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

Anaerobic biodegradation of benzene in contaminated soils.

02 TRIAS/ALW project number or file number

Project number 835.80.009

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

Period: March 2002 - February 2004

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

Prof. dr. ir. Alfons J.M. Stams [1]
Dr. ir. Nico C. G. Tan [1]
Ing. Wim van Doesburg [1]
Dr. ir. Alette A.M. Langenhoff [2]
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The work was mainly carried out at the laboratory of Microbiology.

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

Anaerobic bioremediation is most attractive whenever anaerobic conditions prevail in a polluted soil site. Thus far, anaerobic bioremediation techniques for soils polluted with mobile aromatic hydrocarbons are not applied. The bottleneck in the application of anaerobic techniques is the supposed poor anaerobic biodegradability of benzene. However, anaerobic degradation of benzene under various redox conditions has been described, the microorganisms involved are not known. Only recently the first anaerobic benzene-degrading microorganism was described. The optimal physiological conditions for anaerobic benzene degrading microorganisms and the biodegradation pathway are unknown. Such knowledge is essential to apply anaerobic bioremediation techniques for soils polluted with BTEX compounds.

The aim of this research project was to get insight into the occurrence of anaerobic benzene oxidation in polluted sites by studying microorganisms involved in anaerobic benzene oxidation. Therefore, the aim was to isolate or enrich anaerobic benzene degrading bacteria and study their physiological and phylogenetic properties. This knowledge can be used to determine and stimulate anaerobic benzene oxidation in the field. The acquired knowledge would lead to the development of a novel bioremediation technology and might also result in methods to demonstrate and monitor anaerobic benzene degrading bacteria in polluted soils.

Anaerobic benzene degradation was tested under various redox conditions with different inoculates or pure cultures. The results showed that benzene degradation occurred under nitrate- and chlorate-reducing conditions in batch and column experiments. Other conditions, like sulfidogenic and methanogenic conditions were also tested and did not show degradation of benzene.

Highest conversion rates were obtained under chlorate reducing conditions. A culture was obtained that used chlorate as electron acceptor and was not able to use nitrate or perchlorate as electron acceptor. The benzene degradation rate of the chlorate reducing culture was much higher when compared with other anaerobic benzene degrading enrichment cultures. The rate is in the range of aerobic benzene degrading organisms. Different intermediates of the possible benzene degradation pathway were tested. The results indicated that an initial oxidation to phenol or even catechol is most likely and will be checked in the near future. The mass balance of benzene degradation and chlorate reduction was determined and was stoichiometric if biomass production was taken into account.

The molecular techniques DGGE and 16S rRNA cloning and subsequent sequencing unravelled the bacterial compositions of the chlorate and nitrate-reducing enrichment cultures. The nitrate and chlorate enrichment cultures consist of respectively four and five dominant species. Three clones from the nitrate enrichment culture were closely related to a pivilate (2,2-dimethylpropionate) degrading nitrate reducing organism, a denitrifying *Azoarcus* strains PbN1 and a *Rhodoferax ferrireducens* iron(III) reducing micro-organism. The three clones from the chlorate enrichment culture were closely related to *Acidovorax avenae*, *Zoogloea resinphlia* PIV-3a2Y (pivilate degrading micro-organism) and *Mesorhizobium* sp. WG.

Two chlorate-reducing soil and sediment columns have been operated during this project, and one of them showed benzene removal. Almost stoichiometric amounts of chlorate were used when compared with the amount of benzene degraded. A good recovery

of chloride (product of chlorate reduction) was also observed. The removal of benzene was chlorate dependent. The highest degradation rate determined was 77.8 µmol / $l_{reactor}$ / h.

Two stable enrichment cultures and one soil column that degrade benzene were developed and studied in this project. The isolation of a single anaerobic benzene-degrading microorganism was not achieved.

