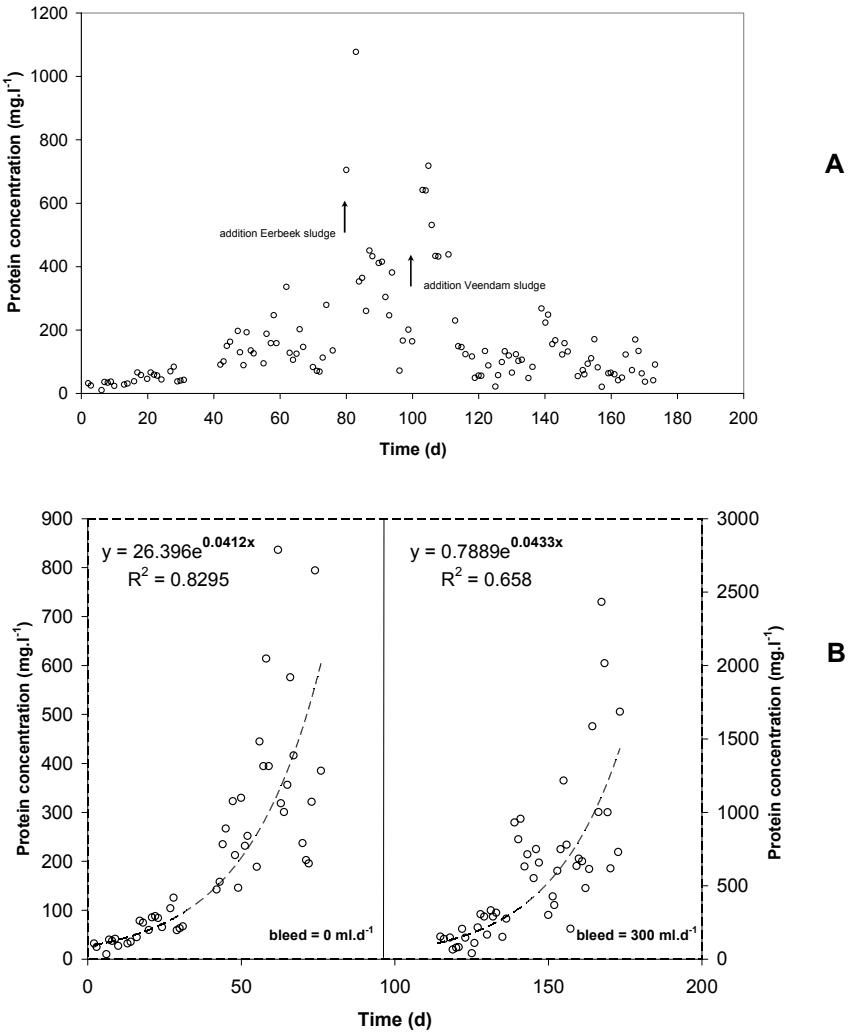

Figure 6

Protein concentration of the bioreactor mixed liquor (A) and accumulation of the protein concentration (B) during the start up period (no bleed stream applied) and during the continuous operation period (measured concentration corrected for the bleed stream).

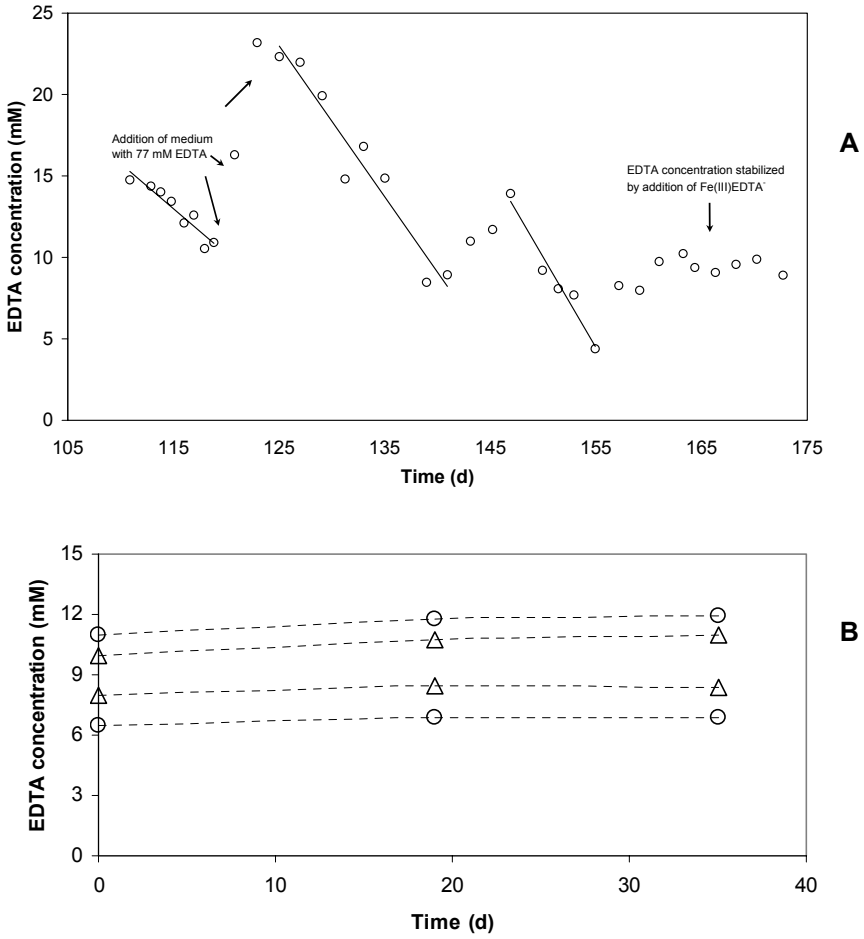

Figure 7

EDTA concentration of the bioreactor mixed liquor during continuous operation with artificial flue gas containing 3.5-3.9 % O_2 and 55-155 ppm NO (A) and during anaerobic batch incubations at 20 ºC (△) and 55 ºC (○) (B).

DISCUSSION

NO removal from the gas phase

The present study shows that the redox potential of the bioreactor mixed liquor needs to be maintained below -150 mV versus Ag/AgCl (pH 7.2 ± 0.2) for achieving the maximal NO removal efficiency from the flue gas under the conditions tested (155 ppm NO, 3.5-3.9 % O_2) (Fig. 2). This can be explained by the relation between the redox potential and the Fe(II)EDTA^{2-} concentration, which is described by the Nernst equation (Kolthoff *et al.*, 1952; Chapter 5). High Fe(II)EDTA^{2-} concentrations result in fast NO absorption into aqueous Fe(II)EDTA^{2-} solutions (reaction 1), since the kinetics are first order for both NO and Fe(II)EDTA^{2-} (Demmink *et al.*, 1997). A sufficiently fast NO absorption obviously is crucial for the economic feasibility of the BioDeNOx concept, since it directly determines the gas residence time (GRT) of the scrubber needed for a given application. In case of a fixed Fe(II)EDTA^{2-} and NO concentration, the NO_x removal from the gas phase is limited by the scrubber configuration, i.e.

contact between scrubber liquor and flue gas. In this study, the gas residence time in the wet scrubber volume amounted to only 1.2 seconds, which gave a NO removal efficiency of 40 % at an $Fe(II)EDTA^{2-}$ and influent NO concentration of 10-15 mM and 155 ppm, respectively. At comparable NO loads and O_2 concentrations, earlier studies with the same bioreactor set-up gave 70-80 % removal at a GRT of 11 seconds (Chapter 4), which is still low compared to the empty bed retention times (EBRT) of 1-2 minutes needed for more conventional biological NO removal techniques which do not utilize $Fe(II)EDTA^{2-}$ (Jin *et al.*, 2005).

Regeneration of the scrubber liquor – $Fe(III)EDTA^-$ reduction

A really stable operation of BioDeNOx reactors can only be achieved in case the regeneration capacity of the bioreactor meets at least its $Fe(III)EDTA^-$ and $Fe(II)EDTA\text{-}NO^{2-}$ load. In this study, the theoretical $Fe(III)EDTA^-$ load was circa 11 $mM.h^{-1}$ or 0.27 $meq.l^{-1}.d^{-1}$ at a flue gas O_2 concentration of 3.5-3.9 % (Fig. 3), based on the mass balance for O_2 and a stoichiometric ratio of 1:4 for the reaction between O_2 and $Fe(II)EDTA^{2-}$ (reaction 3). The volumetric $Fe(III)EDTA^-$ reduction rate was sometimes lower, resulting in elevated redox potentials (> -150 mV versus Ag/AgCl) of the bioreactor mixed liquor (Fig. 4) and, subsequently, unstable operation.

The biological reduction of EDTA chelated Fe(III) was found to be catalyzed by an electron mediating compound (EM), presumably polysulfide, which is generated upon the injection of sulfide into an aqueous $Fe(III)EDTA^-$ solution (Chapter 6). The linear dependence between the relative EM concentration (amount of EM available per bacterium) and the specific $Fe(III)EDTA^-$ reduction rate (Table 3) suggests that a sulfur compound is used as electron acceptor for microbial respiration and that the volumetric conversion rate is limited by the concentration of that electron acceptor. To achieve high volumetric $Fe(III)EDTA^-$ reduction rates it is, therefore, of great importance that this electron mediating compound is available in the BioDeNOx reactor mixed liquor at sufficiently high concentrations. In this study, sulfide additions induced an elevated $Fe(III)EDTA^-$ reduction capacity only for limited periods of time (approximately 4 days). Ideally, oxidation and reduction of the electron mediating compound is a cyclic process in which the total electron mediator concentration (oxidized plus reduced) stays constant. Both low and high redox conditions will, however, result in unbalance of the EM cycling, and thus in unstable BioDeNOx reactor operation.

At low redox potentials below -250 mV versus Ag/AgCl, i.e. when all $Fe(III)EDTA^-$ is reduced, and in the presence of ethanol, the polysulfide will be completely reduced to sulfide (reaction 6). The latter compound can precipitate with Fe^{2+}, which is the predominant valence of iron at these low redox potentials (Fe is present in excess over EDTA). In this way, the polysulfides are ultimately transformed to insoluble FeS according to reaction 7, meaning that the electron mediator has changed to a non or slowly reactive sulfur species.

$$S_n^{2-} + 2\ e^- + H^+ \rightarrow S_{n-1}^{2-} + HS^- \quad (6)$$

$$2\ Fe^{2+} + 2\ HS^- \rightarrow 2\ FeS\ (s) \quad (7)$$

High redox reactor conditions, i.e. low Fe(II)EDTA^{2-} concentrations may also threaten the presence of electron mediating polysulfides. In that case, the scrubber liquor has a relatively low capacity for oxygen scavenging and thus, soluble oxygen may be present in the scrubber liquor. This might induce oxidation of polysulfides to sulfur and thiosulfate according to reactions 8 and 9, which lowers the concentration of the reactive, catalytic sulfur species.

$$S_n^{2-} + ½\ O_2 + 2\ H^+ \rightarrow n\ S^0 \quad (8)$$

$$2\ HS^- + 2\ O_2 \rightarrow\ S_2O_3^{2-} + H_2O \quad (9)$$

Regeneration of the scrubber liquor – NO reduction

Besides Fe(III)EDTA$^-$ reduction, biological reduction of Fe(II)EDTA^{2-} complexed NO is a key reaction within the Fe(II)EDTA^{2-} regeneration. Batch studies showed that NO reduction is coupled to Fe(II)EDTA^{2-} oxidation, i.e., except absorbent, Fe(II)EDTA^{2-} is also an electron donor in BioDeNOx reactors (Chapters 3 and 7). The volumetric NO reduction rate of the bioreactor liquor depended linearly on the biomass (protein) concentration (Table 3). This explains why stable operation of the BioDeNOx reactor at higher NO loadings (> 0.3 mM/h) was only possible after bio-augmentation with denitrifying biomass (at day 98). It is assumable that the biomass (protein) concentration is directly related to the concentration of Nitric Oxide Reductase, the key enzyme in bacterial NO reduction (Wasser *et al.*, 2002). The observed specific NO reduction rate of 1.2 μmol NO.mg_{prot}^{-1}.h^{-1}. (0.34 nmol NO.mg_{prot}^{-1}.s^{-1}) is based on the total protein concentration of the bioreactor mixed liquor. It may be assumed that only part of the biomass contributes to NO reduction, since that conversion covers an electron flow of only 1.8 mmol e^-.l^{-1}.h^{-1} (14 %), against 11 mmol e^-.l^{-1}.h^{-1} (86 %) for Fe(III)EDTA$^-$ reduction. Also with respect to NO reduction, sulfidogenic reactor conditions, i.e. low redox potentials (<-250 mV versus Ag/AgCl) and thus the absence of Fe(III)EDTA$^-$, should be avoided, since already very low sulfide concentrations (0.1 μM) inhibit the second step of NO reduction, i.e. N_2O reduction to N_2 (Manconi *et al.*, 2005; Sörensen *et al.*, 1980).

Biomass growth rate and yield

The assessed values for the growth rate (μ_{obs}) and biomass yield (Y_{obs}), respectively, 0.043 d^{-1} and 0.009 mg protein per mg ethanol are very low. The low net growth rate is presumably due to limitation of the substrates that are used as electron acceptor: NO (aq) and an oxidized electron mediating compound (EM), presumably polysulfides (for Fe(III)EDTA$^-$ reduction). Batch experiments indicated that the K_m value for NO is below 10 nM (Chapter 7), meaning that the specific NO reduction rate is substrate limited at that NO concentration range. It is not clear, however, whether NO reduction is directly coupled to microbial growth, since NO reduction in aqueous Fe(II)EDTA^{2-} solutions might not generate a proton motive force (PMF) (see Chapter 7). Possibly, only Fe(III)EDTA$^-$ serves as electron acceptor for the proton translocating enzyme systems of the denitrifyers (Rosen and Klebanoff, 1981), which thus support the growth of the denitrifying biomass.

$Fe(III)EDTA^-$ reduction is already substrate limited at low EM concentrations, formed by the supply of 0.08 mM Na_2S per mg protein (Fig. Table 2). This means that in a bioreactor with a biomass (protein) concentration of 180 mg/l (Fig. 6A), maximal volumetric $Fe(III)EDTA^-$ reduction rates would be achieved at an EM concentration generated by a sulfide injection of at least 14 mM. In this study, the bioreactor was supplemented four times with 2 mM of sulfide to generate polysulfide according to reaction 5. However, the EM (polysulfides) concentration most likely depleted in time either due to FeS precipitation or due to oxidation to sulfur and thiosulfate (see above). Therefore, it may be expected that also the growth of the microbial population responsible for $Fe(III)EDTA^-$ reduction was limited by the low substrate concentrations.

The low biomass yield found in the present study corresponds well with observations during BioDeNOx pilot studies at full scale applications (Biostar, personal communication). Electron balances of previous batch experiments (Chapter 5) also indicated a low biomass yield during $Fe(III)EDTA^-$ reduction by the methanogenic Eerbeek sludge, i.e. the inoculum used to start up the bioreactor in this study.

EDTA degradation

The observation that EDTA degradation (± 2 $mM.d^{-1}$) only occurred during the continuous operation with flue gas containing 3.5-3.9 % O_2 (Fig. 7A), in contrast to the anaerobic batch incubations (Fig. 7B), suggests that EDTA degradation is induced by the chemical oxidation of $Fe(II)EDTA^{2-}$ by oxygen during gas scrubbing. Batch research indeed showed chemical EDTA degradation during the oxidation of $Fe(II)EDTA^{2-}$ by air (Gambardella and Heeres, 2005).

