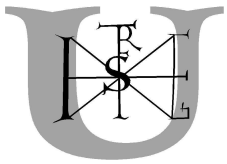

SZENT ISTVÁN UNIVERSITY

Additives enhancing decomposition of different pollutants

Thesis of Ph.D. dissertation

Sára Révész

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The Ph.D School

Denomination: Ph.D. School of Environmental Science
Program: Agricultural and Environmental Microbiology, Soil Biotechnology
Manager: György Heltai , Ph.D. Professor
Head of Department
Szent István University, Faculty of Agriculture and Environmental Science
Institute of Environmental Sciences, Department of Chemistry and Biochemistry
supervisor: Károly, Márialigeti, Ph.D. Professor
Head of Department
Eötvös Loránd University, Faculty of Science
Institute of Biology, Department of Microbiology

.....	.....
Approval of School Manager	Approval of Supervisor

1. Literature summary; Aim of work

1.1 Significance of work

The contamination of our environment has highly increased in the last century. Remediation technologies appropriate for the effective treatment of groundwater and pristine as well as for damage reduction have been applied from the '90s by the national environmental protection system. The experts make decision based on the local conditions to choose the right technology at each contaminated sites. Technologies based on processes of live systems for achieving our goals are increasingly spreading.

Remediation processes - built up from natural or planned processes - are currently used to treat a wide range of chemicals in environmental media including groundwater, soils, and sediments. The remediation technologies using biological processes (bioremediation) cover many organic and inorganic contaminants, often used together with other, physical and chemical remediation techniques. A common element in many engineered bioremediation processes is the stimulation of specific metabolic activity targeting the contaminant(s) of concern.

The microbes have an important role in degradation of organic compounds in the ecosystem. It was already established in the 1950s that every organic compound, generated in natural process, is microbiologically degradable (Kluyver és van Niel 1956). Alexander (1965) completed this theory with thoughts that the microbes have considerable adaptation capability for degrading synthetic compounds too.

Microorganisms have developed a tremendous diversity in metabolism that can be used to degrade naturally occurring organic chemicals. This metabolic diversity also has been shown to extend to many anthropogenic organic chemicals, which are common contaminants of environmental media. It is important to understand, however, that individual microbial species are capable of only a subset (and sometimes a very limited subset) of the collective metabolic diversity known to occur. In nature, microorganisms exist in mixed culture, and bioremediation often seeks to enhance the metabolic expression and activity of a subset of the overall microbial community. (Hughes et al. 2002).

1.2 Aim of work

The huge amount diesel oil released to soil are toxic for organisms, so it is required to develop methods which are suitable for elimination of contaminants rapidly, easily and with appropriate efficience. One of the possible solutions is to enhance hydrocarbon degradation activity of microbes living in soil. Since hydrocarbons are insoluble in water, an important step is enhancing bioavailability of hydrocarbon molecules to microbial cells.

The aim of our studies was to isolate microbes tolerant to hydrocarbon composing molecules, having potential capacity to degrade those compounds and/or use them as sole carbon source. We focused to the isolation of hydrocarbon degrading strains, which have capacity to degrade wide spectra of aromatic compounds. Our goal was also to design primers to detect catechol 1,2-dioxygenase gene in the isolated strains.

Surfactant properties of cyclodextrins (CD) allow enhancing bioavailability of diesel oil components for microbial cells, thereby CDs form complex with the molcules thus the apparent water solubility increase. This process is different for several diesel oil compounds, it depends on their hydrophobicity and size. The inner cavity of CD can complex only a certain size of molecules.

As a result of an equilibrium process, adding appropriate amount CD, the earlier non-available compound become available for the cells.

Our goal was also to demonstrate, that hydroxypropil-β-cyclodextrin (HPCD) can speed up the hydrocarbon degradation by microorganisms. We also compared the effect of HPCD, Tween 80 and starch on diesel oil biodegradation. Beside the contamination originated from fuel hydrocarbons, like diesel oil, other remarkable source of groundwater contamination is the non-appropriate maintenance of chlorinated aliphatic hydrocarbons. Our goal was to develop and optimize molecular biological methods detecting representative microbes of reductive dechlorination process from contaminated sites routinely, like *Dehalococcoides* sp., *Dehalobacter restrictus* and *Desulfuromonas chloroethenica*.

Further aim was to characterize the microbial community of trichloroethylene (TCE) contaminated sites with different geochemical properties and to determine the dominant species. Herewith characteristic parameters of the microbial community composition and the identification of dechlorinating microbes taken from the sites were focused to. Microcosm experiments from groundwater originated from contaminated sites were set up; in order to determine the appropriate substrate for biostimulation technique. Microcosm experiments were designed to enhance the growth of microbes for reductive dechlorination adding different electron donors in anaerobe environment. This method allows the selection of the appropriate substrate for bioremediation.

2. Materials and Methods

Samples were originated from contaminated sites with different geographical properties. For strain isolation we used diesel oil-enriched media, hence only strain with diesel oil degradation capacity were selected. We also applied strains from German Collection of Microorganisms and Cell Cultures (*Pseudomonas putida* DSM50202, *Acinetobacter* genospecies 11 DSM590) and from ELTE Department of Microbiology Collection of Microorganisms (strain signed SM5T4)

Analysis of on-site samples and samples from laboratory experiments was carried out by accredited laboratory. Analysis of BTX compounds was performed by GC-FID using in-house developed method.

Carbon-dioxide production at microcosm experiments was measured in closed system by Oxi-Top OC110 (WTW). The influence of HPCD addition to benzene and toluene degradation was analyzed in serum bottles closed by PTFE-lined septa. For TCE degradation experiments groundwater was collected by a low-flow technique into 2L anaerobically sterilized bottles filled up without headspace under nitrogen atmosphere and closed by PTFE-lined silica septa.

CFU number estimation was done by classical plate count method using 10-fold serial dilution series. Biomass increase was followed by optical density determination. Analysis of community carbon source utilization we used BIOLOG GN2 (Hayward, California, USA) plates. The enzyme-activity measurement was done based on Feist and coworkers' protocol with small changes (Feist és Hegeman 1969).

First step of microbiological ecological molecular biology experiments is the extraction of nucleic acids from organisms in the sample. DNA isolation was done by UltraClean Soil DNA Kit (MoBio), RNA isolation was performed by RNeasy Mini Kit (Qiagen) with protocol described by the manufacturer. For reverse transcription of RNA we used hexamer primers. For amplification we selected different primers which were signed by the 16S rRNA gene sequence of *Escherichia coli* (Brosius et al. 1978) or used the name comes from its designer.

