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 NWO-ALW to finalize the project administratively, e.g. pay the final part of the personnel costs of the project,
- it enables the program commission to check whether the project has met its goals,
- 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

Resilience of the groundwater ecosystem in reaction to anthropogenic disturbances

02 TRIAS/ALW project number or file number

835.80.007

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

October 1, 2001 – October 15, 2005

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

(Note: the names of the PhD students are given here, with indicated people involved at their respective universities)

Traian Brad, Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam The Netherlands.
Also involved: Martin Braster (technician), Boris van Breukelen (advisor hydrogeochemistry), Henk van Verseveld (supervisor till his unfortunate death on 11 July 2003), Wilfred Röling (supervisor), Nico van Straalen (promoter/supervisor/projectleader; Animal Ecology)

Meta van Heusden, Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands.
Also involved: Anton Akkermans (supervisor till December 2002), Willem de Vos (promoter), Hauke Smidt (supervisor).

Sabrina Botton, Earth Surface Processes and Materials, IBED, University of Amsterdam, Nieuwe Achtergracht 166 1018 WV Amsterdam, The Netherlands.
Also involved: John Parsons (supervisor).

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

The quality of groundwater is threatened by the presence of chemicals in, for example, leachates from landfills. Groundwater ecosystems are, however, able to recover from chemical pollution by removing the chemicals by microbial degradation. This self-purifying process is often referred to as natural attenuation and may be monitored and stimulated in order to remediate contaminated sites. The overall objective of this project, executed at the Vrije Universiteit Amsterdam, University of Amsterdam and Wageningen University, was to identify the environmental, biological and chemical prerequisites for biodegradation by groundwater microorganisms (in concert with the existing fauna). The aim was to develop, together with the results of *in situ* degradation potential measurements, an integrated geochemical, ecological and environmental chemical toolbox for the evaluation of the natural attenuation capacity of polluted subsurface environments. Elements of this toolbox, developed in this programme, are listed below:
Opmerking: de navolgende issues sluiten naar formulering niet goed aan bij deze inleidende zin, met name issues 4 en 5 zijn meer beschouwend dan stellend; met het oog op de leesbaarheid graag aanpassen!

1. Evidence for *in situ* anaerobic BTEX degradation in different polluted aquifers, based on measurements in microcosms and on groundwater samples (metabolites, isotope fractionation of BTEX compounds)
2. Identification of …Specific populations (e.g *Geobacter* spp., *Dehalococcoides* spp.) indicative for the occurrence of biodegradation; these species or the genes involved in pollutant degradation can be monitored at other locations (see point 3).
3. A molecular method to quantify the abundance, as well as the metabolic potential and activity of populations involved in biodegradation of chlorinated hydrocarbons using functional markers relating to halorespiration. This allows us to study bioremediation processes in-situ and thus gain knowledge on what are the essential factors of influence
4. Mathematical models, combined with Ecological Control Analysis, can be applied to *suggest* ? alternative strategies in soil protection and bioremediation: for example the (theoretical) potential of bacteria-eating protozoa to enhance the bacteria-mediated degradation of pollutants, via nutrient-recycling.

Vraag: zijn er ‘matehematical models’ ontwikkeld in dit project en hoe past deze issue bij de inleidende zin (‘Elements of this toolbox developed … are:’?

5. Evidence of a large heterogeneity in community structure and microbial abundance ….. Hydrogeochemistry and subsurface sediment-attached microbial community structure (both in general as for (the number of members belonging to) specific groups of microorganisms) can vary dramatically over short distances (meter-scale) as well over time and reveals a large heterogeneity in community structure and microbial abundance. This characteristic has consequences for the sampling design (i.e. a requirement for the number of samples and locations), but on the other hand a high environmental heterogeneity may enhance natural

attenuation because of a large diversity in micro-organisms capable of pollutant degradation, scattered over the area.

N.b.: dat heterogeniteit moet leiden tot een groot aantal monsters is m.i. niet automatisch waar; het volgt in elk geval niet uit de geostatistische theorie. Zowel aantal als plaatsing zijn van belang, zodat je een goed inzicht krijgt in de variatie zelf en het verband met de afstand, de diepte en (in dit geval) met de aard van het sediment en redoxomstandigheden.

These results were obtained using an interdisciplinary experimental approach combining ecology, microbiology, environmental chemistry and statistics. Central in the applied methodology were molecular techniques addressing either rRNA genes or functional genes relating to degradation of pollutants, aiming at both presence (DNA-based) and activity (mRNA-based). The molecular techniques were combined with conventional enrichments or culturing and new techniques such as isotopic labelling of fatty acids, isotope fractionation and metabolite determination. We established the trophic structure in groundwater communities and the relationships between (micro)eukaryotes, prokaryotes and hydrochemistry. With respect to prokaryotes, the focus was especially on anaerobic bacterial degradation of chlorinated and monoaromatic hydrocarbons, important groundwater pollutants in the Netherlands.

Three research locations were examined in detail. These were i) the downstream area of the Banisveld landfill (mixed contamination, mainly BTEX), ii) the Brabant-site (chlorinated ethenes), and iii) the Limburg site (BTEX, chlorinated ethenes).

At the Banisveld location, trophic structure was simple: larger groundwater fauna was absent. Micro-eukaryote communities had a relatively low diversity compared to other environmental settings and this diversity decreased with depth. Bacterivorous protozoa were detected in a few samples. Community profiles of bacteria and eukaryotic communities associated with sediment samples revealed large differences over short distances (meter-scale). This heterogeneity in composition adds to overall diversity at the research location and may be of benefit for natural attenuation, as leachate has a higher chance to encounter a bacterium able to degrade pollutants contained in it. In addition to a large spatial heterogeneity in sediment samples, also a large temporal heterogeneity in the groundwater community structure was observed over a period of 6 years.

The Banisveld location also received major attention with respect to the (potential for) in situ anaerobic degradation of monoaromatic hydrocarbons (BTEX). The biodegradation by groundwater microorganisms from core samples taken from contaminated and uncontaminated sites was assessed in order to compare the effect of previous exposure on the response of microbial communities. For the first time we were able to prove the potential for anaerobic p-xylene degradation under iron-reducing conditions. GC-MS analyses and carbon-13 labeled contaminants were used to identify the metabolites produced during their biodegradation and to investigate the incorporation of carbon from the contaminants into biomass. These results were related to microbial community structure analysis obtained through molecular profiling of BTEX degrading cultures. They suggested a role for Geobacters in degradation. Selected groundwater samples were analysed by GC-IRMS to determine whether isotopic fractionation of the BTEX substrates, indicative of biodegradation, takes place *in situ.* This was the case.