Although the (reaction) mechanism of $Fe(II)EDTA^{2-}$ oxidation and EDTA degradation is beyond the scope of this study, it should be noted that radical formation may occur during flue gas scrubbing via the well known $Fe(II)EDTA^{2-}$ catalysed Haber-Weiss reaction (Buettner *et al.*, 1983; Sutton, 1995; Welch *et al.*, 2002):

$$2\ Fe(II)EDTA^{2-} + 2\ O_2 \rightarrow 2\ Fe(III)EDTA- + 2\ O_2^{\cdot-} \quad (9)$$

$$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2 \quad (10)$$

$$H_2O_2 + Fe(II)EDTA^{2-} \rightarrow Fe(III)EDTA- + OH^- + HO^{\cdot} \quad (11)$$

$$3\ Fe(II)EDTA^{2-} + O_2 + 2H^+ \rightarrow 3\ Fe(III)EDTA^- + OH^- + OH^{\cdot} \quad (12)$$

Reaction 12 shows a $Fe(II)/O_2$ ratio of 3 instead of 4 (reaction 3). The hydroxyl radical formed is very reactive and bactericidal (Wolcott *et al.*, 1994) and may induce a-specific EDTA degradation (Mochidzuki *et al.*, 1999). Further research is needed to illuminate the occurrence and role of radicals in the BioDeNOx process, e.g. their contribution to EDTA degradation as well as to biological stress they induce, i.e. their effect on biomass yield and growth / decay.

EDTA can also be consumed via biological EDTA oxidation, i.e. via aerobic respiration (Witschel *et al.*, 2001; Van Ginkel *et al.*, 1997). It is, however, unlikely that biological EDTA oxidation plays a

major role when the BioDeNOx process is running at redox potentials below -150 mV, since no molecular oxygen is present at these low redox conditions. Based on the kinetics of Fe(II)EDTA^{2-} oxidation (reaction 3) (Wubs and Beenackers, 1993), O_2 (aq) can only be expected at the gas-liquid interface, i.e. in the scrubber and not in the bioreactor.

Optimization of BioDeNOx reactor operation and design

This study reveals that there exists a contradiction between the optimal redox state of the aqueous FeEDTA solution required for NO absorption and biological regeneration. The NO absorption, i.e. NO removal from the gas phase, requires a low redox potential (Fig. 2), thus the Fe(III)EDTA^{-} and Fe(II)EDTA-NO^{2-} concentration should as low as possible. On the other hand, the latter compounds are required in the bioreactor to maintain biological activity and biomass growth (at least to compensate decay), as well as to prevent depletion of the EM compound (polysulfides) and inhibition of NO reduction by S^{2-}. Therefore, some Fe(III)EDTA^{-} (10-20 %) should always be present in the bioreactor mixed liquor, which corresponds with an ORP of between –180 mV and –200 mV versus Ag/AgCl at pH 7.2 (±0.2). This can be achieved when the ethanol supply is properly controlled with the redox potential as steering parameter (Chapter 4). In that way, ethanol (electron donor) is the substrate that limits the biological activity instead of NO and/or Fe(III)EDTA^{-} (electron acceptors).

The scrubber design should be focused on minimization of Fe(II)EDTA^{2-} oxidation by oxygen, since that reaction causes a high Fe(III)EDTA^{-} load and, therefore, an inefficient ethanol consumption (in this study only 14% of the ethanol consumption was linked to NO reduction). Furthermore, minimization of Fe(II)EDTA^{2-} oxidation may lead to less radical formation (via Haber-Weiss) and therefore to lower EDTA degradation rates. Using a mathematic model, Gambardella *et al.* (2005) found that NO absorption is favored compared to oxidation of Fe(II)EDTA^{2-} by oxygen when operating at high mass transfer coefficients. In this respect, a spray tower appears to be a better configuration than a packed column (Gambardella *et al.*, 2005).

With respect to the bioreactor design, well mixed systems as completely stirred tank reactors (CSTR) and gaslift reactors are preferable over plug-flow like systems, e.g. UASB reactors, because of the superior properties for substrate control. By intensive stirring, complete anaerobic zones, e.g. absence of Fe(III)EDTA^{-}, can be avoided. Another advantage of a CSTR / gaslift over a plug-flow system is an equal distribution of the aqueous NO, which prevents the existence of local zones completely depleted of NO, i.e. substrate for the denitrifying biomass.

The availability of polysulfide as electron mediating compound during Fe(III)EDTA^{-} reduction seems to be crucial for the stable operation of BioDeNOx reactors. Next to implementation of a proper ethanol supply, it is therefore also advisable to dose minor amounts sulfide or elemental sulfur to the reactor continuously. The amount required depends on the bioreactor Fe(III)EDTA^{-} load and should be monitored carefully, e.g. with a pS electrode, to prevent overdosing.

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CHAPTER 10

Summary, general discussion and conclusions

INTRODUCTION

The emission of nitrogen oxides (NO_x) to the atmosphere is a major environmental problem. To abate NO_x emissions from industrial flue gases, to date, mainly chemical processes like selective catalytic reduction (SCR) are applied. All these processes require high temperatures (> 300 °C) and expensive catalysts. Therefore, biological NO_x removal techniques using denitrification may represent promising alternatives for the conventional SCR techniques, because denitrification occurs at ambient temperatures and without the consumption of expensive catalysts. However, water based biofiltration requires relatively long scrubber/bioreactor retention times, i.e. big reactor volumes, due to the slow mass transfer of NO from the gas into the liquid phase.

BioDeNOx is in principle a welcome alternative for conventional NO_x removal techniques like SCR and water-based biofiltration, since it does not need high temperatures and catalysts, while scrubber retention times can be very short (< 10 seconds) due to the chemically enhanced NO absorption. In this thesis, the BioDeNOx concept was investigated with special attention to the bioreactor key conversions: NO and $Fe(III)EDTA^-$ reduction. Figure 1 schematically summarizes the aspects of BioDeNOx that were investigated in this thesis.

In this final chapter, the main results obtained are summarized, discussed and related to the research performed at the partner universities of the research project: the University of Groningen (with special emphasis on the scrubber processes) and the Technical University Delft (with special emphasis on the microbial ecology of the bioreactor).

Based on the results obtained, the following main conclusions can be extracted:

1. The NO removal from the gas phase is primary determined by NO absorption kinetics.
2. The bioreactor load (flow of electron acceptors) is mainly determined by $Fe(III)EDTA^-$.
3. $Fe(III)EDTA^-$ reduction is accelerated by an electron mediator (EM), presumably polysulfides.
4. Polysulfides stimulate $Fe(III)EDTA^-$ reduction, but free monosulfides inhibit N_2O and NO reduction.
5. The ORP of BioDeNOx reactors should be steered between -180 and -200 mV versus Ag/AgCl (pH 7.2±0.2).
6. $Fe(II)EDTA^{2-}$ interferes with the biological NO reduction electron transfer chain.
7. The bioreactor regeneration capacity is determined by the biomass and EM/polysulfide concentration.
8. The oxygen sensitivity of $Fe(II)EDTA^{2-}$ leads to high ethanol and EDTA consumption and thus to high operation costs.

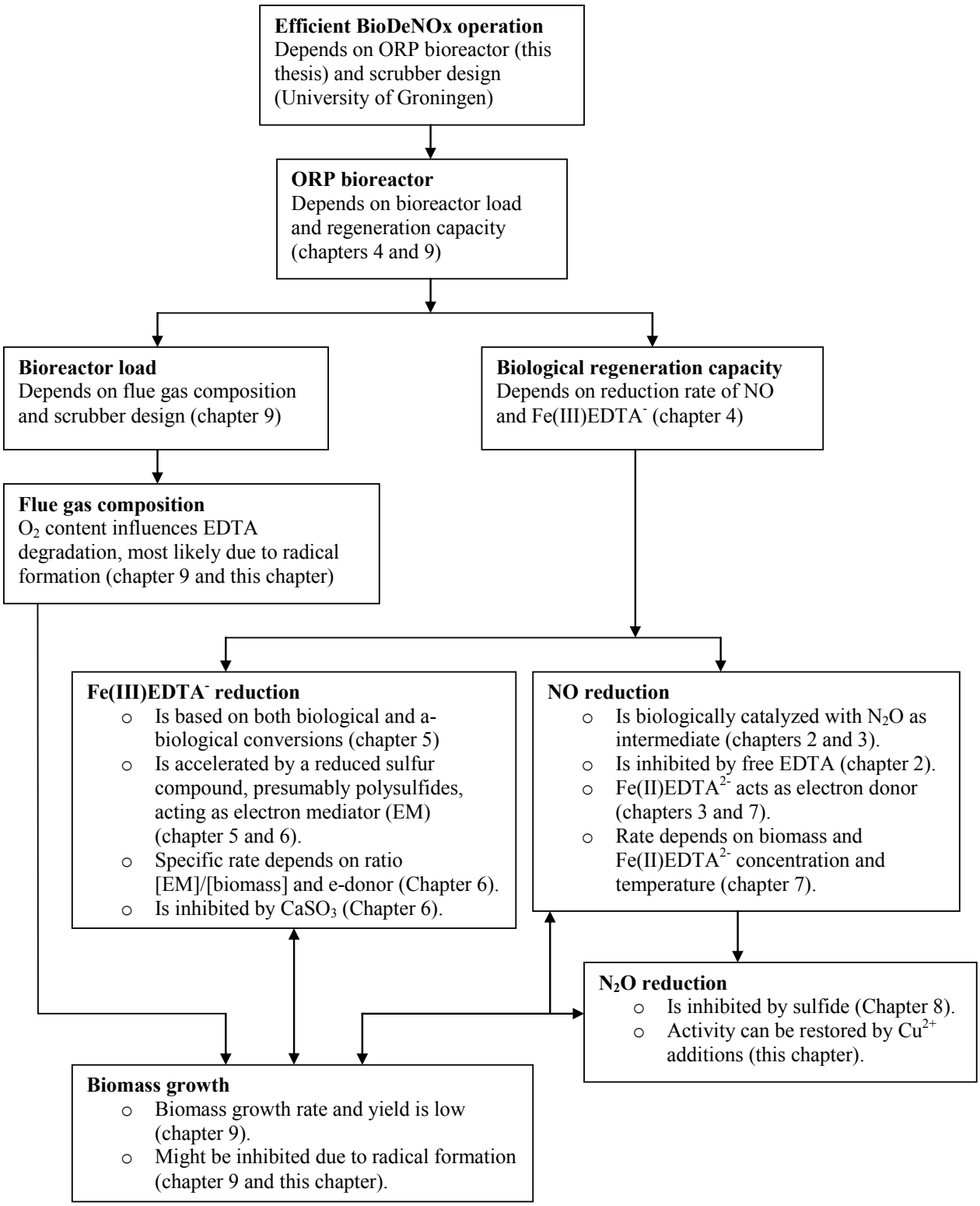

Figure 1

Overview of the main subjects investigated in this thesis.

CONCLUSION 1

NO removal from the gas phase is primary determined by NO absorption kinetics

Comparison of the NO removal efficiency obtained in Chapter 4 and Chapter 9 shows that the NO removal efficiency from the gas phase is primary determined by the $Fe(II)EDTA^{2-}$/NO absorption kinetics. At an empty scrubber retention time (ESRT) of 11 seconds (Chapter 4), stable NO removal with an efficiency of over 80 % was obtained when treating an artificial flue gas containing 3.3 % O_2 and 500 ppm NO. At an ESRT of 1.2 seconds (Chapter 9) and a flue gas composition of 3.5-3.9 % O_2 and 155 ppm NO, the removal efficiency amounted to 39 %. The difference in the NO removal efficiencies can be explained by the kinetics of the reaction of NO with $Fe(II)EDTA^{2-}$, which have been investigated thoroughly in the last two decades. Several authors reported the reaction to be first order in both NO and $Fe(II)EDTA^{2-}$ (Demmink, 2000 and literature cited therein; Schneppensieper *et al.*, 2001). The reaction is intrinsically very fast and mass transfer limitation of NO but also of the $Fe(II)EDTA^{2-}$ complex plays an important role (Demmink, 2000). This is particularly the case in practical BioDeNOx applications, where the presence of biomass and precipitates in scrubber liquors will suppress the NO absorption rate due to mass transfer properties (Fig. 2).

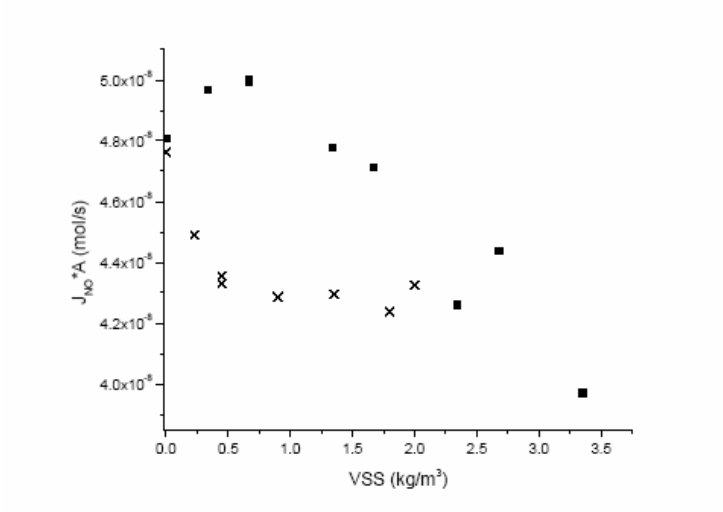

Figure 2

Flux of NO absorbed ($\overline{J}_{NO}$) in 30 mM $Fe(II)EDTA^{2-}$ solution in the presence of biomass obtained from lab-scale BioDeNOx reactors of Wageningen (X) and Delft (■). T = 52 ºC, [NO]in= 250 vppm, pH= 7 (Gambardella *et al.*, 2005c).

The first order dependence between the NO absorption rate and the $Fe(II)EDTA^{2-}$ concentration explains the crucial importance of operating BioDeNOx reactors at redox potentials (ORP) below -140 mV versus Ag/AgCl at pH 7.0 (Chapter 4) or below -150 mV at pH = 7.2±0.2 (Chapter 9). At higher redox potentials, the $Fe(II)EDTA^{2-}$ concentration is apparently too low to have sufficiently fast NO absorption, which results in low NO removal efficiencies (Chapter 4 and Chapter 9). The ORP is shown to be a proper indicator of the $Fe(II)EDTA^{2-}$ concentration (Chapters 4 and 5). The critical ORP

value for BioDeNOx reactor operation (around -150 mV versus Ag/AgCl) corresponds well with the standard redox potential of the $Fe(II)EDTA^{2-}/Fe(III)EDTA^{-}$ system: +96 mV versus Standard Hydrogen Electrode (SHE) (Kolthoff and Auerbach, 1952).

CONCLUSION 2

The bioreactor load (flow of electron acceptors) is mainly determined by $Fe(III)EDTA^{-}$

The evolution of the redox potential during continuous reactor operation indicates the relation between the $Fe(II)EDTA^{2-}$ regeneration capacity (NO and/or $Fe(III)EDTA^{-}$ reduction) and the bioreactor load ($Fe(III)EDTA^{2-}$ and/or NO) of the bioreactor (e.g. Fig. 4 of Chapter 9). An increasing ORP means a decrease of the $Fe(II)EDTA^{2-}$ concentration, i.e. the reactor load exceeds the regeneration (NO and iron reduction) capacity (both expressed as mmol NO or $Fe(III)EDTA^{-}$ per time unit). When the ORP declines, the regeneration capacity exceeds the bioreactor load, while a stable redox potential indicates a well balanced bioreactor load and regeneration capacity.

In both reactor studies (Chapter 4 and Chapter 9), the reactor load was found to be mainly determined by the $Fe(III)EDTA^{-}$ load. Electron balances indicated that 86 % of the total electron flow was related to FeEDTA oxidation and reduction, while only 14 % corresponds to reduction of the $Fe(II)EDTA^{2-}$ absorbed NO. This can be explained by the oxygen sensitivity of aqueous $Fe(II)EDTA^{2-}$: the reaction of $Fe(II)EDTA^{2-}$ with oxygen, known to be irreversible (Wubs and Beenackers, 1993; Zang and Van Eldik, 1990), is first order in oxygen, whereas the order in iron is a function of the iron chelate concentration (Wubs and Beenackers, 1993; Zang and van Eldik, 1990). At low EDTA concentrations (< 10 mol/m^3), the reaction is first order in iron whereas it becomes second order at higher EDTA concentrations. At typical BiodeNOx absorption conditions ([FeEDTA] > 10 mM), the reaction was shown to be first order in oxygen and second order in iron chelate (Gambardella *et al.*, 2005b). Hence, the oxygen sensitivity of $Fe(II)EDTA^{2-}$ leads to major $Fe(III)EDTA^{-}$ loads to the bioreactor. This results in a substantial increase of the required electron donor flow, i.e. ethanol consumption, to regenerate the $Fe(II)EDTA^{2-}$ scrubber solution. Moreover, high volumetric $Fe(III)EDTA^{-}$ reduction rates are a prerequisite for the successful operation of continuous BioDeNOx reactors treating flue gasses with elevated O_2 concentrations (Chapters 4 and 9).