We applied more, alternative methods to analyze community DNA or RNA. One of them was to create clone library to separate the 16S rRNA genes of microbes from the sample and determine their sequences. We also used fingerprint methods, like gradient gel-electrophoresis (DGGE) and terminal restriction length polymorphism (T-RFLP). DGGE was prepared in INGENYphorU (Ingeny) gel-electrophoresis system, T-RFLP results were created with ABI Prism 310 Genetic Analyzer (Applied Biosystems) instrument using GeneMapper software.

To create phylogenetic trees we used ARB-SILVA (Pruesse et al. 2007) internet database and MEGA3 software (Kumar et al. 2004). Result analysis were done by principal component analysis with Syntax 2000 software.

3. Results

3.1 Enhancing diesel oil-degradation at bench scale studies

Based on the CFU number from diesel oil-enriched agar plates, the contamination above a certain TPH level decrease the number of diesel oil degrading microorganisms with one order of magnitude. We have isolated 45 different strains based on colony-morphological properties.

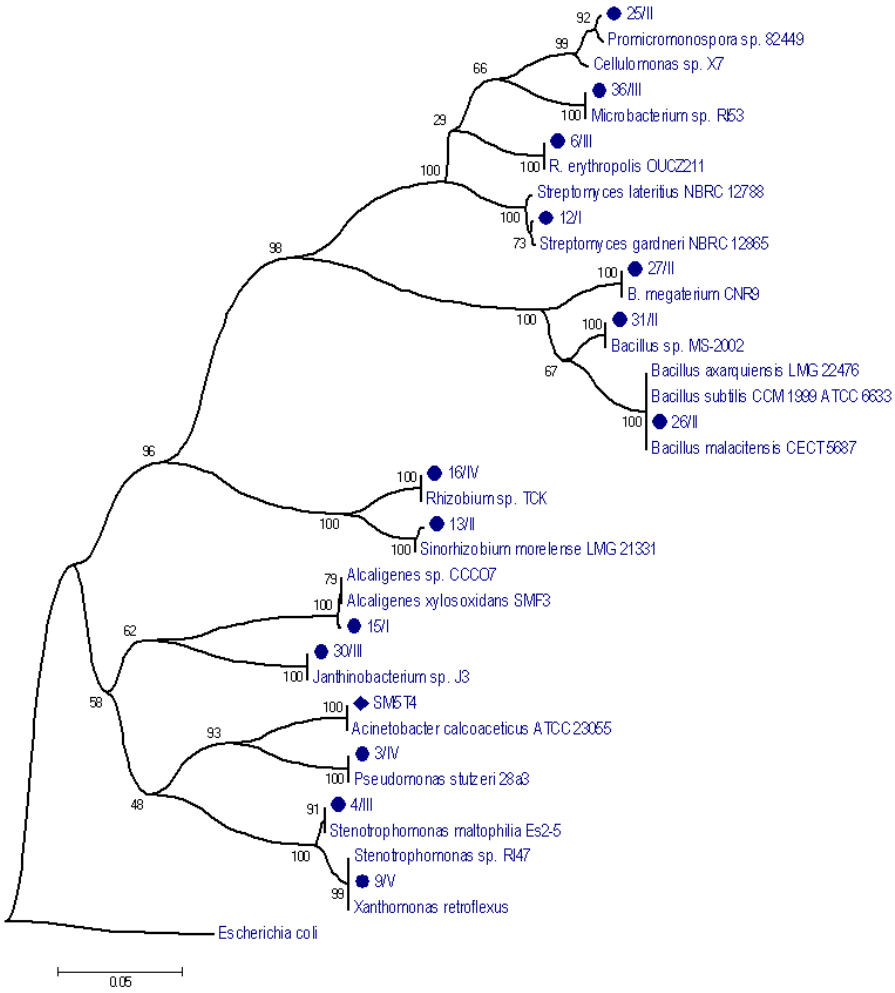

Fig.3.1. . Phylogenetic tree with the isolated and determined strains and their nearest relatives. The tree was created by neighbor-joining method, based on partial 16S rRNA sequences. At branches the bootstrap values (500 repeat) are indicated.

The strains were grouped based on ARDRA analysis. One representative from each group was chosen and submitted to sequence analysis. The sequences was used to identify the strains by RDP II. internet database. All strains had more than 99% homology with already cultured strains. Based on the sequences of our strains and their nearest relatives we created phylogenetic tree (Fig. 3.1).

We investigated the diesel oil degrading capability of isolated and identified group representative strains. We observed, that strain 15/I (*Alcaligenes* sp.); SM5T4 (*Acinetobacter calcoaceticus*); 6/III (*Rhodococcus erythropolis*); 3/IV (*Pseudomonas stutzeri*); 36/III (*Microbacterium* sp.); 9/V (*Xanthomonas* sp.) are able to utilize diesel oil very well, but 4/III (*Stenotrophomonas maltophilia*) only with low rate.

Strains needed different initial *lag* phase period to adapt to the new environment and to start degradation. By the results we created three groups: the first contains strain 3/IV (*Pseudomonas stutzeri*); 36/III (*Microbacterium* sp.) and 9/V (*Xanthomonas* sp.), the second: strain 15/I (*Alcaligenes* sp.); SM5T4 (*Acinetobacter calcoaceticus*) and 6/III (*Rhodococcus erythropolis*). To third group belongs strain 4/III (*Stenotrophomonas maltophilia*).

Diesel oil degrading strains were also able to utilize at least one analyzed aromatic compounds as carbon source. Benzene and toluene were degraded by all strains, mixture of xylene isomers were also utilized by all strains except 15/I – *Alcaligenes*. We have found higher differences at phenol, phthalate and chlorinated aromatics degradation. Phenol can not be utilize by strain 15/I – *Alcaligenes*, 9/V – *Xanthomonas*, and 4/III – *Stenotrophomonas*. We have similar results at phthalate, the only one strain, the 15/I – *Alcaligenes* was able to degrade this compound. All chlorinated aromatic compound were degraded only by strains 6/III – *Rhodococcus*, SM5T4 – *Acinetobacter* and 36/III – *Microbacterium*.

3.1.1 Determination of aromatic ring cleaving enzyme activity

Two strains were chosen, the *Rhodococcus erythropolis* 6/III and *Acinetobacter calcoaceticus* SM5T4, which were able to grow well in liquid media. The reference strain was the *Pseudomonas putida* DSM50202.

Under benzene induction only the "*orto*" metabolic pathway was activated, i.e. only the C12O enzymes show activation. That result was expected based on literature data. The activation order, after 168 hour incubation, was the following: *Pseudomonas putida* DSM50202 > *Rhodococcus erythropolis* 6/III > *Acinetobacter calcoaceticus* SM5T4.