Molecular tools were developed that allow to measure degradation potential and activity of halorespiring microbes. Such knowledge is important to identify subsoil conditions that optimally promote and sustain microbial degradation. Halorespiration by anaerobic bacteria is a naturally occurring process that can be used in the remediation of sites polluted with chlorinated compounds like PCE (tetrachloroethylene). Our interest was focussed on the metabolic function of the microorganisms rather than their identity and thus dehalogenase-encoding genes were chosen as functional markers for their detection. Primers were validated for the amplification of dehalogenase genes from halorespiring isolates and environmental samples. Abundance and metabolic activity of dehalogenating organisms was quantified by specific 16S ribosomal RNA and dehalogenase gene targeted real-time PCR assays, using environmental DNA as well as RNA as templates. Results from the PCR approach were linked to results obtained by community fingerprinting (DGGE) and physico-chemical characteristics of remediated sites under investigation.

The tools and approaches used and developed in this project will make it possible to predict and monitor natural attenuation processes occurring at other sites better and will contribute to the management and remediation of other contaminated groundwater systems.

06 Popular summary to inform the general public (1/2 to 2 pages of text)
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.

Een onderzoeksproject, uitgevoerd aan de Vrije Universiteit, Universiteit van Amsterdam, en Wageningen Universiteit, heeft geleid tot een sterk verbeterd inzicht in biologische processen die van belang zijn bij de afbraak van belangrijke grondwaterverontreinigingen, zoals oliecomponenten en gechloreerde afvalproducten van wasserijen. De resultaten zijn van belang voor bodembescherming en de beoordeling van de natuurlijke afbraak van vervuiling.

Grondwater is een belangrijke bron van drinkwater in Nederland. Daarnaast worden rivieren en natuurgebieden gevoed door opwellend grondwater. Veel grondwater is vervuild, vooral met relatief goed-oplosbare organische stoffen zoals oliecomponenten en gechloreerde koolwaterstoffen. Dit beperkt het gebruik van grondwater en levert mogelijk gevaar op voor mens en natuur. In totaal zijn er naar schatting in Nederland zo'n 600,000 vervuilde locaties. Het actief opruimen of vastleggen van die vervuiling zou de Nederlandse maatschappij vele miljarden kunnen kosten. Echter, in het verleden is gebleken dat de ondergrond, daar waar het grondwater zich bevindt, vaak een natuurlijk vermogen bezit om met name organische verontreinigingen af te breken of vast te leggen. Als dit proces maar voldoende snel verloopt, zou het bij veel verontreinigde locaties niet nodig zijn om in te grijpen: men zou kunnen vertrouwen op de natuurlijke processen, tegen aanzienlijk lagere kosten. Om daadwerkelijk te vertrouwen op natuurlijke afbraak is het belangrijk om inzicht te hebben in welke processen nu belangrijk zijn in die natuurlijke afbraak en hoe men die belangrijke processen zou kunnen meten.

Het doel van deze studie was het in kaart brengen van natuurlijke afbraakprocessen met nadruk op de afbraak onder zuurstofloze omstandigheden van monoaromatische oliecomponenten (dit zijn oliecomponenten bestaande uit moleculen met een enkele ringvorm, zoals benzeen, tolueen, ethylbenzeen en xyleen) en gechloreerde koolwaterstoffen, belangrijke verontreinigingen in Nederland. Veel grondwater in Nederland is zuurstofloos, en de twee genoemde types van verontreiniging hebben een verschillende functie voor microorganismen: monoaromatische oliecomponenten zijn elektronendonoren (vergelijkbaar met de functie van voedsel voor mensen) en gechloreerde koolwaterstoffen zijn elektronenacceptoren (vergelijkbaar met de functie van zuurstof voor mensen.
Het onderzoek is gedaan in een multidisciplinaire samenwerking tussen de drie bovengenoemde universiteiten, waarbij ecologie, microbiologie, milieuchemie en statistiek zijn gecombineerd. In de biologie heeft de toepassing van zgn. moleculaire technieken de laatste jaren een sterke vlucht genomen. Deze technieken richten zich vooral op het specifiek waarnemen van een stukje erfelijke informatie (DNA), of het expressie-product ervan (mRNA), en het slim en snel vermenigvuldigen van die specifieke informatie met de zgn. PCR reactie. Deze aanpak stond ook centraal in dit project, maar werd gecombineerd met conventionele cultivatie van microorganismen en nieuwe technieken zoals labelen van vetzuren met zware isotopen, isotoop fraktionatie en het meten van tussenstoffen in de afbraak, om het inzicht in de afbraakroute van vervuiling te verhogen.

Drie onderzoekslocaties zijn in detail onderzocht, met de meeste aandacht voor het ondergronds gebied stroomafwaarts van de Banisveld vuilstort, Boxtel, Noord-Brabant. Stroomafwaarts van deze vuilstort ligt een belangrijk nutriënt-arm natuurgebied, de Kampina. De structuur van de levensgemeenschappen in de ondergrond bleek vrij simpel, er zijn veel soorten bacteriën aanwezig terwijl het aantal eencellige organismen dat zich zou kunnen voeden met bacteriën, zoals protozoa, gering is. Grotere organismen, zoals kleine kreeftachtigen die in sommige gevallen het grondwater bevolken, waren hier afwezig. Over relatieve korte afstanden (meter schaal) bleek er een grote variatie tussen monsters te zijn qua aanwezigheid van soorten. Deze heterogeniteit in de samenstelling van levensgemeenschappen draagt bij tot de algemene biodiversiteit van de onderzoekslocatie en zou voordelig kunnen zijn voor de natuurlijke afbraak , omdat tijdens zijn loop door de ondergrond het grondwater, met daarin de verontreiniging, een grotere kans zou hebben de juiste omstandigheden en microorganismen tegen te komen die bijdragen aan de afbraak. Behalve een grote ruimtelijke heterogeniteit, werd ook een grote dynamiek in microbiële gemeenschappen over een periode van 6 jaar waargenomen.

Ook de zuurstofloze afbraak van monoaromatische koolwaterstoffen, met ijzer als electronenacceptor is onderzocht voor de Banisveld locatie. De biodegradatie in vervuilde en schone referentie punten werd bepaald om het effect van eerdere blootstelling aan monoaromatische koolwaterstoffen op microbiële gemeenschappen en hun vermogen om die vervuiling af te breken, te achterhalen. We waren de eersten om aan te tonen dat p-xyleen kan worden afgebroken onder ijzerreducerende condities. In dit onderzoek was de toepassing van isotopen belangrijk. Vervuilingscomponenten, zoals tolueen, gelabeld met zware isotopen werden gebruikt om te achterhalen welke organismen die stoffen omzetten en hoe zij dat doen, doordat het label werd ingebouwd in de biomassa, vooral in vetzuren, maar ook in afbraakproducten. Deze gelabelde vetzuren en afbraakproducten werden vervolgens specifiek gedetecteerd en uit het type gelabelde vetzuur werd afgeleid welk soort het was. Deze analyses als ook de moleculaire karakterisatie, suggereren een rol van *Geobacter* in de afbraak. Daarnaast werd de eigenschap van microorganismen om vooral vervuiling die relatief veel lichte isotopen bevat, op te eten, gebruikt om te achterhalen hoe de biologische afbraak van vervuiling bij Banisveld, op basis van veranderingen in de ratio licht en zware isotopen verloopt met de afstand tot de vuilstort (isotoop fractionatie).