CONCLUSION 3

$Fe(III)EDTA^{-}$ reduction is accelerated by an electron mediator, presumably polysulfides

In Chapter 5, the capacity of various inocula to reduce $Fe(III)EDTA^{-}$ (25 mM) was screened using batch experiments. *Escherichia coli*, known to be incapable to reduce crystalline Fe(III) (Lovley *et al.*, 1991), reduced $Fe(III)EDTA^{-}$ to $Fe(II)EDTA^{2-}$ (Fig. 3; Chapter 5). This is in agreement with Tanaka *et al.* (1983), who demonstrated that *E. coli* can be used in fuel cell applications, whith FeEDTA as the electron mediating compound. Addition of small amounts of sulfide greatly accelerated $Fe(III)EDTA^{-}$ reduction by all the inocula tested: anaerobic methanogenic sludges as well as a denitrifying inoculum

(Table 2; Chapter 5). This indicates that biological Fe(III)EDTA$^-$ reduction in BioDeNOx reactor environments (i.e. in presence of sulfur and ethanol) is not a direct, enzymatic conversion, but an indirect reduction with the involvement of an electron mediating compound, presumably polysulfides. Chapter 5 supports the hypothesis that not Fe(III) respiring bacteria, but reducers of elemental sulfur or polysulfides are primary involved in the reduction of EDTA chelated Fe(III) in BioDeNOx reactors.

The mechanism and kinetics of biological Fe(III)EDTA$^-$ reduction was further investigated in Chapter 6, using unadapted anaerobic methanogenic Eerbeek sludge and BioDeNOx reactor mixed liquor. Various electron mediating compounds were screened for their potential to stimulate Fe(III)EDTA$^-$ reduction under BioDeNOx reactor conditions (55 ºC, pH 7.2±0.2). Fe(III)EDTA$^-$ reduction was accelerated by the addition of small amounts (0.5 mM) of sulfide, cysteine or elemental sulfur. This suggests that Fe(III)EDTA$^-$ reduction is catalyzed by a sulfur species, formed by the reaction between Fe(III)EDTA$^-$ and sulfide, and facilitating an electron shuttle between the microbes and Fe(III)EDTA$^-$. Although determination of the chemical structure of this electron mediator (EM) was beyond the scope of this thesis, the linear relation between the amount of sulfide added to the bio-assays and the specific Fe(III)EDTA$^-$ reduction rate (Fig. 3; chapter 6) strongly suggests that polysulfides are the electron mediating compound.

Fe(III)EDTA$^-$ is reported as a rather poor electron acceptor for (direct enzymatic) reduction by dissimilatory iron reducing bacteria (Finneran *et al.*, 2002). Although Fe(III)EDTA$^-$ is a suitable terminal electron acceptor for assimilatory ferric iron reductases (Schroeder *et al.*, 2003), nearly all the enzymes characterized so far show flavin reductase activity and need a flavin as co-factor. The stimulating effect of electron shuttles in various microbial conversions by facilitating the electron transfer to and from bacteria is widely documented in literature, see for instance Hernandez and Newman (2001). Electron shuttling by reduced sulfur compounds has been proposed to catalyze several metal reductions, e.g. chromium (Smillie *et al.*, 1981), technetium (Lloyd *et al.*, 1998) and molybdenum (Tucker *et al.*, 1997). Moreover, this sulfide/elemental sulfur electron shuttle is also involved in the biological decolorization of azo-dyes (Van der Zee *et al.*, 2003).

Using PCR-DGGE analysis and PCR-DNA-sequencing, Kumaraswamy *et al.* (2005b) showed that, besides NO reducing populations, Fe(III) respiring bacteria (closely affiliated to *Deferribacter thermophilus*) were present in BioDeNOx reactor mixed liquors. However, these isolates showed rather slow Fe(III)EDTA$^-$ reduction: at an initial protein concentration of 10 mg.l^{-1}, the conversion of 5 mM Fe(III)EDTA$^-$ to Fe(II)EDTA^{2-} took 7 days (personal communication R. Kumaraswamy), corresponding to a specific reduction rate of below 0.003 mmol.mg$_{prot}^{-1}$.h^{-1}. Besides, in absence of sulfur, Kumaraswamy *et al.* (2005b) observed a volumetric Fe(III)EDTA- reduction rate of 1.8 mM.h^{-1}, corresponding to a specific rate of 0.015 mmol.mg$_{prot}^{-1}$.h^{-1}, during activity tests using biomass from a lab-scale reactor in which the predominant Fe(III)EDTA$^-$ reducing population consisted of bacteria affiliated to *Bacillus infernus* and *Alkaliphilus* spp.. These values are low compared to the rates

observed in Chapter 6 in case small amounts of sulfide were added (± 1 $mmol.mg_{prot}^{-1}.h^{-1}$ when 1 mM sulfide was supplied).

Except by the presence of a suitable electron mediator, the Fe(III)EDTA$^-$ reduction rate is also influenced by the available electron donor. Several electron donors were screened for Fe(III)EDTA$^-$ reduction: ethanol, glucose, acetate, methanol, hydrogen and molasses. The highest Fe-reduction rates were obtained with glucose and molasses, directly followed by ethanol, acetate and hydrogen (Chapter 5 and 6).

Without the addition of external sulfide, the Fe(III)EDTA$^-$ reduction rate by Eerbeek sludge was strongly inhibited by $CaSO_3$ (low concentrations, e.g. 1 mM, showed already a significant inhibition (Fig. 6; Chapter 6). $CaSO_3$ will be present in BioDeNOx reactor mixed liquors at high concentrations (up to 0.8 M) when NO removal is combined with physico-chemical SO_2 removal by means of $CaSO_3$ precipitation (i.e. retrofitting limestone-gypsum plants). The observed inhibition in Chapter 6 is possibly induced by the reaction of SO_3^{2-} with S^{2-} under the formation of thiosulfate, i.e. depletion of the electron-shuttling compound. This implies that SO_3^{2-} inhibition may be suppressed when high concentrations of polysulfide are available.

CONCLUSION 4

Polysulfides stimulate Fe(III)EDTA$^-$ reduction, but free monosulfides inhibit N_2O reduction

The effect of sulfide addition on the Fe(II)EDTA^{2-} regeneration capacity of BioDeNOx reactors was investigated in Chapter 9. Batch experiments showed a linear relation between the relative EM/polysulfide concentration (amount of EM/polysulfide available per bacterium) and the specific Fe(III)EDTA$^-$ reduction rate (Table 2 of Chapter 9), confirming the results of Chapter 6. During continuous reactor operation, sulfide additions induced an elevated Fe(III)EDTA$^-$ reduction capacity only for limited periods of time (approximately 4 days). Ideally, oxidation and reduction of the electron mediating compound is a cyclic process in which the total electron mediator concentration (oxidized plus reduced) stays constant. It is likely to assume, however, that both low and high redox conditions will result in an unbalance of the EM/polysulfide cycling, and thus in unstable BioDeNOx reactor operation.

At low redox potentials below -250 mV versus Ag/AgCl, i.e. when all Fe(III)EDTA$^-$ is reduced, and in the presence of ethanol, the polysulfide will be completely reduced to sulfide. The latter compound can precipitate with Fe^{2+}, which is the predominant valence of iron at these low redox potentials (with magnesium and calcium, the iron concentration exceeds the EDTA concentration to prevent free, uncomplexed EDTA, see Chapter 2). In this way, the polysulfides are ultimately transformed to insoluble FeS, meaning that the electron mediator has changed to a non or slowly reactive sulfur species:

$$S_n^{2-} + 2\ e^- + H^+ \rightarrow S_{n-1}^{2-} + HS^-$$

$$2\ Fe^{2+} + 2\ HS^- \rightarrow 2\ FeS\ (s)$$

Also with respect to NO reduction, sulfidogenic reactor conditions, i.e. redox potentials below -250 mV versus Ag/AgCl and thus the absence of Fe(III)EDTA$^-$, should be avoided. Chapter 8 shows that NO reduction to N_2O was completely inhibited in the presence of 1 mM free sulfide (denitrifying sludge concentration 0.2 gVSS.l^{-1}). The second step of NO reduction, i.e. N_2O reduction to N_2, was already inhibited at very low sulfide concentrations (15 µM). The results of Chapter 8 are in good correspondence with literature, e.g. Sörensen *et al.* (1980), who showed that only 0.3 mM S^{2-} causes a partial inhibition of NO reduction and a strong inhibition of N_2O reduction by *Pseudomonas fluorescens*. Another study reports that N_2O reduction can already be influenced by sulfide concentrations below 1 µM (Sörensen *et al.*, 1987).

Incomplete NO reduction is highly unwanted since it leads to N_2O emissions. The N_2O reducing activity, can, however, be recovered by the supply of small amounts of copper. Manconi *et al.* (2005) showed that the addition of 60 µM Cu restored N_2O reduction in NO reducing batch assays that were inhibited by the sulfide addition (Fig. 3).

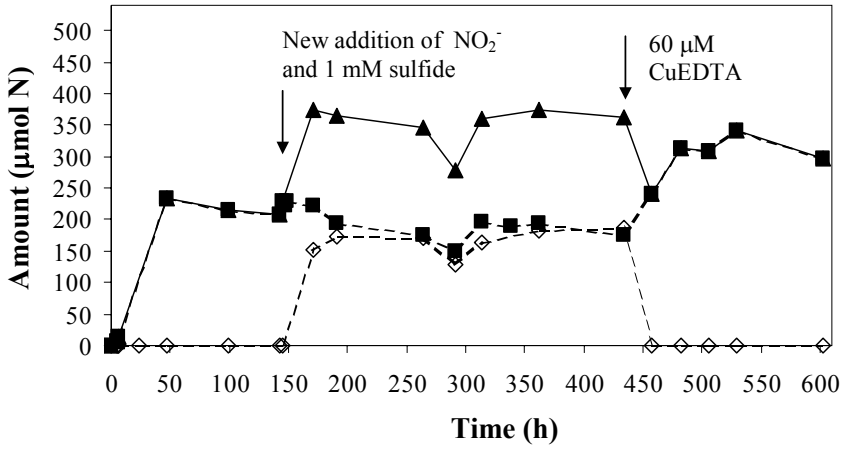

Figure 3

Production of N_2O (◇) and N_2 (■) and total gaseous nitrogen, sum of N_2O and N_2 (▲), from nitrosyl-complex (Fe(II)EDTA-NO^{2-}) reduction by denitrifying sludge (1.3 gVSS/l). Prior to the generation of the nitrosyl-complex by 2 mM nitrite injection at t = 0, the original Fe(III)EDTA$^-$ medium was reduced chemically using 15 mM Fe^0 as reducing agent. At t = 150 hours the nitrosyl complex was generated again using the same procedure, followed by sulfide (1 mM) addition to the vials. At t = 434 hours 60 µM CuEDTA was added to the vials (Manconi *et al.*, 2005).

The restoring effect of copper may be explained by the requirement of Cu for N_2O respiration, because N_2O reductase (N_2OR) is a multicopper enzyme (Coyle *et al.*, 1985; Zumft, 1997). Cu deficient media either do not sustain cell growth on N_2O or at least led to a transient accumulation of N_2O during nitrite reduction (Iwasaki *et al.*, 1980; Iwasaki and Terai, 1982; Matsubara and Zumft, 1982). In this study, 0.22 µM $CuCl_2$ was supplied to all assays with the trace metal solution. This quantity was apparently

too low to prevent the sulfide toxicity observed in Chapter 8, which is possibly based on copper limitation in the medium, induced by its precipitation with sulfide (Manconi *et al.*, 2005).

CONCLUSION 5

The ORP of BioDeNOx reactors should be steered between -180 and -200 mV (pH 7.2±0.2)

Chapter 9 reveals that there is a contrast between the optimal redox state of the aqueous FeEDTA scrubber liquor required for NO absorption and biological regeneration. The NO absorption, i.e. NO removal from the gas phase, requires a low redox potential (see 'Conclusion 1'), thus the $Fe(III)EDTA^-$ and $Fe(II)EDTA\text{-}NO^{2-}$ concentration should be as low as possible. On the other hand, the latter compounds are required in the bioreactor to maintain biological activity and biomass growth (at least to compensate decay), as well as to prevent depletion of the redox mediator and inhibition of NO reduction (See conclusion 4). Therefore, some $Fe(III)EDTA^{2-}$ (10-20 %) should always be present in the bioreactor mixed liquor, which corresponds with an ORP of between –180 mV and –200 mV versus Ag/AgCl at pH 7.2 (±0.2). This can be achieved when the ethanol supply is properly controlled with the redox potential as steering parameter (Chapter 4). In that way, ethanol (electron donor) is the substrate that limits the biological activity instead of NO and/or $Fe(III)EDTA^-$ (electron acceptors).

To minimize substrate limitation with respect to NO and $Fe(III)EDTA^-$, well mixed bioreactors like a completely stirred tank reactor (CSTR) or a gaslift reactor are preferable over plug-flow like systems, e.g. UASB reactors, because of the superior properties for substrate control. By intensive stirring, complete anaerobic zones, e.g. absence of $Fe(III)EDTA^-$ can be avoided. Another advantage of a CSTR or gaslift over a plug-flow system is an equal distribution of the aqueous NO, which prevents the existence of local zones completely free of NO, i.e. substrate for denitrifying biomass.

CONCLUSION 6

$Fe(II)EDTA^{2-}$ interferes with the biological NO reduction electron transfer chain

Reduction of nitric oxide in aqueous $Fe(II)EDTA^{2-}$ solutions was investigated in Chapter 2, 3 and 7. Reduction of NO to N_2 is biologically catalyzed with nitrous oxide (N_2O) as an intermediate. Various sludges from full scale denitrifying and anaerobic reactors are capable to catalyze NO reduction under thermophilic conditions (Chapter 3). The NO reduction rate was not affected by the presence of ethanol or acetate, meaning that $Fe(II)EDTA^{2-}$ acts as electron donor for NO reduction. Indeed, in the absence of ethanol or acetate, which are good electron donors for $Fe(III)EDTA^-$ reduction (Chapter 6), $Fe(III)EDTA^-$ accumulation was observed (Table 3 of Chapter 3).

The NO reduction rate of the denitrifying Veendam sludge was influenced by the $Fe(II)EDTA^{2-}$ concentration (Fig. 6; Chapter 7). This strongly suggests that in BioDeNOx reactors, i.e. in aqueous $Fe(II)EDTA^{2-}$solutions, the EDTA chelated Fe(II) is the direct electron donor for NO reduction, and not ethanol. This is, at first sight, somewhat surprising, since ethanol is known to be an excellent

electron donor for denitrification (Constantin *et al.*, 1997). However, the redox properties of FeEDTA and bacterial Nitric Oxide Reductase (NOR) allow that $Fe(II)EDTA^{2-}$ can be directly involved in NOR reduction, whether direct or indirect via Cytochrome c (Table 1, see also Fig. 8 of Chapter 7).

Table 1

Standard potentials ($E^{0'}$) of various redox couples relevant for NO reduction.