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.*

Het TRIAS project (835.80.009) "Anaerobic biodegradation of benzene in contaminated soils" startte maart 2002 en eindigde februari 2004. Het doel van dit TRIAS-onderzoek was inzicht te krijgen in het verloop van de anaërobe benzeenafbraak en de hierbij betrokken bacteriën. Doormiddel van het isoleren van bacteriën die anaëroob benzeen kunnen afbreken en ze fysiologisch en genetisch te karakteriseren werd getracht het doel te bereiken. Het is bekend uit de wetenschappelijke literatuur dat anaërobe benzeenafbrekende bacteriën niet eenvoudig zijn te isoleren. Mocht het niet mogelijk zijn deze bacteriën te isoleren dan kan ook onderzoek gedaan worden met ophopingsculturen die anaëroob benzeen afbreken.

Het onderzoek werd voornamelijk uitgevoerd in batchexperimenten. Verschillende bacteriële groei media en entmaterialen, zoals vervuilde gronden, sedimenten, grondwater maar ook ophopings- en reinculturen, werden getest op anaërobe benzeenafbraak. Dit werd getest met vier verschillende elektronen-acceptoren, nl. carbonaat (methanogene omstandigheden), sulfaat, nitraat en chloraat. Daarnaast werden ook nog twee continue grondkolommen bestudeerd en getest op de afbraak van benzeen.

Het doel om een anaërobe benzeen afbrekende bacterie te isoleren is helaas niet bereikt. Wel zijn twee ophopingsculturen verkregen die benzeen kunnen afbreken met nitraat of chloraat als elektronen-acceptor. Uit grondwater van de locatie Flebo, waar met behulp van nitraat de benzeenafbraak gestimuleerd is, werd een benzeenafbrekende nitraat reducerende cultuur opgehoopt. De chloraatreducerende ophopingscultuur werd verkregen uit een cultuur afkomstig van TNO-MEP (Dr. Jan Gerritse). Op deze cultuur werd verder onderzoek uitgevoerd. Tevens werd een benzeen-afbrekende grondkolom, gevuld met grond van een vervuilde locatie (Van Velde Buren), onderzocht onder chloraat-reducerende condities.

Het stimuleren van afbraak van benzeen door middel van chloraat-toediening is nog weinig onderzocht maar dit onderzoek heeft duidelijk aangetoond dat dit een interessante en mogelijk nieuwe en snelle methode is om persistente verontreinigingen af te breken. Bij de reductie van chloraat komt naast chloride ook moleculaire zuurstof vrij. Dit is een verbinding die niet aanwezig is onder anaërobe condities maar voor een eenvoudige afbraak van verbindingen zoals benzeen wel essentieel is.

In dit project werd in batches gekeken naar een ophopingsculturen die benzeen afbrak met chloraat als elektronen acceptoren in een chloraat een grondkolom waaraan chloraat en benzeen werden toegevoegd. In beide systemen werd chloraat afhankelijke

afbraak van benzeen waargenomen. De chloraat-reducerende ophopingscultuur bleek niet de aan chloraat nauw verwante elektronen acceptoren nitraat en perchloraat te kunnen gebruiken. Deze ophopingscultuur kon naast benzeen ook fenol, catechol en tolueen afbreken. Echter, de tolueenafbraak is niet zo snel als de afbraak van benzeen, fenol en catechol. Benzoaat, dat een mogelijk intermediair is van de anaërobe benzeenafbraak, werd niet afgebroken. De benzeen-afbraaksnelheid van de chloraat ophopingscultuur is 1650 µM /d. Dit is veel hoger dan de afbraaksnelheid van vergelijkbare anaërobe ophopingsculturen en geïsoleerde organismen. De afbraaksnelheid van deze ophopingscultuur ligt in de buurt van die van een aërobe benzeenafbrekende cultuur. Doordat de potentiaal van de redox koppels chloraat/chloride en zuurstof/water dicht bij elkaar in de buurt liggen is het niet verwonderlijk dat de afbraaksnelheden bij elkaar in de buurt liggen. De massabalans van deze cultuur werd ook opgesteld en bleek sluitend wanneer met biomassaproductie rekening werd gehouden. Tevens werd alle chloraat volledig omgezet naar chloride. Na tiende keer doorenten bestaat de cultuur waarschijnlijk nog uit vier verschillende bacteriën. Door middel van kloneren en sequencen werden drie van de bacteriën op basis van het 16S rRNA gen vergeleken met bekende bacteriën uit een database. De drie klonen hadden nauwe verwantschap met *Acidovorax avenae*, *Zoogloea resinphlia* PIV-3a2Y en *Mesorhizobium* sp. WG.

