SZENT ISTVÁN UNIVERSITY

Ph.D. THESIS

Characterizing of microbe populations on hydrocarbon contaminated sites

Szabó István

Gödöllő

2011

Ph. D. school

Name:	**Ph.D. School of Environmental Sciences**
Discipline:	**Environmental Sciences**
Leader:	**Dr. György Heltai, D.Sc.**
	professor and head of department
	Szent István University,
	Faculty of Agricultural and Environmental Sciences
	Institute of Environmental Sciences
	Department of Chemistry and Biochemistry
Supervisor:	**Dr. Sándor Szoboszlay, Ph.D.**
	associate professor
	Szent István University,
	Faculty of Agricultural and Environmental Sciences
	Institute of Environmental and Landscape Management
	Department of Environmental Protection and Environmental Safety

....

Approval of School Leader **Approval of Supervisor**

TABLE OF CONTENTS

1. BACKGROUND

Crude oil and its derivatives are the most common contaminates everywhere in the world, including Hungary. The cleanup of groundwater and soil pollution caused by hydrocarbon compounds is the utmost challenge in environmental remediation. The proportion of contaminated sites are increasing worldwide despite environmental efforts against it. In the 21st century most of the operational functions in our artificial civilization require energy based on fossil resources including oil products. The growing demand for crude oil and the depletion of former oil fields lead to oil explorations in difficult-to-access or environmentally protected areas such as, nature reserves and seashores. Thus oil fields that were not worth exploiting previously due to environmental and ecological complications now are becoming possible oil production areas. Unfortunately, these changes mean that we will have to contend with oil contamination caused environmental disasters and it will happen not only in faraway areas but in Europe or even in Hungary.

When sites are polluted with oil, the environmental elements of the sites severely harmed as remediation requires considerable financial resources and energy, if the clean-up is even possible at all. A lot of different remediation technologies could be used to eliminate this kind of pollution. These technologies should be based on physical, chemical, thermal, isolating or biological methods. The main focus of the biological methods is the use of the xenobiotic degrading ability of natural resources, for example, procariotic microorganisms (bacteria), to eliminate contamination on the site. The simplest method of bioremediation is the utilization of natural attenuation that is the assessment and use of the hydrocarbon degrading capacity of the indigenous bacterial community. Thus the determination of composition of the bacterial community and diversity that is caused by oil contamination has a great importance for exploiting the possibility of natural attenuatation. The other widely used method for bioremediation is bioaugmentation which uses hydrocarbon degraders (bacterial strains) for the elimination of contaminants. The degradation capacity of these bacteria is verified by *in vitro* laboratory experiments.

It is well known that the key organisms of hydrocarbon degradation are not only bacteria but fungi, too. However, in my Ph.D. dissertation the biodegrading bacterial community of contaminated sites was examined only.

2. AIMS AND SCOPE

Three objectives were formulated in this PhD dissertation:

1. A statistical examination of the changes in a bacterial community and its diversity caused by hydrocarbon contaminations in polluted groundwater samples from Hungarian sites.
2. Operational taxonomic units (OTUs) based on molecular biological methods (T-RFLPs and clone libraries) that exclusively mark pollution in groundwater were looked for that could be used as biomarkers of contaminated or uncontaminated groundwater in environmental practice.
3. The isolation and identification of new hydrocarbon degrading bacterial strains from contaminated sites that are able to be used in bioaugmentation processes. Verification of hydrocarbon degradation ability of the isolated strains.

For the first and second objectives biological and geochemical monitoring was performed in three, hydrocarbon-contaminated, Hungarian sites between 2006 and 2008. Results of the bacterial community were established by the outcomes of several different analyses that were fulfilled with the samples from contaminated and background (from the results of pollutant concentration and groundwater flow) groundwater monitoring wells from these sites. Samples (n=31) were taken on different dates during monitoring for examination.

The total petrol hydrocarbon (TPH) and aromatic hydrocarbon (BTEX) concentrations of the samples were compared with the results of *Aliivibrio fischeri* toxicological tests by statistic analysis. It was assumed that the appearance of contamination would be traceable in the changes of bacterial diversity and community. The verification of this hypothesis was examined by the results of genetic methods (T-RFLP, clone libraries) and statistical analyses (Man Whitney U test, cluster analysis). Using the results of these genetic methods, biomarkers were looked for that could obviously and exclusively mark the samples by contamination type (contaminated or background).

For the third objective, different samples were taken from seven, oil-polluted, Hungarian sites. In the first step, gravimetrical and, with the results, gas-chromatographic analysis on a contaminated sample verified the biodegrading ability of the strains. The strains were identified on the species level by 16S rDNA sequencing in order to determine their pathogenic features for future use as members of inocula.

3. MATERIALS AND METHODS

3.1. Biological and geochemical monitoring

The phrase 'biological and geochemical monitoring' was used in this work as it was in the environmental practice in this Ph.D. dissertation. According to this denomination the methods that measured groundwater samples were: physical parameters (pH, temperature, dissolved oxygen, redox potential and conductance); chemical analytical parameters [TPH, BTEX and general water parameters (Wessling Hungary Ltd.)]; measurement of alternative electron acceptors; determination of cell number and hydrocarbon degrading cell number with most probable number (MPN) method, OxiTop™ soil respiration and ToxAlert 100™ *Aliivibrio fischeri* ecotoxicological test.

3.1.1. Sites and sampling

The three Hungarian, monitoring sites were filling stations in Biharkeresztes and Zalaegerszeg and, in Ópusztaszer, an oil product pipeline brake. Groundwater sampling was carried out on five different occasions in Biharkeresztes (22 February, 12 June, 28 August, 27 November, 2007 and 19 February, 2008), three times in Zalaegerszeg (18 May, 7 November, 2006 and 6 February, 2007), and also three times in Ópusztaszer (May 31, August 15, November 21, 2006). Based on the results of preliminary chemical analysis, samples were taken from contaminated and background sampling points from the same sites. Two contaminated (Z5, Z9) and one background (Z4) from Zalaegerszeg, two contaminated (OM7, OM8) and a background (OM3) from Ópusztaszer and one contaminated (BM7) and one background (BM1) samples were taken in Biharkeresztes from the monitoring wells of the sites. Samples are background not just because of the TPH and BTEX analytical results but from the direction of the previously determined groundwater flow. The groundwater flowed from the background wells to the contaminated ones. The groundwater wells were sampled using the instructions of MSZ 21464:1998 Hungarian standard.