Redox couple	**$E^{0'}$ (mV)**	**Reference**
NO/N_2O	+1177	Zumft, 1997
N_2O/N_2	+1352	Zumft, 1997
NOR_{ox}/NOR_{red}	between +280 and +320	Wasser *et al.*, 2002
N_2OR_{ox}/N_2OR_{red}	+260	Coyle *et al.*, 1985
Cyt c_{ox}/Cyt c_{red}	between +148 and +253	Gray *et al.*, 1986
amicyanin	+294	Gray *et al.*, 1986
$Fe(III)EDTA^{-}/Fe(II)EDTA^{2-}$	+96	Kolthoff and Auerbach, 1952

Note that the data obtained in Chapter 7 do not exclude NO reduction via the more conventional electron supply chain, i.e. via the Cytochrome *bc*1 complex and prior redox couples, following ethanol oxidation (Fig. 8; Chapter 7, pathway 3). Kumuraswamy *et al.* (2005a) observed $Fe(III)EDTA^{-}$ accumulation as a result of anaerobic ferrous iron oxidation coupled to NO_2^-/NO reduction at 55 ºC by strain KT-1, which was isolated from a lab scale BioDeNOx reactor. However, Fe(III)EDTA-accumulation was only observed when low amounts of ethanol (initial concentration 1 mM) were available (Fig. 4A). At higher ethanol concentrations (initial concentration 3 mM), no Fe(III)EDTA-accumulation was observed (Fig. 4B). Their results suggest that pathway of electron supply depends on the concentrations of the available electron donors. Quantification of the electron flow via ethanol oxidation (coupled to NO reduction) in redox buffered media as $Fe(II)EDTA^{2-}$ requires further research, e.g. by using Cytochrome *bc*1 deficient mutants or specific inhibition of the Cytochrome *bc*1 complex by e.g. antimycin (Slater *et al.*, 1973).

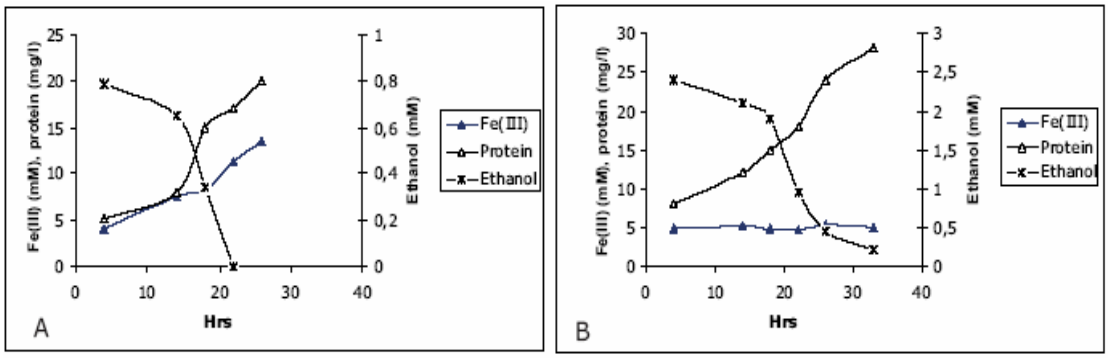

Figure 4

Fe(III)EDTA$^-$ (▲) and protein (△) accumulation and ethanol (✱) consumption during NO_2^-/NO reduction by strain KT-1 with 25 mM Fe(II)EDTA2-, 5 mM nitrite and 1 mM (A) or 3 mM ethanol (B) (Kumaraswamy *et al.*, 2005b).

CONCLUSION 7

The bioreactor regeneration capacity depends on the biomass concentration and EM/polysulfide concentration

As mentioned at 'conclusion 2', the capacity of Fe(II)EDTA^{2-} regeneration is determined by the rate of NO and Fe(III)EDTA$^-$ reduction. Good results were obtained when the bioreactor was inoculated with a mixture of denitrifying sludge (for NO reduction) and anaerobic granular sludge (for Fe(III)EDTA^{2-} reduction) (Chapter 4). Crushing of the anaerobic granules gave higher Fe-reduction rates (Chapter 6). Stable bioreactor operation was obtained when the reactor was inoculated with ca. 1 g VSS/l of Veendam sludge, enriched with the same VSS amount of crushed Eerbeek granules (Chapter 9). However, lower concentrations of Eerbeek sludge likely also give satisfying results, since, under the conditions investigated, the volumetric Fe-reduction rate is not limited by the VSS, but the electron mediator concentration (Chapter 6).

The BioDeNOx reactor mixed liquor showed a specific NO reduction rate of 1.3 $\mu mol.h^{-1}.mg_{prot}^{-1}$, which is in agreement with literature data (Chapter 7). The volumetric NO reduction capacity of BioDeNOx reactors was shown to be linearly related with denitrifying biomass concentration. Elevation of the volumetric NO reduction capacity can thus be obtained by reactor operation at higher biomass concentrations (Fig. 3 of Chapter 7 and Table 1 of Chapter 9).

High rate Fe(III)EDTA$^-$ reduction (1.0 $mmol.mg_{prot}^{-1}.h^{-1}$) was observed in case 1 mM sulfide was added to 25 mM Fe(III)EDTA$^-$ medium (with ethanol as electron donor). The specific Fe(III)EDTA$^-$ reduction rate showed a linear relation with the amount of sulfide supplied to the batch assays, suggesting a linear relation between the [EM/polysulfide] : [biomass] ratio and the volumetric Fe(III)EDTA$^-$ reduction rate (Fig. 3 of Chapter 6).

Hence, the volumetric Fe(II)EDTA^{2-} regeneration capacity of BioDeNOx reactors is a result of (i) the concentration of (both denitrifying and Fe(III)EDTA$^-$/polysulfide reducing) biomass and (ii) the EM/polysulfide concentration (Chapter 9). In our reactor studies (Chapters 4 and 9), the latter factor

was limiting the O_2 (i.e. Fe(III)EDTA$^-$) load that could be treated by the bioreactor. As discussed at 'conclusion 5', it is a pitfall to operate BioDeNOx reactors at redox potentials below -200 mV, as it was done in Chapter 9. Steering the redox potential between -180 and -200 mV avoids depletion of the electron mediating compound (polysulfide), resulting in a sustained and high Fe(III)EDTA$^-$ reduction capacity.

Elevation of the NO reduction capacity can be achieved by biomass growth (Chapter 4), which takes time, or by bio-augmentation with denitrifying sludge (Chapter 9). To avoid biomass loss via a medium bleed stream, the application of membrane filtration, i.e. micro- or ultrafiltration, may be an interesting option. The volumetric Fe(III)EDTA$^-$ reduction capacity can be increased by elevating the concentration of polysulfides in the bioreactor. In case the flue gas contains SO_2, that compound may be the source for a continuous generation of sulfur/polysulfides. In case of treatment of a sulfur free flue gas, substrate (sulfur/polysulfide) limitation can easily occur, e.g. due to sulfide precipitation with Fe^{2+}. This may be overcome by continuous addition of (poly)sulfide via the medium supply / bleed.
Only relatively low biomass concentrations could be maintained in the continuously operating BioDeNOx reactor: ±90 and ±180 mg protein.l^{-1} in Chapter 4 and Chapter 9, respectively. Also very low values for growth and biomass yield were found, respectively, 0.043 d^{-1} and 0.009 mg protein per mg ethanol (Chapter 9), equal to 0.03 mg protein per mmol electron oxidized/reduced. That value, obtained under thermophilic reactor conditions (55 ºC), is extremely low compared to the biomass yield (1.8 mg/mmol electrons) observed by Kumaraswamy *et al.* (2005a) during NO reduction at 30 ºC by the newly described bacterium *Paracoccus ferrooxidans*). However, the latter value is most likely influenced by biological nitrite reduction (Kumaraswamy *et al.*, 2005a), whereas NO reduction is the conversion in BioDeNOx reactors. Moreover, the thermophilic conditions applied in BioDeNOx reactors (Chapter 9) likely induce a higher biomass decay and lysis rate compared to the mesophilic conditions applied by Kumaraswamy *et al.* (2005a).
The low biomass yield can also be due to substrate limitations, i.e. the electron acceptors NO and presumably polysulfide, that occur in continuous BioDeNOx reactors, in contrast to batch assays. Besides physiological stress conditions in continuous BioDeNOx reactors might contribute to low biomass yields and growth rates. Especially radical formation during Fe(II)EDTA^{2-} oxidation by oxygen present in the flue gas, possibly via the Haber-Weiss reaction (Chapter 9) is a point of concern and of special interest for future research, since the radicals formed are very reactive and bactericidal (Wolcott *et al.*, 1994).

CONCLUSION 8

The oxygen sensitivity of Fe(II)EDTA^{2-} leads to high ethanol and EDTA consumption and thus high operation costs

Radicals (e.g. hydroxyl) may not only inhibit bacterial growth (conclusion 7), but possibly also induce EDTA degradation (Mochidzuki *et al.*, 1999). A substantial EDTA degradation was observed in

Chapter 9, the rate amounted to 2.1 (± 0.1) $mM.d^{-1}$ when the flue gas contained 3.5-3.9 % O_2. A comparison between the results of Chapter 4 (no substantial EDTA degradation observed at 0.9 % O_2), Chapter 9 and Utomo (2004), indicates that the EDTA degradation rate is linearly related with the flue gas O_2 content (Fig. 5). These observations are in good agreement with data of Gambardella *et al.* (2005a), who investigated EDTA degradation using batch experiments. They found also a close link between the rates of $Fe(II)EDTA^{2-}$ oxidation and EDTA degradation. The main degradation product was shown to be ED3A. Furthermore, an excess of EDTA over iron (ratio 1.5-2) was found to considerably decrease the EDTA degradation rate (Gambardella *et al.*, 2005a). The presence of free, uncomplexed EDTA is, however, not allowable in BioDeNOx reactors, since it strongly inhibits NO and $Fe(III)EDTA^-$ reduction (Chapters 2 and 5).

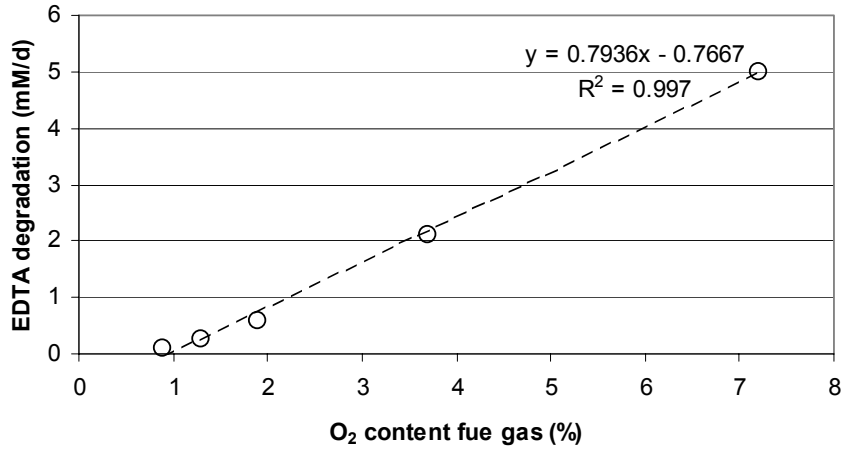

Figure 5

EDTA degradation rate observed at variable O_2 concentrations in Chapter 4 (0.9 %), Chapter 9 (3.5-3.9 %) and Utomo (2004) (1.3, 1.9 and 7.2 % O_2).

The oxygen sensitivity of $Fe(II)EDTA^{2-}$ can be considered as the key problem for launching BioDeNOx into practice. $Fe(II)EDTA^{2-}$ oxidation by O_2 not only leads to radical formation, which most likely induces EDTA degradation, but also results in substantial elevated ethanol consumption rates. Apart from ethanol consumption that is linked to $Fe(II)EDTA^{2-}$ oxidation and reduction, ethanol may be consumed via the reaction with hydroxyl radicals that are possibly formed via the Haber Weiss reaction (Winston and Cederbaum., 1983). In fact ethanol acts as a radical scavenger in that case. An alternative radical scavengers to surpress EDTA degradation is e.g. mannitol (Chen and Schopfer, 1999).

Both EDTA degradation and ethanol consumption lead to high operation costs, as illustrated by a case concerning a flue gas stream of 2 million $Nm^3.h^{-1}$, containing 250 ppm NO and 5 % O_2, treated by a BioDeNOx scrubber with 90 % removal efficiency. This generates a bioreactor load of 20 kmol $NO.h^-$

[1]. The Fe(III)EDTA$^-$ load can be estimated by the selectivity coefficient (σ), which is determined by the ratio of the average NO ($\bar{J}_A$) and O_2 ($\bar{J}_O$) flux from the gas phase to the aqueous Fe(II)EDTA^{2-} scrubber liquor (Gambardella *et al.*, 2005b):

$$\sigma = \frac{\bar{J}_A}{\bar{J}_O}$$

Assuming a selectivity coefficient (σ) of 0.4, i.e. the NO flux to the aqueous Fe(II)EDTA^{2-} amounts 40 % of the O_2 flux, the Fe(III)EDTA$^-$ load amounts to 200 kmol.h^{-1}. The NO and Fe(III)EDTA$^-$ load generates a total ethanol consumption of 240 kmol e$^-$.h^{-1}, which requires an ethanol flow of 20 kmol.h^{-1} or 920 kg.h^{-1}. Note that the amount of ethanol needed as carbon source for cell synthesis is neglected, which is justified by the low biomass yields observed in Chapter 4 and Chapter 9).
It should be stressed that a selectivity coefficient (σ) of 0.4 is presumably higher than the selectivity under the experimental conditions of Chapter 4 and Chapter 9. From the electron flow partitioning found in these studies (14/86), a selectivity coefficient of 0.33 can be estimated. The latter value corresponds well with values found by Gambardella *et al.* (2005b), who used a stirred cell reactor (Fig. 6). Fig. 6B shows that the selectivity of the absorption process is strongly affected by the presence of oxygen (Gambardella *et al.*, 2005b).

Assuming the relative degradation amounts to 0.9 % (mol EDTA degraded per mol Fe(II)EDTA^{2-} oxidized), i.e. the value observed in Chapter 9 (where the flue gas contained 3.5-3.9 % O_2), the EDTA degradation rate amounts to 200*0.9 = 1.80 kmol.h^{-1} or 670 kg.h^{-1}, corresponding to 1.1 ton EDTA per ton NO_x removed. This high EDTA consumption rate suppresses the sustainability of the BioDeNOx concept. Moreover, based on a bulk price of, respectively, € 500,- and € 2.000,- per ton ethanol and EDTA (Biostar, personal communication), a specific cost level of € 3.000,- per ton NO_x removed can be estimated (only for ethanol and EDTA consumption). A comparison with the cost levels of competing NO_x removal techniques like SCR (€ 500,- to € 2.300,- per ton NO_x incl. capital and operation, level 1997 (EPA, 1997)) makes clear that the economic potential of the BioDeNOx technology is presently still poor.

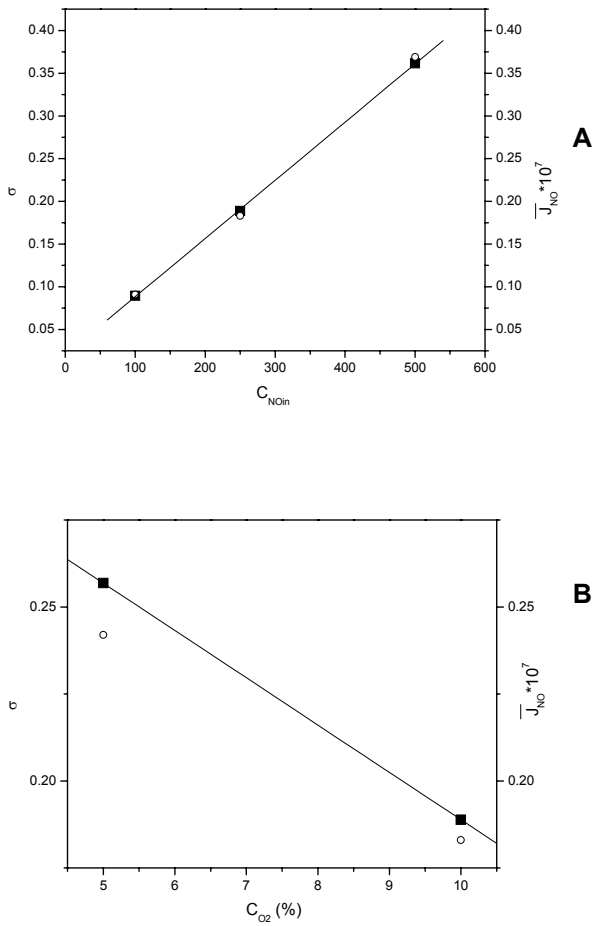

Figure 6

Effect of the NO concentration (C_{NOin}) with a fixed O_2 concentration (C_{O2in}) of 10 % (A) and effect of the O_2 concentration with a fixed NO concentration of 250 ppm (B) on the selectivity coefficient σ (■) and the NO flux $\bar{J}_{NO}$ (○). [Fe(II)EDTA^{2-}] = 10 mM, T = 323 K. Lines: for illustrative purposes only (Gambardella *et al.*, 2005b).

