Eindrapportage-formulier TRIAS projecten
Final report format for TRIAS projects.

When a TRIAS project has finished, or is about to finish, a Final Report is required. This report serves several goals simultaneously:

- it enables the program commission to check whether the project has met its goals,
- it enables NWO-ALW to finalize the project administratively, e.g. pay the final part of the personnel costs of the project,
- it provides some of the information needed for evaluation purposes,
- it provides information which can be publicized, e.g. via a web site.

We have integrated the questionnaires from TRIAS and ALW into one, in order to prevent the need to fill in the same answers twice.

Please send in the filled out forms within a month after the project is completed to:

Netherlands Organisation for Scientific Research
Earth and Life Sciences
Carmen van Meerkerk and/or Theo Saat
P.O. Box 93510
2509 AM The Hague

Part I

General information, also intended for publication through the TRIAS website

01 Project Title.

Redox reactivity and bioavailability of iron oxyhydroxides in the subsurface

02 TRIAS/ALW project number or file number

835.80.004

03 Research period, at what date did the project start, at what date did it end.

Start: 1 January 2001
End: 1 March 2005 (end of contract last research (Bin Lin))

04 Names of the researchers involved, the names and addresses of the institutes where the research work was carried out.

Steeve Bonneville, Department of Earth Sciences-Geochemistry, Faulty of Geosciences, Utrecht University P.O. Box 80.021, 3508 TA Utrecht, The Netherlands.

Bin Lin, Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam The Netherlands.

Wilfred Röling, Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands.

05 Short scientific summary (500 words) in English of: main research objective, research methods, results and conclusion

The geochemical and ecological importance of enzymatically-mediated reduction of iron oxyhydroxides in subsurface systems is widely recognized, but little understood. The overall objective of this research project, executed at the University of Utrecht and the Vrije Universiteit Amsterdam, was to advance the mechanistic understanding of the geochemical, environmental and ecophysiological controls on the activity of Fe(III) reducing microbial populations in the subsurface. The major impacts and relevant outcomes for applications in soil protection and remediation are:

1. Present mathematical groundwater-models can be optimised by including the established kinetics of microbial iron reduction

2. The large redundancy in iron reducers observed suggest that upon pollution there is a large pool of iron reducers from which suitable (with respect to degradation) ironreducers are naturally selected

3. Some species indicative for the occurrence of biodegradation have been identified, these species can be monitored at other iron-reducing environments.

4. Insight in the functioning of the total system allows for alternative strategies in soil protection and bioremediation: e.g. the addition of bacteria-eating protozoa to enhance bacteria-mediated processes.

5. A rapid chemical extraction method for determining the bioavailability of iron for microbial reduction was developed

These results were obtained using an interdisciplinary approach combining experimental and theoretical methods from geochemistry and microbiology. We established the relationships between the bioavailability of natural iron oxyhydroxides and their chemical redox reactivity. We further determined the community composition and physiological abilities of iron-reducing communities in a set of iron-reducing environments. An integrating activity was the development of quantitative models capable of describing the rates of enzymatic reduction of iron oxyhydroxides in biogeochemical systems.

The community structure of iron reducing microbial populations is complex. Research at 3 locations revealed very different communities. In a landfill-leachate polluted aquifer *Geobacter* dominated and exhibited a high diversity in unpolluted parts of the aquifer. Pollution appeared to select for a subset of the *Geobacters*. Also in enrichments from this site *Geobacters* dominated, which allowed for the physiological characterization of the dominating bacteria. In contrast, in two sediments of the Scheldt estuary, no dominance by *Geobacter* was observed. Iron reducers belonging to *Shewanella* and other genera (e.g. *Alkaliphilus*, *Clostridium*, *Ralstonia*) were also encountered. Recovered consortia and isolates were very versatile with respect to pH and temperature ranges, and the use of electron acceptors. Detailed physiological characterization was performed for *Geobacter metallireducens.* Cultivation in retentostat showed very low maintenance energy demand and flexibility in electron acceptor use: cells growing slowly on humic acid analogs were able to use directly several iron oxides and nitrate. Initial rates of iron oxide reduction by G. metallireducens were very similar to those obtained with *S. putrefaciens.*

Many previous studies have attempted to clarify the kinetic controls on microbial iron reduction. However, no definitive mechanistic model of microbial iron reduction has yet emerged. Our approach has relied on careful characterization of the thermodynamic properties of synthetic Fe(III) oxyhydroxides and anaerobic incubations with the model microorganism *Shewanella putrefaciens*. We were able to demonstrate that microbial iron reduction follows the well-known Michaelis-Menten rate expression. Furthermore, we showed that the maximum reduction rate per cell is related to the Fe(III) oxyhydroxide solubility via a linear free energy relationship. Based on this result and sorption experiments of colloidal Fe(III) oxyhydroxide onto *S. putrefaciens*, we developed a conceptual model for microbial iron reduction kinetics accounting for the physical association of cell surface and iron particles. In addition to the study with pure synthetic Fe(III) oxyhydroxides, we successfully applied our kinetic approach to Fe(III) reduction in natural freshwater and brackish estuarine sediments (Scheldt estuary).

Experimental results were implemented in additional kinetic models, which account for the three different mechanisms to reduce iron (direct attachment, chelation, electron shuttling), competition for different iron oxides, inhibition of iron reduction by iron(II) sorption to iron(III)oxides and to cells, cell growth and mineral heterogeneity. Modeling results indicate that:

- The contribution of chelation to total iron reduction rate can be considerable, in contrast to electron-shuttling.
- Sequential use of iron oxides is correctly described using experimental data.
- The maintenance energy concept in microbial physiology provides an alternative explanation for the (partial) resistance of crystalline oxides to reduction.
- Predation can stimulate iron reduction under phosphate or nitrogen limiting conditions.

Ecological Control Analysis was applied in order to reveal the relative importance of different physiological groups in iron reduction. This knowledge can be applied in monitoring or remediation strategies. In contrast to currently hold opinions, control on a certain flux can be distributed over several groups, and control over intermediate concentrations is always shared. Most flux control resides with the fermenting bacteria, not with the iron reducing bacteria.