Based on literature, under toluene induction the activation of "*meta*" metabolic pathway was expected (Farhadian et al. 2007). Contrary, at all of three strains the "*orto*" pathway was activated. The order of enzyme activity was the same: *Pseudomonas putida* DSM50202 > *Rhodococcus erythropolis* 6/III > *Acinetobacter calcoaceticus* SM5T4. We also observed lower enzyme activity at toluene than benzene degradation in case of all strains.

3.1.2 C12O gene detection using PCR technique

At primer design we try to detect the required gene from our newly isolated strains, so we emphasized the group-specific primer design. Accordingly, we designed genus-specific primers for *Rhodococcus* and *Acinetobacter* species.

To show the diversity of C12O gene, we created phylogenetic tree from sequences originated of genes from our newly isolated strains and from GeneBank database.

The phylogenetic tree shows (Fig. 3.2), that the Gram positive and Gram negative bacteria split into two group based on their catabolic genes. We can observe that the clustering based on Gram dyeing is also very similar, if we compare this phylogenetic tree to the tree based on the same strains' 16S rRNA gene (Fig. 3.3).

3.2 Enhancing hydrocarbon degradation using cyclodextrin

At this experiment we analyzed how the amount of degraded diesel oil increases if HPCD is added to the medium.

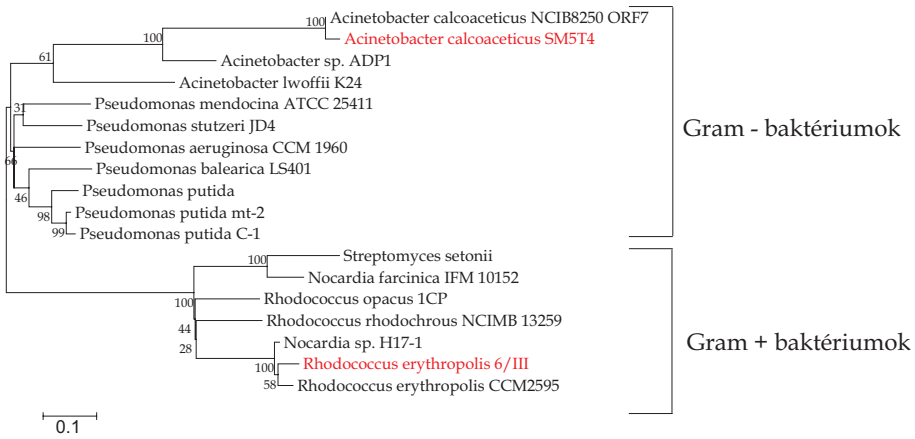

Fig.3.2. . Phylogenetic tree based on C12O catabolic gene. Sequences identified by us are signed with red color, the black ones originated from GenBank. Phylogenetic tree was created by neighbor-joining method. At branches are represented the bootstrap values (500 repeat)

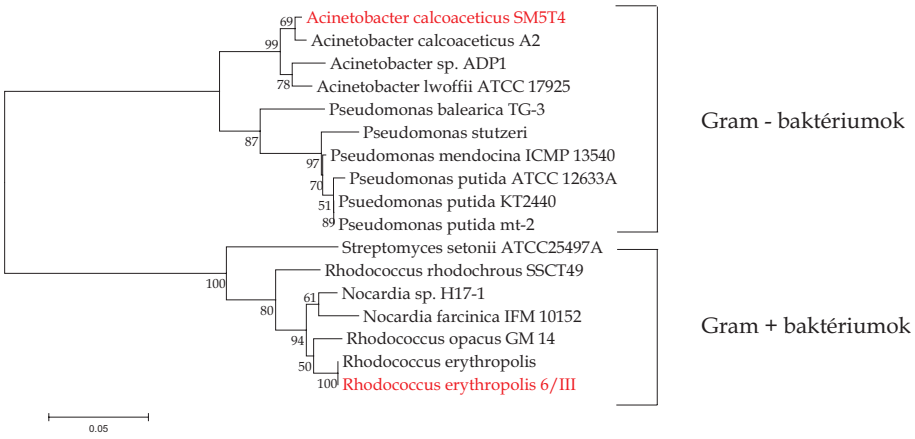

Fig.3.3. . Phylogenetic tree based on 16S rRNA gene. Sequences identified by us are signed with red color, the black ones originated from GenBank. Phylogenetic tree was created by neighbor-joining method. At branches are represented the bootstrap values (500 repeat)

More CO_2 created in the three times more (1200 mg/L) diesel oil containing medium that at control (400 mg/L) or two times more (800 mg/L) diesel oil concentration (125 and 204 mg, respectively). At the same time, from the data it was also concluded, that the three times more amount of diesel oil did not increase the CO_2 production at three times.

The higher amount of diesel oil added to the medium has delayed the start of degradation processes, so supposedly it inhibited the microbe. In the next period, the degradation rate was approximately the same at all case. After that, we can observe the slow of degradation rate at all the three diesel oil concentration. At control and 2x concentration, this slower rate was observed from hour 48. and 96., respectively. At 3x diesel oil concentration this period started from hour 144.

After cyclodextrin addition, the CO_2 production has increased at all case. The smallest increase of organic compound utilization was at 1:1 molar cyclodextrin : diesel oil ratio, the highest rate at 1:2 molar ratio. Not only the amount of CO_2 production has changed, but the timing of degradation

rate. The cyclodextrin addition applied in 1:0.5 and 1:2 molar ratio has increased the initial rate of degradation and also the amount of degraded compounds. The maximum of degradation rate was at hour 96 in case of 1:0.5 molar ratio and after that the CD utilization as carbon source was detected. At 1:2 molar ratio, the maximum was at hour 48. and after that point no significant CO_2 production was detected.

A certain amount of cyclodextrin effects the diesel oil degradation at different way. At 3x diesel oil concentration the *lag* phase would be longer and the CO_2 production decreased significantly. At the other two concentration (1x, 2x diesel oil concentration) the *lag* phase would be shorter and more intensive degradation rate was described. Summary, the highest initial degradation rate was observed at 2:1, 1:2 and 1:0.5 molar ratio, significant higher effectiveness was detected at control, i.e. low diesel oil concentration.

Enhancing diesel oil degradation capacity by HPCD in case of microbial community, the cyclodextrin has shortened the *lag* phase and increased the CO_2 production at 100 mg/l diesel oil concentration. However we could detect changes at higher concentrations to control microcosms, that was not significant. It can explain with the amount of the added cyclodextrin, which has 1:1 molar ratio at 100 mg/L diesel oil concentration.