DIRECTIONS FOR FURTHER DEVELOPMENT

In this context it is evident that further development of the BioDeNOx technology should primary be focussed on favouring NO absorption while suppressing Fe(II)EDTA^{2-} oxidation by oxygen. One way to do so is to operate at lower temperatures, since it enhances the rate of NO absorption, thereby elevating the NO flux from the gas to the liquid phase (Gambardella *et al.*, 2005b). Lowering operation temperatures for BioDeNOx reactors will, however, lead to lower volumetric NO reduction rates (Chapter 7) and therefore, bigger bioreactor volumes.

Another way to minimize Fe(II)EDTA^{2-} oxidation is by means of the scrubber design. A model developed by Gambardella *et al.* (2005b) indicates that NO absorption using typical BiodeNOx conditions is favored, e.g. high selectivity coefficients (σ), up to 1.0 at Fe(II)EDTA^{2-} concentrations of

100 mM (i.e. much higher than the Fe(II)EDTA^{2-} concentrations used in this thesis) when operating at high mass transfer coefficients for both the gas and liquid phase. In this view, a spray tower appears to be a better choice for the scrubber unit than a packed column (Gambardella *et al.*, 2005b).

An alternative research direction may be the development of an iron chelate (Fe-L) that is, compared to Fe(II)EDTA^{2-}, less sensitive for oxidation, without concessions to the NO absorption potential. This will not be easy, since the oxygen sensitivity of various iron chelates was shown to be positively related with the NO absorption capacity (Fig. 7). However, when such an absorbent can be found, this implies that:

1. high rate Fe(III)-L reduction and thus the presence of polysulfides/EM in the BioDeNOx reactor becomes less important.
2. the use of ethanol is more efficient, i.e. ethanol will mainly be used for NO reduction instead of for Fe(III)-L reduction.
3. the ligand degradation rate will most likely drop, based on the observation that, in case of EDTA, the ligand degradation is closely related to the Fe(II) oxidation rate (Gambardella *et al.*, 2005a).

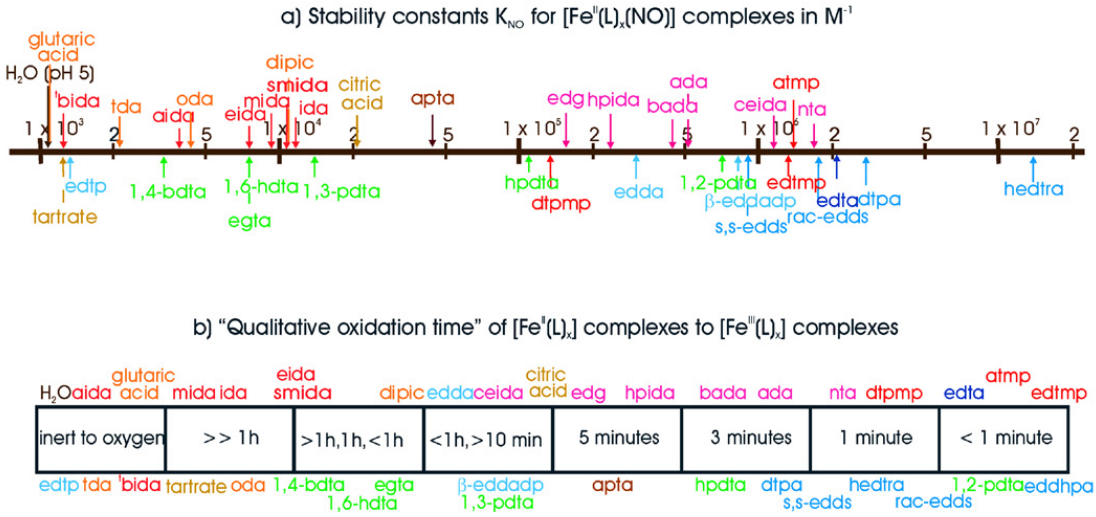

Figure 7

Stability constants of nitrosyl complexes (a) and the oxygen sensitivity (b) of various Fe(II)-L systems (Schneppensieper *et al.*, 2001).

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Hoofdstuk 10

Samenvatting, algemene discussie en conclusies

INLEIDING

De uitstoot van stikstofoxiden (NO_x, bestaande uit 95% NO en 5% NO_2) naar de atmosfeer is een omvangrijk milieuprobleem. Om de uitstoot via industriële rookgassen tegen te gaan worden op dit moment vooral chemische zuiveringsprocessen toegepast, zoals selectieve katalytische reductie (SCR). Deze zuiveringsprocessen werken echter bij hoge temperaturen (> 300 ºC) en verbruiken dure katalysatoren. Uit dat oogpunt zijn biologische NOx verwijderingstechnieken mogelijk een welkom alternatief. Ten eerste omdat biologische processen bij lagere (omgevings) temperaturen werken, zonder het verbruik van dure katalysatoren. Daartegenover staat dat biologische filtratietechnieken in het algemeen lange reactor verblijftijden vereisen, vooral vanwege de trage overdracht van NO van de gas- naar de vloeistoffase (in dit geval water).

De overdracht van NO van de gas- naar de vloeistoffase kan worden versneld door gebruikmaking van een waterige oplossing van $Fe(II)EDTA^{2-}$. Deze wasvloeistof absorbeert NO, waarbij een stabiel nitrosylcomplex $Fe(II)EDTA\text{-}NO^{2-}$ wordt gevormd. In het BioDeNOx proces wordt NO absorptie door $Fe(II)EDTA^{2-}$ gecombineerd met de biologische regeneratie van $Fe(II)EDTA^{2-}$.

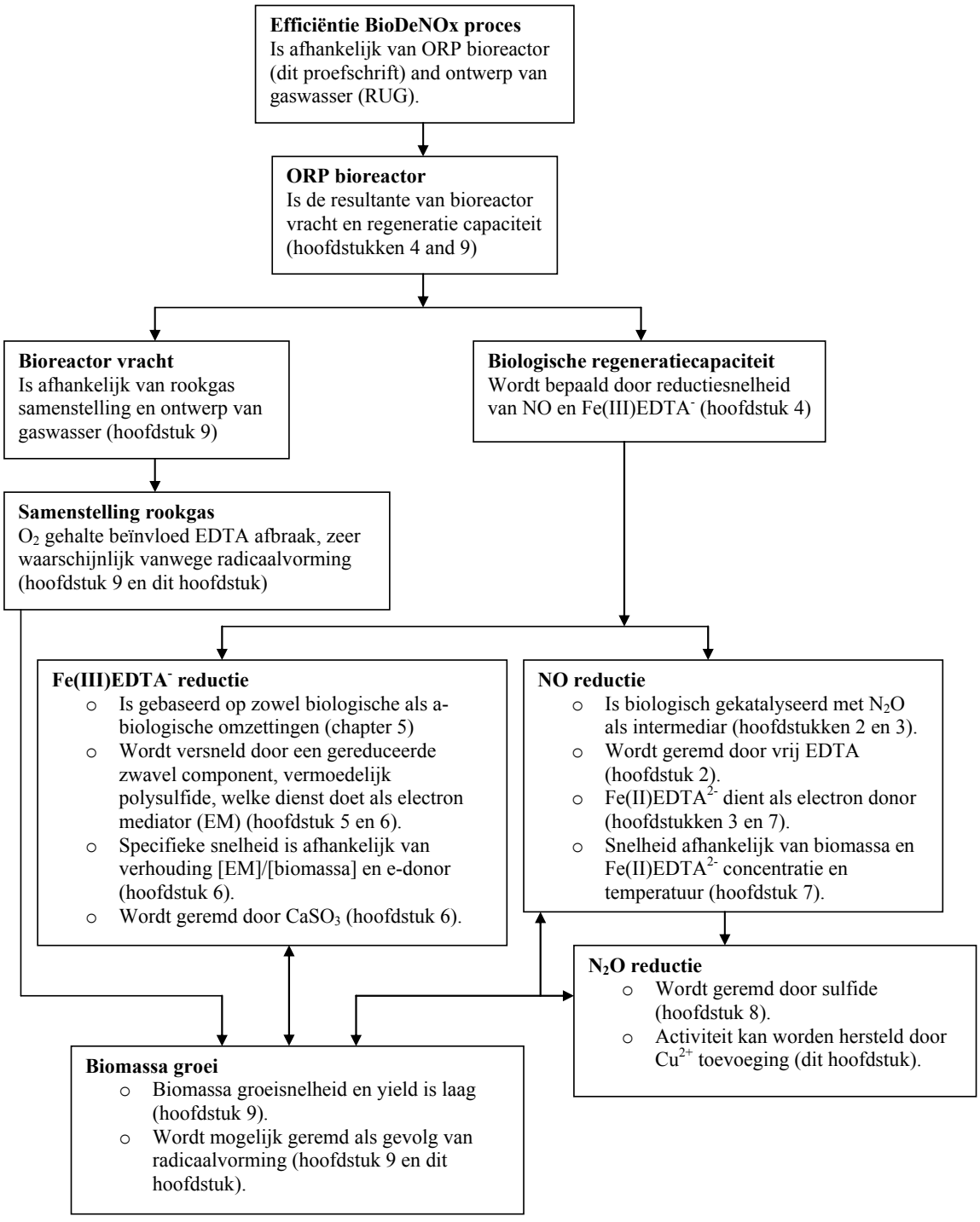
BioDeNOx is in beginsel een aantrekkelijk alternatief voor bestaande NO_x verwijderingstechnieken zoals SCR en briofiltratie. Dit vanwege het feit dat hoge temperaturen en dure katalysatoren niet nodig zijn, terwijl de benodigde contacttijden in gaswasser beperkt kunnen blijven (< 10 seconden) vanwege de snelle NO absorptie door $Fe(II)EDTA^{2-}$. In dit proefschrift is het BioDeNOx concept onderzocht, waarbij speciale aandacht is uitgegaan naar de belangrijkste processen in de bioreactor: de reductie van stikstofmonoxide (NO) en $Fe(III)EDTA^{-}$. Figuur 1 vat schematisch de aspecten van het BioDeNOx proces samen welke in dit proefschrift zijn onderzocht.

In dit hoofdstuk worden de belangrijkste resultaten samengevat, bediscussieerd en gerelateerd aan het onderzoek dat is uitgevoerd bij de partner-universiteiten: de Rijks Universiteit Groningen (RUG, met speciale aandacht voor de gaswas-processen) en de Technische Universiteit Delft (TUD, met speciale aandacht voor de microbiële ecologie van de bioreactor).

Op basis van de verkregen resultaten kunnen de volgende conclusies worden getrokken:

1. De mate van NO verwijdering uit de gasfase wordt in de eerste plaats bepaald door de NO absorptie kinetiek.
2. De belasting van de bioreactor wordt (voor wat betreft electron acceptoren) voornamelijk bepaald door $Fe(III)EDTA^{-}$.
3. De reductie van $Fe(III)EDTA^{-}$ wordt versneld door de betrokkenheid van een electron mediator, vermoedelijk polysulfides.
4. Polysulfides stimuleren de $Fe(III)EDTA^{-}$ reductie, daarentegen remmen monosulfides de reductie van N_2O en NO.
5. De redox-potentiaal (ORP) van BioDeNOx reactoren moet worden gestuurd tussen -180 en -200 mV (pH 7.2 ± 0.2).
6. $Fe(II)EDTA^{2-}$ interfereert met de electron overdracht in het NO reductie proces.

7. De capaciteit van de bioreactor hangt af van de biomassa concentratie, als wel van de electron mediator (polysulfide) concentratie.
8. De gevoeligheid van Fe(II)EDTA^{2-} voor oxidatie met zuurstof leidt tot een hoge consumptie van ethanol en EDTA en daarmee tot hoge bedrijfskosten.



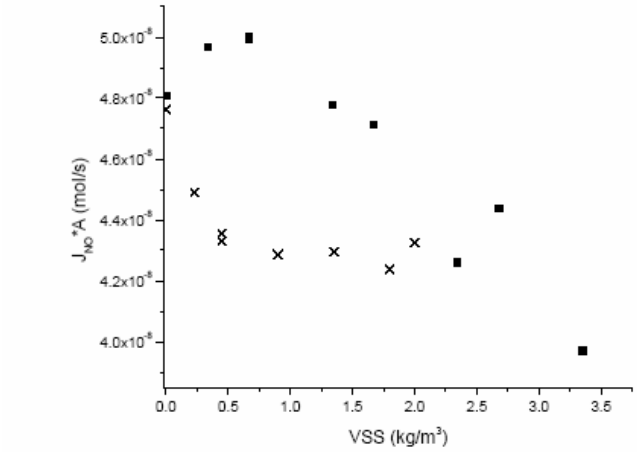
Figuur 1

Overzicht van de belangrijkste aspecten die zijn onderzocht in dit proefschrift.

CONCLUSIE 1

De mate van NO verwijdering uit de gasfase wordt in de eerste plaats bepaald door de NO absorptie kinetiek

Vergelijking van de NO verwijderingsefficiëntie zoals gevonden in hoofdstuk 4 en hoofdstuk 9 laat zien dat de NO verwijderingsefficiëntie van de gasfase in de eerste plaats wordt bepaald door de Fe(II)EDTA^{2-}/NO absorptie kinetiek. Bij een gaswasser retentie tijd (ESRT) van 11 seconden (hoofdstuk 4) bedroeg de NO verwijdering bij stabiele bedrijfsvoering meer dan 80 % bij de behandeling van een kunstmatig rookgas met 3.3 % O_2 en 500 ppm NO. Bij een ESRT van 1.2 seconden (hoofdstuk 9) en een rookgas met 3.5-3.9 % O_2 en 155 ppm NO bedroeg de NO verwijdering 39 %. Het verschil in de NO verwijderingsefficiency kan worden verklaard met behulp van de reactiekinetiek van de absorptie van NO met Fe(II)EDTA^{2-}, een reactie die uitvoerig is onderzocht in de laatste 20 jaar. Verschillende onderzoekers rapporteerden dat de reactie 1^{e} orde is in zowel NO als Fe(II)EDTA^{2-} (Demmink, 2000 en daarin geciteerde literatuur; Schneppensieper *et al.*, 2001). De reactie is erg snel en limitatie van de stofoverdracht speelt een belangrijke rol bij NO maar ook bij Fe(II)EDTA^{2-} (Demmink, 2000). Dit is zeker het geval bij BioDeNOx, waar de aanwezigheid van biomassa en deeltjes (precipitaten) in de gaswasser vloeistoffen de NO absorptie drukt vanwege stofoverdracht eigenschappen (Fig. 2).



Figuur 2

Flux van geabsorbeerd NO ($\overline{J}_{NO}$) in 30 mM Fe(II)EDTA^{2-} oplossing in the aanwezigheid van biomassa uit lab-schaal BioDeNOx reactoren in Wageningen (X) en Delft (■). T = 52 ºC, [NO]in= 250 vppm, pH= 7 (Gambardella *et al.*, 2005c).

De eerste orde afhankelijkheid tussen de NO absorptiesnelheid en de Fe(II)EDTA^{2-} concentratie verklaart de cruciale importantie van het bedrijven van BioDeNOx reactoren bij een redoxpotentiaal van lager dan -140 mV (tegen een Ag/AgCl electrode en bij pH 7.0, hoofdstuk 4) of lager dan -150 mV bij pH 7.2 ± 0.2 (hoofdstuk 9). Bij hogere redox potentialen is de Fe(II)EDTA^{2-} concentratie blijkbaar te laag voor een voldoend snelle NO absorptie, hetgeen resulteert in lage NO verwijderingdeficiënties

(hoofdstuk 4 en hoofdstuk 9). Aangetoond is dat de redox potentiaal een goede indicator is voor de $Fe(II)EDTA^{2-}$ concentratie (hoofdstuk 4 en 5). De kritieke redox potentiaal voor het bedrijven van BioDeNOx reactoren (omstreeks -150 mV tegen Ag/AgCl) komt goed overeen met de standaard redox potentiaal van $Fe(III)EDTA^{-}/Fe(II)EDTA^{2-}$: +96 mV tegen stadaard waterstof electrode (SHE) (Kolthoff and Auerbach, 1952).

