Socio-microbiology of biofilm forming, hydrocarbon degrading and ureolytic bacteria for bioremediation purposes

INTRODUCTION

The primary aim of this research project was to engineer a bacterial consortium, consisting of a petroleum-hydrocarbon degrading, a prolific biofilm forming and a ureolytic calcium carbonate precipitating bacteria. The engineered consortium could be applied for the development of durable semipermeable reactive biobarriers resistant to environmental perturbation. In general, biobarriers are the greenest and the cheapest *in situ* passive bioremediation solutions used for the decontamination and containment of groundwaters polluted either with organic (petroleum-hydrocarbons, chlorinated compounds etc.) or inorganic toxic contaminants (heavy metals, nitrate etc.) (Careghini et al. 2013)

To reach the primary goal of the research the following tasks have been performed:

a. Assessing the biofilm forming ability of members of a bacterial strain collection originating from a bacterial biofilm community enriched on petroleum-hydrocarbons as sole source of carbon and energy (naphthalene or BTEX).

b. The selection of bacterial isolates, which show elevated biofilm forming and petroleum-hydrocarbon degrading capabilities, for the development of the envisaged bacterial consortium.

c. Socio-microbiology, i.e. co-cultivation studies of the selected biofilm forming and petroleum-hydrocarbon degrading bacteria together with *Sporosarcina pasteurii* DSM 33 the model organism of ureolytic, microbially induced calcium-carbonate precipitation (MICP).

d. Establishment of laboratory scale mineral-biobarrier systems using the petroleum-hydrocarbon degrading, biofilm forming and calcium-carbonate precipitating bacteria; performing durable tests on the porous matrix at different pH, temperature levels and flowrates.

e. Establishment of laboratory scale mineral-biobarrier systems again, by using the three aforementioned bacteria, to investigate the applicability of the inoculated porous matrix in the elimination of petroleum-hydrocarbons.

Research questions and hypotheses were the following:

1. Will the development of a mineral-biofilm barrier be more stable than a solely biofilm-barrier?
2. How will the hydraulic conductivity and durability of biofilm-barriers vs. mineral-biofilm barriers change due to changing environmental conditions (pH change, desiccation, high volumetric flow rate)?
3. Will the mineral precipitation impede the hydrocarbon degradation ability of the consortium?

4. In turn, will the presence of massive biofilm forming and hydrocarbon degrading bacteria impede or enhance the ability of ureolytic bacteria to catalyze MICP?
5. Will the presence of EPS and biofilm bacteria promote a mineral biobarrier that completely plugs the formation?

It was hypothesized that the EPS produced by biofilm bacteria on the one hand will provide additional nucleation sites for calcite precipitation and on the other hand will ensure protection to ureolytic bacteria against environmental perturbations (e.g. pH alterations). In turn, the precipitated calcium-carbonate will maintain the fluid retaining capacity of mineral biobarriers even after desiccation or washout of biofilm EPS. Moreover, another hypothesis is that perhaps a mature biofilm does not need to be grown up if MICP occurs and sequesters the active degrading bacterial species in a stable mineral barrier. In addition, by combining biofilm formation with the MICP cementing technique biobarriers can be built faster with less use of nutrients contributing thus to reduction of costs.

THE OBTAINED RESULTS

During the first stage of the research (01.12.2018-30.11.2019) (i) the biofilm forming ability of the most promising biofilm originating bacterial strains, belonging to the strain collection of the Home Institution, was assessed; (ii) the co-cultivability/social behavior of the selected strains (biofilm producer, hydrocarbon degrader and MICP bacteria) was determined; (iii) and laboratory scale MICP-biobarrier systems were established and (iv) eventually endurance tests were conducted.