3.1.2. Processing bacterial community DNA for terminal fragment length polimorphism (T-RFLP)

The common DNA of the bacterial community was taken during biological and geochemical monitoring and, as a first step, T-RFLP method was carried out to investigate the microbial diversity of the groundwater samples. Clone libraries were prepared from the bacterial community DNA for the taxonomical identification of terminal fragments (TRFs). The first step of this work was cloning, i.e. the isolation and amplification of individual DNA

compartments. Clone sequencing was carried out for the identification of individual members of the bacterial community. T-RFLP was performed again to determine the terminal fragment length of the individual cloned DNA fragments. From these results the fragment peaks of the individual bacterial clones were determined and these peaks were named in the common T-RFLP. The polimerase chain reaction (PCR) for the T-RFLP was performed by 5-carboxyfluorescein labeled 27f (5'-AGAGTTTGATCCTGGCTCAG-3') VIC and 519r (5'-GWATTACCGCGGCKGCTG-3') 16S rDNA primers. For the bacterial diversity analysis based on the T-RFLP, the primer amplified DNA of the bacterial community was digested by MspI restriction endonuclease enzyme. During the T-RFLP method the MspI digested PCR products were analysed by capillary gel electrophoresis.

3.1.3. Examination and processing of clone libraries

As stated previously, the PCR of the 16S rRNA genes from the bacterial community DNA was amplified by 27f and 519r primers. The PCR products were purified by QIAGEN PCR Clean up Kit and ligased into the p-GemT Easy vector (Promega Co.) and transformed into competent *Escherichia coli* cells. The vectors containing cells were separated by blue white screen selection. For the preparation of the clone libraries, the sequences were amplified by nested PCR method with the utilization of M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primers. After the nested PCR only the transformed 16S rDNA region was amplified by fluorescent labeled 27f VIC and 519r primers. The terminal fragments of the clones were examined by T-RFLP with the same MspI restriction enzyme. The 27f 519r amplified region of 16S rDNA of the clones was also used for determination on the species level by sequencing.

3.1.4. Statistical methods

The outcomes of the biological and geochemical monitoring were analyzed and compared using statistical methods. These methods were performed by STATISTICA 7 and Microsoft EXCEL (Microsoft Inc., Redmond, USA). The applied statistical methods were: Spearman rank correlation, Shannon diversity, T-probe, Mann-Whitney U test, and cluster and biomarker analysis.

3.2. Isolation of hydrocarbon degrading bacteria

Contaminated samples were taken from seven, hydrocarbon-polluted, Hungarian sites (Table 1). It was thought to be more possible to isolate hydrocarbon degrading bacteria from these kinds of samples.

Table 1. Samples from seven , oil-polluted, Hungarian sites for isolation biodegrading bacterial strains

Sampling site	**Site type**	**Source of the sample(s)**	**Number of samples**	**Name of samples**
Bátonyterenye	Petrol station	soil	1	CHBT
Sásd	Petrol station	groundwater	4	4S, 5S, 7S, 8S
Ópusztaszer	Pipeline brake	groundwater	2	OM-7, OM-8
Szarvas	Petrol station	groundwater	1	SZM5
Former soviet air base		biofilter	2	TBF1, TBF2
Zalaegerszeg	Petrol station	groundwater	2	Z5, Z9
Hungarian oil refinery		Industrial waste water	2	ZFM 19, ZFM23
Sum of sites: 7		**Sum of samples:**	**14**	

As the first step of the selection, 10 ml of the groundwater or 10 g from the biofilter and the soil samples were put in 100 ml OIR-III broth. This broth contained 2 cm^3 gasoline and crude oil mixture as the only carbon source. The aim of carbon source diminution was to select and multiply the bacteria in the solution that were able to tolerate or utilize (degrade) the hydrocarbon contamination. The bacteria were isolated on nutrient agar plates after the selective assay. Degrading ability of the isolated strains was analyzed by gravimetric degradation tests.

3.2.1. Identification of microbes

The strains with well degrading ability were determined on the species level by genetic methods, i.e. 16S rDNA sequencing analysis. The partial 16S rRNA gene sequences were compared with the items from on-line databases [NCBI GenBank and leBibi (Bio Informatic Bacteria Identification)]. If the compared strains showed more than 97% 16S rRNA gene sequence similarity it was concluded that they were the same at the species level.

3.2.2. Hydrocarbon degrading experiment on a contaminated groundwater sample

The hydrocarbon degrading ability of the isolated strains was attempted to be verified by *in vitro* complied solution and a contaminated groundwater sample. According to this expectation a self-developed degradation experiment was made with an extractable, petroleum, hydrocarbon (EPH)-contaminated groundwater sample from a Hungarian, oil-polluted site. During this experiment the pre-sterilized sample was inoculated with the selected hydrocarbon degrading strains. Degradation ability of TBF2/20.2, ZFM23.1 and 4S-8 strains was investigated *in vitro* with a contaminated environmental sample. The negative control of the experiment was pre-sterilized groundwater that was not inoculated but instead

incubated for five days under the same circumstances as the treated samples. For positive control the same groundwater sample was inoculated with strains from the members of an *in vivo* applied strain collection of biodegraders.

3.3. Detailed description of a new bacterium species

During the isolation of new oil degraders it was found that a bacterial strain, TBF2.20.2, showed only around 93% homology with the 16S rDNA sequences of identified strains in the reference databases (NCBI GenBank, leBibi). Consequently, this strain was an unknown and novel candidate bacterial species at the time. For a description of this new species other assays had to be completed:

- Genetic examinations: philogenetic analysis (almost complete 16S rDNS sequencing), guanine and cytosine analysis
- Phenotipical examinations: morphological description, fatty acid analysis of the cell membrane, respiratory lipoquinon analysis, polar lipid assays, electron microscopic analysis.

4. RESULTS AND DISCUSSION

4.1. Results of biological and geochemical monitoring on contaminated sites

Statistical relationships were attempted to be determined between the accredited laboratory measured contaminant (TPH, BTEX) concentrations and the results of ToxAlert 100™, *Aliivibrio fischeri* based ecotoxicological microbe test (ISO 11348) of the groundwater samples from the three polluted, Hungarian sites. Spearman rank correlation was calculated by STATISTICA 7 software (Table 2).

Table 2. Statistical comparison of contaminant concentrations and the Tox Alert 100™ calculated toxicity (EC_{50}) of contaminated and background groundwater samples (n=31) from three polluted, Hungarian sites with Spearman rank correlation by STATISTICA 7 software. The value of rank correlation is between -1 and +1. The closer value was calculated to +1 or -1, the closer the coherency was between the two variables. From the results of the statistical comparison significant and close coherency was found between the examined variables.