Overall, our research has significantly advanced insight in many aspects of iron-reduction. Our results and those of others reveal that iron reduction is more complex than initially assumed, indicating new research areas.

06 Popular summary to inform the general public (1/2 to 2 pages of text) **in Dutch.**
The funding organisations of TRIAS (SKB, NWO-ALW and Delft Cluster) want to inform a more general audience about the results of the TRIAS Research projects. That is why we ask you to give an executive summary of the project in a popularising way and written in the Dutch language.

Een onderzoeksproject, uitgevoerd aan de Universiteit Utrecht (Geochemie) en Vrije Universiteit (Moleculaire celfysiologie) heeft geleidt tot een sterk verbeterd inzicht in de gecombineerde geochemie en microbiologie van ijzer-reduktie, een belangrijk proces in (vervuilde) bodems en water. De resultaten zijn van belang voor bodembescherming en het evalueren van de natuurlijke afbraak van vervuiling:

1. Huidige grondwatermodellen kunnen worden verbeterd door het invoegen van de reactie kinetiek van microbiële ijzerreductie.
2. Inzicht in het functioneren van het gehele ecologische systeem kan leiden tot verrassende en nieuwe strategieën in bioremediatie, zoals het toevoegen van bacterie-etende organismen die, indirect, leiden tot een stimulatie van de bacteriële degradatieactiviteit.
3. De waargenomen grote verscheidenheid in micro-organismen betrokken bij ijzerreductie geeft aan dat er een groot reservoir van ijzer-reduceerders is waaruit

door natuurlijke processen, eenvoudig geschikte ijzer-reducerende microorganismen kunnen worden geselecteerd die de vervuiling te lijf gaan.

4. De aanwezigheid van sommige soorten van ijzerreduceerders correleert met afbraak, deze indicatieve soorten kunnen worden nu worden gedetecteerd op andere locaties.

5. Een snelle chemische extractie methode voor het bepalen van de beschikbaarheid van ijzer voor microbiële reductie is ontwikkeld.

Wat is ijzerreduktie en waarom is het belangrijk? Mensen hebben zuurstof nodig om te kunnen leven. Zuurstof functioneert als een elektronen acceptor, het neemt electronen op. Tijdens de vertering van voedsel, worden elektronen en protonen vrijgemaakt in de vorm van de redox transporter NADH. NADH op zijn beurt staat via een elektronentransport keten de elektronen af aan zuurstof. Tijdens dit transport wordt een chemische vorm van energie (ATP) geproduceerd. Zuurstof wordt hierbij gereduceerd tot water. Het ATP wordt gebruikt om allerlei fysiologische processen te laten plaatsvinden. Terwijl mensen slechts zuurstof als elektronenacceptor kunnen gebruiken, zijn micro-organismen in staat een enorme reeks van elektronenacceptoren te gebruiken.

Een van deze elektronenacceptoren is ijzer. Dat micro-organismen in staat zijn om ijzer als elektronenacceptor te gebruiken is nog niet lang bekend, maar het blijkt dat biologische ijzerreductie van groot belang is. Het was waarschijnlijk de elektronenacceptor die gebruikt wordt door de eerste organismen op aarde, miljarden jaren geleden. In veel huidige zuurstofloze omgevingen is ijzerreductie het dominante elektron-accepterende proces, doordat ijzer een veel-voorkomend element op aarde is. Ijzerreduktie treedt vooral op in vervuilde grondwater ecosystemen. In deze omgevingen verdwijnen de voor organismen voordeliger elektronenacceptors zuurstof en nitraat snel en worden niet makkelijk aangevuld op natuurlijke wijze.

Hoewel belang van biologische ijzerreductie in grondwater ecosystemen algemeen onderkend wordt, is er nog maar weinig wetenschappelijke kennis over. Het doel van deze studie was om de kennis over de factoren die de activiteit van ijzerreducerende micro-organismen in de ondergrond controleren, te verbeteren. Dit is gedaan door een combinatie van experimenten en wiskundige modellering. Hierbij hebben we de relaties vastgesteld tussen de beschikbaarheid van natuurlijke ijzervormen voor reductie door micro-organismen (ijzeroxides) en hun chemische redox reactiviteit. Ook hebben we bepaald voor een aantal omgevingen welke micro-organismen betrokken zijn bij ijzerreductie en hoe zij dit doen. Het geochemische en microbiologische onderzoek werd geïntegreerd door de ontwikkeling van kwantitatieve wiskundige modellen die in staat zijn de snelheid van biologische ijzerreductie te beschrijven.

Het bleek dat de samenstelling van de ijzerreducerende microbiële gemeenschappen complex was en sterk varieerde van locatie tot locatie. In grondwater dat vervuild was door een lekkende vuilstort werden vooral zgn. *Geobacter* soorten aangetroffen. In twee ondiepe ijzerreducerende omgevingen in de Schelde werden vele andere soorten aangetroffen. Dit werd vastgesteld met een aantal technieken. Allereerst met zeer snelle moleculaire ‘fingerprint’ technieken waarbij het niet nodig is om eerst micro-organismen te groeien. Daarnaast ook met de veel langzamere, traditionele kweek technieken. Deze kweken hadden als voordeel dat de gegroeide micro-organismen weer konden worden gebruikt voor aanvullende groei-testen die leidden tot een beter inzicht in de fysiologie van ijzerreduceerders. Binnen een locatie troffen we meerdere ijzerreducerende soorten aan, wat een indicatie is dat natuurlijke omgevingen een grote flexibiliteit hebben met respect tot het aanpassen aan en afbraak van vervuiling. De grote diversiteit vormt een reservoir waaruit de natuur makkelijk een micro-organisme kan selecteren die in staat is om de vervuiling af te breken onder ijzer-reduceerde condities. Met behulp van zeer ge-advanceerde groeitechnieken konden de ijzerreduceerders worden gegroeid onder condities die zeer sterk overeen komen met de condities welke ze ervaren in het echt; erg lage groeisnelheden en lage substraat concentraties. Dit leidde tot inzicht in hoe deze micro-organismen in het echt leven: ze spenderen slechts weinig energie aan cel-onderhoud en zijn in staat flexibel om te springen met beschikbare elektronen acceptoren.