Selection of bacterial strains for the development of the engineered consortium

In total, a set of 63 bacterial strains had been preliminary tested for biofilm production (crystal-violet, 96 well microplate assay) and petroleum-hydrocarbon (BTEX, PAH) degradation (GC-MS). Based on the obtained results, as well as on the basis of the scientific literature three strains have been selected for further studies:

- ***Variovorax paradoxus* strain BFB1_13**
- ***Pseudomonas veronii* strain BFHA4_7**
- ***Sporosarcina pasteurii* strain DSM 33**

The reason why ***V. paradoxus* strain BFB1_13** was selected, as one of the members of the engineered consortium was the fact that, as it was determined during this project, it is capable of degrading all the six BTEX-compounds (the most toxic petroleum hydrocarbons and one of the most common groundwater pollutants in Hungary as well as worldwide) with the same efficiency under both aerobic and oxygen-limited conditions (Figure 1). It has to be mentioned that, despite the fact that *Variovorax* species are often identified at BTEX-contaminated sites (Hendrickx et al. 2006; Benedek et al. 2016 and 2018; Posman et al. 2017), to the best of our knowledge no *Variovorax* isolates capable of degrading all the six BTEX have been reported so far. It has to be highlighted, that in the literature, until today, only a handful of isolates have been reported capable of degrading alone all the six BTEX, including all the three xylene isomers: *Pseudoxanthomonas spadix* BDa-59 (Choi et al. 2013), *Paraburkholderia aromaticivorans* BN5 (Lee et al. 2019), *Dechloromonas* sp. RCB (anaerobic, Chakraborty et al. 2005), *Ralstonia* sp. PHS1 (Sung-Kuk and Lee 2002), *Ralstonia pickettii* PKO1 (Leahy et al. 2003), *Rhodococcus* sp. ZJUT312 (You et al. 2018); *Rhodococcus rhodochrous* (Deeb and Alvarez-Cohen 1999); *Pseudomonas putida* YNS1 (You et al. 2012). Although no BTEX-degrading *Variovorax paradoxus* pure isolates have been reported so far, according to Satola et al. (2013) *V. paradoxus* strains can be characterized by a broad metabolic capacity, being capable of degrading a wide range of xenobiotic compounds. The finding, according to which no statistically significant difference was observed between aerobic and micro-aerobic BTEX-degradation of strain BFB1_13, promotes the applicability of the strain in the biodegradation of BTEX at oxygen concentrations as low as 0.5 mg l^{-1}. Most probably, the *ex* or *in situ* use of strain BFB1_13 for BTEX removal would remarkably reduce the costs of interventions, since no substantial oxygen supply or additional operational costs related to aeration are required to achieve high degradation efficiencies, and in order to maintain the most efficient aerobic degradation routes (not to mention that uncontrolled aeration could lead to the transfer of volatile organic compounds, such as carcinogenic BTEX, to the atmosphere). This can be particularly advantageous during the application of strain BFB1_13 in *in situ* bioremediation (bioaugmentation, biobarriers) of petroleum hydrocarbon contaminated shallow groundwaters, where the concentration of oxygen is generally low, found in the hypoxic range (≤ 2 mgl-1; Benedek et al. 2016; Marić et al. 2020).

Moreover, usually *V. paradoxus* strains, e.g. strain EPS, are capable of prolific biofilm production too. Also, as it turned out during this study, *V. paradoxus* strain BFB1_13 is capable of producing massive biofilms on artificial biofilm forming surfaces as assessed by using CDC-reactors. Strain BFB1_13 produced prolific biofilms on hydroxyapatite, polycarbonate, polypropylene and polyvinyl chloride. Notable biofilm production was observed also in the case of copper, stainless steel,

polytetrafluoroethylene and natural rubber. No remarkable biofilm formation occurred on polished stainless steel, viton rubber, ductile iron, titanium and glass surfaces (Figure 2).