Contaminants	EC_{50}
Benzene	-0.617135
Toluene	-0.686002
Ethylbenzene	-0.650600
Xilenes	-0.763680
Other alkyl benzene	-0.766920
VAPH (C_6-C_{12})	-0.747321
VPH (C_5-C_{12})	-0.719477
VALPH (C_5-C_{12})	-0.798951
EPH (C_{10}-C_{40})	-0.809142
TPH (C_5-C_{40})	-0.812834

NOVEL SCIENTIFIC RESULT (I. thesis, from the results of chapter 4.1.): Significant and directly proportional coherency was verified between the *Aliivibrio fischeri*-based toxicological results and the TPH and BTEX analytical results at different collection times and from both background and contaminated groundwater samples from the three, hydrocarbon-polluted, Hungarian sites using Spearman rank correlation analysis.

Henceforth T-RFLP based Shannon diversity index was determined and significant coherency was attempted to be found between the diversity values and contaminant concentrations by Man Whitney U statistical analysis. Cluster analysis was also done based on the T-RFLP. From the results of the statistical analyses of samples from the three Hungarian, oil-polluted sites there was no significant definable coherency between the terminal restriction fragments (TRFs) based microbial diversity and the concentrations of contaminants. Moreover in most cases the T-RFLP profiles of same, but taken at different

times, samples were substantially varied (Table 3., Figure 1.). TRFs were used as *operational taxonomic units* (OTUs) in these analyses.

Table 3. The averages and the comparison of terminal restriction fragment length polimorphism (T-RFLP) analyses based Shannon indexes of the groundwater samples (n=31) from three Hungarian oil contaminated sites in connection with the contamination by the STATISTICA software calculated p values. Difference is significant if p value is over 0.05.

	Average of Shannon index	Background	Contaminated
Background	2.40		0.128736
Contaminated	2.80	0.128736	

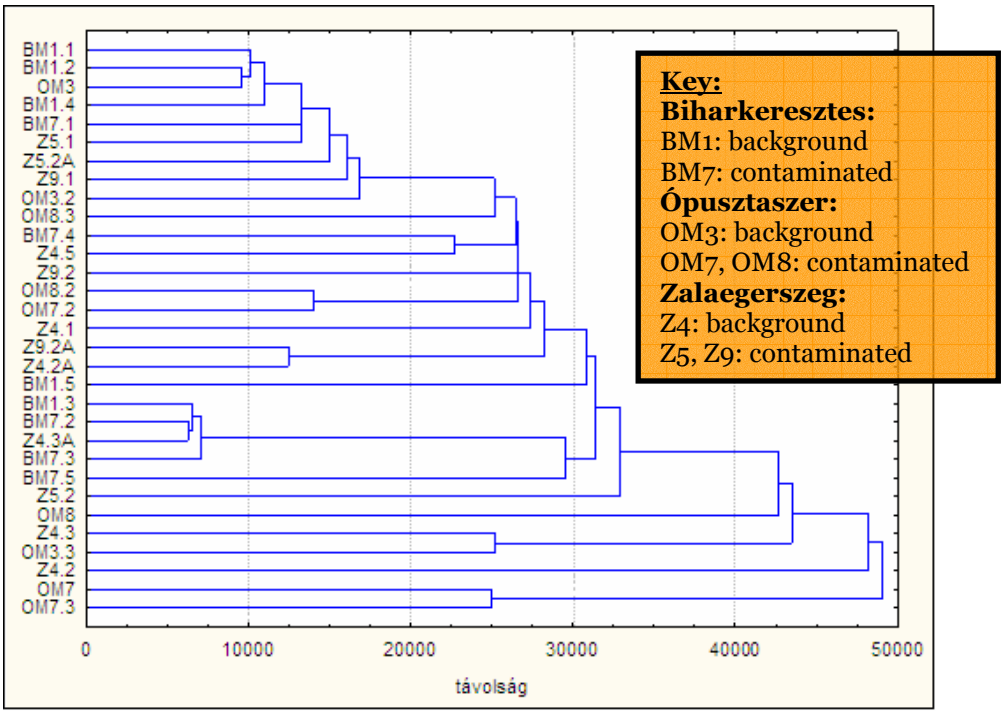

Figure 1. Comparison of bacterial communities of contaminated and background groundwater samples from three Hungarian hydrocarbon contaminated sites by cluster analysis based on the restriction fragment length polymorphism (T-RFLP) profiles. The name of the background and contaminated samples are found in the table's key. (Last number of the sample name marks the time of sampling: same number, same date)

4.1.1. Quest for biomarkers

Two TRFs, 123 and 202, were of acceptable precision to mark contaminated samples. These TRFs had high level specificity thus they gave minimal pseudo-positive, but, due to the low level of sensitivity, too many pseudo-negative results. This means that the appearance of the previously mentioned fragments in the T-RFLP profile do not unambiguously mark the background samples. In particular, the samples that were marked negative (background) by the TRFs could be positive (contaminated) in some cases, while at the same time most of the

positive (contaminated) marked samples were really positive (contaminated) in the analytical results.

4.1.2. Analyzing the bacterial community to the results of clone libraries

Clone libraries were made to fulfill the first and second objectives and examined around 100 clones per samples. At the time of writing, two samples from the Biharkeresztes site, two from Zalaegerszeg and three from Ópusztaszer have been prepared (Table 4).

Table 4. Summary of notable* 16S rDNA clone sequences from different samples based on the NCBI GenBank and leBibi database matches.

* Abundant clones and/or clones that possessed relevant metabolic properties (e.g. indicating hypoxic conditions of groundwater or degradation of aromatic hydrocarbons).

** Class of Proteobacteria marked with Greek letters.

In brackets: Total number of investigated clones.