Eerdere studies hadden zich al bezig gehouden met de kinetiek van biologische ijzerreduktie. Echter nog geen definitief mechanisch model van ijzerreductie was opgesteld. Onze aanpak richtte zich op de gedetailleerde karakterisering van de thermodynamische eigenschappen van synthetische ijzeroxidesen zuurstofloze incubatie met een model ijzerreduceerder, de bacterie *Shewanella putrefaciens*. We waren in staat aan te tonen dat biologische ijzerreductie zeer goed kan worden beschreven met de welbekende Michaelis-Menten snelheidsvergelijking. Daarnaast toonden we aan dat de maximale reductie snelheid is gerelateerd aan de ijzeroxide oplosbaarheid via een lineaire vrije energie relatie. Gebaseerd op dit resultaat en sorbtie experimenten van colloïdaal ijzeroxides op de bacterie, hebben we een conceptueel model ontwikkeld voor de kinetiek van microbiële ijzerreductie dat zich richt op de fysische associatie van het celoppervlak met ijzer deeltjes. Deze kinetiek kan nu worden ingebouwd in huidige wiskundige modellen van grondwater, om beter natuurlijke processen te beschrijven en te voorspellen.

De experimentele resultaten werden ook gebruikt voor andere kinetische modellen, die het gehele proces van ijzerreductie beschrijven (directe hechting aan ijzeroxides, het gebruik van oplosbaar ijzer en elektronen-shuttles, competitie voor elektronen door verschillende ijzeroxides, microbiële groei) maar ook de interactie van ijzer-reduceerders met andere micro-organismen. Een zeer opvallend resultaat was dat de consumptie van ijzer-reduceerders door predatoren, zoals protozoa, kan leiden tot een stimulatie van organische stof afbraak en de daaraan gekoppelde ijzerreduktie. Ook bleek dat hoewel uitsluitend bacteriën in staat zijn om vervuiling af te breken, en om ijzer te reduceren, niet uitsluitend zij de snelheid van deze processen bepalen. Dit is meer afhankelijk van de staat van het totale ecosysteem waarvan zij deel uit maken. Deze kennis kan worden toegepast in alternatieve bioremediatie-strategieën (bv. toevoeging van bv. bacterie-etende protozoa om bacteriële degradatie te stimuleren, het in de gaten houden van die factoren die daadwerkelijk de afbraak controleren)

Het onderzoek heeft aanzienlijk de kennis verbeterd op het gebied van ijzerreductie. Onze resultaten, en die van anderen, laten zien dat ijzer reduktie complexer is dan voorheen aangenomen en opent de weg voor aanvullend onderzoek.

07 What impact and relevance has this project's outcome for practicing soil protection and / or soil remediation? Again, please motivate.

In short the major impacts and relevant outcomes for applications in soil protection and remediation are:

1. A rapid chemical extraction method for determining the bioavailability of iron for microbial reduction was developed.
2. Present mathematical groundwater-models can be optimised by including the established kinetics of microbial iron reduction.
3. The large redundancy in iron reducers observed suggest that upon pollution there is a large pool of iron reducers from which suitable (with respect to degradation) ironreducers are naturally selected.
4. Some species indicative for the occurrence of biodegradation have been identified, these species can be monitored at other iron-reducing environments.
5. Insight in the functioning of the total system allows for (surprising) alternative strategies in soil protection and bioremediation: for example the addition of bacteria-eating protozoa in order to enhance the bacteria-mediated degradation of pollutants and the monitoring of those factors that really control the biodegradation (the process-controlling microorganisms are not necessarily the microorganisms that degrade the pollutants).

These impacts are explained below in more detail:

The reductive dissolution of Fe(III) oxyhydroxides is an important pathway for the degradation of mono-aromatic hydrocarbons, such as toluene, as well as other organic contaminants. Hence, quantitative insight into the reactivity of Fe(III) oxyhydroxides in subsurface environments, and insight in the microorganisms involved, is essential to estimate the potential for natural attenuation at contaminated sites and to design or optimise in situ (bio)remediation strategies. Furthermore, phosphate and certain toxic metals such as arsenic are preferentially associated with Fe(III) oxyhydroxides. Iron reduction can thus lead to eutrophication and contamination of ground and surface waters.

Knowledge of the kinetics of microbial iron reduction is essential to quantify the potential for bioremediation of polluted sites and also to estimate the time scale of natural attenuation. Our research on Fe(III) oxyhydroxide microbial reactivity is of great interest to address these issues. The approach derived from experimental work with *S. putrefaciens* was shown to be applicable for evaluating the *in situ* potential of microbial iron reduction in natural sediments. We further developed an innovative combination of microbial and chemical extraction (based on pH 7.5-buffered ascorbate solution), which allows us to determine the pool of Fe(III) oxyhydroxides available for microbial iron reduction in natural environments. Thus, in view of application, our research provided information on how fast individual Fe(III) oxyhydroxides are utilised over time and based on the type and content of the pool of iron oxyhydroxides in a certain environment, we are now able to estimate much better the potential and capacity of iron reduction

Our microbial ecological research indicates the presence of a large pool of iron-reducers in the 3 environments investigated, hence, confirming the widespread occurrence of this metabolic capacity in the subsurface. Furthermore, the presence of some *Geobacter* species correlates with biodegradation and monitoring of these species could be included in (the development of) monitoring strategies.

Mathematical modelling and Ecological Control Analysis provide a means to identify those physiological groups of microorganisms that control process rates or concentrations of intermediates. ECA also revealed that the activity of (micro)organisms not directly degrading or consuming a particular compound (e.g. bacterial predators), may influence the rate of degradation. Bacteria-predation can in fact stimulate bacteria-mediated degradation processes under suitable environmental conditions (nitrogen- and/or phosphate limitation). Thus, in soil remediation also these activities need to be considered. Approaches to enhance the activity of iron-reducing microorganisms such as injection of electron shuttles or chelators could be used to enhance *in situ* bioremediation, as well as the addition of bacteria-predators. The experimental data and developed models can assist in the prediction of the conditions under which these approaches could be successfully applied.