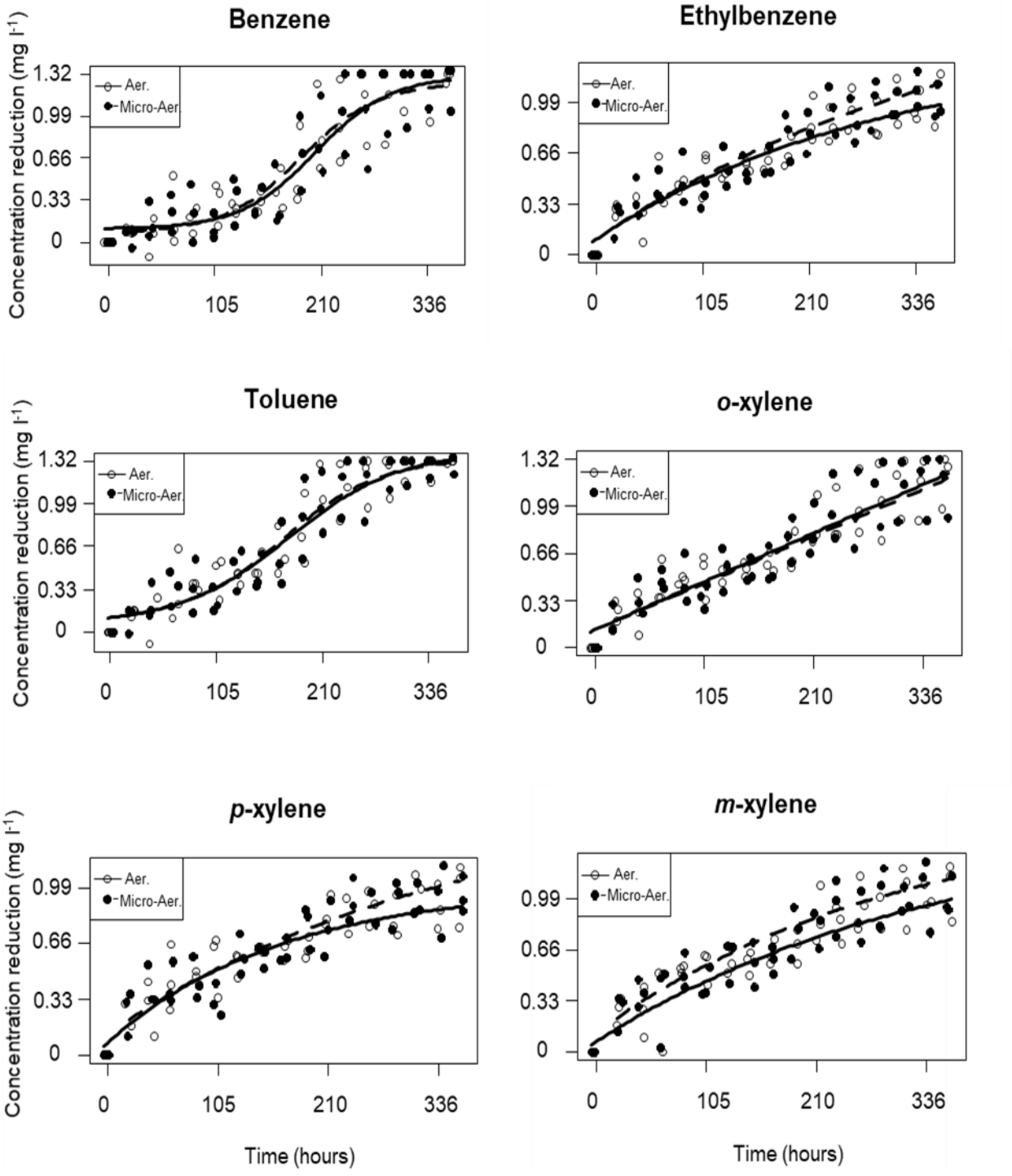

Figure 1. Aerobic and oxygen-limited/micro-aerobic BTEX degradation capacity of *V. paradoxus* strain BFB1_13. No statistically significant difference was observed between BTEX-compounds degradation under full aerobic and oxygen-limited conditions.