Samples and number of clones	Closest relative on the species level*	T-RF (Msp)	Similarity (%)	Taxonomic class**
OM3.3 (115)				
8	*Albidoferax ferrireducens*	484-486	96.5 - 98.7	Beta (β-)
6	*Vogesella indigofera*	424, 484	96.7 - 100	Beta (β-)
5	*Pseudomonas jessenii*	484	99.1 - 100	Gamma (γ-)
4	*Rhodoferax antarcticus*	486, 490	97.4 - 99.1	Beta (β-)
4	*Pseudomonas anguilliseptica*	484, 487, 489	98.1 - 99.6	Gamma (γ-)
3	*Rhodoferax fermentans*	478, 479	98.5 - 99.1	Beta (β-)
3	*Leptothrix cholodnii*	132, 140	95.8 - 99.5	Beta (β-)
1	*Pseudomonas frederiksbergensis*	490	100	Gamma (γ-)
OM8.3 (112)				
32	*Malikia spinosa*	481,482	95.5 - 100	Beta (β-)
8	*Dechloromonas aromatica*	430, 431	99.3	Beta (β-)
7	*Gallionella capsiferriformans*	119	92.6 - 99.5	Beta (β-)
5	*Albidoferax ferrireducens*	488, 489	90.9 - 97.9	Beta (β-)
4	*Geothrix fermentans*	484	98.0	*Holophagae*
3	*Rhodoferax antarcticus*	486, 490	95.1 - 98.7	Beta (β-)
3	*Denitratisoma oestradiolicum*	485, 486	91.8 - 93.0	Beta (β-)
ZM4.1 (106)				
3	*Dehalobacter restrictus*	141, 489	99.0 - 99.5	Epszilon (ε-)
2	*Novosphingobium aromaticivorans*	150	98.0 – 98.5	Alfa (α-)
2	*Flavobacterium xinjaingense*	85	99.1 - 100	*Flavobacteria*
2	*Denitratisoma oestradiolicum*	486	91.7 - 93.3	Beta (β-)
1	*Sphingomonas dokdonensis*	150	97.9	Alpha (α-)
1	*Sphingomonas jaspsi*	150	97.0	Alpha (α-)
1	*Sphingomonas xenophaga*	162	99.9	Alpha (α-)
1	*Sphingomonas mucosissima*	150	96.3	Alpha (α-)
1	*Albidoferax ferrireducens*	487	98.7	Beta (β-)
1	*Azoarcus aromaticum*	79	98.5	Beta (β-)
1	*Hydrogenophaga flava*	162	98.8	Beta (β-)
1	*Malikia granosa*	293	96.0	Beta (β-)
1	*Flavobacterium johnsoniae*	85	97.9	*Flavobacteria*
1	*Pseudomonas jessenii*	137	100	Gamma (γ-)
ZM9.1 (104)				
4	*Asticcacaulis benevestitus*	436	98.1 - 98.5	Alpha (α-)
3	*Albidoferax ferrireducens*	488, 489	97.2 - 99.4	Beta (β-)
3	*Nevskia ramosa*	129, 131	92.5 - 97.7	Gamma (γ-)
2	*Oleomonas sagaranensis*	440	100	Alpha (α-)
2	*Curvibacter gracilis*	485, 487	98.7 - 99.0	Beta (β-)

Samples and number of clones	Closest relative on the species level*	T-RF (Msp)	Similarity (%)	Taxonomic class**
1	*Asticcacaulis excentricus*	435	97.5	Alpha (α-)
1	*Novosphingobium aromaticivorans*	150	98.5	Alpha (α-)
1	*Rhodoferax fermentans*	489	97.3	Beta (β-)
1	*Denitratisoma oestradiolicum*	481	90.5	Beta (β-)
1	*Polaromonas vacuolata*	480	95.3	Beta (β-)
1	*Hydrogenophaga atypica*	472	99.9	Beta (β-)
BM1.1 (68)				
6	*Malikia spinosa*	483	99.4 - 100	Beta (β-)
4	*Acidovorax delafieldii*	481	97.7 - 100	Beta (β-)
4	*Pseudomonas anguilliseptica*	487	99.5 - 99.9	Gamma (γ-)
3	*Acinetobacter lwoffii*	488	98.9 - 99.2	Gamma (γ-)
3	*Herbaspirillum sp.* AKB-2008-TE24	486	95.1 - 99.5	Beta (β-)
2	*Acidovorax defluvii*	481	98.8 - 99.5	Beta (β-)
2	*Acinetobacter beijerinckii*	488	99.5 - 100	Gamma (γ-)
1	*Hydrogenophaga atypica*	n/a	99.5	Beta (β-)
BM7.1 (86)				
12	*Malikia spinosa*	481, 482, 483	99.6 - 100	Beta (β-)
9	*Albidoferax ferrireducens*	484, 489	95.2 - 98.0	Beta (β-)
2	*Leptothrix cholodnii*	131	99.5	Beta (β-)
2	*Pseudomonas putida*	486	99.3	Gamma (γ-)
1	*Pseudomonas anguilliseptica*	487	99.9	Gamma (γ-)
1	*Rhodoferax antarcticus*	486	95.7	Beta (β-)

NOVEL SCIENTIFIC RESULT (II. thesis by the results of chapter 4.1.): By the results of statistical analyses (Man Whitney U test, cluster analysis) of the T-RFLP profiles of background and contaminated groundwater samples (n=31) from three Hungarian hydrocarbon polluted sites, significant coherency was not verified between the concentration of pollutants and bacterial diversity.

NOVEL SCIENTIFIC RESULT (III. thesis by the results of chapter 4.1.): Based on 16S rDNA clone library analysis of groundwater samples from three Hungarian hydrocarbon polluted sites, *Betaproteobacteria* dominated the community. These results were published in an international publication (Táncsics et al., 2010).

4.2. Quest for new hydrocarbon degraders

Forty-seven strains were isolated from seven, oil-polluted, Hungarian sites. Correlated to the control, six of these isolated strains reduced the concentration of the gasoline and crude oil mixture concentration by more than 20% in three, repeated, gravimetric analyses (Table 5).

Table 5. The hydrocarbon degrading ability of the seven, oil-contaminated ,Hungarian-site isolated strains from the results of a three times repeated gravimetric analysis.

Strains	Hydrocarbon degradation rate in percentage correlated to the control (average of the three times repeated results)
Control	0
TBF2/20.1	64.62
TBF2/20.2	21.09
ZFM 23.1	47.94
SZM5/4.2	44.94
OM 7-2	31.15
4S-8	33.10

4.2.1. Taxonomic identification of hydrocarbon degrading bacteria

The six bacterial strains with hydrocarbon degrading ability were identified on the species level by 16S rDNA sequencing analysis. The sequences were compared with the results of international databases (NCBI GenBank, leBibi, Table 6).

Table 6. The results of identification based on 16S rDNA sequencing of the six hydrocarbon degrading strains. Hydrocarbon degradation was examined by gravimetric analysis.

Strains	Result of identification	Similarity
TBF2/20.1	*Acinetobacter rhizosphaerae*	98.14%
TBF2/20.2	*Olivibacter sp. nov.*	93.25%
ZFM 23.1	*Rhodococcus erythropolis*	99.60%
SZM5/4.2	*Pseudomonas mandelii*	99.31%
OM 7-2	*Rhodococcus erythropolis*	99.55%
4S-8	*Rhodococcus ruber*	100.00%

4.2.2. Results of the hydrocarbon degrading experiment

An *in vitro* degradation experiment was performed with an EPH contaminated groundwater sample from a Hungarian site. From the results shown in Table 7, the examined, novel isolated bacterial strains were able to considerably decrease the concentration of aliphatic hydrocarbon contaminations (TPH).