De grondkolom die benzeen afbreekt gekoppeld aan chloraatreductie bleek voor de afbraak van benzeen chloraat nodig te hebben. Massabalans berekeningen laten zien dat benzeenafbraak stoichiometrisch gekoppeld was aan de reductie van chloraat en dat chloride bijna volledig werd teruggevonden in het effluent. De maximale afbraaksnelheid van dit systeem werd getest en ligt rond de 80 µmol / $l_{reactor}$ / h.

De nitraat-reducerende benzeen-afbrekende ophopingscultuur bleek benzeen veel langzamer af te breken dan de chloraat ophopingscultuur. De benzeen-afbraaksnelheid van deze cultuur is 0.5 µM /d. De benzeenafbraak bleek nitraatafhankelijk te zijn. Als nitraat werd weggelaten, vond er geen afbraak meer plaats. Tevens werd met behulp van moleculaire technieken aangetoond dat deze cultuur waarschijnlijk uit vijf soorten bacteriën bestaat. Drie hiervan werden gekloneerd en gesequenced. Deze waren nauw verwant aan een pivilaat (2,2-dimethylpropionaat) afbrekende nitraat reducerende bacterie, een denitrificerende *Azoarcus* stam PbN1 en een *Rhodoferax ferrireducens* bacterie.

In dit project zijn twee benzeenafbrekende ophopingsculturen verkregen. Deze werden bestudeerd op fysiologisch en genetisch niveau. Tevens bleek de toepassing van chloraat dosering in een grondkolom de benzeenafbraak te stimuleren. De isolatie van anaërobe benzeenafbrekende micro-organismen. is dit niet gelukt.

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

The impact of this project for practising soil remediation is substantial. Thus far the method to stimulate benzene degradation coupled with chlorate reduction has received little attention so far. This research showed the feasibility of this method. It was proven on lab scale in soil column experiments. Furthermore, the microorganisms involved in the biodegradation of benzene coupled with chlorate and nitrate reduction were studied. Furthermore, the different physiological properties of these enrichments were examined. The research conducted indicated that in both enrichment cultures bacteria were present that have a high homology with anaerobic pivilate (2,2-dimethypropinonate) degrading microorganisms.

08 Please list the presentations held in connection to this project.

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M, Gerritse, J. and Stams, A.J.M. (2004) Microbial analysis of nitrate and chlorate reducing benzene degrading enrichment cultures. Oral presentation at the 10th International Symposium on Molecular Ecology, 22-27 August 2004, Cancun.

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M, and Stams, A.J.M. (2004). Benzene degradation in soil columns coupled with chlorate reduction. Oral presentation at the 3th National Scientific Soil and Water Symposium, 2-3 June 2004, Woudschoten, Zeist.

Weelink, S.A.B, Tan, N.C.G., Van Doesburg, W., and Stams, A.J.M. (2004). Isolation of a novel toluene-degrading Fe(III)-reducing bacterium . Oral presentation at the 3th National Scientific Soil and Water Symposium, 2-3 June 2004, Woudschoten, Zeist.

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M, Gerritse, J. and Stams, A.J.M. (2004). Benzene degradation coupled with chlorate reduction. Poster presentation at the European Symposium on Environmental Biotechnology, April 25-28, Oostende.

Tan, N.C.G, Van Doesburg, W., and Stams, A.J.M. (2003). Anaerobe benzeen afbraak met nitraat en chloraat als elektronen acceptor. SKB Workshop Anaerobe Afbraak Benzeen, 4 november 2003, Utrecht.