08 Please list the presentations held in connection to this project

oral presentations (only co-researchers of this project mentioned as co-authors):

S. Bonneville, Microbial Fe^{III} reduction: effect of solubility. i11th International Symposium on Water-Rock Interaction, New-York, USA, June 2004.

S. Bonneville, Thermodynamic constraints on microbial Fe(III) oxide reduction. EGS-AGU-EUG Joint Assembly, Nice, France, April 2003.

S.Bonneville, Redox reactivity of Fe(III) oxides.Geochemische Kring Symposium, Utrecht, The Netherlands, October 2003.

S Bonneville Redox reactivity and bioavailability of iron oxyhydroxides in the subsurface. 1st National Scientific Symposium BodemDiep, Zeist, The Netherlands June 2002.

B. Lin. *Geobacter* diversity and its relationships to hydrochemistry in an iron-reducing landfill leachate-polluted aquifer. June, 2004. National symposium, Soil & Water. The Netherlands. (with S. Bonneville, W.F.M. Röling).

W.F.M. Röling. Forzungscentrum Jülich, Germany. December 2002 (invited seminar)

W.F.M. Röling. Bodemdiep 2003, Woudschoten, NL. June 2003 (with S. Bonneville, B. Lin)

W.F.M. Röling. International Conference on Environmental and Urban Management, Semarang, Indonesia. August 2003.

W.F.M. Röling. Satya Wacana Christian University, Salatiga, Indonesia. August 2003 (invited seminar).

W.F.M. Röling. Centre for Ecology and Hydrology, Oxford, UK. May 2004 (invited seminar)

W.F.M. Röling. Soil and Water, Woudschoten, NL. June 2004. (with S. Bonneville, B. Lin)

W.F.M. Röling. International Symposium on Microbial Ecology 10, Cancun, Mexico. August 2004.

W.F.M. Röling. UNESCO Workshop, München, Germany. December 2004 (invited speaker, with B. Lin).

P. Van Cappellen. Incorporating geomicrobial processes in subsurface reactive transport models. US Federal Interagency Workshop on Conceptual Model Development for Subsurface Reactive Transport Modeling in Inorganic Contaminants, Radionuclides and Nutrients, Albuquerque, New Mexico, April 2004 (invited speaker).

P. Van Cappellen. Reactive Transport Modeling of Biogeochemical Systems. COST Action 629. Workshop on Saturated and Unsaturated Zone. Rome, May 2004 (invited speaker).

Miscellaneous:
W.F.M. Röling: co-author of 5 other oral presentations, relating to this TRIAS project and the resilience-TRIAS project.

This list does not include oral presentations hold within the departments (B. Lin, 5 W.F.M. Röling 5) or during the half-yearly TRIAS meetings (B. Lin 6, S. Bonneville 6, W.F.M. Röling, 5)

Poster presentations:

1. Bin Lin, Martin Braster, Boris van Breukelen, Henk van Verseveld, Hans Westerhoff, Wilfred F.M. Röling. *Geobacter* diversity and its relationships to hydrochemistry in an iron-reducing landfill leachate-polluted aquifer. June 2004, poster in the 4th GW international conference (July 19th- 22nd, 2004) in Waterloo University, Canada

2. Bin Lin, Wilfred F.M. Roling, Martin Braster, Bonneville Steeve, Philippe Van Cappellen, Henk Van Verseveld. Redox Reactivity and Bioavailability of Iron Oxyhydroxides in the subsurface: Structure and activity of microbial iron-reducing communities. June 2003. Bodemdiep,The Netherlands.

3. Bonneville S., Behrends T., Van Cappellen P. Microbial iron(III) oxide reduction: Effect of solubility. Workshop on "Biogeochemical Processes involving Iron Minerals in Natural Waters", November 2003, Ascona, Switzerland.

4. Bonneville S., Behrends T, Van Cappellen P. Microbial reduction of colloidal iron oxide. In: Zesde Nederlands Aardwentenschappelijk Congres, Veldhoven, The Netherlands April 2002.

5. Bonneville S., Behrends T., Van Cappellen P. Thermodynamic contraints on microbial iron reduction. In: NSG symposium, Amsterdam, December 2004.

W.F.M. Röling. In total 10 poster presentations (3 first authored).

09 Please list publications (published and submitted) in connection to this project. Please indicate publication took place in either a refereed journal, a non-refereed journal (incl. conference proceedings); whether it was published as a chapter of a book, as a monography or as a dissertation.

Refereed journals

S. Bonneville, P. Van Cappellen, T. Behrends. Microbial iron reduction of iron(III) oxyhydroxides: effect of mineral solubility and availability. Chemical Geology, in press (2004).

S. Bonneville, T. Behrends, P. Van Cappellen. Microbial Fe^{III} reduction : effect of solubility. In: Wanty, R. B., Seal II, R. R. (Eds.), Water-Rock Interaction, A. A. Balkema publishers, London, pp. 1173-1177.

C. Hyacinthe and P. Van Cappellen. An authigenic iron phosphate phase in estuarine sediments: composition, formation and chemical reactivity. Marine Chemistry, **91**, 227-251 (2004).

S. Bonneville, T. Behrends, C. Hyacinthe, P. Van Cappellen. Microbial reduction of Fe(III) colloids: a kinetic model. Environmental Science and Technology, to be submitted shortly.

S. Bonneville, T. Behrends, C. Hyacinthe, P. Van Cappellen. Linear free energy relationship for microbial iron reduction. Geochimica et Cosmochimica Acta, to be submitted shortly.

C. Hyacinthe, S. Bonneville and P. Van Cappellen. Reactive iron in sediments: chemical versus microbial extractions. Geochimica Cosmochimica Acta, to be submitted shortly.

C. Hyacinthe, S. Bonneville, P. Chevrier, P. Van Cappellen. Effect of adsorbed Fe(II) on initial rates of microbial Fe(III) reduction. In preparation.