Also as performed during the course of this project, the whole-genome sequencing revealed that *V. paradoxus* BFB1_13 has a large genome of 9,581,132 bps. The total number of coding DNA sequences without artefacts was 9586. Strain BFB1_13 proved to be a toolbox of catabolic genes, harbors genes encoding 32 monooxygenases and 60 dioxygenases, that may be involved in the biodegradation of BTEX, terephthalate, benzoate/toluate, phenol, naphthalene, biphenyl and other hydrocarbons. Five catechol 2, 3-dioxygenase genes (*xylE*) could be identified in the genome.

Besides, genes most probably involved in biofilm formation were also detected in its genome e.g. *pga*C, *pil*AB, *flg*K, *clfA* etc.

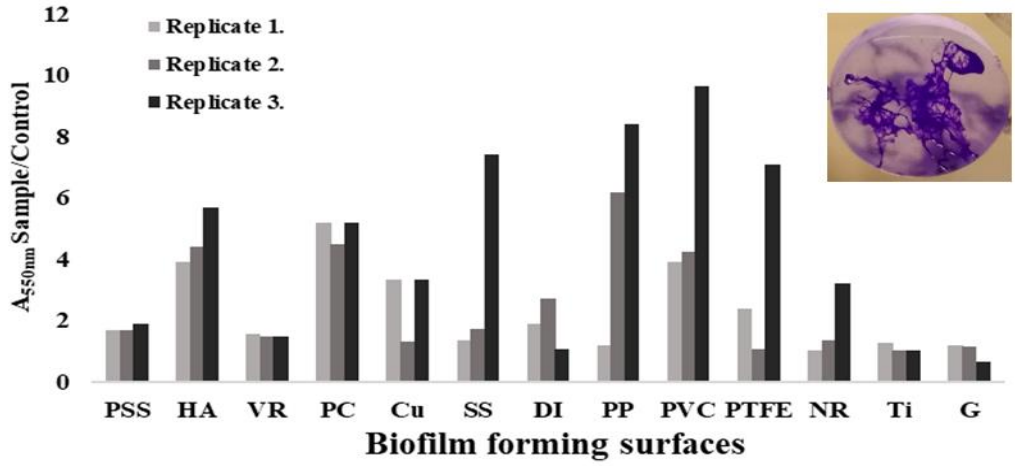

Figure 2. Biofilm forming ability of *V. paradoxus* strain BFB1_13 on polished stainless steel (**PSS**), hydroxyapatite (**HA**), viton rubber (**VR**), polycarbonate (**PC**), copper (**Cu**), stainless steel (**SS**), ductile iron (**DI**), polypropylene (**PP**), polyvinyl chloride (**PVC**), polytetrafluoroethylene (**PTFE**), natural rubber (**NR**), titanium (**Ti**) and glass (**G**). The ratio of absorbance values obtained at 550 nm (A_{550nm}) of biofilm containing coupons (Sample) and negative control coupons (Control) is shown. In the top right-hand corner, a crystal-violet stained biofilm of strain BFB1_13, developed on polycarbonate surface is shown.

***P. veronii* BFHA4_7** was selected as the second member of the consortium because, as it was demonstrated in microcosm studies, it is capable of degrading efficiently and very fast toluene, *m*- and *p*-xylene (biodegradation of these compounds occurred already after 48 hours of incubation, data not shown). Moreover, it is also able to produce massive biofilms on hydroxyapatite, polycarbonate, polypropylene, polyvinyl-chloride, polytetrafluoroethylene and titanium (Figure 3). Although, it needs to be mentioned that the biofilm producing capacity of strain BFHA4_7 was lower than that of strain BFB1_13. A series of studies discuss the hydrocarbon degradation ability (including both aliphatic- and aromatic) and biofilm forming potential of *P. veronii/P.extremaustralis* lineage affiliating bacteria (Tribelli et al. 2018; Tribelli et al. 2012; Wald et al. 2015).