At community analysis the effectiveness of HPCD was compared to the effectiveness of other, generally used surfactant, the Tween 80. We have observed, that Tween 80 was used as primary carbon source, it resulted the slowing of diesel oil degradation. Presumably, the starch could not used at alternative carbon source, because at that microcosms the diesel oil degradation rate was similar to controls.

The amount of biological degraded benzene and toluene has increased by cyclodextrin addition at strain *Acinetobacter calcoaceticus* SM5T4, in all case. The microorganisms can utilize the benzene from the water phase only (Prenafeta-Boldu et al. 2002), so the water solubility can be limitation factor. If we add HPCD to the medium, the apparent solubility of benzene increases through molecular complex creation with HPCD molecules.

A certain amount CD is not able to create molecular complexes above a certain amount of benzene, so the more and more benzene concentration resulted decreasing effectiveness also at HPCD treated microcosms. The same tendency was observed also at toluene with the only difference, that the effect of cyclodextrin was not so significant because of the much lower water solubility of the latter compound.

When we added the mixture of the two aromatic compounds, it was observed, that the biologically degraded contaminants rate increased to the same, one-component (only benzene or toluene containing) microcosms at same final concentration. The tendency has not changed, the HPCD increased the rate of biologically degraded hydrocarbons, but at higher concentrations the effect was not so significant as at lower concentrations.

3.3 Enhancing degradation of halogenated hydrocarbons in microcosms experiments

3.3.1 Comparing samples from different contaminated sites

The chlorinated aliphatic hydrocarbons can be electron acceptors at metabolic pathways in anaerobe environments. For that electron donors are needed. The ideal additives enhance dechlorinating organisms the most directly, promote their population specially.

The most surely we could detect *Dehalococcoides* species by nested PCR, and we detected 16S rRNA gene copies of *Dehalobacter restrictus* from 10^3 order of magnitude.

The contamination of the analyzed five sites was especially different. Three sites (*J*, *B* and some wells from site *T*) were highly contaminated, while the other two sites (*K* and *A*) contain the halogenated hydrocarbons in lower concentration. The sites also differ in contamination type, at site *K* the cDCE, at site *J* and *T* the TCE, while at site *B* the PCE was the main pollutant. The sites *A* and *B* have high soluble iron containing wells. Significant nitrate and nitrite concentration was only detected at sites *J* and *T*. The highest sulfate concentration was observed at wells from site *K*, the lowest concentration was measured in wells from site *J*.

We analyzed every sample for dehalorespiring organism present on the sites using genus-specific PCR. At site *T*, we have not detect any, analyzed dehalorespiring organisms. Against that, at site *B*, from all wells we could detect *Dehalococcoides* sp. at 10^5 order of magnitude and the *Desulfuromonas* sp. test was also positive in all case. The results of test for present of *Dehalobacter* sp. was not clear, but we suppose, that near to the detection limit these organisms were also present. At wells from site *A*, the *Dehalococcoides* sp. was detected only by nested PCR, which result means that amount of that microbes were between 10^5 and 10^2 in order to magnitude. After that, the *Desulfuromonas* sp. specific tests were also positive. The results from site *J* and *K* showed more heterogeneous picture. There were wells, which were positive after DHC1 test, others became positive after DHC2 test and there were wells also from what any *Dehalococcoides* sp. were not detected. The results were also similar at *Desulfuromonas* sp. At site *J*, 60% of the wells were positive, at site *K* this value was 40%. The *Dehalobacter* sp. was not detected at site *K* and at site *J* we detected those organisms only from well J18.2.

The composition of microbial community of each samples were analyzed by a fast fingerprint method, the T-RFLP. We observed from the biplot (Fig. 3.4) originated from principal component analysis which TRFs resulted the most significant differences at T-RFLP profiles. There are three important peaks along the samples, these are TRFs with length 384 bp, 386 bp and 201 bp. Sites *T* and *K* grouping TRF-386 bp mainly and sites *J* and *A* with TRF-201 bp. Wells from site *B* belongs to TRF-384 bp together with some wells from other sites.

3.3.2 Selection of the appropriate substrate in microcosms experiments

The aim of microcosm experiments was to select additive, which enhance biological degradation. We sampled two wells, the *K7* and *J18.2*. The biotic control (K) was without any substrates, at the other three type of microcosms we added *A*, *B* or *C* substrates in 500-500 mg/L dry matter concentration. The experiment was done during 318 days, sampling was on day 24., 54., and 318.

At microcosms *B* and *C*, where we observed significant degradation processes, we analyzed not only chemical but the biological parameters also.

The *Dehalococcoides* sp. were detected from the original samples,but on day 318 at microcosms *K* and *B* we could detect only by nested PCR. This result means, that the rate of these organisms decreased in the community. The other two dehalorespirating microbes were not detectable at initial *K7* samples, but were present in *J18.2* samples. From day 54. diminish their rate at all microcosms, they was not detectable, but on day 318. *Dehalobacter* sp. were detected again in all microcosms, and *Desulfuromonas chloroethenica* was detected in microcosms *J18.2/K*, *K7/B* and *K7/C*. The withdraw of these microbes can explain with the necessary disturbance of the community during microcosm setup. The continuous presence of *Dehalococcoides* sp. in the