W.F.M. Röling and H.W. van Verseveld. Natural Attenuation: What does the subsurface have in store? Biodegradation, **13**, 53-64 (2002).

van Breukelen, B.M., Röling, W.F.M., J. Groen, J. Griffioen and van Verseveld, H.W. Biogeochemistry and isotope chemistry of a landfill leachate plume (Banisveld landfill, the Netherlands). Journal of Contaminant Hydrology, **65**, 245-268 (2003).

van Breukelen, B.M., Griffioen, J., Röling, W.F.M. and van Verseveld, H.W. Reactive modeling of biogeochemical processes inside a landfill leachate plume. Journal of Contaminant Hydrology, **70**, 249-269 (2004).

Bin Lin, M. Braster, B.M. van Breukelen, H.W. van Verseveld, H.V. Westerhoff, W.F.M. Röling. *Geobacteraceae* community composition relates to hydrochemistry and biodegradation in an iron-reducing landfill leachate-polluted aquifer, to be submitted shortly.

W.F.M. Röling, B.M. van Breukelen, H.W. van Verseveld, H.V. Westerhoff. Ecological Control Analysis: Being(s) in control of substrate flux and metabolite concentrations in anaerobic syntrophic degradation processes, To be submitted shortly to Environmental Science and Technology.

W.F.M.Röling et al.: Interactions between iron-reducers and their environment: a modelling approach, in preparation.

W.F.M. Röling: Stimulation of degradation processes by protozoan predation, in preparation.

Bin Lin, M. Braster, H.V. Westerhoff, W.F.M. Röling. Physiology of iron reducer *Geobacter metallireducens* grown on humic acid analog-AQDS/acetate limited retention cultivation, in preparation

Bin Lin, S. Bonneville, C. Hyacinthe, M. Braster, H.V. Westerhoff, W.F.M. Röling. Biological contributions of iron-reducing microorganisms to iron reduction in estuarine sediments. In preparation

Non-refereed (only first-authored papers mentioned):

Bin Lin, Wilfred F.M. Röling, Martin Braster, Bonneville Steeve, Philippe Van Cappellen, Henk Van Verseveld. Redox Reactivity and Bioavailability of iron oxyhrdroxides in the subsurface: structure and activity of microbial iron-reducing communities. 2nd National Scientific Soil Symposium, 2003.

Bin Lin, Martin Braster, Bonneville Steeve, Boris M. van Breukelen, Hans Westerhoff, Wilfred F.M. Röling. Structure and activity of microbial iron-reducing communities in a landfill laeachate-polluted aquifer and iron bogs. Netherlands Scientific Symposium, Soil & Water, 2004.

Bin Lin,Martin Braster, Boris van Breukelen, Henk van Verseveld, Hans Westerhoff, Wilfred F.M. Röling. "*Geobacter* diversity and its relationships to hydrochemistry in an iron-reducing landfill leachate-polluted aquifer" 2004, 4th ground water international conference, Canada.

W.F.M. Röling et al. Redox reactivity and bioavailability of iron oxyhydroxides in the subsurface: modelling iron respiration in anaerobic environments. BodemDiep proceedings (June 2003). P. 37-40.

W.F.M. Röling et al. Interaction and communication between cells and their environment in a landfill-leachate polluted Dutch aquifer. ICEUM Proceedings, Semarang, Indonesia (August 2003), CD.

W.F.M. Röling et al. Interactions between iron-reducers and their biotic and abiotic environment: a modelling approach. Proceedings of the Netherlands Scientific Symposium Soil and Water (June 2004). P. 51.

S.Bonneville, T.Behrends, R.Haese, P.Van Cappellen Thermodynamic constraints on microbial Fe(III) oxide reduction, in: EGS-AGU-EUG Joint Assembly, Nice, April 2003.

10 Please list Patent applications or other professional products (including contracts, articles in the popular media, contributions to documentaries or scientific television or radio programs, CD-ROMS, DVD or other (electronic) media).

none

TRIAS

Part II

Detailed information, primarily intended for administrative and statistical use by NWO-ALW

11a Under item 5 you have filled in the main research objectives. Please list all the original research objectives as indicated in the project's application and both indicate as well as motivate, to what extent these goals were realised, and/or whether the original research objectives had to be adapted.

Here, the detailed objectives are given. As question 12 appears to overlap with the second part of this question (realisation of objectives), the realisation or matching of results to objective is given only under question 12.

The general research objective of the project was to advance the mechanistic understanding of the geochemical, environmental and ecophysiological controls on the activity of Fe(III) reducing microbial populations in the subsurface. More specifically, it was proposed to:

(1) quantitatively establish the relationships between the bioavailability of iron oxyhydroxides and their mineralogical, physical and chemical properties (project 1, S. Bonneville).
   a. Assess the microbial reactivity of synthetic Fe(III) oxyhydroxide, sand artificially coated with ironoxide and Fe(III) rich sedimentary assemblages using *Shewanella putrefaciens*.
   b. Investigate the inhibition effect of Fe(II) adsorption to Fe(III) oxyhydroxide on microbial iron reduction.
   c. Compare microbial reactivity of Fe(III) oxyhydroxides with the abiotic reactivity using H_2 as reductant.

(2) determine the relative importance of Fe(III) bioavailability and metabolic activity levels on the kinetics of dissimilatory Fe(III) reduction (project 2, B. Lin)
   a. Determine the enzymatic Fe(III) reduction by natural microbial consortia of iron reducers obtained from field samples, using the same Fe(III) substrates as in Project 1
   b. Characterize the community structure of the natural consortia as well as the response of the community structure to changes in the nature and abundance of iron oxyhydroxides and organic matter availability.

(3) develop a comprehensive model for microbial Fe(III) reduction in subsurface environments (Project 3, W.F.M. Röling).
   a. Develop a quantitative framework in which the experimental results of the entire study are integrated. In addition to explicitly representing Fe(III) bioavailability and microbial community structure, the model accounts for the nature and abundance of organic (energy) substrates, and competition by alternative pathways for organic carbon degradation, in particular, sulfate reduction and methanogenesis. Thus, the model provides a tool to

predict the rate of enzymatic Fe(III) reduction under conditions representative of soils, sediments and aquifers.

b. Development of Ecological Control Analysis (ECA) and application to the models mentioned above, but also models that include competing processes, such as sulfate reduction.
c. Use the ECA models to further test the generality (or lack thereof) of simplified (empirical) kinetic expressions used in multicomponent reactive transport models of redox-stratified environments.