Table 7. Analytical results of TPH contaminants after the degradation experiment on a hydrocarbon polluted groundwater sample (measured by: Wessling Hungary Kft.). Comparison of novel isolated strains and the members of an *in vivo* bioremediation applied bacterial collection.
* Members of i*n vivo* bioremediation applied bacterial collection
** Novel isolates
SK: sterilized control with no inoculation

Component	NCP3 - AK38*	AK40 - CHB15*	AK38 - AK35*	TBF2/20.2**	ZFM23.1**	4S-8**	SK
Unit	$\mu g/dm^3$						
TPH (C5-C40)	1370	345	648	592	654	559	2530
TPH degradation percentage	45.85%	86.36%	74.39%	76.60%	74.15%	77.90%	-

4.3. The taxonomic classification of TBF2/20.2 strain

The TBF2/20.2 strain from novel isolates was not able to be identified taxonomically on the species level because of low similarity (93% with *Olivibacter ginsengisoli*) of the examined 16S rDNA sequence. Therefore, it was considered a novel species candidate. Other examinations of the strain and its genetically closest relatives (*Olivibacter sp.)* which were shown in 'Materials and Methods,' had to be completed to bear out this result. Results are shown in Tables 8 and 9 and in Figures 2-4.

Table 8 Distinctive characteristics of strains TBF2/20.2^T, *Olivibacter soli* Gsoil 034^T, *O. ginsengisoli* Gsoil 060^T, *O. terrae* Jip13^T and O. *sitiensis* AW-6^T

Key: +: growth/assimilation; -:no growth/assimilation; +w: weak; G+C: Guanine and Cytosine

Characteristic	TBF2/20.2	*O. soli* Gsoil 034	*O. ginsengisoli* GSoil 060	*O. terrae* Jip13	*O. sitiensis* AW-6
Growth at 5ºC	+	-	-	-	+
Growth at 15ºC	+	+	+	+	+
Growth at 30ºC	+	+	+	+	+
Growth at 37ºC	+	+	-	-	+
Growth at 42ºC	+	-	-	-	+
Growth with 4% NaCl	-	+	+	+	-
Growth on MacConkey agar plates	-	-	-	-	-
Enzymatic activity:					
Gelatin hydrolysis	-	+	+	-	-
Esterase (C4)	-	-	+	+	+
Esterase lipase (C8)	-	-	-	+	-
Naphtol-AS-BI-phosphohydrolase	+	-	-	-	+
β-galactosidase	+	-	-	-	-
β-glucoronidase	-	-	-	+	-
α-mannosidase	+	-	-	-	-
α-fucosidase	-	+	-	-	-
Assimilation of:					
D-Adonitol	+	+	-	-	-
D-Lyxose	+	+	-	-	-
D-Mannitol	-	+	-	-	-
D-Melezitose	+	+w	-	-	-
D-Turanose	+	+w	-	+w	-
Inositol	-	-	+	-	-
L-Arabinose	+	+	-	+w	-
L-Histidine	-	-	-	-	+
N-Acetyl-glucosamine	+	-	-	-	-
DNA G+C content (mol%)	41.2	39.2	43.6	40.6	45.6

Table 9 Cellular fatty acid components of strains TBF2/20.2^T, *Olivibacter soli* Gsoil 034^T, *O. ginsengisoli* Gsoil 060^T, *O. terrae* Jip13^T and *O. sitiensis* AW-6^T.

Values are percentages of total fatty acids; -, not detected; ECL, Equivalent chain length.

*Unknown: Unknown fatty acid component; ECL: equivalent chain length
** Summed feature was not able to be separated by Microbial Identification System (Microbial ID)
Summed feature 1: iso-$C_{15:1}$ H and/or $C_{13:0}$ 3OH.
Summed feature 3: iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7c.
Summed feature 4: iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

Fatty acid	**TBF2/20.2**	***O. soli* Gsoil 034^T**	***O. ginsengisoli* GSoil 060^T**	***O. terrae* Jip13^T**	***O. sitiensis* AW-6^T**
$C_{14:0}$	1.57	1.12	1.59	2.10	0.34
$C_{16:0}$	**4.54**	**4.88**	**5.30**	**4.22**	**4.54**
$C_{14:1}$ω5c	0.24	-	-	-	-
$C_{16:1}$ω5c	0.54	1.53	0.58	-	1.17
$C_{15:1}$ω6c	0.30	-	-	-	-
iso-$C_{15:0}$	**39.43**	**29.60**	**28.24**	**27.61**	**31.18**
anteiso-$C_{15:0}$	0.30	0.66	-	0.32	-
iso-$C_{15:0}$ 3-OH	1.94	2.51	2.51	2.49	1.87
iso-$C_{15:1}$ F	0.13	-	-	-	-
iso-$C_{16:0}$ 3-OH	0.24	0.34	0.39	-	-
iso-$C_{17:0}$	0.97	0.13	0.37	0.43	0.31
iso-$C_{17:0}$ 3-OH	**14.46**	**12.86**	**13.97**	**12.34**	**10.97**
iso-$C_{17:1}$ω9c	1.88	1.16	0.96	1.29	1.75
C $_{14:0}$ 2-OH	0.68	0.27	0.31	0.34	-
C $_{15:0}$ 2-OH	0.26	-	-	-	-
C $_{16:0}$ 2-OH	1.68	2.23	2.62	2.87	0.59
C $_{16:0}$ 3-OH	0.7	2.18	2.85	1.64	0.63
C $_{16:1}$ 2-OH	0.14	-	0.33	0.48	-
C $_{17:0}$ 2-OH	-	0.33	-	-	-
$C_{17:0}$ CYCLO	0.86	-	-	-	-
Unknown*					
ECL 13.565	0.71	0.64	0.80	0.80	1.21
ECL 16.582	1.18	0.96	0.91	0.92	0.81
Summed feature**					
1	0.33	-	-	-	-
3	**26.01**	**38.42**	**38.26**	**42.16**	**44.23**
4	0.38	0.19	-	-	0.41

```
   1 gctcaggatg aacgctagcg gcaggcctat acatgcaagt cgaacgggat cgggggcttc
  61 ggtccccgtg agagtggcgc acgggtgcgt aacgcgtgag caacctgccc gtcccagggg
 121 gatagcccgg agaaatccgg attaataccg catgagaatg caggtccgca tgggccttcg
 181 tttaaatatt catagggggac ggatgggctc gcgtgacatt agcttgttgg cgggggtaacg
 241 gcccaccaag gcgacgatgt ctaggggctc tgagaggaga aacccccaca ctggtactga
 301 gacacggacc agactcctac gggaggcagc agtaaggaat attggtcaat gggggggaacc
 361 ctgaaccagc catgccgcgt gcaggaagac ggccctacgg gttgtaaact gcttttgcac
 421 gagaataacc ccggtatgta taccgggctg aatgtaccgt gagaataagg atcggctaac
 481 tccgtgccag cagccgcggt aatacggagg atccgagcgt tatccggaat tattgggttt
 541 aaagggtgcg taggcggcgc gctaagtcag gggtgaaaga cggtggctca accatcgcag
 601 tgcctttgat actggcgcgc ttgaattgta cctgaggtag gcggaatgtg gcaagtagcg
 661 gtgaaatgca tagatatgcc acagaacacc gattgcgaag gcagcttacc aaagtacgat
 721 tgacgctgag gcacgaaagc gtggggatcg aacaggatta gataccctgg tagtccacgc
 781 cctaaacgat gaacactcga tgtcggcgat agactggtcg gcgtccaagc gaaagcgtta
 841 agtgttccac ctgggagtac gcccgcaagg gtgaaactca aaggaattga cgggggcccg
 901 cacaagcgga ggagcatgtg gtttaattcg atgatacgcg aggaacctta cccgggcttg
 961 aaagttactg aattgtccag agatggacaa ggtccttcgg gacaggaaac taggtgctgc
1021 atggctgtcg tcagctcgtg ccgtgaggtg ttgggttaag tcccgcaacg agcgcaaccc
1081 ctatgtttag ttgccagcac gtcaaggtgg ggactctaaa cagactgcct gtgcaaacag
1141 agaggaaggc ggggacgacg tcaagtcatc atggcccttа cgtccggggc tacacacgtg
1201 ctacaatggg gggtacagag ggcagcgaca ccgcgaggtg aagccaatct cagaaagccc
1261 ctcacagttc ggatcggggt ctgcaactcg accccgtgaa gttggattcg ctagtaatcg
1321 cgtatcagca atgacgcggt gaatacgttc ccgggccttg tacacaccgc ccgtcaagcc
1381 atgaaagccg ggggtaccta aagcatgtaa ccgcaaggag cggtcagg
```