CONCLUSIE 2

De belasting van de bioreactor wordt (voor wat betreft electron acceptoren) voornamelijk bepaald door $Fe(III)EDTA^{-}$

Het verloop van de redox potentiaal in BioDeNOx reactoren geeft de relatie aan tussen de $Fe(II)EDTA^{2-}$ regeneratiecapaciteit (NO en/of $Fe(III)EDTA^{-}$ reductie) en de belasting (met $Fe(III)EDTA^{-}$ en/of NO) van de bioreactor (zie bijv. Fig. 4 van hoofdstuk 9). Een stijgende redox potentiaal (> -250 mV) betekent een afname van de $Fe(II)EDTA^{2-}$ concentratie, d.w.z. dat de reactor belasting groter is dan de regeneratiecapaciteit (uitgedrukt als mmol NO of $Fe(III)EDTA^{-}$ per tijdseenheid). Wanneer de redox potentiaal daalt, is de regeneratiecapaciteit groter dan de belasting, terwijl een stabiele redox potentiaal duidt op een evenwicht tussen de belasting en de regeneratiecapaciteit van de bioreactor.

In beide reactor studies (hoofdstuk 4 en hoofdstuk 9) bleek de reactor belasting voornamelijk te worden bepaald door de $Fe(III)EDTA^{-}$ vracht. Electronen balansen gaven aan dat 86 % van de electronenvracht gerelateerd was aan de oxidatie en reductie van ijzer, terwijl slechts 14 % verbonden was met NO reductie. Dit kan worden verklaard door de gevoeligheid van $Fe(II)EDTA^{2-}$ voor oxidatie met zuurstof. De reactie van $Fe(II)EDTA^{2-}$ met zuurstof is irreversibel (Wubs en Beenackers, 1993; Zang en Van Eldik, 1990) en eerste orde in zuurstof, terwijl de $Fe(II)EDTA^{2-}$ afhankelijkheid wordt bepaald door de $Fe(II)EDTA^{2-}$ concentratie. Bij lage EDTA concentraties (< 10 mM) de reactie is eerste orde in ijzer, terwijl de reactie tweede orde wordt bij hogere concentraties. Bij BioDeNOx condities ([FeEDTA] > 10 mM), de oxidatie van $Fe(II)EDTA^{2-}$ bleek eerste orde in zuurstof en tweede orde in $Fe(II)EDTA^{2-}$ (Gambardella *et al*., 2005b). Hieruit kan worden afgeleid dat de zuurstofgevoeligheid van $Fe(II)EDTA^{2-}$ een relatief grote $Fe(III)EDTA^{-}$ vracht voor de bioreactor tot gevolg heeft. Dit laatste resulteert in een stijging van de electron donor vraag, d.w.z. een stijging van de ethanol consumptie, teneinde de $Fe(II)EDTA^{2-}$ gaswasvloeistof te regenereren. Bovendien wordt een hoge volumetrische $Fe(III)EDTA^{-}$ reductiesnelheid een belangrijke voorwaarde voor het succesvol bedrijven van BioDeNOx reactoren wanneer rookgassen met verhoogde O_2 concentraties worden behandeld.

CONCLUSIE 3

De reductie van Fe(III)EDTA$^-$ wordt versneld door de betrokkenheid van een electron mediator, vermoedelijk polysulfides

In hoofdstuk 5 zijn m.b.v. batch experimenten verschillende inocula gescreend op hun capaciteit om Fe(III)EDTA$^-$ (25 mM) te reduceren. *Escherichia coli*, een bacterie die niet in staat is om kristallijn ijzer te reduceren (Lovley *et al.*, 1991), was wel in staat tot de reductie van Fe(III)EDTA$^-$ tot Fe(II)EDTA^{2-} (Fig. 3 van hoofdstuk 5). Dit laatste is in overeenstemming met onderzoeksresultaten van Tanaka *et al.* (1983), waarbij werd gedemonstreerd dat *E. coli* kan worden toegepast in brandstofcellen waarbij FeEDTA als electron mediator wordt gebruikt. Toevoeging van kleine hoeveelheden sulfide leidde tot een sterke toename in de Fe(III)EDTA$^-$ reductie snelheid bij alle geteste inocula: anaërobe methanogene slibsoorten alsook een denitrificerend slib (Tabel 2 van hoofdstuk 5). Dit geeft aan dat de biologische Fe(III)EDTA$^-$ reductie (onder de experimentele omstandigheden) niet een directe omzetting is, maar een indirecte reductie met behulp van een electron mediator, waarschijnlijk polysulfides. Hoofdstuk 5 ondersteunt de hypothese dat niet Fe(III) respirerende bacteriën, maar reduceerders van elementair zwavel of polysulfides in belangrijke mate zijn betrokken bij de reductie van Fe(III)EDTA$^-$ in BioDeNOx reactoren.

Het mechanisme en de kinetiek van biologische Fe(III)EDTA$^-$ reductie is verder onderzocht in Hoofdstuk 6, waarbij niet geadapteerd methanogeen Eerbeek slib en BioDeNOx slib als inoculum zijn gebruikt. Verschillende electron mediatoren zijn gescreend op stimulatie van Fe(III)EDTA$^-$ reductie onder BioDeNOx omstandigheden (55 ºC, pH 7.2±0.2). Fe(III)EDTA$^-$ reductie werd versneld door toevoeging van een kleine hoeveelheid (0.5 mM) sulfide, cysteïne of elementair zwavel. Dit suggereert dat de reductie van Fe(III)EDTA$^-$ wordt gekatalyseerd door een zwavelcomponent die wordt gevormd bij de reactie tussen Fe(III)EDTA$^-$ en sulfide en die betrokken is bij de electron overdracht tussen de micro-organismen en Fe(III)EDTA$^-$. Hoewel de bepaling van de chemische structuur van deze electron mediator buiten het onderwerp van dit proefschrift valt, suggereert de lineaire relatie tussen de hoeveelheid toegevoegde sulfide en de specifieke Fe(III)EDTA$^-$ reductiesnelheid (Fig. 3 van hoofdstuk 6) dat polysulfide de electron mediator is.

Volgens de literatuur is Fe(III)EDTA$^-$ een tamelijk zwakke electron acceptor voor Fe(III) respirerende bacteriën (Finneran *et al.*, 2002). In tegenstelling tot Fe(III) reductases die betrokken zijn in assimilatieprocessen (Schroeder *et al.*, 2003) tonen bijna alle gekarakteriseerde enzymen die betrokken zijn bij Fe(III) respiratie flavine reductase activiteit: ze hebben een flavine nodig als co-factor. Het stimulerende effect van electron shuttles in verschillende microbiële conversies a.g.v. het vergemakkelijken van de electron overdracht van en naar bacteriën is uitgebreid gedocumenteerd in de literatuur (zie bijvoorbeeld Hernandez en Newman, 2001). Verschillende auteurs rapporteren electron overdracht via gereduceerde zwavel componenten in de reductie van diverse metalen: chroom (Smillie *et al.*, 1981), technetium (Lloyd *et al.*, 1998) en molybdeen (Tucker *et al.*, 1997). Bovendien is het sulfide / elementair zwavel koppel betrokken in de biologische ontkleuring van azo-kleurstoffen (Van der Zee *et al.*, 2003).

Gebruikmakend van PCR-DGGE analyse en PCR-DNA sequencing, toonden Kumaraswamy *et al.* (2005b), naast NO reducerende populaties, de aanwezigheid van Fe(III) respirerende bacteriën (nauw verwant aan *Deferribacter thermophilus*) in BioDeNOx reactoren. Deze reinculturen lieten echter een tamelijk langzame Fe(III)EDTA$^-$ reductie zien: bij een initiële eiwit concentratie van 10 mg.l^{-1} nam de conversie van 10 mM Fe(III)EDTA$^-$ naar Fe(II)EDTA^{2-} 7 dagen in beslag (persoonlijke communicatie R. Kumaraswamy), overeenkomend met een specifieke reductie snelheid van minder dan 0.003 mmol.mg$_{prot}^{-1}$.h^{-1}. Daarnaast vonden Kumaraswamy *et al.* (2005b), in afwezigheid van zwavel, een volumetrische Fe(III)EDTA$^-$ reductiesnelheid van 1.8 mM.h^{-1}, overeenkomend met een specifieke snelheid van 0.015 mmol.mg$_{prot}^{-1}$.h$^-$1 tijdens activiteitstesten met biomassa uit een laboratorium schaal reactor, waarin de dominante Fe(III)EDTA$^-$ reducerende populatie bestond uit bacteriën verwant *Bacillus infernus* and *Alkaliphilus* spp.. Deze waarde is laag vergeleken met de snelheden gevonden in hoofdstuk 6 (± 1 mmol.mg$_{prot}^{-1}$.h^{-1} ingeval 1 mM sulfide was toegediend).

Behalve de aanwezigheid van een geschikte electron mediator wordt de Fe(III)EDTA$^-$ reductiesnelheid ook beïnvloed door de beschikbare electron donor. Verschillende electron donoren zijn getest op toepasbaarheid voor Fe(III)EDTA$^-$ reductie: ethanol, glucose, acetaat, methanol, waterstof en melasse. De hoogste Fe reductiesnelheden werden verkregen met glucose en melasse, directe gevolgd door ethanol, acetaat en waterstof (hoofdstuk 5 en 6).

Zonder de toevoeging van extern sulfide bleek de Fe(III)EDTA$^-$ reductie door Eerbeek slib sterk geremd door $CaSO_3$ (lage concentraties, bijv. 1 mM, toonden al een sterke remming, zie Fig. 6 van hoofdstuk 6). $CaSO_3$ is in hoge concentraties aanwezig in BioDeNOx reactoren wanneer NO verwijdering wordt gecombineerd met fysisch chemische SO_2 verwijdering door middel van $CaSO_3$ precipitatie (d.w.z. inpassing van het kalksteen gips proces). De waargenomen remming in hoofdstuk 6 is mogelijk veroorzaakt door de reactie van SO_3^{2-} met S^{2-}, waarbij thiosulfaat wordt gevormd maar de electron mediator (polysulfide) verdwijnt. Dit houdt in dat remming door SO_3^{2-} mogelijk kan worden onderdrukt in geval van hoge concentraties polysulfide.

CONCLUSIE 4

Polysulfides stimuleren de Fe(III)EDTA- reductie, daarentegen remmen monosulfides de reductie van N_2O en NO

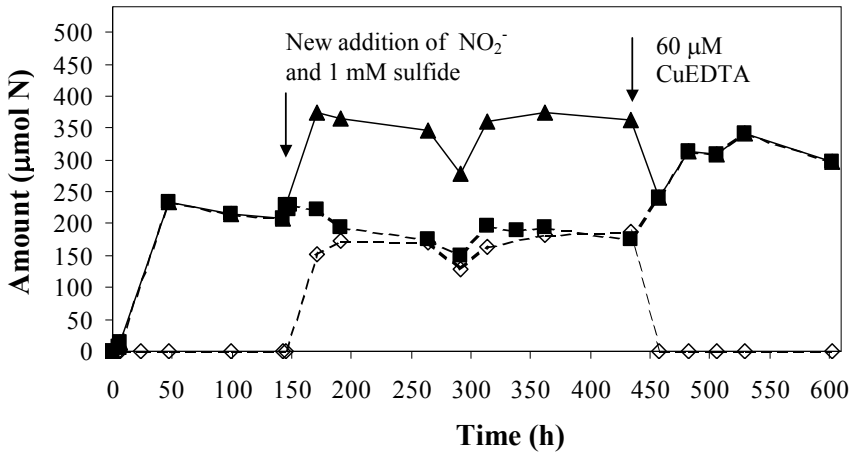
In hoofdstuk 9 is het effect van sulfide additie op de Fe(II)EDTA^{2-} regeneratiecapaciteit van BioDeNOx reactoren onderzocht. Batch experimenten toonden een lineaire relatie tussen de relatieve EM concentratie (hoeveelheid EM/polysulfide) beschikbaar per bacterie en de specifieke Fe(III)EDTA$^-$ reductie snelheid (Tabel 2 van hoofdstuk 9), hetgeen de resultaten van hoofdstuk 6 bevestigt. Bij het continue bedrijf van de bioreactor induceerde de toevoeging van sulfide (puls van 2 mM) een verhoogde Fe(III)EDTA$^-$ reductiecapaciteit, echter voor een beperkte periode (ongeveer 4 dagen). In het ideale geval is de oxidatie en reductie van de electron mediator een cyclisch proces, waarbij de concentratie van deze component constant blijft. Het is echter aannemelijk dat zowel hoge als lage

redox potentialen zullen resulteren in onbalans van de EM cyclus, en daarmee in een instabiel reactor bedrijf.
Bij redox potentialen beneden -250 mV tegen Ag/AgCl, d.w.z. wanneer alle Fe(III)EDTA$^-$ is gereduceerd, zal in aanwezigheid van ethanol de aanwezige polysulfide compleet worden gereduceerd tot monosulfide. Deze laatste component kan precipiteren met Fe^{2+}, hetgeen de belangrijkste valentie van ijzer is bij deze lage redox potentialen (samen met magnesium en calcium overstijgt de Fe concentratie de EDTA concentratie om vrij, niet gecomplexeerd EDTA te voorkomen, zie hoofdstuk 2). Op die manier worden de polysulfides uiteindelijk omgezet in onoplosbaar FeS, zodat de electron mediator is veranderd in een niet of nauwelijks reactieve vorm van zwavel:

$S_n^{2-} + 2\ e^- + H^+ \rightarrow S_{n-1}^{2-} + HS^-$
$2\ Fe^{2+} + 2\ HS^- \rightarrow 2\ FeS\ (s)$

Ook met betrekking tot NO reductie moeten condities waarbij accumulatie van sulfide kan optreden (bij redox potentialen beneden -250 mV versus Ag/AgCl) worden voorkomen. Hoofdstuk 8 toont dat NO reductie tot N_2O volledig is stilgelegd bij aanwezigheid van 1 mM vrij sulfide (concentratie denitrificerend slib 0.2 $gVSS.l^{-1}$). De tweede stap van NO reductie, d.w.z. N_2O reductie tot N_2, is veel gevoeliger voor remming door sulfide: reeds bij 15 µM S^{2-} werd remming aangetoond. De resultaten van hoofdstuk 8 komen goed overeen met de literatuur. Sörensen *et al.* (1980) rapporteerde bijvoorbeeld dat 0.3 mM S^{2-} reeds gedeeltelijke remming van NO reductie en sterke remming van N_2O reductie veroorzaakt bij *Psudomonas florescens*. Volgens een andere studie kan N_2O reductie al door sulfide worden beïnvloed bij concentraties beneden 1 µM (Sörensen *et al.*, 1987).

Een onvolledige NO reductie is zeer ongewenst omdat het tot de uitstoot van N_2O leidt. De N_2O reducerende activiteit kan echter worden hersteld door middel van toediening van kleine hoeveelheden koper. Manconi *et al.* (2005) toonde dat additie van 60 µM Cu de N_2O reductie herstelde in een batch experiment waarbij de N_2O reductie was geremd door de toevoeging van sulfide (Fig. 3). Het herstellende effect van koper kan worden verklaard door de Cu behoefte ten behoeve van N_2O respiratie, omdat het betrokken enzym, N_2O reductase, een koper enzym is (Coyle *et al.*, 1985, Zumft, 1997). Media met een tekort aan koper leidde tot stopzetting van celgroei of tenminste tot N_2O accumulatie tijdens nitriet reductie (Iwasaki *et al.*, 1980; Iwasaki en Terai, 1982; Matsubara en Zumft, 1982). In hoofdstuk 8 bedroeg de Cu concentratie 0.22 µM (toegevoegd via de spoorelementen oplossing). Deze hoeveelheid was blijkbaar te laag om de waargenomen sulfide toxiciteit tegen te gaan. Deze sulfide toxiciteit is mogelijk gebaseerd op koper limitatie in het medium, geïnduceerd door precipitatie met sulfide (Manconi *et al.*, 2005).