11b Did the project also include objectives which were not scientific? For instance, did the project also intend to apply research results, or strengthen the economic position of certain businesses?

No

11c Did the project's aims include the expanding the (international) network of contacts (at what level), providing education, improve communication, serve as input for policy drafting or policy decisions, etc.? Please motivate.

No

12 Do the results obtained match the original objectives?
Please provide a short motivation why they do or don't.

The matching is indicated per project as mentioned under 11a.

Project 1 (S. Bonneville)

The overall goal of determining the relationship between bioavailability of Fe(III) oxyhydroxides and their physical, chemical and morphological properties was achieved. In particular the microbial iron reduction rates were related to the thermodynamic solubilities of the mineral phases.

Ad 1a. The microbial reactivity of ten different Fe(III) oxyhydroxides, 2 ferric phosphates, and two Fe(III)-rich natural sediments (Scheldt estuary) have been characterized in batch experiments using *S. putrefaciens*. Sand coated with Fe(III) oxyhydroxide has been prepared and will be used in the near future.

Ad 1b. The inhibitory effect of Fe(II) adsorbed on Fe(III) mineral surfaces has been investigated for ferric phosphates and ferrihydrite. Thermodynamic constraints on microbial iron reduction due to the accumulation of Fe^{2+} in the system have also been investigated.

In terms of characterization of the various Fe(III) oxyhydroxide, our efforts have focused on the determination of the solubility of the Fe(III) solids, which appears to be a key factor in controlling the Fe(III) reduction rate by *S. putrefaciens*. In addition, we analysed specific surface area, morphology and grain size (TEM and SEM imaging), cristallinity and purity (XRD, pe-pH titrations and Mössbauer spectroscopy).

The abiotic reductive dissolution of Fe(III) oxyhydroxide by H_2(g) was found to be very slow and not suitable for this study. Therefore, we developed a new chemical extraction by ascorbate at pH 7.5. This method calibrated with Fe(III) oxyhydroxides and applied to natural sediments has been showed to specifically extract the bioavailable pool of Fe(III) from the sediments. This method will be of great value for application in soil protection and remediation strategies.

Project 2 (B. Lin).

Ad a. The Physiology, phylogenetics and community of iron-reducing bacteria population in three iron-reducing environments were determined by culture-dependent and independent approaches. 16S rRNA gene based techniques played an important role in the latter part of the work. The response of communities to different iron oxides was determined in culturing-dependent enrichments. Metabolic versatility was tested in batch cultures. A surprising large biodiversity in iron-reducers was observed, which is of importance with respect to resilience to pollution.

Ad b. Kinetic experiments on a limited number of isolated consortia were performed in batch and continuous reactors (retentostats). Due to slow growth of the isolates and enrichments, characterisation was mainly qualitative. Furthermore, long-term cultivation under environmentally relevant conditions (low growth rates in retentostats) was hampered by blockage of filters by iron precipitates and abiotic changes affecting the iron oxides. To compensate for this the physiology of *Geobacter metallireducens*, a model strain corresponding to dominantly occurring iron reducers, was compared in detail to another model strain, *Shewanella putrefaciens*, by growing it on the well-soluble humic acid analog AQDS. Energetic requirements for cell maintenance are very low, and under slow-growth conditions the microorganisms expressed a large flexibility towards the use of electron-acceptors.

Project 3 (W.F.M. Röling).

Due to the sudden death of Dr. Henk van Verseveld (VUA projectleader of projects 2 and 3), Dr. W.F.M. Röling has taken over most of his duties, including teaching duties to undergraduates and supervision of Drs. B. Lin and three other PhD students (including Traian Brad, PhD student in the resilience-TRIAS project). This left less time for modelling and objective c was not yet achieved, but as Dr. Röling currently holds an assistant professorship at VUA and is involved in a BSIK-funded ecogenomics programme where he is responsible for further developing Ecological Control Analysis, the objectives of this project will be achieved in the future. Iron reduction models and Ecological Control Analysis were developed and tested by using literature data and results from projects 1 and 2. parameterisation was often difficult since data on iron reduction are still limited despite considerable progress obtained in this project as well by other researchers since 2000. It was observed that the models are very suitable to determine directions for further research (e.g. iron oxide mineral heterogeneity, kinetics of chelation, the kinetic formalism representing microbial activity) and which are required to yield more precise data. ECA also proved useful to describe other environmental processes and determine the controlling steps. This was done for the nitrogen-cycle.

13 Will the results of this project serve as input for an initiative integrating/and or generalizing input from several projects, for instance into a (numerical) model, or into more understanding at the higher/system level? If so, was this intended and optimised from the beginning or did it occur by chance/ spontaneous? Please elaborate.

The following results and approaches serve(d) as input for integrating several projects:

1. Derived kinetics laws for iron reduction can now be incorporated in general reactive transport models, and related to the kinetics of other metals (As, U(VI)).
2. Quantitative insight in iron oxide reactivity and microorganisms involved is essential to estimate NA potential, and potential negative effects (heavy metal and phosphate release).
3. Control analysis can be applied to determine those groups of microorganisms or processes that control the rate of a certain process, providing monitoring and remediation tools. Ecological Control analysis is a major subject of a large project (Ecogenomics) that recently started.
4. Co-sampling on Banisveld with another TRIAS project, in order to get an in-dept characterisation of the microbial processes.

In more detail (some bullet-headings are explained in next sections, in order to avoid repetition): The goal of this project was to provide kinetic laws for microbial iron reduction. The study of the microbial reduction of Fe(III) oxyhydroxides is closely related to ongoing research on iron biominerals (magnetite) and remediation of (radionuclide) U(VI) and As (T. Behrends, UU). In a broader context, these studies are integrated in the research thrust on redox stratified environments, which is a core activity of the biogeochemistry group at UU. Especially, the rate model for Fe(III) reduction developed in this study is being incorporated in a general reactive transport model for early diagenetic processes (the so-called Biogeochemical Reaction Network Simulator or BRNS).