Het tweede type vervuiling dat werd onderzocht, waren de gechloreerde koolwaterstoffen. Moleculaire methoden werden ontwikkeld om het afbraakpotentiaal van microorganismen die ademhalen met gechloreerde alifaten (anaërobe dehalogeneerders) te evalueren. Uniek in deze aanpak was dat we de functie van de microorganismen in hun natuurlijke omgeving onderzochten en niet zozeer hun identiteit. Dit werd gedaan door specifiek te kijken naar een gen dat essentieel is in de ademhaling met gechloreerde koolwaterstoffen en codeert voor het enzym dehalogenase. Een methodiek voor de snelle detectie en moleculaire vermenigvuldiging via een PCR is opgezet en gevalideerd aan de hand van een aantal laboratoriumstammen die ademhalen met gechloreerde alkanen (m.b.: zijn het alkanen of alkenen?), en monsters uit het veld. Aantallen en activiteit van de anaërobe dehalogeneerders zijn bepaald op basis van het 16S ribosomale RNA gen dat in elke bacterie aanwezig is en wordt gebruikt om evolutionaire verwantschap tussen organismen te bepalen, en het dehalogenase-gen dat het vermogen tot afbraak van gehalogeneerde koolwaterstoffen aangeeft. Hieruit is gebleken, dat hogere aantallen aan dehalogenerende populaties goed korreleerden met een verhoogd afbraakactiviteit in zogenoemde bioschermen, waar het afbraakproces door middel van substraatinjectie in de ondergrond gestimuleerd werd.

De methodieken gebruikt en ontwikkeld in dit project kunnen bijdragen aan het voorspellen en volgen van natuurlijke afbraak bij andere vervuilde locaties en zullen bijdragen aan de management en actieve remediatie van andere vervuilde grondwater ecosystemen.

De vervuilingspluim bij de vuilstort Banisveld lijkt zich gestabiliseerd te hebben, maar er is ook een indicatie verkregen, dat de pluim in de laatste jaren meer naar het oppervlakte is gekomen. Een systeem van extensieve monitoring lijkt gewenst.

De resultaten zijn van belang voor bodembescherming en het evalueren van de natuurlijke afbraak van vervuiling:

n.b.: zie mijn opmerking bij onderdeel 5 m.b.t. de navolgende issues!

1. Er is bewijs verkregen voor de *in situ* (ter plekke) anaërobe afbraak van monoaromatische oliecomponenten in een aantal vervuilde grondwater ecosystemen, op basis van het meten van koolstofisotoop-fractionatie en afbraakproducten in het veld en in vergelijking tot laboratoriumexperimenten.
2. Een aantal (mengsels van) bacterie-soorten die betrokken zijn bij de afbraak van belangrijke verontreinigingen zijn geïdentificeerd en gekarakteriseerd. De aanwezigheid van deze soorten kan worden gemeten bij andere locaties.
3. Met behulp van nieuw ontwikkelde, zogenaamde moleculaire methodes, is het nu mogelijk de hoeveelheden en activiteiten van bacteriën betrokken bij biodegradatie

te bepalen op basis van functionele markers (genen betrokken bij de afbraak). Dit maakt het mogelijk om bioremediatie processen *in situ* te bestuderen en kennis te verkrijgen over wat de belangrijke factoren zijn die een gewenst proces (bv. afbraak) beïnvloeden.

4. Mathematische modellen, gecombineerd met Ecologische Controle Analyse, kunnen bijdragen tot nieuwe inzichten in het functioneren van het grondwaterecosysteem. Het inzicht in het functioneren van het geheel systeem kan worden gebruikt om verrassende nieuwe mogelijkheden in bodembescherming en bioremediatie te ontwikkelen en te testen. Een voorbeeld daarvan is de (theoretische) mogelijkheid van toepassing van bacterie-etende protozoa om bacteriële processen te versnellen.

5. Hydrogeochemie en de samenstelling van microbiële gemeenschappen (zowel in het algemeen, als gericht op (aantallen behorende tot) een bepaalde groep) die gehecht zijn aan sediment in de ondergrond *kan* (n.b. is gebleken ?) sterk variëren over korte afstanden (meter-schaal) en met de tijd. Dit heeft consequenties voor het nemen van monsters (het aantal monsters nodig om een vervuilde locatie goed te kunnen beschrijven). Aan de andere kant, zou de grote heterogeniteit grotere biodiversiteit kunnen onderhouden welke in zijn totaal beter in staat is om vervuiling af te breken (n.b.: is deze laatste zin een vraag of een antwoord?).

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 can be divided in two groups: new knowledge and insights and new tools (or what not to do):

New knowledge and insights

1. Insight in the functioning of the system as a whole allows for alternative strategies in soil protection and bioremediation: for example the (theoretical) potential of bacteria-feeding protozoa to enhance the bacteria-mediated degradation of pollutants, via nutrient-recycling.

2. Hydrogeochemistry and subsurface sediment-attached microbial community structure can vary dramatically over short distances (meter-scale) and reveals a large heterogeneity in the pollutant-degrading community structure. This should be reflected in sampling design (i.e. a requirement *for a high number of samples n.b.: zie mijn eerdere opmerking!*). On the other hand the high environmental heterogeneity may support a larger diversity of microorganisms capable of pollutant degradation and therewith enhance natural attenuation.

New (potential for) tools (or what not to do):

3. Evidence was obtained for *in situ* BTEX degradation in different polluted aquifers, based on measurements in microcosms and on groundwater samples (metabolites, isotope fractionation)

4. Some (consortia of) species (e.g *Geobacter* spp., *Dehalococcoides* spp.) indicative for the occurrence of biodegradation have been identified; these species or their genes involved in pollutant degradation can be monitored at other locations (see point 3).

5. A method has been developed to quantify the abundance, as well as the metabolic potential and activity of populations involved in biodegradation using functional markers. It provides an important tool. This has allowed us to study bioremediation processes in-situ and thus allowing to identify physico-chemical as well as biological conditions that are required to promote and sustain effective clean-up.