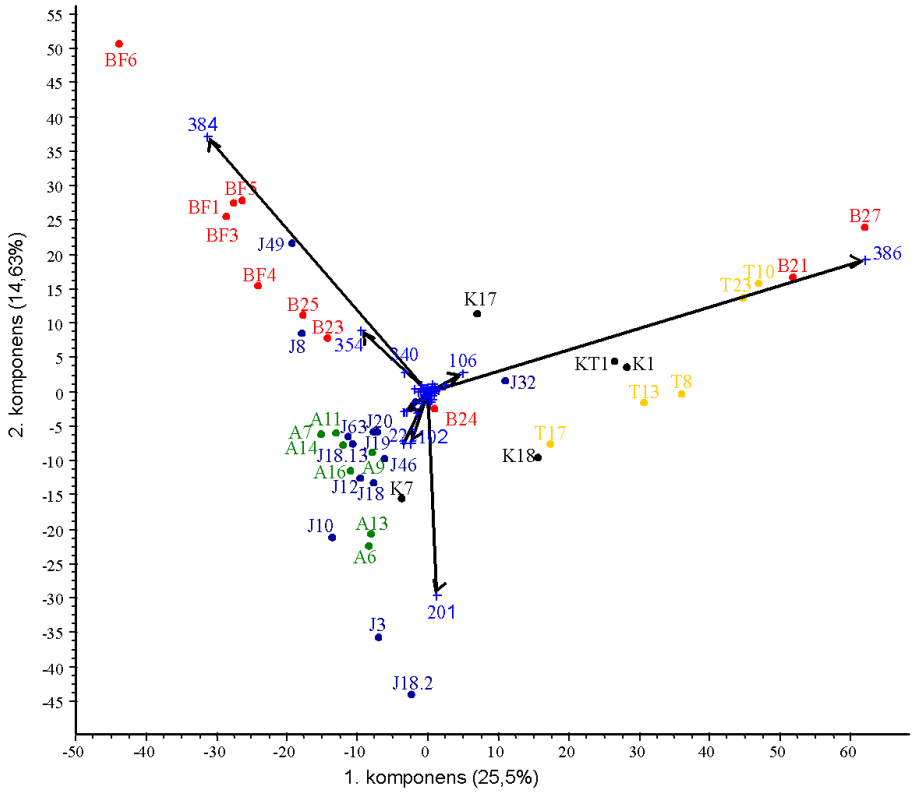

Fig.3.4. . Principal component analysis of T-RFLP pattern from groundwater samples. The colored, filled circles sign the samples, the crosses with numbers sign the TRFs.

microcosms suggest, that the detection of those microbes not sufficient condition for reduction of PCE to ethane, contrast to Hendrickson and coworkers (2002). Jointly analysis more dehalorespiring organisms can serve more precise results.

Based on Shannon diversity index, the treatments cause diversity increase, i.e. the number of the detectable microbes increased.

We followed the microbial community changes by fingerprint methods. On day 318. 16S rDNA based sample analysis was completed with 16S rRNA analysis. RNA from the successful microcosms *J18.2/C - day 318* was analyzed not only by T-RFLP, but also clone library was created. We have defined the TRF's of identified organisms, after that we could identify the most important peaks in community T-RFLP (Fig. 3.5).

To determine the dominant members of the other microcosm communities we have chosen the DGGE method to get quickly sequence information. DGGE was done from samples on day 318. based on 16S rRNA and 16S rDNA also.

The analysis of T-RFLP pattern by principal component analysis shows well (Fig. 3.5), that the microbial community of initial samples highly differ from the treated communities. The dominant organism in the initial samples was the organism with 202 bp length TRF, which was later identified (*Acidovorax* sp., DGGE band 27.). This taxon was changed to the organism with 386 bp length TRF in the untreated microcosms by day 318. (*Pseudomonas* sp., DGGE and 2,3,4,5,7 and 8). It is also evident, that certain treatments at samples from the two different wells did not developed the same microbial community. At microcosms *J18.2/C*, *K7/B* and *K7/C* some new organisms

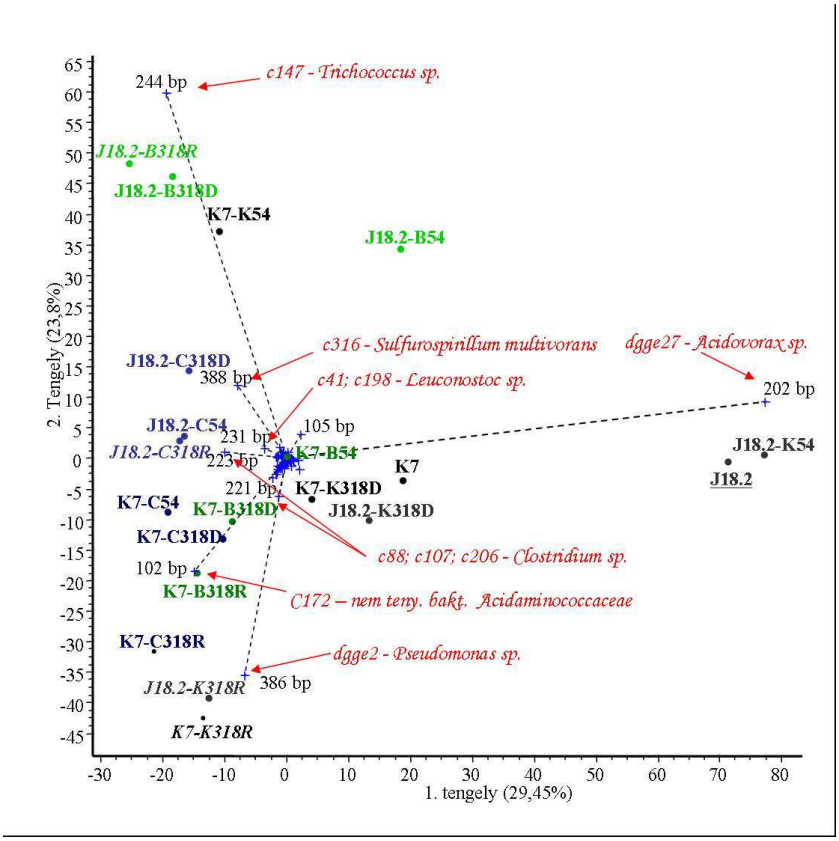

Fig.3.5. . Principal component analysis of T-RFLP pattern from microcosms

ha appeared, which are unambiguously representative of the community. These are microbes described by TRFs with length 102 bp, 221 bp, 223 bp (*Clostridium* spp., clone c88, c206 and c107) and 231 bp (*Leuconostoc* spp.). It is very fascinating results from principal component analysis, that microcosms `J18.2/B` has a unique, 244 bp length TRF (*Trichococcus* sp., clone c147). That organism is present also in other type microcosms, but only in those microcosms is dominant. The clone, described by 388 bp length TRF (*Sulfurospirillum multivorans*, clone c316) was found first chiefly in *J18.2/c* microcosms.

At `J18.2/K` microcosms, same as at initial samples, the *Acidovorax* sp. was the only and dominant strain detectable by fingerprint methods. After one year, at the end of the experiment there were species belong to *Acidovorax*, *Clostridium*, *Pseudomonas* and *Trichococcus* genera. The community structure did not changed significantly, the dominant organisms were the same.

At microcosms `K7` we detected significant sulfate reduction and the decrease of cDCE and VC concentration at the first 54 day. The increasing rate of sulfate reduction could happen because of developing anaerobe environment in microcosms in such degree what could not happen on site. After day 54. the metabolic processes were stopped. We could identify an organism, which has 98% similarity to *Tolumonas ausensis* (DGGE bands 13, 14 and 22). *T. ausensis* is a toluene producing bacteria, isolated from anoxic environment. At microcosm `K7/A` and `J18.2/A` the metabolic processes were similar to the biotic controls, the additive could not speed up the reductive dechlorination.