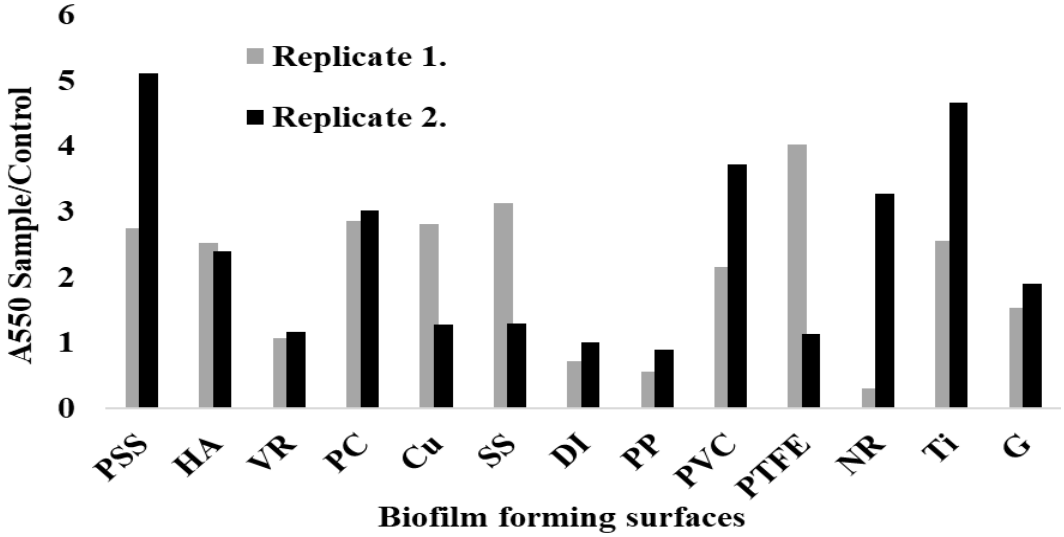

Figure 3. Biofilm forming ability of *P. veronii* strain BFHA4_7 on polished stainless steel (**PSS**), hydroxyapatite (**HA**), viton rubber (**VR**), polycarbonate (**PC**), copper (**Cu**), stainless steel (**SS**), ductile iron (**DI**), polypropylene (**PP**), polyvinyl chloride (**PVC**), polytetrafluoroethylene (**PTFE**), natural rubber (**NR**), titanium (**Ti**) and glass (**G**). The ratio of absorbance values obtained at 550 nm (A_{550nm}) of biofilm containing coupons (Sample) and negative control coupons (Control) is shown. In the top right-hand corner, a crystal-violet stained biofilm of strain BFB1_13, developed on polycarbonate surface is shown.

By using two approaches, urea hydrolysis test by using Christensen's medium and a spectrophotometric assay (Jung-assay), it was also determined if strains BFB1_13 and BFHA4_7 were capable of urea hydrolysis and were able to resist the presence of urea or not. According to the results, strains BFB1_13 and BFHA4_7 can slightly hydrolyse urea and can tolerate high urea concentrations (20g/l). All the above mentioned, as well as literature data supported the selection of strains BFB1_13 and BFHA4_7 for further studies. These two strains will be the hydrocarbon degrading (principally BTEX-degrading) and biofilm forming members of the engineered consortium.

For the engineering of the proposed bacterial consortium, the third selected bacterium was ***Sporosarcina pasteurii* DSM 33,** a model ureolytic bacterium capable of MICP (for a review of MICP technique please see Philips et al. 2013). The role of strain DSM 33 in the consortium was to cement the porous matrix, without inhibiting the activity of the two previously mentioned bacteria, the petroleum-hydrocarbon (especially BTEX) degrading and biofilm forming organisms. If the three selected organisms do not inhibit each other's growth and activity, by using the engineered consortium stable, semipermeable reactive mineral-biobarriers could be developed, resistant to harsh environmental conditions, applicable in the *in situ* bioremediation of BTEX-contaminated groundwater. *Sporosarcina pasteurii* DSM 33 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ),