Tan, N.C.G, Weelink, S.A.B., Van Doesburg, W., Langenhoff A.A.M and Stams, A.J.M. (2003). Anaerobic biodegradation of benzene in contaminated soils. Oral presentation the 2nd National Scientific Soil Symposium, 4-5 June 2003, Woudschoten, Zeist.

Tan, N.C.G, Weelink, S.A.B., Van Doesburg, W., Langenhoff, A.A.M and Stams, A.J.M. (2003). Anaerobic biodegradation of benzene in contaminated soils. Poster presentation at Consoil 2003, 12-16 May 2003, Gent.

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.

Scientific publication submitted:

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M and Stams, A.J.M. (2004). Benzene degradation coupled with chlorate reduction in soil column study. Submitted for publication in Biodegradation.

Scientific publications in preparation:

Tan, N.C.G, Van Doesburg, W., Langenhoff A.A.M, Gerritse J. and Stams, A.J.M. (2004). Benzene degradation coupled with chlorate reduction.

Tan, N.C.G, Van Doesburg W., Langenhoff A.A.M, and Stams, A.J.M. (2004). Anaerobic benzene degradation coupled with nitrate reduction.

Conference proceedings:

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M and Stams, A.J.M. (2004). Benzene degradation in soil columns coupled with chlorate reduction. Proceedings of the 3th

National Scientific Soil and Water Symposium, 2-3 June 2004, Woudschoten, Zeist, pp 19-21.

Weelink, S.A.B, Tan, N.C.G, Van Doesburg, W. and Stams, A.J.M. (2004). Isolation of a novel toluene-degrading Fe(III)-reducing bacterium. Proceedings of the 3th National Scientific Soil and Water Symposium, 2-3 June 2004, Woudschoten, Zeist, pp 22-24.

Tan, N.C.G, Van Doesburg, W., Langenhoff, A.A.M, Gerritse, J. and Stams, A.J.M. (2004). Benzene degradation coupled with chlorate reduction. Proceedings of the European Symposium on Environmental Biotechnology, April 25-28, Oostende. pp 803-805.

Tan, N.C.G, Weelink, S.A.B., Van Doesburg W., Langenhoff, A.A.M and Stams, A.J.M. (2003). Anaerobic biodegradation of benzene in contaminated soils. Proceedings of the 2nd National Scientific Soil Symposium, 4-5 June 2003, Woudschoten, Zeist, pp 55-58.

Tan, N.C.G, Weelink, S.A.B., Van Doesburg, W., Langenhoff, A.A.M and Stams, A.J.M. (2003). Anaerobic biodegradation of benzene in contaminated soils. Proceedings of Consoil 2003, 12-16 May 2003, Gent.

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.

"*The aim of our research is to study the anaerobic benzene degradation in detail. This research will give insight into:*

- *The biochemical mechanisms of anaerobic benzene degradation*
- *The physiological properties of anaerobic benzene degrading bacteria*
- *The phylogenetic properties of anaerobic benzene degrading bacteria*
- *Benzene degradation in anaerobic polluted soils with different redox potentials*"

The biochemical mechanisms were only briefly studied and the initial degradation step in the chlorate reducing benzene degrading enrichment culture is an oxidation. The main focus of this project the isolation of bacteria from the enrichment cultures resulting in a less thorough investigation of the involved biochemical mechanisms. However, the physiological and phylogenetic properties (bacterial composition) of both enrichments were studied in detail. Benzene degradation in anaerobic polluted soil was also studied in soil column systems. No benzene degradation was observed in the nitrate and sulfate stimulated columns. However, a chlorate stimulated column showed fast and good degradation of benzene. Therefore, this column was studied in more detail in this research project.

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

No, but eventually insight in and application of anaerobic *in situ* bioremediation of benzene was the applied goal of this project. This project helped to understand and also showed new possibilities for the applicability of anaerobic *in situ* bioremediation of benzene.

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.