Figuur 3

Accumulatie van N_2O (◇) en N_2 (■) en totaal van gasvormige stikstofcomponenten (som van N_2O en N_2) (▲), als gevolg van reductie van het nitrosyl-complex (Fe(II)EDTA-NO^{2-}) reductie door denitrificerend slib (1.3 gVSS/l). Voorafgaand aan de vorming van het nitrosyl-complex via injectie van 2 mM nitriet op t = 0, was het aanvankelijke Fe(III)$EDTA^-$ medium gereduceerd m.b.v. 15 mM Fe^0 als reductor. Op t = 150 h werd het nitrosyl weer gevormd via nitriet injectie, nu gevolgd door toevoeging van sulfide (1 mM) aan de flesjes. Op t = 434 h werd 60 µM CuEDTA toegevoegd aan de flesjes (Manconi *et al.*, 2005).

CONCLUSIE 5

De redox-potentiaal (ORP) van BioDeNOx reactoren moet worden gestuurd tussen -180 en -200 mV (pH 7.2 ± 0.2)

Hoofdstuk 9 laat zien dat er een tegenstelling bestaat tussen de optimale redox potentiaal van de FeEDTA scrubber/bioreactor vloeistof met het oog op NO absorptie en biologische regeneratie. De absorptie van NO, d.w.z. NO verwijdering uit de gasfase, is gebaat bij een lage redox potentiaal (zie Conclusie 1). Dus de Fe(III)$EDTA^-$ en Fe(II)$EDTA^{2-}$ concentratie moet zo laag mogelijk zijn. Aan de andere kant moeten deze electron acceptoren in de bioreactor aanwezig zijn om biologische activiteit en groei te onderhouden, zodat ten minste de afsterving wordt gecompenseerd. Ook kan sulfide accumuleren bij complete afwezigheid van Fe(III)$EDTA^-$ hetgeen ongewenst is (zie Conclusie 4). Daarom moet altijd een zekere hoeveelheid (10-20 %) Fe(III)$EDTA^-$ in de bioreactor vloeistof aanwezig zijn, hetgeen overeenkomt met een redox potentiaal van tussen -180 en -200 mV tegen Ag/AgCl bij pH 7.2 (± 0.2). Dit kan worden bereikt door de ethanol dosering aan de reactor te sturen op de redox potentiaal (zie hoofdstuk 4). Op die manier is ethanol (electron donor) het substraat dat de biologische activiteit limiteert in plaats van NO en/of Fe(III)$EDTA^-$ (electron acceptoren).

Teneinde substraatlimitatie m.b.t. NO en Fe(III)$EDTA^-$ te minimaliseren zijn goed gemengde bioreactoren zoals een compleet geroerde tank reactor (CSTR) of een gaslift reactor een betere keuze dan propstroom-achtige systemen, zoals de UASB, reactor vanwege de superieure eigenschappen

m.b.t. substraat controle. Door intensieve menging kunnen compleet anaërobe zones, d.w.z. in afwezigheid van Fe(III)EDTA$^-$, worden voorkomen. Daarnaast zal bij intensief gemengde systemen een gelijkmatige verdeling van NO beter zijn gewaarborgd, hetgeen NO-vrije zones helpt te voorkomen (NO is substraat voor denitrificerende biomassa).

CONCLUSIE 6

Fe(II)EDTA^{2-} interfereert met electron overdracht in het NO reductie proces

NO reductie in Fe(II)EDTA^{2-} oplossingen was het onderwerp van de hoofdstukken 2, 3 en 7. Reductie van stikstofmonoxide tot stikstofgas is biologisch gekatalyseerd met lachgas (N_2O) als intermediair. Verschillende slibsoorten afkomstig van anaërobe en denitrificerende praktijkinstallaties bleken in staat om onder thermofiele condities (55 °C) NO te reduceren. De NO reductiesnelheid was niet afhankelijk van de aanwezigheid van ethanol of acetaat, hetgeen betekent dat Fe(II)EDTA^{2-} als electron donor optreedt. Inderdaad werd bij afwezigheid van ethanol of acetaat (dit zijn goede electron donoren voor Fe(III)EDTA$^-$ reductie, zie hoofdstuk 6) de accumulatie van Fe(III)EDTA$^-$ waargenomen (Tabel 3 van hoofdstuk 3).

De snelheid van NO reductie van het denitrificerende Veendam slib werd beïnvloed door de Fe(II)EDTA^{2-} concentratie (Fig. 6 van hoofdstuk 7). Dit suggereert dat in BioDeNOx reactoren, Fe(II)EDTA^{2-} als directe electron donor optreedt bij NO reductie, in tegenstelling tot ethanol. Dit is, op het eerste oog, enigszins verassend omdat ethanol te boek staat als een uitstekende electron donor voor denitrificatie (Constantin *et al.*, 1997). Echter, de redox eigenschappen van FeEDTA en het Nitric Oxide Reductase (NOR) enzym, maken een directe betrokkenheid van Fe(II)EDTA^{2-} bij NOR reductie mogelijk, hetzij via direct of indirect via Cytochrome c (Tabel 1; zie ook Fig. 8 van hoofdstuk 7).

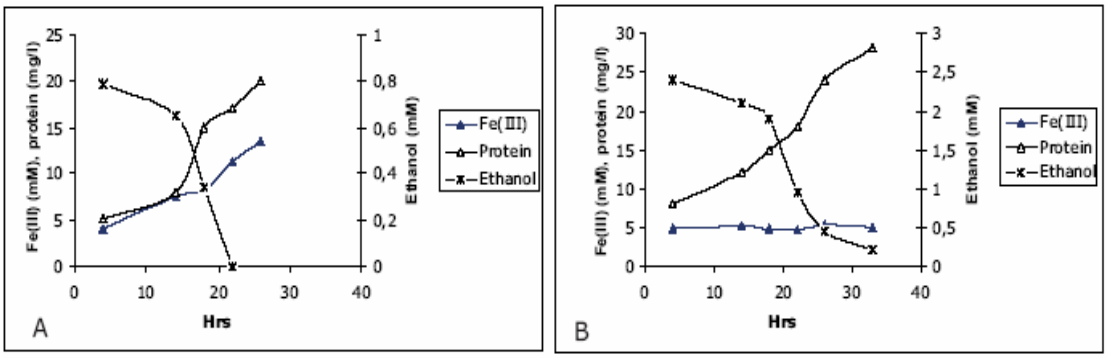
Tabel 1

Standaard potentialen ($E^{0'}$) van verschillende redox koppels die relevant zijn voor NO reductie.

Redox koppel	$E^{0'}$ (mV)	Referentie
NO/N_2O	+1177	Zumft, 1997
N_2O/N_2	+1352	Zumft, 1997
NOR_{ox}/NOR_{red}	tussen +280 en +320	Wasser *et al.*, 2002
N_2OR_{ox}/N_2OR_{red}	+260	Coyle *et al.*, 1985
Cyt c_{ox}/Cyt c_{red}	tussen +148 en +253	Gray *et al.*, 1986
amicyanin	+294	Gray *et al.*, 1986
Fe(III)EDTA$^-$/Fe(II)EDTA^{2-}	+96	Kolthoff en Auerbach, 1952

Let wel: de resultaten van hoofdstuk 7 sluit NO reductie via de meer conventionele electron overdracht, d.w.z. via het Cytochrom bc1 complex en voorliggende redox koppels (Fig. 8 van hoofdstuk 7), niet uit. Kumaraswamy *et al.* (2005a) zagen Fe(III)EDTA$^-$ accumulatie als gevolg van anoxische Fe(II) oxidatie gekoppeld aan NO_2^-/NO reductie bij 55 °C door de reincultuur KT-1, welke

was geïsoleerd uit een lab-schaal BioDeNOx reactor. Echter, Fe(III)EDTA$^-$ accumulatie werd alleen waargenomen indien lage concentraties (initiële concentratie 1 mM) ethanol beschikbaar waren (Fig. 4A). Bij hogere ethanol concentraties (initiële concentratie 3 mM) werd deze Fe(III)EDTA$^-$ accumulatie niet waargenomen (Fig. 4B). Hun resultaten suggereren dat de weg van electron levering afhangt van de concentraties van de beschikbare electron donoren. Kwantificering van de electron stroom via ethanol oxidatie (gekoppeld aan NO reductie) in redox buffers als Fe(II)EDTA^{2-} /Fe(III)EDTA$^-$ vergt verder onderzoek, bijvoorbeeld met gebruikmaking van mutanten die het Cytochrome bc1 complex missen of m.b.v. specifieke remmers van het Cytochrome bc1 complex, zoals antimycine (Slater *et al.*, 1973).



Figuur 4

Fe(III)EDTA$^-$ (▲) en eiwit (△) accumulatie en ethanol (✱) consumptie tijdens NO_2^-/NO reductie door strain KT-1 met 25 mM Fe(II)EDTA^{2-}, 5 mM nitriet en 1 mM (A) of 3 mM ethanol (B) (Kumaraswamy *et al.*, 2005b).

CONCLUSIE 7

De capaciteit van de bioreactor hangt af van de biomassa concentratie, als wel van de electron mediator (polysulfide) concentratie

Zoals bediscussieerd bij 'Conclusie 2' wordt de Fe(II)EDTA^{2-} regeneratie capaciteit bepaald door de volumetrische snelheid van NO en Fe(III)EDTA$^-$ reductie. Goede resultaten werden verkregen waneer de bioreactor was geïnoculeerd met een mengsel van denitrificerend slib (voor NO reductie) en anaëroob korrelslib (voor Fe(III)EDTA$^-$ reductie) (zie hoofdstuk 4). Het vermalen van het korrelslib resulteerde in hogere Fe(III)EDTA$^-$ snelheden (hoofdstuk 6). Een stabiel reactorbedrijf werd behaald in geval de reactor was geënt met ca. 1 g VSS.l^{-1} Veendam slib, verrijkt met een zelfde hoeveelheid gemalen Eerbeek korrelslib (hoofdstuk 9). Het is echter aannemelijk dat lagere concentraties Eerbeek slib ook bevredigende resultaten zullen geven omdat de volumetrische Fe reductiesnelheid niet werd gelimiteerd door de VSS concentratie, maar door de electron mediator concentratie (hoofdstuk 6).

De BioDeNOx reactor vloeistof liet een specifieke NO reductie snelheid zien van 1.3µmol..mg$_{prot}^{-1}$.h^{-1}, hetgeen qua orde grootte overeenkomt met literatuur data (hoofdstuk 7). De volumetrische NO reductiecapaciteit van was lineair gerelateerd aan de concentratie denitrificerende biomassa. Verhoging

van de volumetrische NO reductie capaciteit kan daarom alleen worden verkregen door hogere biomassa concentraties (Fig. 3 van hoofdstuk 7 en Tabel 1 van hoofdstuk 9).
Een hoge Fe(III)EDTA$^-$ reductiesnelheid (1.0 mmol.mg_{prot}^{-1}.h^{-1}) werd waargenomen bij toevoeging van 1 mM sulfide aan het 25 mM Fe(III)EDTA^{2-} medium (met ethanol als electron donor). De specifieke Fe(III)EDTA$^-$ reductiesnelheid toonde een lineaire relatie met de aan de batchflesjes hoeveelheid toegevoegde sulfide, hetgeen een lineaire relatie suggereert tussen de hoeveelheid EM (polysulfide) die beschikbaar is per hoeveelheid biomassa en de volumetrische Fe(III)EDTA$^-$ reductiesnelheid (Fig. 3 van hoofdstuk 6).
Op grond van bovenstaande is de volumetrische Fe(II)EDTA^{2-} regeneratiesnelheid een resultante van (i) de concentratie biomassa (zowel NO reducerende als Fe(III)EDTA$^-$/polysulfide reducerende) en (ii) de EM (polysulfide) concentratie (hoofdstuk 9). Deze laatste factor limiteerde in onze reactor experimenten (hoofdstukken 4 en 9) de zuurstof (d.w.z. de Fe(III)EDTA$^-$) vracht die kon worden behandeld door de bioreactor. Zoals bediscussieerd bij ‘Conclusie 5’ is het een valkuil om BioDeNOx reactoren te bedrijven bij redoxpotentialen beneden -200 mV, zoals is gebeurd in hoofdstuk 9. Door middel van sturing op een redoxpotentiaal tussen -180 en -200 mV kan depletie van de electron mediator (polysulfide) worden voorkomen, resulterend in een blijvend hoge Fe(III)EDTA$^-$ reductiecapciteit.

Verhoging van de NO reductiecapaciteit kan worden verkregen door biomassa groei (hoofdstuk 4), hetgeen tijd vergt, of via enting met denitrificerend slib (hoofdstuk 9). Om verlies van biomassa via een ‘bleed’ stroom te voorkomen, is toepassing van membraanfiltratie, d.w.z. micro- of ultrafiltratie, wellicht een interessante optie. De volumetrische Fe(III)EDTA$^-$ reductie capaciteit kan worden verhoogd d.m.v. verhoging van de polysulfide concentratie in de bioreactor. In geval het rookgas SO_2 bevat, kan deze component de bron zijn voor een continue aanmaak van elementair zwavel en polysulfides. Wanneer een SO_2 vrij rookgas wordt behandeld kan substraat (polysulfide) limitatie gemakkelijk optreden als gevolg van sulfide precipitatie met Fe^{2+} (bij lage redoxpotentialen !). In dat geval kan de Fe(III)EDTA$^-$ reductiecapaciteit worden hersteld door dosering van (poly)sulfide aan het Fe(III)EDTA$^-$ medium.

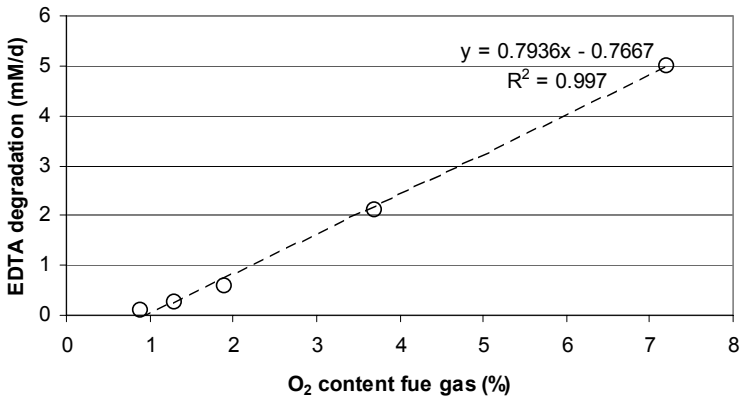
Alleen relatief lage biomassa concentraties konden worden bereikt tijdens het continue bedrijf van de BioDeNOx reactor: ± 90 en ± 180 mg eiwit per liter in, respectievelijk, hoofdstuk 4 en hoofdstuk 9. Ook werden zeer lage waarden gevonden voor de biomassa groeisnelheid en yield: 0.043 d^{-1} en 0.009 mg eiwit per mg ethanol (hoofdstuk 9), overeenkomend met 0.03 mg eiwit per mmol electronen (geoxideerd of gereduceerd). Deze laatste waarde, verkregen onder thermofiele condities (55 °C), is extreem laag in vergelijking met de biomassa yield (1.8 mg per mmol electronen) die werd waargenomen door Kumaraswamy *et al.* (2005a) tijdens NO reductie bij 30 °C door de nieuw beschreven bacterie *Paracoccus ferrooxidans*. Daarbij moet worden gerealiseerd dat de door Kumaraswamy waargenomen yield zeer waarschijnlijk is beïnvloed door biologische nitriet reductie, hetgeen niet voorkomt in BioDeNOx reactoren. Bovendien dragen de thermofiele condities in de

BioDeNOx reactor (hoofdstuk 9) hoogstwaarschijnlijk bij aan een hogere afsterving en lyse van biomassa vergeleken met de mesofiele condities zoals toegepast door Kumaraswamy *et al.* (2005a).
Ook kan de lage biomassa yield een gevolg zijn van substraatlimitatie, d.w.z. van de electron acceptoren NO en waarschijnlijk polysulfide, dat plaatsvindt in continue BioDeNOx reactoren in tegenstelling tot batch experimenten. Daarnaast kunnen fysiologische stress condities in BioDeNOx reactoren debet zijn aan een lage biomassa yield en lage groeisnelheden. In het bijzonder radicaal vorming tijdens $Fe(II)EDTA^{2-}$ oxidatie met zuurstof (aanwezig in het rookgas), mogelijk via de Haber-Weiss reactie, is een punt van aandacht en van belang bij toekomstig onderzoek. De gevormde radicalen zijn namelijk zeer reactief en bacteriedodend (Wolcott *et al.*, 1994).