6. Monitoring the presence of multicellular eukaryotes in Dutch groundwater is not a suitable tool to determine groundwater quality. Groundwater fauna was *never* ? observed in fine ? sandy sediments (the majority of Dutch sediments where one can expect pollution) and only infrequently in coarse sediments (e.g. gravel).

n.b.: heeft Jos Notenboom ze nooit in grof zand aangetroffen? Als dat zo is, dan is het uitgangspunt van dit onderzoek, zoals destijds is geformuleerd m.b.t. een inventarisatie van een groot aantal soorten sedimenten op aanwezigheid van grondwaterfauna om de resilience vraag te beantwoorden, een verkeerde geweest!

n.b.: hoe sluit het navolgende gedeelte aan bij het vorige en bij de vraagstelling van vraag 07 ? BTEX and chlorinated alkenes are characterised by a relatively high solubility in water and slow biodegradation rates under anaerobic conditions: they can pose a serious risk to the environment and, in the case of benzene, to human health.
Therefore, the investigation of the ability of groundwater ecosystems to limit the effects of those pollutants is particularly relevant with respect to the decontamination or biodegradation activity that indigenous microorganisms can accomplish.
The involvement of indigenous and relatively widespread micro-organisms in the removal of BTEX could surely have a positive effect in contaminated soil management, because if it can be demonstrated that the pollutants do not pose an immediate health threat and if they remain localised, the sediment does not need to be further treated.
Laboratory incubations data must be corroborated by field investigations in order to prove that BTEX degradation is occurring. Different tools can be applied: pollutant and/or metabolite detection along the groundwater flow or natural isotopic fractionation. Both approaches were tested and the latter surely offers a novel and easy tool for determining whether *in situ* degradation takes place. These measurements are easily combined with molecular analysis, to determine the presence and activity of specific types of micro-organisms to obtain a far more complete picture on what is happening in groundwater systems.

08 Please list the presentations held in connection to this project

1. **Brad, T., Braster, M**., van Breukelen, B., M., **van Straalen, N. M., Röling, W. F. M. van Verseveld, H**. W., 2002, *Effects of pollution on the biodiversity of*

groundwater fauna and related microbial communities (preliminary results) - Poster, First National Scientific Symposium BodemDiep, June 5-6, 2002, Zeist, The Netherlands. Poster presentation

2. **Brad, T., Braster, M**., van Breukelen, B., M., **van Straalen, N., M., Röling, W., F.,** M., van Verseveld, H., W., 2003 *Eukaryotic diversity and influence of protozoan grazing on microbial processes in a contaminated aquifer - Poster*, International Conference on the Molecular Biology and Biotechnology of Ciliates and Anaerobic Protozoa, March 4-6, 2003, Nijmegen, The Netherlands. Poster presentation.

3. **Brad, T., Braster, M**., van Breukelen, B., M., **van Straalen, N., M., Röling, W., F., M**., van Verseveld, H., W., 2003 *Eukaryotic diversity and influence of protozoan grazing on microbial processes in a contaminated aquifer - Poster*, 2nd National Scientific Soil Symposium BodemDiep, June 4-5 2003, Zeist, The Netherlands. Poster presentation.

4. **Brad**, **T., Braster, M**., van Breukelen, B., M., **van Straalen, N., M., Röling, W., F., M.**, 2003 International Symposium *Structure and Function of Soil Microbiota*, Philipps-University Marburg, Germany, September 18-20, 2003. Poster presentation.

5. **Brad, T., Braster**, M., van Breukelen, B., M**., van Straalen, N., M., Röling, W., F., M.,** 2004, *Eukaryotic community structure in a landfill-leachate contaminated aquifer and its relationship to pollution and Natural Attenuation*, Netherlands Scientific Symposium “Soil and Water”, June 2-3 2004, Zeist, The Netherlands. Oral presentation

6. **Brad, T., Braster, M.,** Van Breukelen, B., M., **Van Straalen, N., M., Röling, W., F., M.**, 2005, *Relationships and contribution of eukaryotes to subsurface contaminant biodegradation*, Netherlands Scientific Symposium “Soil and Water”, June 1-2 2005, Zeist, The Netherlands. Oral presentation

7. **Brad, T., Braster**, M., Van Breukelen, B., M., **Van Straalen, N., M., Röling, W., F., M.**, 2004, *Eukaryotic community structure in a landfill-leachate contaminated aquifer and its relationship to pollution and Natural Attenuation*, 10th International Symposium on Microbial Ecology ISME-10, Cancun, Mexico, August 22-27, 2004. Poster presentation

8. **Brad, T**., **Braster, M**., Van Breukelen, B., M., **Van Straalen, N., M., Röling, W., F., M.,** 2005, *Relationships and contribution of eukaryotes to Natural Attenuation in a landfill leachate-contaminated aquifer*, The Joint International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII), Jackson Hole, Wyoming, USA, August 14-19, 2005. Oral presentation

9. **M. van Heusden, A.D.L. Akkermans**, Dehalogenation in deep groundwater ecosystems. oral presentation, National Bodemdiep Symposium, June 2002.

10. **M. van Heusden, H. Smidt, A.D.L. Akkermans**, Dehalogenation in deep groundwater ecosystems. poster presentation , ISSM (International Symposium on Subsurface Microbiology), Denmark, September 2002.

11. **M. van Heusden, A.D.L. Akkermans, H. Smidt** and W. M. de Vos, Molecular tools for the prediction and monitoring of dehalogenation in deep groundwater ecosystems, oral presentation, National Bodemdiep Symposium, June 2003.

12. **M. van Heusden, H. Smidt** and W. M. de Vos, Molecular detection of dehalogenation in groundwater ecosystems. oral presentation, International symposium on Structure and Function of Soil Microbiota, Marburg, Germany, September 2003

13. **M. van Heusden, H. Smidt** and W. M. de Vos, In-situ molecular detection of the dehalogenating potential and activity, poster presentation, National Bodemdiep Symposium, June 2004.

14. **M. van Heusden**, M. Luijten, J. Gerritse, **H. Smidt** and W. M. de Vos, In-situ molecular detection of the dehalogenating potaential and activity, poster presentation, ISME Cancun, Mexico, August 2004.

15. **M. van Heusden, H. Smidt** and W. M. de Vos, Resilience of a groundwater ecosystem to anthropogenic disturbances: *In-situ* molecular detection of reductive dehalogenation potential in contaminated soils, poster presentation, national Bodemdiep symposium, June 2005.

16. **S. Botton, J. R. Parsons**. Bodem Breed, Lunteren, November 26-27, 2001. Oral presentation: “Resilience of groundwater ecosystems in reaction to anthropogenic disturbances”.

17. **S. Botton, J. R. Parsons**. Bodem Diep, Zeist, June 5-6, 2002. Oral presentation: ”Biodegradation process of BTEX in a range of groundwater ecosystems”.