Figure 2. The almost complete 16s rDNS sequence of the novel species candidate TBF2/20.2 bacterial strain as it was published in the NCBI GenBank (GenBank No.: HM021721).

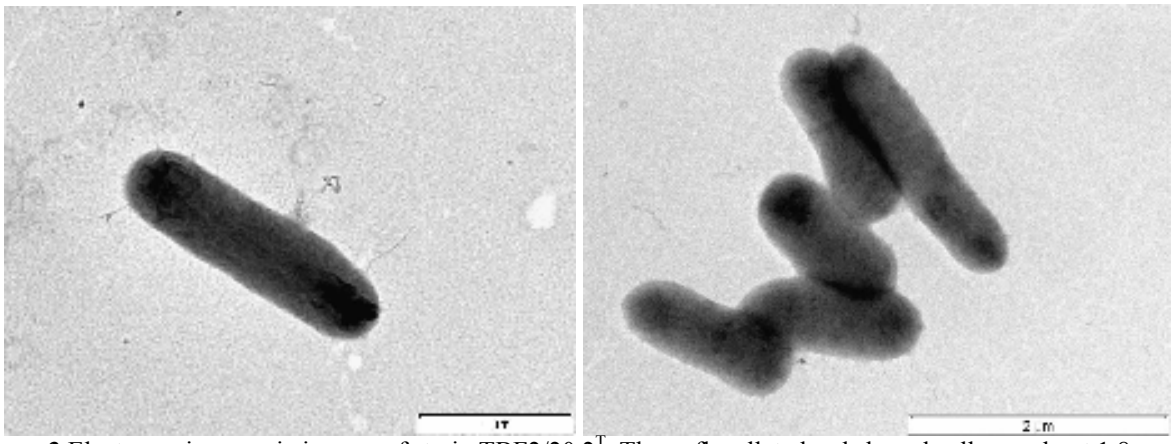

Figure 3 Electron microscopic images of strain TBF2/20.2^T. The unflagellated rod shaped cells are about 1.8 µm long and 0.6 µm in diameter.

From the results of major respiratory quinone analysis, MK-7 was predominant (98%) and a trace amount of MK-6 (2%) was also observed. The majority of MK-7 matches with the results of other *Olivibacter* species. Phosphatidylethanolamine (PE), aminophospholipids (PN1-PN2), phospholipids (PL1-PL3), lipids (L1-L4) and an atypical glycolipid (GL1) are present as polar lipids.