Integration with other projects has been achieved from the start, especially with another TRIAS project (845.080.007, Resilience of the groundwater ecosystem in reaction to anthropogenic disturbances). The resilience project also deals with one of the research sites of this project, the Banisveld landfill leachate polluted aquifer, Boxtel. Field sampling has occurred simultaneously, and PhD students of the two projects have worked on the same samples, in order to get very detailed and integrative information on microbial communities and activities. Sabrina Botton (UvA) works on BTEX degradation under iron-reducing conditions, work that closely relates to that of Bin Lin. Traian Brad (VUA) works on eukaryotic communities, with emphasis on predation. As combination between both projects Wilfred Röling has modelled the effects of predation on bacteria-mediated degradation processes.

Ecological Control Analysis is a tool that is very suitable to achieve understanding at the higher/system level; this was kept in mind from the start of the project. Recently a BSIK-funded Ecogenomics programme has started. Ecological Control Analysis is an integral part of this programme and the tools developed in this TRIAS project will be applied and further extended in this new programme. Wilfred Röling will work on this subject. Another part of the Ecogenomics program concerns bioremediation of the subsurface, allowing the generability of the data on microbial community structure collected by Bin Lin on the Banisveld landfill leachate polluted aquifer to be tested at other sites.

14 To what extent has this research project pointed the way in which further research has to be undertaken? Please motivate the guiding role perceived.

This research project has pointed the way in which further research has to be undertaken to answer the following questions:

1. what is the nature of the solubility control on the iron reduction rate?
2. in how far can the results on iron reduction by *Shewanella* be generalised to other iron-reducers, especially the iron reducers that dominate in iron-reducing environments?
3. which cell-cell and cell-environment interactions in ecosystems control its qualitative and quantitative functioning, and how?

The knowledge acquired in this project is crucial for the process-based understanding of the redox reactivity and bioavailability of iron phases in natural environments. Three major outcomes can be identified: (i) Fe(III) oxyhydroxide solubility controls the maximum specific reduction rate by *S. putrefaciens*, (ii) microbial iron reduction can be described by a Michaelis-Menten rate expression, (iii) a kinetic model links microbial iron reduction to the extent of oxide-cell association.

These results also raise new questions, in particular concerning the nature of the solubility control on the reduction rate. In this respect, more detailed studies of the rate of electron transfer from membrane-bound reductase to the surface of the Fe(III) oxyhydroxide are greatly needed. In addition, the fate of Fe(II) from its formation at the surface of Fe(III) minerals to the its ultimate release in solution needs to be further characterized. Further studies exploring the (bio)chemical nature of the association between Fe(III) minerals and microbes are also needed.
From a broader point of view, the results obtained in batch experiments with *S. putrefaciens* as model organism need to be extended to iron reducing communities, Fe(III) mineral assemblages and electron donors found in natural environments.

To understand ecological processes, the system as a whole (community structure, activities of individual members and interactions between the different members, as well as with their environment, the chemical and physical structure of the environment) should be studied, not just its parts, or just those parts which are now considered important (e.g. the pollutant degrading microorganisms in biodegradation). Ecological Control Analysis and modelling led to this conclusion. The research indicated that not only the activity of contaminant-degrading (micro)organisms should be considered in natural attenuation and biodegradation, but also the microorganisms that affect the activities of these microorganisms (e.g. predators, competitors, electron-accepting microorganisms).

Finally, there is still a lack of suitable kinetic parameters for several important pathways in iron reduction (e.g. chelation). Iron reduction research has so far been mainly performed under idealised situations (controlled batch incubations of limited complexity). Environments are very complex, and this complexity needs to be addressed in future experiments, as also indicated by some of the modelling results

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15 In what way, and to what extent, are the results reached of importance to research done by others? Please motivate or elaborate.

See also previous answer. Systems complexity needs to be addressed. Modelling and ECA are important aspects of this. ECA is also suitable to determine what rate 'limiting' steps control environmental processes.

There is disagreement in the scientific community about which type of iron-reducing microorganisms are dominating/important (*Geobacter* vs *Shewanella*). The results actually indicate that the composition of the iron-reducing community is environment-dependent, and since different mechanisms of iron reduction are employed, this is expected to affect iron reduction capacities.

The outcomes of the project are of great interest for the entire field of microbial ecology, geomicrobiology and biogeochemistry (see question 5). A general kinetic expression, the Michaelis-Menten expression, has been shown to describe microbial iron reduction. This formalism has been used in biogeochemical reactive transport models, however our study is the first to provide direct experimental proof. Further, a linear free energy relationship between maximum reduction rate per cell and mineral solubility has been established, hence rationalizing the large differences in Fe(III) reduction rate in nature. More generally, the kinetic approach and analysis developed can be extended to other microbe-mineral reactive systems. These include, for instance, dissimilatory reduction of manganese oxyhydroxides, reductive precipitation of U(VI) and As and oxidative precipitation of Fe and Mn (hydr-)oxides. These processes play important roles in the cycles of carbon, nutrients and metals.

16 Are you aware of any essential gaps or obstacles standing in the way of applying the results from your research project? Please elaborate.

The goal of the project was to quantitatively describe the bioavailability of Fe(III) oxyhydroxides. Microbial iron reduction also requires suitable electron donor, e.g. organic carbon, which, in our experiments, was always lactate. In soil and sediments, the quality and the availability of organic matter are highly variable and they may exert a dominant influence on the iron reduction kinetic as indicated by the modelling results.

The quantitative application of kinetic models is still limited by the lack of critical parameter values. Furthermore, the exact nature of the interactions between microorganism and their biotic (other species) and abiotic environment are not well known. The modelling has however indicated which parameters/processes should be included and which not.

Part II - continued

Detailed information, primarily intended for administrative and statistical use by NWO-ALW

17 Which new research questions were generated through this project? Were these new questions addressed within this research project itself? Or will these new questions, or the results from your research project lead to new research projects (to be) funded by either 1st , 2nd,or 3rd category funding or funding through international funding agencies? Please elaborate.