18. **S. Botton, J. R. Parsons, H. van Verseveld**. International Symposium on Subsurface Microbiology (ISSM), Copenhagen, September 8-13, 2002. Poster presentation: “Biodegradation of BTEX in subsurface environments in reaction to the geochemistry and microbial ecology of the ecosystem”

19. **S. Botton, J. R. Parsons, H. van Verseveld**. Setac, Hamburg, April 27-May 1, 2003. Poster presentation: “Biodegradation of BTEX in subsurface environments in reaction to the geochemistry and microbial ecology of the ecosystem”.

20. **S. Botton, J. R. Parsons**. Consoil, Gent, May 16-16, 2003. Poster presentation: “Resilience of groundwater ecosystems in reaction to anthropogenic disturbances”.

21. **S. Botton, J. R. Parsons**, M. van Harmelen, **H. van Verseveld, W. Röling**, N. Driessen. Bodem Diep, Zeist, June 4-5, 2003. Poster presentation: "Resilience of subsurface anaerobic environments in reaction to BTEX contamination".

22. **S. Botton, J. R. Parsons, W. Röling**. European Symposium on Environmental Biotechnology (ESEB), Oostende, April 25-28, 2004. Oral presentation: "Natural attenuation of landfill leachate in reaction to BTEX contamination".

23. **S. Botton, J. R. Parsons**. Soil and water, Zeist, June 2-3, 2004. Oral presentation: "Natural attenuation of landfill leachate in reaction to BTEX contamination".

24. **S. Botton, J. R. Parsons, W. Röling**. International Symposium of Microbial Ecology (ISME), Cancun 22-27 August, 2004. Poster presentation: Resilience of subsurface anaerobic environments in reaction to BTEX contamination".

Oral presentations by supervisors, related to the project:

1. **N.M. van Straalen.** "Assessment of soil pollution - a functional perspective". Lecture at Yokohama National University, 29 May 2003.

2. **N.M. van Straalen**. Assessing the living soil". Lecture at the Soil and Water Conference, Woudschoten, 4 June 2004.

3. **N.M. van Straalen.** "The elusive concept of ecological quality status". Lecture at an ECETOC workshop, Budapest, 27 November 2004.

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

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

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

7. **W.F.M. Röling**. Soil and Water, Woudschoten, NL. June 2004. (with T. Brad).

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

9. **W.F.M. Röling**. UNESCO Workshop, München, Germany. December 2004 (invited speaker, with **T. Brad and S. Botton**).

10. **W.F.M. Röling**. Satya Wacana Christian University, Salatiga, Indonesia. July 2005 (invited seminar).

11. **J. Parsons, S. Botton, W. Röling, T. Brad,** B. van Breukelen, Benzene workshop Anaerobe afbraak van benzeen in de ondergrond van de voormalige vuilstort Banisveld, Workshop Anaërobe Afbraak van benzeen, Utrecht, 4 november 2003

12. **H. Smidt**. Michigan State University, East Lansing, USA, October 2003 (invited seminar).

13. **H. Smidt**. Max-Planck Institute for Terrestrial Microbiology, November 2004 (invited seminar).

14. **H. Smidt**. SETAC Europe 15th Annual Meeting, Lille, France, May 2005 (invited speaker).

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

Botton, S. Potential for BTEX degradation in an iron reducing aquifer. Co-author: J. Parsons (submitted)

Publications in preparation:

Besides three PhD theses the following publications are in preparation:
N.b.: zijn de titels al bekend?

1. **Brad**, T., Braster, M., Van Breukelen, B., M., Van Straalen, N., M., Röling, W., F., M., *Heterogeneity in eukaryotic and bacteria community structure in a landfill leachate-contaminated aquifer.*
2. **Brad**, T., Braster, M., Van Breukelen, B., M., Van Straalen, N., M., Röling, W., F., M., *Study of the eukaryotic diversity by 18S-rRNA gene fragments cloning and sequencing in a landfill leachate-contaminated aquifer*
3. **Brad**, T., Braster, M., Van Breukelen, B., M., Van Straalen, N., M., Röling, W., F., M., *Dynamics of the plume of pollution in a landfill leachate-contaminated aquifer based on variation of bacteria and eukaryotic community DGGE profiles*
4. **Brad**, T., Braster, M., Van Straalen, N., M., Röling, W., F., M., *Influence of protozoan grazing/predation on organic matter biodegradation under different nutrient limitations*
5. **Botton, S,** J. Parsons: BTEX biodegradation by dissimilatory iron reducing cultures.
6. **Botton, S** , J. Parsons, W. Röling: Physiological profiling of microbial communities involved in BTEX removal.
7. ***Botton, S*** ,J. Parsons, H. Richnow*: In situ* BTEX degradation in iron reducing aquifer.
8. **Botton, S** , J. Parsons, W. Röling: Degradation of BTEX under nitrate reducing condition. Co-authors:

9. **van Heusden, M. Botton, S**. and co-authors: Resilience of microbial systems towards anthropogenic disturbances.

10. **van Heusden, M**., J. Gerritse, W.M. de Vos, **H. Smidt**, Q-PCR based detection of dehalogenase genes and their mRNA in environmental samples.

11. **van Heusden, M**., M. Luijten, J. Gerritse, W.M. de Vos, **H. Smidt**, In-situ assesment of dehalogenation - a comprehensive study of an actively dechlorinating site over time.

12. **van Heusden, M.**, Maphosa, F., de Vos, W.M., **Smidt, H**., Development of molecular detection approaches for detection of halorespiring potential and activity.

13. **van Heusden, M.**, W.M. de Vos, **H. Smidt**, Genetic mechanisms involved in loss of dehalogenating capacity in *S.halorespirans*.

Publications in refereed journals, associated to the project

Van Straalen, N.M. Assessment of soil contamination a functional perspective. Biodegradation, 13, 41-52 (2002)

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

Lin, B., van Verseveld, H.W. and **Röling, W.F.M.**: Microbial aspects of anaerobic BTEX degradation. Biomedical and Environmental Sciences, **15**, 130-144 (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, Applied and Environmental Microbiology, 71, 5983-5991 (2005).

Mouser, P.J., Rizzo, D.M**., Röling, W.F.M.,** and Van Breukelen, B.M.: Multivariate geostatistical approach to spatial representation of groundwater contamination using hydrochemistry and microbial community profiles. Environmental Science and Technology, 39, 7551-7559 (2005).

Smidt, H., and de Vos, W. M.. Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. 58, 43-73 (2004).

Conference proceedings

S.Botton, J. Parsons, W. Röling an M. Braster. Natural attenuation of landfill leachate in reaction to BTEX contamination. Proceedings of the European symposium on Environmental Biotechnology, 2004. W Verstraete ed. University of Ghent.

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

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 extracted from the research proposal. 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 here.

The overall objectives of the proposed research were to characterise the in situ biodegradation of selected contaminants in subsurface (groundwater) ecosystems in relationship to the geochemistry and microbial ecology of these systems and to contribute to the development of a combined geochemical, ecological and environmental chemical framework for the evaluation of the natural attenuation capacity of subsurface environments.