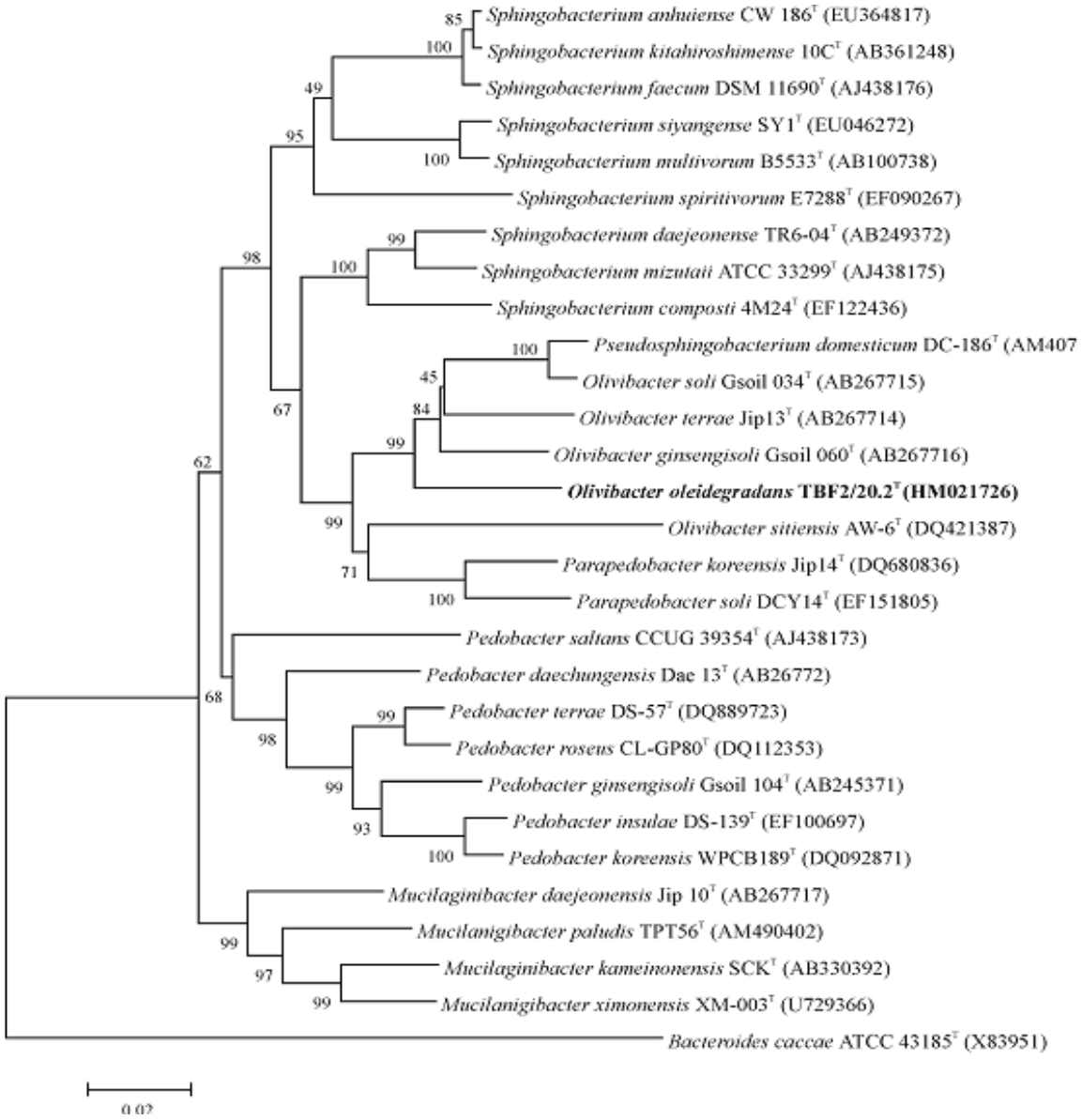

Figure 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the relative position of *Olivibacter oleidegradans* TBF2/20.2^T (edited with MEGA4 software).

According to the results of genetic and phenotypical examinations, it could be definitely declared that the novel isolated TBF2/20.2 bacterial strain is an agent of an unknown species. This species is a member of *Olivibacter* genus, but unequivocally distinct from the others by its morphological profile.

4.3.1. Description of Olivibacter oleidegradans

Olivibacter oleidegradans [o.le.i.de.gra'dans., L. n. *oleum* oil; L. part. adj. *Degradans* returning to the original order referring to the ability of the type strain to degrade oil (hydrocarbons); N.L. part. adj. *oleidegradans* capable of degrading oil (hydrocarbons)].

Cells are Gram-negative, obligate aerobe, non-spore forming, non-motile rods, devoid of flagella and 0.6 × 1.8-2.0 μm in size. Colonies grown on R2A agar plates are smooth, circular, convex, non-luminescent, opaque and yellowish in color within 5 days at 30 ºC. Good growth is observed on TGE, tryptic soy and nutrient agars but not on MacConkey agar. Growth is observed at temperatures between 15-45 ºC and pH 6.0-9.0. The optimal growth temperature and pH are 30-37ºC and pH 6.5–7.0. It is unable to grow in the presence of 2% NaCl. Enzymatic and assimilation tests for a detailed description were done. Major fatty acids are iso-$C_{15:0}$, summed feature 3 (consisted of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7c), iso-$C_{17:0}$ 3-OH and $C_{16:0}$. Menaquinone 7 (MK-7) is the predominant (98%) respiratory quinone, MK-6 occurs in small amounts (2%). Phosphatidylethanolamine (PE), aminophospholipids (PN1-PN2), phospholipids (PL1-PL3), lipids (L1-L4) and an atypical glycolipid (GL1) are present as polar lipids.

NOVEL SCIENTIFIC RESULT (IV. thesis by the results of chapter 4.3.): The TBF2/20.2, Hungarian, hydrocarbon contaminated, site originated, isolated bacterial strain is a novel species and a member of *Olivibacter* genus. Detailed description of the new species was done and it was named, due to its oil degrading ability, *Olivibacter oleidegradans*.

NOVEL SCIENTIFIC RESULT (V. thesis by the results of chapter 4.2. and 4.3.): The *in vitro* hydrocarbon degrading ability in the case of *Olivibacter* genus was primarily verified from the results of gravimetric and gas chromatographic (bio)degradation experiments. These results were published in an international publication (Szabó et al, 2011).

5. CONCLUSIONS AND SUGGESTIONS

According to the first objective of my dissertation, the bacterial diversity of groundwater samples from three, contaminated, Hungarian sites was examined. Conclusions about the bacterial community were established from the results of several analyses that were fulfilled by the above presented, different times taken, groundwater samples. On these sites (with a minimum of three times) biological and geochemical monitoring was performed to determine the changes caused by the examined contaminants on the bacterial community and diversity. During this study not only contaminated but background (from the results of pollutant concentration and groundwater flow) groundwater monitoring wells were sampled on the same sites and examined similarly. Significant and directly proportional coherency was verified between the *Aliivibrio fischeri* based toxicological (EC_{50}) and TPH and BTEX analytical results of contaminated and background groundwater samples of the examined sites. According to the above presented outcomes, **significant coherence was primarily verified between the analytical and toxicological results of contaminated and background groundwater samples of polluted sites.**

Based on this close coherency between the toxicological and analytical results, it was thought that the bacterial diversity or the composition of the community in the samples was highly influenced by the contamination concentration. According to this hypothesis, the contaminated and background samples (taken at the same time as for the toxicological examinations) were evaluated by T-RFLP method (like a genetic fingerprint). It was found by the results of statistical analyses (Man Whitney U test, cluster analysis) that there was no significant coherency between the TRFs (as OTUs) based bacterial diversity and concentration of contaminations. Moreover in most cases the T-RFLP profiles of same, but taken at different time samples were substantially variant of each other. **According to the analysis of contaminated and background groundwater samples from the three, Hungarian, hydrocarbon-polluted sites, it can be said that measured concentrations of the BTEX and TPH contaminants are not causing significant changes in the bacterial diversity or the composition of the community**.

Despite this result and in accordance with the second objective of this dissertation, TRFs were looked for that could exclusively mark the contaminated and background samples. The aim was to determine the fragments whose appearance in the profile marked the same type (contaminated or background) of samples and could be use as biomarkers. Only the TRFs were examined that were able to be found at a minimum of in 25% of all (n=31)

groundwater samples. From the results of all (contaminated and background) groundwater samples from the three Hungarian sites, two TRFs, namely 123 and 202, had acceptable precision to mark contamination. These TRFs had high level specificity, but a low level of sensitivity. These TRFs gave a few pseudo-positive results but, due to a low level of sensitivity, too many pseudo-negative results. As a result of the biomarkers quest it can be said that, from the statistical analyses, there were some fragments that are able to give information about the contamination of samples. **As expected, the later results of the primarily-used biomarkers quest with advanced number of groundwater samples could lead to TRFs that have as high a specificity as ours but higher sensitivity.** Development of this method could be advised from the results in this objective.