Social behavior analysis of the selected strains

Prior to social behavior analyses of the selected organisms, it was necessary (i) to determine the optimal cultivation conditions and (ii) the most appropriate growth media for co-cultivation of all the three organisms. In the case of strains BFB1_13 and BFHA4_7 nine different liquid and solidified culture media, nine different pH, three different temperatures and eleven different NaCl concentrations have been tested. The most appropriate conditions for the cultivation of these strains were the following: liquid or solidified Nutrient medium, 25-30 ºC, pH 5-9 and 0-2% NaCl concentration (Figure 4 A and B). For strain DSM 33 no culture medium optimization was needed, it had already been assessed before deposition of the strain in the bacterial cell culture (DSMZ). From the studies it turned out that, for the co-cultivation of the selected three bacterial strains Caso Agar (DSM 220 medium according to DSMZ) supplemented with 20 g/l is the most appropriate. All the three strains showed a good growth while using this growth medium either in liquid or solid form.

For the social behavior analysis of the organisms, **first**, the cross streaking-plate method was applied on urea containing Caso Agar plates. According to the results, between strains BFB1_13 and DSM 33 no inhibition was observed. However, between strains BFHA4_7 and DSM 33 a slight antibiotic effect was observed, after cross streaking a slight clearing zone appeared on the plate between the cultures of the two bacteria (data not shown). Between strains BFHA4_7 and BFBA_13 no antagonistic effect was observed by using streaking-plate technique.

Second, a molecular biological fingerprinting approach, the Terminal Restriction Fragment Length Polymorphism (T-RFLP), was developed to study further the social behavior of strains in co-cultures. Prior community T-RFLP analyses, the individual T-RFLP profiles for each microorganism were determined. According to the results (by using *Alu*I restriction enzyme) on the community T-RFLP electropherograms strain BFHA4_7 will be represented by a ~70 bp sized T-RF, strain BFB1_13 by a 148 bp sized T-RF and strain DSM 33 by 236, 237, 239, 420 and 941 bp T-RFs. *S. pasteurii* DSM 33, as it turned out from these studies, contains in its genome more than one copy of *16S rRNA* genes, that is why on the electropherograms more than one T-RF will indicate the presence of the strain).

Third, to confirm the co-cultivability and symbiotic interaction between strains BFB1_13 and BFHA4_7 in a BTEX-contaminated environment, a microcosm experiment was initiated in mineral salts medium containing a mixture of BTEX compounds as sole source of carbon and energy (total concentration of BTEX was 10-20 mg l^{-}). For the sake of completeness, it has to be mentioned, that

in a previous study, during which the aforementioned two bacteria were inoculated simultaneously and grown in a nutrient rich medium, after one week of incubation strain BFHA4_7 overproliferated strain BFB1_13. It was found that, in a growth medium, where the nutrients are unlimited strain BFHA4_7, due to its faster reproduction rate, outcompetes strain BFB1_13 which is characterized with a slower growth rate. The BTEX amended microcosms were co-inoculated in equal cell numbers (10^6 cells/ml) with strains BFB1_13 and BFHA4_7, simultaneously. The microcosm experiments were conducted for 8 days. The concentration of BTEX was continuously measured by GC-MS, and in the case of BTEX depletion supplementation/addition took place. At the end of the experiment the number of the bacterial cells was determined, as well as the relative abundance of the strains was assessed by using T-RFLP.