The proposed research originally consisted of four main activities:

1. Determination of biogeochemical redox-reactivity of the subsurface in relation to sediment characteristics (lithofacies data). Research analist at VUA/TNO-NITG (de Gans, van Verseveld).
2. Using the established biogeochemical redox-reactivity of the subsurface sediments for the determination of their relation to occurring eukaryotic and microbial communities. AIO subproject *a* at VUA (van Straalen, van Verseveld/Röling)
3. Detection of the occurrence and *in-situ* activity of bacteria in deep groundwater and subsoil samples by using molecular methods. AIO subproject *b* at WU (Akkermans/Smidt)
4. Determination of resilience/reactivity of specific populations in microbial communities using 13C tracer analysis and labelling of DNA with various nucleotides of DNA upon growth after a stimulus. AIO subproject c at UvA/VUA (Parsons , Lingeman, van Verseveld).

Below the research objectives are given per subproject, in italics, and their achievement and/or modifications.

Ad (2). Project of Traian Brad (VUA) + analist (VUA/TNO). Eukaryote community structure in relation to microbial community structure and hydrochemistry

1. *Generate data from a comparison of sites with different anthrophogenic activities at the surface.* The request for an analist for doing hydrochemical analysis was not awarded. Therefore, it was decided to analyse two sites in detail instead of 20 sites.
2. *Sampling, identification and molecular characterization of groundwater fauna.* Metazoa were only encountered at one research location, and there only at one groundwater sampling well. Therefore this research objective has been adapted towards analyzing the spatial and temporal dynamics in community structure of smaller eukaryotes (e.g. protozoa). A detailed characterization was obtained for Banisveld landfill polluted aquifer. Flagellate protists have been identified in the most polluted part of the aquifer at Banisveld, and this suggests the indirect, but important contribution of protists to biodegradation, by feeding on bacteria and recycling of limiting nutrients, stimulating bacterial growth, the substrate uptake rate, biodegradation.
3. *Characterisation of microbial groundwater communities using molecular and physiological fingerprinting methods.* The spatial and temporal dynamics in bacterial community structure were analysed in large detail for the aquifer downstream of the Banisveld landfill. In addition the interaction between protozoa and bacteria with respect to the rate of biodegradation of pollutants were determined.
4. *Analysing the microbial diversity in animal guts.* See point 2, since only in one case metazoa were found, in low numbers, this objective was not further addressed.
5. *To contribute to the ecological theory on the relationship between genetic (and physiological) biodiversity and ecosystem resilience.* We have obtained complex bacterial and eukaryotic communities and relatively high (genetic) diversity of both bacteria and eukaryotes in the polluted aquifer from Banisveld. Ecological theory says that the higher the biodiversity, the higher resilience of ecosystems. The longer the food chains are the higher resilience. Since only a limited number of sites were characterized (see point 1), it is difficult to generalize the results. An essay addressing the ecological basis of the resilience concept and its application to groundwater remediation is part of the PhD thesis of the candidates

n.b.: doet Trajan mee aan de voorgenomen publicatie m.b.t. resilience van Meta en Sabrina (zie publikatielijst, nr 9)?.

Ad (3). Project by Meta van Heusden (WUR). Detection of the occurrence and *in-situ* activity of bacteria in deep groundwater and subsoil samples by using molecular methods. The main objective has been to detect the in-situ activity of bacteria in deep groundwater and subsoil samples by detecting the mRNA of the key enzymes in respiration with halogenated compounds, nitrate or sulphate as electron acceptors:

1. *extraction and characterisation of reductive dehalogenase mRNA's (optimise DNA and RNA extraction procedures; amplification by existing primer sets and study diversity of gene fragments by DGGE and cloning and sequencing, use competitive PCR for quantification and construct expression gene libraries).* The optimisation of DNA and RNA extraction was successful. With respect to the application of existing primers for catabolic gene amplification, however, it was found in the course of the project that existing primer sets were not entirely suitable for reaching the goals. This related mainly to the fact that new sequence information was increasingly becoming available and it was

decided to redesign the existing primers and design new primers using the new database of information. The primers were validated also for use in real-time PCR and used on environmental (soil and groundwater) samples.

2. *characterisation of key genes involved in nitrate and sulphate respiration (using available primer sets; design specific PCR primers using the newly available sequence information; correlate the occurrence of genes involved in nitrate and sulphate respiration and their mRNAs with the redox condition of the sample).* Because redesigning and validating required significant attention and time, it was decided to focus only on the detection of halorespiring organisms. The strategy included the detection based on 16S rRNA genes of certain phylogenetic groups of halorespiring bacteria. DGGE profiling of the dehalogenase genes proved practically not possible, and different methods were considered, including RFLP. This remark also applies to the next two points.
3. *development of expression libraries (from contaminated and uncontaminated sites).*
4. *development of DNA arrays for key genes in anaerobic respiration (using the expertise obtained from arrays on 16S rDNA aimed at detecting bacteria in anaerobic sludge).*

Ad (4). Project by Sabrina Botton (UvA). Determination of resilience/reactivity of specific populations in microbial communities using 13C tracer analysis and labeling of DNA with various nucleotides of DNA upon growth after a stimulus.

1. *Characterisation of microbial consortia obtained from subsurface core samples from contaminated and uncontaminated sites under laboratory conditions for their ability to degrade contaminants.* The potential for BTEX biodegradation was investigated in two polluted aquifers, Banisveld and Limburg, characterised by iron and nitrate reducing conditions respectively. Evidence for degradation of benzene, toluene and the three xylene isomers with iron as electron acceptor was obtained in sediment and groundwater microcosms incubations and bacteria involved in the BTX oxidation were further enriched. Benzene, ethylbenzene and p-xylene removal with nitrate as electron acceptor was detected in groundwater microcosms for Limburg and maintained in enrichment cultures.
2. *Identification of BTEX degrading cultures.* ^{13}C trace analysis in combination with PLFA analysis, and molecular analysis (16S rRNA gene-based DGGE) confirmed the involvement of *Geobacteraceae* in toluene degradation. The methodology applied for identifying BTEX degrading bacteria (16S rDNA-based DGGE) was different from the originally proposed one (immuno-capture of labelled nucleotides): in fact we found DGGE profiling to be more suitable to answer the research question.
3. *Evaluation of the occurrence of biodegradation processes in situ.* Chemical and carbon isotope analysis of groundwater samples indicated very low BTEX levels (µg/l) and enrichment in ^{13}C benzene and ethylbenzene along the plume, indicating biological removal of those pollutants.

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?

The projects had a clear applied aspect, being directed towards the role of natural attenuation in the management of landfill plumes. However, we have pursued the scientific basis of natural attenuation rather than pursuing commercial applications.