From the results of TRFLP profiles and the quest for biomarkers, it was thought that the taxonomic identification of the found notable TRFs was an important task to determine the domination of bacterial groups at the examined sites. Hence, in the case of the seven groundwater samples, clone libraries were prepared based on the isolated bacterial community 16S rDNA. Despite our expectations the TRFs that were found during the biomarker quest were not able to identify on the species level, but more information was obtained about the dominant community member microbes. According to the results, biodegrading bacteria (based on the publications) are able to be found in groundwater samples from all the sites, not only in the contaminated, but in the background samples, too. Presumably the composition of the bacterial community is primary not determined by the amount of hydrocarbon contaminants but instead by mostly one or more environmental effects or the interaction with each other or with the contaminants. Based on the results of publications it is thought that the oxygen supply of ambient media could have outstanding importance.

For the third objective, bacterial strains which are able to degrade hydrocarbons were isolated from different samples that had been taken from seven, oil-polluted, Hungarian sites. Forty-seven bacterial strains were isolated from the contaminated samples by classical microbiological (multiplying) methods on selective media (gasoline and crude oil mixture as the only carbon source). *In vitro* hydrocarbon degrading ability was verified in the case of six bacterial strains from the results of gravimetric analysis. In the case of TBF2/20.2 *Olivibacter oleidegradans sp. nov.*, ZFM 23.1, *Rhodococcus erythropolis*, and 4S-8 *Rhodococcus ruber* bacterial strains, the degrading ability was also verified with a groundwater sample from an oil polluted site (degradation experiment, Table 10).

Table 10. The results of 16S rDNA identification and hydrocarbon degrading ability of novel isolated strains from oil contaminated Hungarian sites from the results of three times repeated gravimetric analyses and contaminated groundwater based degradation experiment. Degradation rate was compared to the control that was used in the examinations.

Strains	Result of identification (NCBI GenBank, LeBibi)	Gravimetric	Degradation experiment
		Degradation rate (%)	
TBF2/20.1	*Acinetobacter rhizosphaerae*	64.62	n/a
TBF2/20.2	*Olivibacter sp. nov.*	21.09	76.60
ZFM 23.1	*Rhodococcus erythropolis*	47.94	74.15
SZM5/4.2	*Pseudomonas mandelii*	44.94	n/a
OM 7-2	*Rhodococcus erythropolis*	31.15	n/a
4S-8	*Rhodococcus ruber*	33.10	77.90

The six novel isolated strains were identified on the species level by 16S rDNA sequencing analysis by observing the international identification rules. An unexpected result of biodegraders identification was that the **TBF2/20.2 had been determined as a novel, unknown species of *Olivibacter* genus. After detailed description of the new species, it was named, due to of its oil degrading ability, *Olivibacter oleidegradans*. This result also foremost verified the hydrocarbon-degrading ability in case of a member of *Olivibacter* genus.** Suggestions from the results of dissertation:

- Continuation of the biomarkers quest and focus on TRFs that, based on high specificity and sensitivity, are able to mark the appearance of hydrocarbon contamination on the sites.
- The identification of the found biomarkers by genetic methods. Thus the pollution-caused appearance and disappearance of taxons could be determined on the species level.
- The determination of T-RFLP profile of the novel isolated hydrocarbon degraders before bioaugmentation in order to use it as a genetic marker for following the changes of amount and viability of the inoculated strains in the bacterial community of the site (e.g. RNS based T-RFLP methods).
- Determination of the sensitivity of the hydrocarbon degraders against environmental factors and optimilization of fermentation technologies for the strains according to the expectations of bioaugmentation.
- Bioaugmentation on contaminated sites based on the novel, isolated, bacterial strains.
- Examination of hydrocarbon biodegrading ability with the other members of *Olivibacter* genus. Description of the enzyme background of hydrocarbon degrading ability of *Olivibacter oleidegradans* by genetic methods. Designation of *Olivibacter* specific primers for quick and exact identification.

6. PUBLICATIONS

Journal articles:

Szoboszlay S., Atzél B., Cserháti M., **Szabó I.** (2004): Szénhidrogénnel szennyezett talajok bioremediációja. *Biokémia*, 28 (2) 37-40

Kaszab, E., Bedros, J. R., Szoboszlay, S., Atzél, B., **Szabó, I.,** Cserháti, M., Kriszt, B. (2006): Problems with environmental safety on bioremediated sites. *AARMS, (Academic and Applied Research in Military Science)*, 5 (3) 383-397.

Kaszab, E., Kriszt, B., Atzél, B., Szabó, G., **Szabó, I.,** Harkai, P., Szoboszlay, S. (2010): The occurrence of multidrug resistant *Pseudomonas aeruginosa* on hydrocarbon contaminated sites. *Microbial Ecology*, 59 (1): 37-45. [IF(2009): 3,251]

Táncsics, A.[§], **Szabó, I.[§],** Baka, E., Szoboszlay, S., Kukolya, J., Kriszt, B., Márialigeti, K. (2010): Investigation of catechol 2,3-dioxygenase and 16S rRNA gene diversity in hypoxic, petroleum hydrocarbon contaminated groundwater. *Systematic and Applied Microbiology*, 33, 398-406 p. [IF(2010): 2,643] (§: Both authors contributed equally to this study.)

Szabó I., Szoboszlay, S., Kriszt, B., Háhn, J., Harkai P., Baka E., Táncsics, A., Kaszab, E., Privler, Z., Kukolya, J. (2011): *Olivibacter oleidegradans* sp. nov. hydrocarbon degrading strain isolated from a clean-up facility (biofilter) set up on a Hungarian hydrocarbon contaminated site, *International Journal of Systematic and Evolutionary Microbiology*, first published on January 21, 2011 as doi: 10.1099/ijs.0.026641-0, [IF(2009): 2,113]

Proceedings:

I. Szabó, S. Szoboszlay, B. Atzél, M. Cserháti, S. Rúzs-Molnár, B. Kriszt (2007): Tests of microbiological hydrocarbon degradation on contaminated groundwater samples. *Acta Microbiologica et Immunologica Hungarica,* 54 (Suppl.): 121.

Szabó, I., Háhn, J., Harkai, P., Kaszab, E., Szoboszlay, S. (2009): Effects of heavy metal components on hydrocarbon degrading bacteria as abiotic stress on hydrocarbon contaminated sites. *Cereal Research Communications* 37 (Suppl 4): 561-564.

Cummulative impact factors of all publications (IF): **14.345**