See also the answer to question 14.
Research on iron reduction is still at an early stage. There are a couple of outstanding issues:

1. further characterization of microorganisms capable of iron reduction,
2. the relationship between different physiological types of microorganism involved in the iron reduction process (how do they use the iron oxides),
3. what factors ultimately control the reduction process,
4. the relationship between biotic and abiotic reactions which may contribute to the cycling of the electron acceptor and donor.

Our kinetic study of the chemical and microbial reactivity of Fe(III) oxyhydroxides raises the interesting possibility of a close coupling between microbial iron reduction and iron oxidation. Microbially precipitated Fe(III) oxyhydroxides may form preferential substrates for iron reducing bacteria. A new study of the oxidative precipitation of iron will start in the near future at Utrecht University. This study, which will built on the results of the TRIAS project, is funded through the EU-FP6 Integrated project FUNMIG. Another study on the cycling of iron in wetland soils will also begin shortly. It will be performed jointly by the biogeochemistry group of UU and the microbial ecology group of NIOO (Laanbroek). It is funded through the Center of Wetland Ecology (CWE), a consortium of Dutch universities and the NIOO.

Modelling results indicated that predation under some circumstances could give rise to enhanced degradation, compared to situations without predation. This question will be further address in another TRIAS project, which is not yet finished (resilience project 007).

The need for more precise determination of *in situ* reduction rates and which iron oxides are degraded was identified. Advances in measuring iron-isotopes have opened ways to use isotopic fractionation as a tool for specifically measuring iron reduction *in situ* and in microcosms. A joint research proposal (Röling, Van Cappellen) has been prepared for funding within the new Darwin Biogeology centre (main applicant is Dr. Vroon, VUA).

18 In what way did you link this project to other projects within the TRIAS-program or link it to projects outside TRIAS? Did you cooperate within the TRIAS-program and did this cooperation lead to integrated results?

See also answer to question 12,13. We cooperated with the resilience TRIAS project (007; Brad, Botton) and this is leading to integrated results and co-authored manuscripts (Röling, Lin) are in preparation. Furthermore, at the VUA two projects, performed in Indonesia, are ongoing, which deal with landfill leachate pollution and associated redox processes, such as iron reduction. The Ecological Control Analysis research will be continued in the BSIK-founded Ecogenomics programme, while monitoring of activity of iron reducing microorganisms will also part of this programme.

The project is of direct relevance to a number of interrelated TRIAS programme themes (underlined). The results of the research aimed to quantify the redox reactivity and bioavailability of iron oxyhydroxides. The latter represent important reactive interfaces, which affect the redox cycles and mobility of many inorganic and organic constituents. Iron oxyhydroxides also buffer the composition of soil and ground waters, via sorption and dissolution-precipitation reactions, and they enhance the natural attenuation of certain organic contaminants. Because iron oxyhydroxides play a central role in the biogeochemical reaction networks and microbial ecology of subsurface environments, the research also fitted in the TRIAS research area 'Ecology and Soil Quality'.

19 Can you elaborate on the impact on society as a whole of your results (e.g. societal organisations, NGO's, businesses, schools, municipal authorities, etc.)

Several potential applications for soil protection and remediation, of use for problem owners, have already be mentioned above (e.g. in the general summary and in the Dutch Summary), and are discussed there in more detail:

1. A rapid chemical extraction method for determining the bioavailability of iron for microbial reduction was developed.
2. Present mathematical groundwater-models can be optimised by including the established kinetics of microbial iron reduction.
3. The large redundancy in iron reducers observed suggest that upon pollution there is a large pool of iron reducers from which suitable (with respect to degradation) ironreducers are naturally selected.
4. Some species indicative for the occurrence of biodegradation have been identified, these species can be monitored at other iron-reducing environments.
5. Insight in the functioning of the total system allows for (surprising) alternative strategies in soil protection and bioremediation: for example the addition of bacteria-eating protozoa in order to enhance the bacteria-mediated degradation of pollutants and the monitoring of those factors that really control the biodegradation

(the process-controlling microorganisms are not necessarily the microorganisms that degrade the pollutants).

In terms of world-wide economic impact, the presence of high concentrations of Fe(II) in groundwaters is the most significant water quality issue. It is a common problem encountered in Dutch aquifers that are utilized for drinking water winning. The development of tools to quantify the oxidation capacity and bioavailability of Fe(III) oxyhydroxides is also relevant to the assessment of the environmental impact of flood control options that are currently under consideration in The Netherlands. The latter include the storage of excess water in floodplains and polders. Flooding of unsaturated soils with river water has the potential to mobilize large amounts of iron and associated trace elements.

Furthermore, it has been found that iron-reducing bacteria are very useful and efficient in the generation of electricity, by their binding to cathodes and transfer of electrons to the electrode in bioelectricity-cells. Bioelectricity cells may offer an alternative for fossil fuels in the future (see also point 20), and electrodes to which organic matter-degrading iron-reducers are bound can be used in bioremediation.

The last two issues also require an interdisciplinary approach, like was used in this research project.

20 What actions were taken to disseminate the results in the direction of the general public, besides the usual scientific channels?

Data and results of the project have been made available via a public webpage: http://www.bio.vu.nl/geomicrob/TRIAS/
As a spin-off of this project, VUA was contacted by two students who won a price to include their experiment on a upcoming the Soljoez mission this April (with Andre Kuipers). Their experiment consists of a bioelectricity-cell (electricity generation by cathode-reducing bacteria, such as iron-reducing bacteria like Geobacter). This experiment received major attention in the press. See also http://www.phys.uu.nl/~bugnrg/

21 Have the researchers involved obtained a new position or employment after the project came to an end? Please specify and elaborate!

Steeve is currently looking for a suitable postdoctoral position in the general area of geomicrobiology. His research is being well received and should permit him to find a position in the near future.
Bin Lin has not found a new position yet. Hopes to find a research fellow position in a university or research institute or biotech/environmental relevant company to continue his academic career with the techniques and knowledge obtained during the involvement in this TRIAS project.
Wilfred Röling holds presently an assistant professorship at the Vrije Universiteit Amsterdam. He teaches geomicrobiology and continues the research started in this TRIAS project as well in the resilience TRIAS project. His focus is on further developing Ecological

Control Analysis and developing monitoring tools for degradation of pollution and associated processes.