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.

The results of this programme have been disseminated widely in the scientific community (see list of publications above). In addition, direct contacts with consultancy companies and organisations for strategic applied research have been maintained by regular meetings of the utilization committee supervising the programme.

n.b.: + 2 SKB/TRIAS-workshops m.b.t. NA met deelname van onderzoekers en deskundigen van adviesbureaus

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

This question largely repeats question 11. In short, results obtained match the original objectives, but some objectives needed to be modified seriously. These modifications were discussed with the programme committee. Since an analyst was not awarded in the project, far less locations were investigated than stated in the research proposal and obliterated answering the resilience question as originally intended.

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 and understanding at the higher/system level:

1. As the three PhD students in this project worked together on samples obtained from two research sites, the natural attenuation could be addressed in large detail (especially for Banisveld) and knowledge was obtained on the system level (tropic structure, community structure and activity, isotope fractionation, biodegradation)

2. Co-sampling on Banisveld with another TRIAS project (845.080.004) was performed, in order to achieve an even more in-depth characterisation of the microbial communities and processes.
3. In another TRIAS project (845.080.004), work was done on a new theoretical framework in microbial ecology (Ecological control analysis). Especially, prey-predator interactions were experimentally tested together, and to a lesser degree syntrophic interactions (e.g. fermenting microorganisms and halorespirers).
4. Links to new projects, including the BSik-supported programme on Ecogenomics and several EU-funded projects were established.

Integration with other projects has been achieved from the start, especially with two other TRIAS projects (845.080.004, Reactivity of iron-oxyhydroxides, involving among others Bin Lin and Wilfred Röling (both VUA); 835.080.121, CORONA: Confidence in forecasting of natural attenuation, involving among other Maurice Luijten and Jan Gerritse (TNO-MEP)). In the latter project work was also done on halorespiration, in close collaboration with Meta van Heusden and Hauke Smidt (WU) and joined TRIAS meetings were held to enhance optimal integration of the data. The former project also dealt 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. Bin Lin (VUA) worked on iron-reduction, work that closely related to that of Sabrina Botton (UvA) on BTEX degradation under iron-reducing conditions. Traian Brad (VUA) worked on eukaryotic communities, with emphasis on predation. Combining the two projects Wilfred Röling (VU) has modelled the effects of predation on bacteria-mediated degradation processes. Within the 'resilience' cooperation it was attempted as much as possible to work on the same samples from two research locations, in order to be able to integrate the data as much as possible to get fuller insight into the process of natural attenuation. The molecular tools for the detection of abundance, as well as biodegradative potential and activity of anaerobic dehalogenating populations, which were developed by Meta van Heusden (WUR) formed the basis for the integration with recently initiated programmes in which the group of Hauke Smidt (WUR) is involved. These include the Ecogenomics programme, in which also the VU researchers participate, as well as two EU-funded projects. The integrative analysis of data collected in the framework of these different projects will be useful to further define characteristics of subsurface microbiota for successful bioremediation. This can be seen as the definition and exploitation of the bioremediative capacity of the living soil.

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 or achieve the following requirements:

1. How does subsurface heterogeneity in microbial community structure and hydrogeochemistry affect the potential for natural attenuation?

2. At which sub-meter scale (cm, mm, um) are microbial communities auto-correlated and what are the underlying causes for spatial and temporal heterogeneity in microbial communities.
3. Development of DNA arrays for high throughput phylogenetic and functional profiling of subsurface populations relevant for bioremediation is required to link intracellular processes to intercellular processes and interactions with the abiotic environment.
4. Integrative analysis of contaminated and unpolluted sites to get insight in how processes are influenced and how they can be managed to get a desired end result.
5. How versatile are microorganisms towards the degradation of different BTEX compounds?
6. Is degradation of a mixture of compounds affected by synergistic or antagonistic interactions?
7. What are the *threshold values* for degradation of pollutants, and in how far to these depend on environmental conditions (redox process, presence of other pollutants, groundwater chemistry)?

   n.b.: ik meen, dat destijds door Marijke Tros en anderen al is aangetoond, dat drempelwaarden niet bestaan, maar zeer trage afbraak of restconcentraties vooral moeten worden verklaard door geringe mobiliteit of opsluiting van verontreiniging in bodemdeeltjes, waardoor ze onbereikbaar zijn voor micro-organismen. Hoe zit dat?

8. What determines the stability of genetic elements involved in biodegradation within bacterial cells, and under which conditions are these elements lost?
9. Theory needs to be tested in practice, in an iterative manner. Once proven in the laboratory, evidence for in situ occurrence needs to be obtained.

In order to enhance the understanding of complex 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). 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) and environmental conditions (e.g. presence of more favourable substrates for pollutant degrading microorganisms, subsurface heterogeneity). This can only be achieved by a multidisciplinary approach, such as employed in this project, preferentially extended with the inclusion of theoretical ecology and hydrology/hydrochemistry.

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 the previous answer. Systems complexity (such as processes like natural attenuation) needs to be addressed in a multidisciplinary manner, involving microbiologists, environmental chemists, hydrologists, geochemists, ecologists, molecular biologists and environmental engineerers.

Also, this project has contributed to the development of a toolbox for the cultivation-independent detection of organisms involved in biodegradative processes. The general setup of the toolbox can be adapted for other groups of organisms.

The research has provided new insight in the ability of anaerobic microorganisms to degrade different pollutants (para-xylene) under iron reducing conditions. This important novel finding needs to be further addressed in order to understand which bacteria are responsible of this process.

Moreover, it is also important to further evaluate the role of a specific group of microorganisms *(Geobacteraceae)* that seems to be involved in the removal of more than one pollutant.

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

We have identified the following gaps:

1. Technical limitations of the methods like DNA and RNA extraction greatly influence the results, for example by the detection limits etc.
2. To be able to interpret results fully in relation to determining *in situ* conditions, an integrative approach is needed, combining results from different approaches and different toolboxes (e.g. determination of isotope fractionation and degradation metabolites, pollutant degradation rates, molecular assessment of numbers and activities of specific groups of microorganisms, trophic/microbial interactions in ecological networks)

Answers to the research questions stated under question 17 will certainly also contribute to optimal application of our results.

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.

New questions that still need to be addressed:

1. At which sub-meter scale (cm, mm, um) are microbial communities auto-correlated in subsurface aquifers, and at Banisveld in particular. Are similar degrees of temporal and spatial heterogeneity in microbial communities and hydrochemistry also found in other polluted aquifers, or does it for example depend on the type of aquifer (e.g. sandy aquifer directly under the surface vs. sandy aquifer underneath a layer of clay)?

2. What are the underlying causes for temporal and spatial heterogeneity in subsurface microbial communities (e.g. does it depend on the size of sediment particles, the organic matter bound to sediment particles, changes in groundwater chemistry, does it relate to chance processes).