In case of the other substrates, the developed community highly differed from the biotic cont-

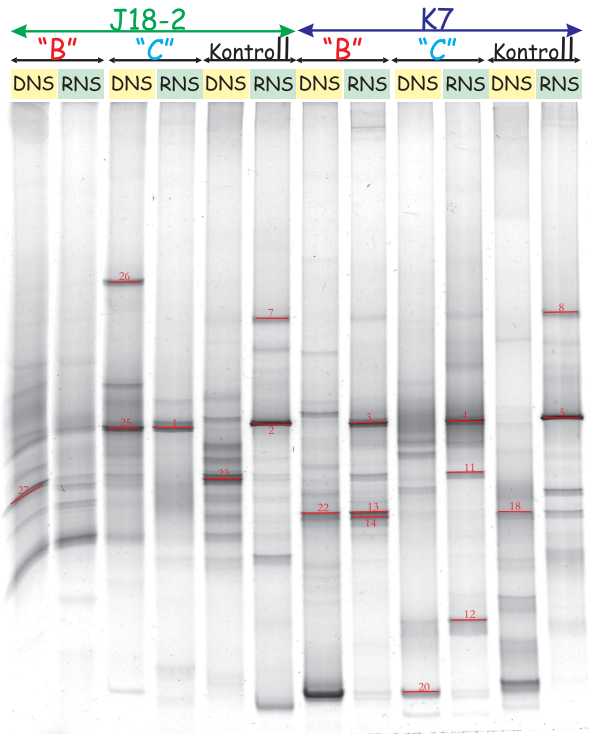

Fig.3.6. . RNA and DNA based DGGE analysis of microcosms *J18.2* and *K7* from day 318.

rol, the main difference appeared in the activity of fermenting bacteria. In microcosms treated with substrate *B* both fermenting *Trichococcus* sp. and dechlorinating *Sulfurospirillum multivorans* were present.

At microcosms *K7/B* we detected the reductive dechlorination process, the concentration of a typical product, the cDCE increased for day 54. After depletion of TCE, the cDCE concentration also decreased after day 155. Very important, that at this treatment the processes of sulfate reduction and of dechlorination happened parallel. The substrate depleted for day 155., what causeed stops all biological process. At those microcosms we also identified the bacterium similar to *Tolumonas ausensis*.

A treatment *J18.2/B* the dechlorination process was very similar to *K7* microcosms, but the degradation rate was higher. Presumably the most important electron donor was the molecular hydrogen for all the dechlorinating, sulfate-reducing and methanogen microbes. Band 1 and 25 from DGGE analysis were the same as the clone 10 from the clone library. This clone was detected both at DNA and RNA level, which mean, that this organism had a high biomass and had done active metabolism.

At treatment *K7/C*, TCE reduced to cDCE, but the concentration of the latter compound did not change. Substrate *C* also had depleted for day 155 and resulted stopping all biological process. Before that, the main process was the sulfate reduction instead dechlorination. From DGGE band 20, we identified a microbe similar to *Pedobacter heparinus* DSM2366 in 91%. *Pedobacter heparinus* is psychrophilic, utilize different sugars as carbon source and also able to degrade heparin.

At microcosms *J18.2/C* we detected all steps of reductive dechlorination, including ethane and ethene formation. Less than 10% of the contaminant was residual after day 155. The depletion

of sulfate, the pH and redox-potential changes slow those processes, and the only active biological process was reductive dechlorination. In microcosms more fermentating bacteria were present. More genera from *Clostridia*, the *Leuconostoc* (clone 41 and 198) species were identified and *Trichococcus* also did not disappear because of treatment. Only in this microcosms was present clone 10. This clone was present both at RNA and DNA level. We do not know about its function, it is non-cultured, the taxon started to describe nowadays.

In addition, *Sulfurospirillum multivorans* were also present. The band 26 from DGGE covered bacteria was only in these microcosms, but did not show any activity (there is no band in the same range in RNA samples). The closest relative is *Paludibacter propionicigenes* (96%). Clone 1 belongs to order *Myxococcales*. More detailed identification not possible, because there in no close relative between cultured microbes. In case of clone 3, also not exists any close, culture relative. The closest, cultured relative is *Victivallis vadensis* type strain, which is the only one cultured member of the group. The clone 172 has 92% sequence similarity with strain *Pelosinus fermentans* R7, so in that case the species identification was also unsuccessful. The clone 229 also belongs to family *Acidaminococcaceae*, but closer relative are with genus *Aminobacterium*.

All biological changes were confirmed by chemical results. We assume, that the high amount of organic substrate was degraded quickly by aerobe microbes, which resulted oxygen depletion and low redox potential. Hence microcosms are close systems, there no oxygen supply. After that the anaerobe biological processes started up. First step was fermentation the added organic substrate resulted molecular hydrogen and organic acid production. Latter caused the decrease of pH from day 54 in treated microcosms.

Treatment with substrate `B` and `C` developed different microbial community; certain microbes in either or other type of microcosms were present. The effectiveness of microcosms varied also, the pollutant degraded faster to ethene and ethane in microcosms treated with `C` substrate. This result can explain, that the two substrate are utilized in different way by the microbes or by different microorganisms and so resulted different metabolic products.

3.4 New scientific accomplishments

1. 15 new bacterial strains were identified and it was determined the range of aromatic hydrocarbons utilized as sole carbon source. Also their diesel oil degrading capacities were established based on carbon dioxide production.
2. The hydroxypropyl-β-cylcodextrin accelerated and enhanced diesel oil degradation by *Acinetobacter calcoaceticus* SM5T4 and microbial community isolated from contaminated sites. The addition of hydroxypropyl-β-cylcodextrin to media increased the degradation potential of benzene and toluene at *Acinetobacter calcoaceticus* SM5T4.
3. Substrate `C` enhanced best TCE degradation in bench scale microcosms experiment, so TCE depleted for day 54. This substrate can be appropriately used on field scale, also.
4. Diversity analysis of microbial communities from chlorinated aliphatic hydrocarbon contaminated sites concluded that the composition of microbial community is related to the characteristic chemical parameters of site.
5. It is not possible to design universal primers to detect enzymes for every bacteria, therefore we designed primers for specific genera, like *Pseudomonas*, *Acinetobacter* and *Rhodococcus*.

6. Next to the dehalorespiring bacteria, the fermenting bacteria play an important role in degradation of halogenated hydrocarbons, because of the conversion of complex organic compounds to simples organic substrate for dehalorespiring bacteria.