Yes, one of the project partners was Rainer Meckenstock from Germany. We are conducting an experiment in which their knowledge and equipment for isotopic fractionation will be used. Alette Langenhoff paid a visited Dr. Meckenstock and his group and has other collaborations with Rainer Meckenstock. Winnie Dejonghe and Johan Gemoets were members of the steering committee. Both are working at the Flemish Institute for Technological Research (VITO) in Mol (Belgium). Finally, two symposia (Consoil 2003 and European Symposium on Environmental Biotechnology 2004) in Gent and Oostende (Belgium) were visited and a conference (10th International Symposium Microbial Ecology 2004) in Cancun (Mexico) will be visited this summer.

Presenting twice at the soil conference "Bodemdiep" (2003 and 2004), visiting the symposium "Bodembreed" (2002 and 2003) twice and presenting at the SKB workshop "Anaerobe afbraak benzeen" (November 2003) expanded the national network.

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

To our opinion the results obtained do match the original objectives. The objective was to study the anaerobic benzene degradation and this was done. The isolation of a single anaerobic benzene degrading microorganisms was not achieved. Two enriched benzene degrading cultures and chlorate-amended benzene-degrading soil column were obtained. The results obtained are more scientific publications.

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.

No

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 and work conducted at TNO gave better insight in the application of chlorate as stimulation for bioremediation.

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

The research conducted is certainly of value for research done by others. The fact that benzene was degraded coupled with chlorate reduction is not new. But application of chlorate to degrade benzene in a soil column experiments is to our knowledge not often conducted. In the United States there is a big environmental issue of the occurrence of too high levels of perchlorate and chlorate in drinking water. This research is an example how mixtures of pollutants can be successfully treated together.

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 application of chlorate to stimulate benzene degradation is not used in full-scale bioremediation yet, has only been tested in a pilot by TNO. One of the obstacles is the legal issue if chlorate can be used in the environment (how toxic is chlorate; is chlorite found). Furthermore, to know how chlorate reacts with other reducing agents such as sulfate or nitrate in the groundwater is essential for the application of chlorate.

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.

The role of the generated oxygen in the chlorate reduction is still not clear and has to be tackled for the successful implementation of chlorate-stimulated bioremediation. Also the initial aim to isolate single microorganisms still remains. Probably this is very difficult because a consortium of bacteria degrade benzene and not a single microorganism. If no single organism can be isolated the interaction and role of the different organisms present in the enrichment cultures has to be evaluated.

A pre-proposal was submitted in NWO water program with the title "Interspecies oxygen transfer; a novel mechanism for anaerobic bioremediation of polluted soil and groundwater". Unfortunately, this project did not pass the first selection round. Probably a STW project will be written in order to get additional funding for this research.

During this project a Ph.D. project (1st category funding) from Sander Weelink has started. The aim of his project is to get insight in anaerobic conversions of xenobiotic compounds for which oxygenases are required in aerobic bacteria to activate these compounds. Benzene is a possible example and is also studied in this project. Sander did also join the steering committee meetings and his results and experiments were frequently discussed.

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?

We had contacts with John Parsons (steering committee member), and colleagues Hauke Smidt and Meta van Heusden, who are involved in the TRIAS project "Resilience of the groundwater-ecosystem in reaction to anthropogenic disturbances" (TRIAS project 835.80.007). We also exchanged knowledge and know-how with Sabrina Botton who is supervised by John Parsons. This exchange did not yet lead to common and integrated results.

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.)

This question is probably difficult to answer due to the fact that the relevance for the society as a whole is not so clear. Stimulated *in situ* anaerobic soil bioremediation of BTEX is financially interesting compared to excavation or other more expensive techniques. As mentioned, the anaerobic benzene biodegradation is often a bottleneck in bioremediation. Therefore, this project have led to better understanding of the anaerobic benzene biodegradation, but still the not everything is clear yet how benzene can be treated best.

Part II - continued

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

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

Other then internet web page from TRIAS where all progress reports can be retrieved, no action were undertaken to disseminate the results in the direction of the general public.

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

Nico Tan did not yet find a new position but various job applications are made. But these did not lead to a positive result yet.

Wim van Doesburg got an extension of his contract for three years at the laboratory of Microbiology as a technician and analytical chemist on other projects.

Wageningen, 25 juni 2004.