3. Do spatial and temporal heterogeneity indeed contribute to a better attenuation of pollutants compared to a relatively more homogeneous environment?

4. Although it is possible to enrich iron-reducing consortia capable of benzene degradation, benzene appears relatively recalcitrant at Banisveld (present in 30 year old groundwater). What are the underlying causes for this. How versatile are micro-organisms towards the degradation of different compounds (e.g. which compounds can they consume besides benzene, what are the sources of these compounds)? How is degradation of a mixture of compounds affected by synergistic or antagonistic interactions? Up till which threshold is benzene still degraded and which factors influence these thresholds?

Some questions have been addressed within the project or are being addressed in new projects. Already addressed in this project:

Due to the absence of large groundwater fauna a shift was made to the characterisation of microeukaryotes. This led also to new questions regarding how microeukaryotes could influence microbial degradation of organic matter, and under which environmental conditions (especially the type of nutrient limitation), which were subsequently addressed in the project.

Other new research questions, already being addressed new in other 3rd category programmes, relate to:

1. Involvement of genetic, potentially mobile elements in the evolution and adaptive capacity of halorespiring organisms – as a link to understanding the adaptive capacity of the soil in response to the pollution wit halogenated compounds. This is now addressed in the Ecogenomics programme (Smidt, WU)
2. Biogeography: how universal are the results obtained in this project: are the same types of halorespirers or Geobacters found everywhere. This is also addressed in the Ecogenomics programme (Smidt, WU/Röling, 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 answers to question 12, 13. We cooperated with the TRIAS project on the Reactivity of iron-oxyhydroxides (845.080.004; involving Bin, Röling). This is leading to integrated results and co-authored manuscripts (Röling/Brad/Van Straalen,

Botton/Parsons/Röling) are in preparation. The same holds true for TRIAS project 835.80.121 (Luijten, Gerritse), which was linked to our project via combined progress report meetings. Furthermore, actual integration with this project was achieved on the research level, which will lead to joint scientific output (Luijten/Gerritse/van Heusden/Smidt).
Furthermore, at the VUA two projects, performed in Indonesia, are ongoing, which deal with landfill leachate pollution and associated redox processes. The research on molecular monitoring of the potential and occurrence of BTEX and PCE/DCE degradation will be continued at the VU (Röling) and WU (Smidt) in the BSIK-founded Ecogenomics programme, while monitoring of presence of microeukaryotes will also be part of this programme.
Research results obtained in this project has also led to the participation of WU (Smidt) in two EU projects within the 6th framework programme. Specifically, within the AQUATERRA project, molecular ecological approaches are used to assess the impact of (globally) changing environmental conditions affect microbial degradation potential and activity, while in the SEDBARCAH project similar methods are used to investigate the role of the sediment zone as a barrier against the infiltration of chlorinated aliphatic pollutants into surface water.

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 been mentioned above (e.g. in the general summary and in the Dutch Summary).

Overall, our results have contributed to a better insight in natural attenuation and how to monitor its potential and *in situ* occurrence. This allows now for better assessing Natural Attenuation and determining whether a polluted sites needs to be remediated or not. This should help to diminish the number of sites that need to be cleaned up and therewith lower costs for society as a whole.

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/

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

Traian Brad has gotten an extension of his contract at VUA till 31 December 2005. In 2006 he will resume working at his former Romanian employer: Institute of Speleology 'Emil racovita', Department of Cluj. http://www.speleological-institute-cluj.org/ .
Sabrina Botton is looking for a postdoc position. Meta van Heusden will be employed till mid December.
All other scientific staff involved will continue their work at their respective universities.

Amsterdam, 21 October 2005

APPENDIX:

As part of the programme, the three PhD students wrote an essay on the concept of resilience and its application to natural attenuation of pollution from landfill plumes. A summary of this essay, which is part of the three PhD thesis, is attached to this report.

Essay on the resilience of ecosystems: Emphasis on the groundwater ecosystem

M. van Heusden, S. Botton & T. Brad

SUMMARY

In the context of our project, studying the resilience of the groundwater ecosystem in reaction to anthropogenic disturbances, it is important to look at what determines the resilience of an ecosystem. Resilience is a term that, in the case of groundwater systems contamination, implies more than assessing the degradation potential of a specific pollutant, but rather aims at describing the effects of the introduction of such pollutant on the whole ecosystem functioning.

Two different definitions of resilience are commonly used in ecology and this distinction reflects which aspect of stability is emphasized: “engineering resilience” concentrates on stability near an equilibrium steady state; resistance to disturbance and speed or time of return to the equilibrium are used to measure the property. In contrast “ecological resilience” relates to conditions far from equilibrium steady state and resilience is the magnitude of disturbance that can be absorbed before the system changes its structure by changing the variables and processes that control its behaviour.

Resilience of ecosystems may be studied in theory by determining a stable point, applying a disturbance, measuring the time needed for the community to return to the previous state or to a new stable one and then repeating the experiment for communities in different environments. In practice, however, it is not such an easy task.

It is clear that the definition of resilience is intimately linked to the definition of stability.

When looking at the groundwater fauna the groundwater ecosystem is assumed to be relatively little resilient, because of the limited number of links in the food web and low overall biodiversity. Populations of groundwater fauna typically have grouped distribution and present K type-selection strategy. In many cases only one population of a species is present, leaving little means for recovery once affected by pollution. Groundwater organisms are generally stenobiontic, they only tolerate narrow environmental fluctuations, and thus they are vulnerable to disturbance. The degree of resilience also depends on the type of groundwater environment examined, but a return of the ecosystem to the original state is less feasible, due to the occurrence of irreversible processes (e.g. redox processes). Adaptation and speciation in aquifers (groundwater ecosystem) occur mainly due to this geographic isolation. This means that every aquifer is unique, and a nominal state after a perturbation can never be regained. The groundwater ecosystem cleans sooner or later, but the community may never be the same again. Secondary succession may occur when the environment is not severely isolated.

When assessing resilience in subsoil and groundwater ecosystems with respect to the microbial community structure, not many studies have described this, moreover, the necessary simplification required to approach such studies has inevitably brought to “collapse” the microbial community into one (bacteria) or few functional groups (for example, nitrogen fixers, decomposers, methanogens) and very few studies have described stability and resilience in a microbe dominated ecosystem. An additional dimension of the role of microbial diversity in natural systems is related to the microbial adaptations to spatial and temporal variations in the environment. It is difficult to study the adaptation of these

organisms through genetic evolution in whole ecosystem context; however, it could be a very important factor in any resilience study.

We discuss the actual applicability of the existing concepts and hypotheses of resilience in relation to groundwater fauna and its possible translation for those ecosystems in which microbial communities should not be ignored, as in the case of groundwater ecosystem.