4. Conclusions and recommendations

Colony forming unit (CFU) estimation of the samples deriving from hydrocarbon contaminated sites leads to the conclusion that diesel oil can even promote the growth of microbial communities at an enough low concentration. CFU decreased above a certain concentration value, which can be accounted (among others) for the toxic effect of diesel oil components or the decrease of dissolved oxygen content. Several similar results were also reported in the literature (Singh és Ward 2004).

Two-third of the group representative strains was able to grow and to be metabolically active on diesel oil as sole carbon source in the OxiTop respirometric measuring system. Such strains were the 15/I (*Alcaligenes* sp.), SM5T4 (*Acinetobacter calcoaceticus*), 6/III (*Rhodococcus erythropolis*), 3/IV (*Pseudomonas stutzeri*), 36/III (*Microbacterium* sp.), 9/V (*Xanthomonas* sp.), and 4/III (*Stenotrophomonas maltophilia*).

All of the diesel oil degrading microbial strains were able to utilize benzene and toluene, as well as xylenes (except 15/I *Alcaligenes* sp. in the latter case) as carbon source. Results of phenol- and chlorinated aromatic hydrocarbon degradation were not yet so uniform, but e.g. strain 6/III (*Rhodococcus erythropolis*) was able to break down all chlorinated aromatic compounds.

A method, comprising enzyme activity tests, was elaborated for the investigation of aromatic compound degrading microbial groups, determining *(i)* the activated metabolic pathway and *(ii)* the mode on how the examined isolate degrades the actual contaminant compared to given reference strains . On the other hand, as an alternative to the culturing method, probes based on molecular biology technique (PCR) were designed, allowing an easy establishment of the presence of main genera (*Pseudomonas*, *Rhodococcus*, *Acinetobacter*) even without culturing. Base sequences of the detected genes showed that these genes may be primarily coded on chromosomes.

Cyclodextrin applied in 3:1 (oil:cyclodextrin) molar ratio inhibited the degradation of diesel oil. 2:1 ratio application increased the initial degradation speed significantly, but the effectiveness remained constant. The highest increase of effectiveness was obtained by 1:2 and 1:0.5 ratio cyclodextrin application. The simultaneous success of the various ratios may probably be explained by the inclusion complexes formed with the cyclodextrin and other molecule types. HPCD for instance, builds the inclusion complexes with mono- and polyaromatic compounds on different ways, respectively (Szejtli 1988); however this hypothesis should be confirmed through several further investigations.

Biological remediation of pollutions caused by chlorinated hydrocarbons represents a serious challenge to professionals, among others due to the anaerobic metabolism of numerous microbes being able to effectively degrade such type of pollution; as the cultivation and investigation thereof is really hard and time-consuming. In the first step, a molecular biological method was developed for the routine detection of reductive dechlorination from the contaminated sites through characteristic microorganisms; such as *Dehalococcoides* sp., *Dehalobacter restrictus* and *Desulfuromonas chloroethenica*. With the help of this method, it was established that there were more types of dehalorespiring organisms deriving from more (monitoring) wells in the samples comprising higher amount of intermediates even initially, rather than in samples not bearing an active decomposition.

Today it is already known from the microbes having dealt with, that those accept only simple organic compounds and/or molecular hydrogen as electron donor (Smidt és de Vos 2004). It was therefore assumed that other members of the microbial community in the site, producing these simple compounds by their metabolism, also may influence the effectiveness of decomposition processes. The latter was investigated by microcosm experiments set up with different additives.

The most important result is that on-site interventions have been justified through the microcosm experiments. Two of the three selected additives, comprising more complex organic matter

admixture, were able to start decomposition processes in case of both samples. The main difference between the two samples was the sulfate concentration; and as a consequence of that, degradation processes were different. High sulfate concentration supplied sulfate-reducing organisms with electron acceptor, thus the microbes turned even more active with the help of the added electron donor, while inhibited dechlorinating bacteria. On the other hand, dechlorinating organisms could proliferate in case of sulfate lack, because the organic matter serving as electron donor was not used by any other kind of microbes (Révész et al. 2006). Another competitive group of dechlorinating organisms, the methanogens, primarily use molecular hydrogen as electron donor (Zinder 1993), however methane genesis was experienced after starting decomposition alone, therefore it is probable that the molecular hydrogen evolved only afterwards in adequate quantity from the added organic matter.

A detailed community analysis was done for the active (working) microcosms. Generally speaking, community structure in all cases changed significantly in response to the treatments. Additives induced the predominance of fermentative microbe groups decomposing complex carbon sources (e.g. *Leuconostoc*, *Trichococcus*, *Clostridium*, *Acidaminococcaceae*). Dechlorination processes start with the activation of dehalorespiring organisms utilizing simple organic acids and molecular hydrogen. The latter are formed from complex organic compounds by the fermentative organisms through different metabolic pathways. Nevertheless fermenting microbe groups can replace each other; their taxonomic classification is irrelevant from process viewpoint. Though the original endemic microbiota determines the range of fermentative bacteria proliferating on the additive substrates, yet this feature not influences the success of biodegradation process. It is questionable that how does the end-product of fermentation, accumulating in the environment, influence decomposition processes. Assumingly, this may play a bigger role in the successful TCE degradation, since dehalorespiring organisms are able to utilize only a narrow range of the possible electron donors, although the documentation thereof requires further investigations.

This work highlights also the important point, that there live many microbe species at the contaminated sites, which are not only non-cultivated but they have not any close relatives among the cultured microorganisms. This observation attracts attention to two things: *(i)* only very few microbes are cultivated although detailed analysis (e.g. metabolic analysis) are based on cultivation method indeed *(ii)* toxic territories, like trichloroethylene contaminated soil, could have microbiota with active metabolic processes.