CONCLUSIE 8

De gevoeligheid van $Fe(II)EDTA^{2-}$ voor oxidatie met zuurstof leidt tot een hoge consumptie van ethanol en EDTA en daarmee tot hoge bedrijfsvoeringskosten

Radicalen (bijvoorbeeld hydroxyl) zijn, naast remming van microbiële groei, mogelijk ook de oorzaak van EDTA afbraak (Mochidzuki *et al.*, 1999). In hoofdstuk 9 werd een substantiële EDTA afbraak waargenomen: 2.1 (± 0.1) $mM.d^{-1}$ bij behandeling van rookgas met een zuurstofconcentratie van 3.5-3.9 %. Vergelijking van de resultaten van hoofdstuk 4 (geen substantiële EDTA afbraak waargenomen bij 0.9 % O2), hoofdstuk 9 en Utomo (2004), geeft aan dat de EDTA afbraaksnelheid een lineaire relatie vertoond met het zuurstof gehalte van het rookgas (Fig. 5). Dit komt goed overeen met data van Gambardella *et al.* (2005a), die EDTA degradatie onderzochten m.b.v. batch experimenten. Zij vonden eveneens een grote afhankelijkheid tussen (de snelheden van) $Fe(II)EDTA^{2-}$ oxidatie en EDTA afbraak. Het voornaamste degradatieproduct bleek ED3A te zijn. Bovendien werd aangetoond dat een overmaat EDTA t.o.v. ijzer (ratio 1.5-2) de EDTA afbraaksnelheid remt (Gambardella *et al.*, 2005a). De aanwezigheid van vrij, niet gecomplexeerd EDTA is echter niet toegestaan in verband met de sterke remming van NO en $Fe(III)EDTA^{-}$ reductie (hoofdstukken 2 en 5).



Figuur 5

Waargenomen EDTA afbraaksnelheid bij variabele O_2 concentraties in hoofdstuk 4 (0.9 %), hoofdstuk 9 (3.5-3.9 %) en Utomo (2004) (1.3, 1.9 and 7.2 % O_2).

De zuurstofgevoeligheid van Fe(II)EDTA^{2-} kan als het belangrijkste probleem worden beschouwd voor introductie van BioDeNOx in de praktijk. Behalve radicaalvorming en de (hoogstwaarschijnlijk) daarmee samenhangende EDTA afbraak, leidt de oxidatie van Fe(II)EDTA^{2-} door zuurstof tot een sterk verhoogde ethanol consumptie. Naast microbiële ethanol consumptie ten behoeve van Fe(III)EDTA^{-} reductie, wordt ethanol mogelijk ook verbruikt a.g.v. de reactie met hydroxyl radicalen die worden gevormd a.g.v. Fe(II)EDTA^{2-} oxidatie via de Haber-Weiss reactie (Winston en Cederbaum, 1983). In feite is dient ethanol in dat geval als 'radical scavenger'.

Zowel EDTA afbraak als ethanol consumptie leidt tot hoge operationele kosten, zoals geïllustreerd door middel van een denkbeeldige toepassing, waarbij een rookgas (debiet 2 miljoen $Nm^3.h^{-1}$) met 250 ppm NO en 5% O_2 wordt behandeld door een BioDeNOx gaswasser met 90% NO verwijderingsrendement. Dit genereert een bioreactor vracht van 20 kmol $NO.h^{-1}$. De Fe(III)EDTA^{-} vracht kan worden geschat m.b.v. de selectiviteitscoëfficiënt (σ), die wordt bepaald door de verhouding tussen de gemiddelde NO ($\bar{J}_A$) en O_2 ($\bar{J}_O$) flux van de gasfase naar de Fe(II)EDTA^{2-} gaswasvloeistof (Gambardella *et al.*, 2005b):

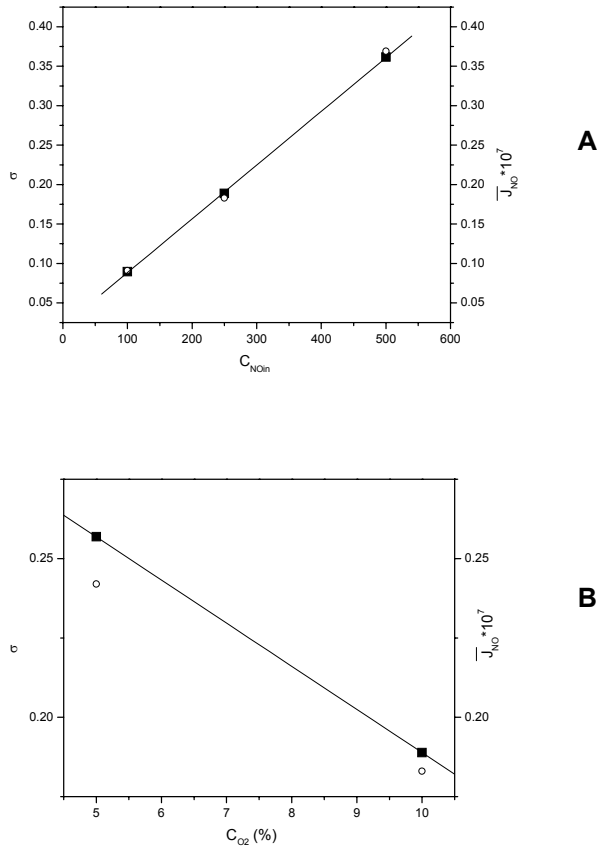
$$\sigma = \frac{\bar{J}_A}{\bar{J}_O}$$

Bij aanname van een selectiviteitscoëfficiënt van 0.4, d.w.z. de NO flux bedraagt 40 % van de O_2 flux, bedraagt de Fe(III)EDTA^{-} vracht 200 $kmol.h^{-1}$. In totaal vraagt de NO en Fe(III)EDTA^{-} vracht een electron gift van 240 kmol $e^{-}.h^{-1}$, overeenkomend met een ethanol consumptie van 20 kmol of 920 kg per uur. Hierbij is de hoeveelheid ethanol die benodigd is voor cel synthese buiten beschouwing gelaten, hetgeen gerechtvaardigd is gezien de lage biomassa yield die is gevonden in de hoofdstukken 4 en 9.

Nadrukkelijk wordt gesteld dat een selectiviteitscoëfficiënt (σ) van 0.4 waarschijnlijk hoger ligt dan het geval was onder de experimentele condities van de studies in hoofdstuk 4 en 9. Daar lag de verdeling van electron flow op 14/86, op grond waarvan een selectiviteitscoëfficiënt van 0.33 kan worden geschat. Deze waarde komt goed overeen met de coëfficiënten van Gambardella *et al.* (2005b), die gebruik maakten van een geroerde cel reactor (Fig. 6). Fig. 6B laat zien dat de selectiviteit van de absorptie sterk wordt beïnvloed door de aanwezigheid van zuurstof.

Wat EDTA afbraak betreft wordt in dit rekenvoorbeeld uitgegaan van een relatieve degradatie van 0.9 % (mol EDTA per mol Fe(II)EDTA^{2-} geoxideerd), d.w.z. de waarde zoals gevonden in hoofdstuk 9 (3.5-3.9 % O_2 in rookgas). In dat geval bedraagt de EDTA afbraaksnelheid 200 * 0.9 % = 1.80 kmol of 670 kg per uur, overeenkomend met een EDTA consumptie van 1.1 ton per ton verwijderde NO_x. Deze hoge EDTA consumptie komt de duurzaamheid van het BioDeNOx proces niet ten goede. Bovendien leidt het tot hoge kosten. Uitgaande van bulk prijzen van € 500,- per ton ethanol en € 2.000,- per ton EDTA (Biostar, persoonlijke communicatie), kan een specifiek prijspeil worden berekend van (orde-grootte) € 3.000,- per ton verwijderde NOx. Een vergelijking met SCR (€ 500,- tot 2300,- per ton NOx

incl. kapitaals- en onderhouds/bedieningskosten, prijspeil 1997 (EPA, 1997)) maakt duidelijk dat economische potentie van BioDeNOx op dit moment nog beperkt is.



Figuur 6

Invloed van de NO concentratie (C_{NOin}) bij een vaste O_2 concentratie (C_{O2in}) van 10 % (A) en de invloed van de O_2 concentratie bij een vaste NO concentratie van 250 ppm (B) op de selectiviteitscoefficiënt σ (■) en de NO flux $\overline{J}_{NO}$ (○). [Fe(II)EDTA^{2-}] = 10 mM, T = 323 K. Lijnen: alleen ter illustratie (Gambardella *et al.*, 2005b).

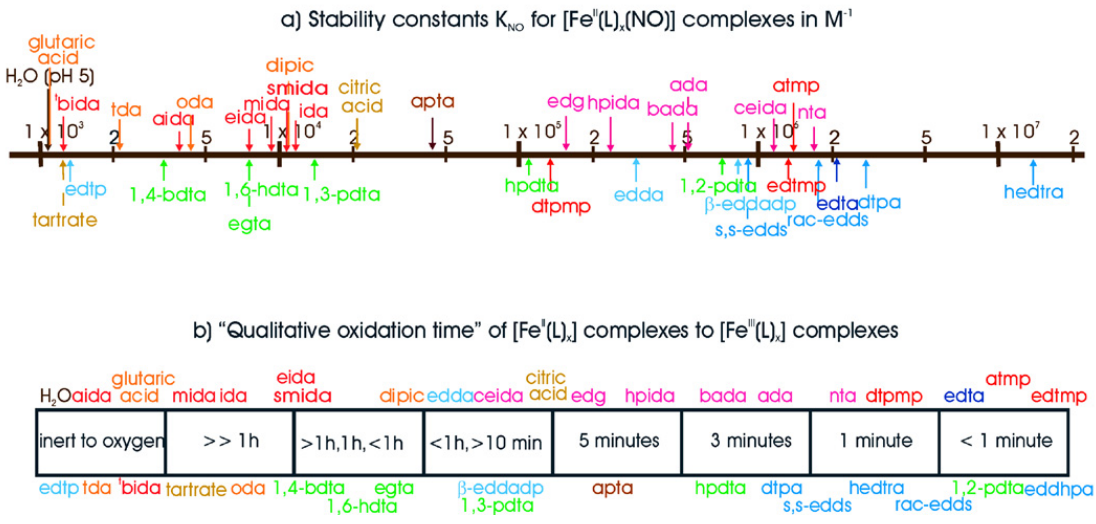
RICHTINGEN VOOR VERDERE ONTWIKKELING

In de bovenstaande context is het duidelijk dat verdere ontwikkeling van de BioDeNOx technologie primair gericht moet zijn op het verhogen van de selectiviteitscoëfficiënt voor NO absorptie. Dit zou kunnen worden bereikt door verlaging van de operationele temperatuur omdat zodoende de NO absorptiesnelheid wordt verhoogd hetgeen leidt tot een hogere NO flux van de gas- naar de vloeistoffase (Gambardella *et al.*, 2005b). Aan de andere kant resulteert temperatuursverlaging tot een lagere NO reductiesnelheid in de bioreactor (hoofdstuk 7), waardoor (bij gelijke biomassaconcentratie) grotere reactorvolumes nodig zijn.

Ook een goed ontwerp van de gaswasser kan de Fe(II)EDTA^{2-} oxidatie door zuurstof minimaliseren. Volgens een model ontwikkeld door Gambardella *et al.* (2005b) wordt NO absorptie bevoordeeld t.o.v.

Fe(II)EDTA^{2-} oxidatie, d.w.z. een hoge selectiviteitsconstante (σ) tot 1.0 bij een Fe(II)EDTA^{2-} concentratie van 100 mM (veel hoger dan in dit proefschrift), indien wordt gewerkt met hoge stofoverdrachtscoëfficiënten voor zowel de gas- als de vloeistoffase. Vanuit dit oogpunt lijkt een sproeitoren voor gaswassing een betere keuze dan een gepakte kolom (Gambardella *et al.*, 2005b).
Een alternatieve onderzoeksrichting is mogelijk de ontwikkeling van een ijzercomplex (Fe-L) dat, vergeleken met Fe(II)EDTA^{2-}, minder gevoelig is voor oxidatie, zonder concessies te doen aan de NO absorptie. Het zal niet makkelijk zijn om een dergelijke ligand te vinden, gezien het feit dat de O_2 gevoeligheid van verschillende Fe-L complexen een positieve relatie vertonen met de NO absorptiecapaciteit (Fig. 7). Echter, wanneer een dergelijke absorbent wordt gevonden houdt dat in dat:

1. Een hoge Fe(III)-L reductiesnelheid en dus de aanwezigheid van polysulfides / electronmediatoren in de BioDeNOx reactor minder belangrijk wordt.
2. Het gebruik van ethanol efficiënter wordt, d.w.z. dat ethanol voornamelijk wordt gebruikt voor NO reductie in plaats van Fe(III)-L reductie.
3. De ligand degradatiesnelheid hoogstwaarschijnlijk zal afnemen. Deze verwachting is gebaseerd op de waargenomen relatie tussen de Fe(II) oxidatiesnelheid en de afbraaksnelheid van de EDTA (Gambardella *et al.*, 2005a).



Figuur 7

Stabiliteitsconstanten van nitrosylcomplexen (a) en de zuurstofgevoeligheid (b) van verschillende Fe(II)-L systemen (Schneppensieper *et al.*, 2001).

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List of Abbreviations

AQDS	anthraquinone-2,6-disulfonate
DGGE	denaturing gradient gel electrophoresis
E^0	midpoint redox potential
EDTA	ethylenediaminetetraacetic acid
EM	electron mediator
ESRT	empty scrubber retention time
Fe(II)	ferrous iron
Fe(III)	ferric iron
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FISH	fluorescence in situ hybridization
HRT	hydraulic retention time
K_m	affinity constant
M	molar
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
N_2	dinitrogen
N_2O	nitrous oxide
NO	nitric oxide
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
NOR	nitric oxide reductase
N_2OR	nitrous oxide reductase
NTA	nitrilotriacetate
ORP	oxidation reduction potential
ORP_c	oxidation reduction potential corrected for pH 7.0
PCR	polymerase chain reaction
S^{2-}	monosulfide
S_n^{2-}	polysulfide
S^0	elemental sulfur
SCR	selective catalytic reduction
SNCR	selective noncatalytic reduction
SRT	sludge retention time
T	temperature
UASB	upflow anaerobic sludge blanket
VSS	volatile suspended solids
Y	biomass yield

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"Elke waarheid is alleen maar waarheid tot op zekere hoogte; komt zij aan haar grens dan komt er een contrapunt en wordt zij onwaarheid".

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Peter van der Maas
Oosterbeek, november 2005

Curriculum Vitae

Petrus Maria Franciscus van der Maas werd geboren te Oldenzaal op 15 oktober 1968. In 1987 behaalde hij zijn Atheneum diploma aan het Thijcollege te Oldenzaal, om vervolgens Milieukunde te studeren aan het Prof. H.C. van Hall Instituut te Groningen. In 1991 werd deze opleiding afgerond en in 1993 behaalde hij het doctoraaldiploma Milieuhygiëne aan de Landbouwuniversiteit Wageningen met als specialisatie Behandeling en hergebruik van afvalwater. Zijn afstudeeronderzoek in Wageningen werd uitgevoerd in samenwerking met Kiwa te Nieuwegein en was gewijd aan de wiskundige modellering van het Kalksteen Zwavel Denitrificatieproces.

In 1993 begon de auteur als zuiveringstechnoloog bij het Zuiveringsschap Drenthe te Assen. Vanaf 1997 is hij werkzaam bij Waterlaboratorium Noord (WLN) te Glimmen. In mei 2000 werd gestart met het promotie-onderzoek bij de sectie Milieutechnologie van Wageningen Universiteit, waarvan dit proefschrift het eindresultaat is. Sinds oktober 2004 werkt hij bij WLN als adviseur watertechnologie.

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