5. Publications related to the thesis

- Articles in refereed books

    1. Sipos, R., Székely, A., **Révész, S.**, Márialigeti K., Addressing PCR Biases in Environmental Microbiology Studies. Chapter 3. *Bioremediation Methods and Protocols, in series: Methods in Molecular Biology* (Vol. 599) editor: S. P. Cummings, Humana Press, ISBN: 978-1-60761-438-8 *(in press)*

- Refereed publication in journals

    1. Táncsics, A, Szoboszlay, S., Kriszt, B., Kukolya, J., Baka, E., Márialigeti, K., **Révész, S.** (**2008**) Applicability of the functional gene catechol 1,2-dioxygenase as a biomarker in the detection of BTEX-degrading Rhodococcus species *Journal of Applied Microbiology* 105 (4) 1026-1033 (IF 2.501)
    2. Sipos, R., Székely, A. J., Palatinszky, M., **Révész, S.**, Márialigeti, K., Nikolausz M. (**2007**) Effect of primer mismatch, annealing temperature and PCR cycle numbers on 16S rRNA gene targeting bacterial community analysis. *FEMS Microbiology Ecology* Vol. Issue 2: 341-350. (IF: 2.787)
    3. **Révész, S.**, Sipos, R., Kende, A., Rikker, T., Romsics, Cs., Mészáros, É., Mohr, A., Táncsics, A., Márialigeti, K. (**2006**) Bacterial community changes at TCE biodegradation detected in microcosm experiments. *International Biodeterioration and Biodegradation* 58: 239-247 (IF: 1.209)
    4. Nikolausz, M.; Sipos, R.; **Révész, S.**; Székely, A. J.; Márialigeti, K. (**2005**) Observation of bias associated with re-amplification of DNA isolated from denaturing gradient gels. *FEMS Microbiology Letters*, 244 (2):385-390 (IF:2.057)

- Articles in vetted periodicals

    1. **Révész, S**., Sipos, R., Romsics, Cs., Kende, A., Rikker, T., Mészáros, É., Táncsics, A., Márialigeti K. (**2005**) Bacteria and Archea community changes at TCE biodegradation as revealed in microcosm experiments. *3rd European Bioremediation Conference, Chania, Crete, 4-7. July*.

- Articles in vetted periodicals in Hungarian.

    1. **Révész Sára**, Romsics Csaba, Pór Tamás, Mansour Mashregi, A. A. Khalif, Márialigeti Károly (2002): Enhance diesel-oil degradation using hydroxypropyl-ß-cyclodextrin *Az MTA Szabolcs-Szatmár-Bereg megyei Tudományos Testületének X. - Közgyűléssel egybekötött - Jubileumi Tudományos Ülése; Közleménykötet*. Nyíregyháza, 28-29 September 2002.
    2. Pór Tamás, **Révész Sára**, Márialigeti Károly (2003): Effect of the dihydroxypropyl-ß-cyclodextrin on gasoline degradation ability of bacterial community isolated from contaminated soil. *MTA Szabolcs-Szatmár-Bereg Megyei Testületének XI. - Közgyűléssel egybekötött -Tudományos Ülése; Közleménykötet. Nyíregyháza, 26-27 September 2003.*

- Oral presentations

    1. **Révész, S.**, Romsics, Cs., Mészáros, É., Mohr, A., Rikker, T., Kende, A., Márialigeti K. (2006) Microbial community analysis of TCE contaminated sites and technology improvement for enhanced bioremediation. *ISEB-ESEB-JSEB 2006 International Conference on Environmental Biotechnology, Leipzig, Germany*, *9-13 Júl. 2006*.

- Poster presentations

1. **Révész, S.**, Pór, T., Masreghi, M., Romsics, Cs., Márialigeti K. (2002): The influence of hydroxypropyl- -cyclodextrin on hydrocarbon degradation of isolated hydrocarbon-degrading microorganisms. *International Conference on "Power of Microbes in Industry and Environment" Opatija, 7-9 Jún. 2002, Croatia.*
2. **Révész, S.**, Romsics, Cs., Pór, T., Sipos, R., Palatinszky, M., Márialigeti K. (2003): Stimulation of microbial degradation with cyclodextrins at BTX compounds. FEMS 2003 - 1st Congress of European Microbiologists. Ljubljana, Cankarjev Dom, 29 Jún. - 3 Júl., 2003, Slovenia.
3. Pór, T., **Révész, S.**, Romsics, Cs., Márialigeti, K. (2003): Comparison of the effect of three supplementary compounds on the ability of a microbial community isolated from contaminated soil to biodegrade diesel oil. *FEMS 2003 - 1st Congress of European Microbiologists. Ljubljana, Cankarjev Dom, 29 Jún. - 3 Júl., 2003, Slovenia.*
4. **Révész, S.**, Sipos, R., Kende, A., Rikker, T., Mészáros, É., Márialigeti, K. (2005) Bacterial community changes at TCE biodegradation in microcosms experiments. *IBBS-13; 13th International Biodeteriaration and Biodegradation Symposium. 4-9 Sept., 2005 - Madrid, Spain.*
5. **Révész, S.**, Romsics, Cs., Mohr, A., Kende, A., Rikker, T., Márialigeti, K. (2006) Bacterial community changes at TCE biodegradation in microcosms experiments. *11th International Symposium in Microbial Ecology - ISME-11, Vienna, Austria, 20-25 Aug. 2006.*
6. Táncsics, A., **Révész, S.**, Pór, T., Márialigeti K. (2006) Investigation of aromatic hydrocarbon degradation by bacteria with molecular and enzyme kinetical methods. *ISEB-ESEB-JSEB 2006 International Conference on Environmental Biotechnology, Leipzig, Germany, 9-13 July 2006.*
7. Romsics, Cs., **Révész, S.**, Mészáros, É., Mohr, A., Rikker, T., Kende, A., Márialigeti, K. (2006) Prokaryote diversity of TCE contaminated sites in Hungary. *ISEB-ESEB-JSEB 2006 International Conference on Environmental Biotechnology, Leipzig, Germany, 9-13 July 2006.*
8. Mohr, A., Mészáros, É., Rikker, T., Márialigeti, K., **Révész, S.** (2007) Monitoring the chemical and biological features of a TCE contaminated site in Hungary during in situ biostimulation process *BioMicroWorld 2007 - II. International Conference on Environmental, Industrial and Applied Microbiology Seville, Spain, 28. Nov. - 1. Dec. 2007.*
9. Varga, K., Mészáros, É., Mohr, A., Romsics, Cs., Rikker, T., Márialigeti, K., **Révész, S.** (2007) Prokaryote diversity of TCE contaminated sites in Hungary *15th International Congress of the Hungarian Society for Microbiology Budapest, July 18-20, 2007.*
10. Tóth, Á., Mohr, A., Mészáros, É., Romsics, Cs., Rikker, T., Márialigeti, K., **Révész, S.** (2007) Microbial community analysis of TCE contaminated sites and technology improvement for enhanced bioremediation *15th International Congress of the Hungarian Society for Microbiology Budapest, July 18-20, 2007.*
11. Varga, K., Mészáros, É., Mohr, A., Romsics, Cs., Tóth, Á., Rikker, T., Márialigeti, K., **Révész, S.**, (2008) Cultivation-based approaches to characterization of TCE contaminated sites *Congress Year 2008 of the Hungarian Society for Microbiology Budapest, Oct. 15-17, 2008.*

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