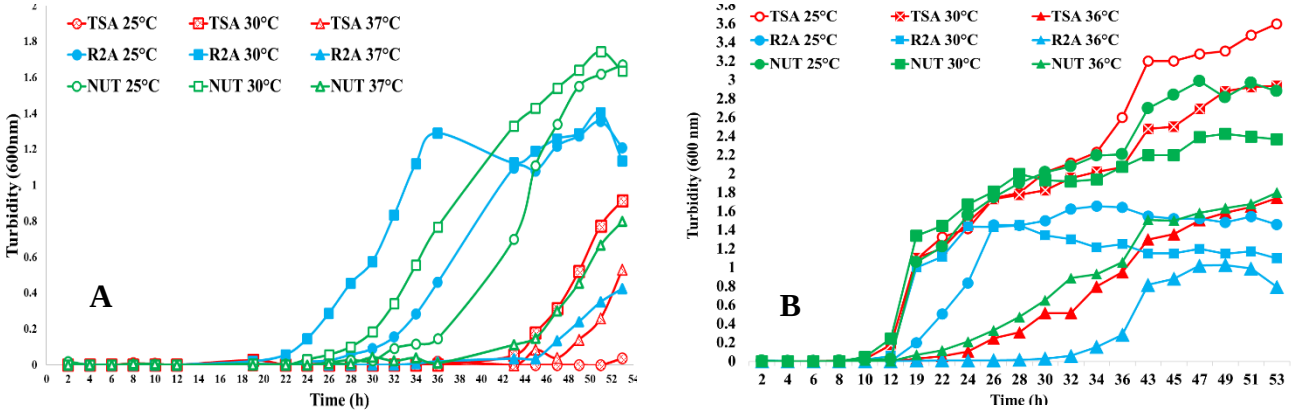

Figure 4. Optimal liquid growth media and temperature for the cultivation of *V. paradoxus* strain BFB1_13 and *P. veronii* BFHA4_7 assessed at pH 7, NaCl 1%.

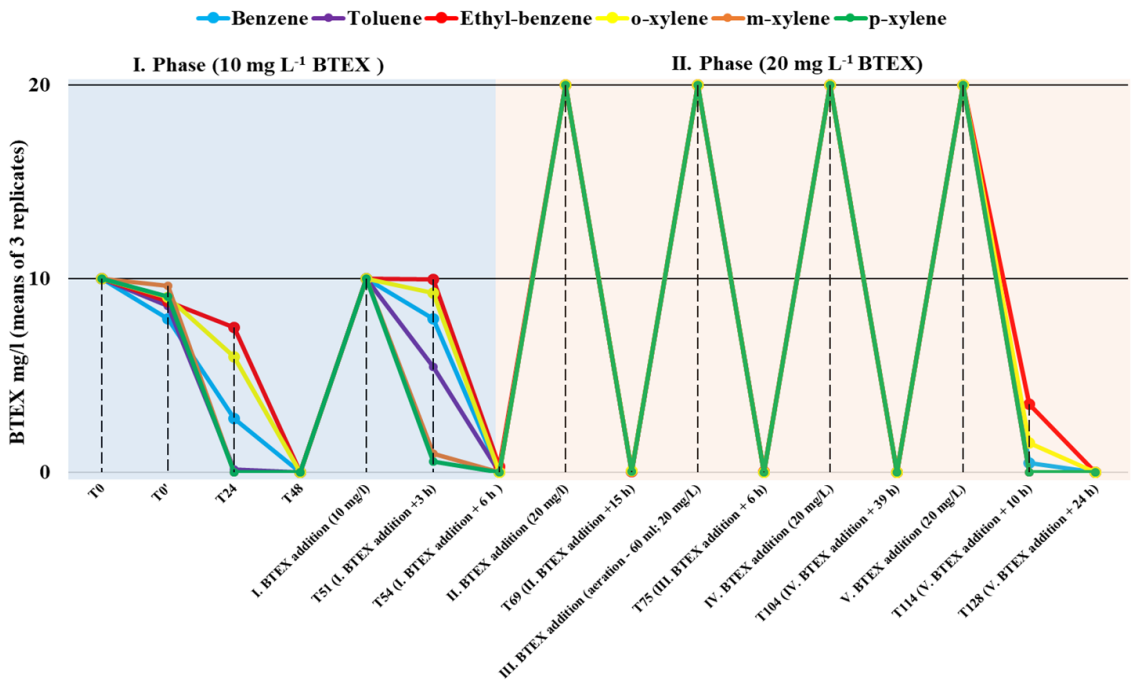

Figure 5. BTEX-biodegradation capacity of the bacterial consortium made of *V. paradoxus* BFB1_13 and *P. veronii* BFHA4_7. T_n – time in hours (n).

It was found that, the two strains in the BTEX amended microcosms could grow together and were able to completely degrade simultaneously all the six BTEX-compounds. For instance, after 3 days of adaptation the two-member consortium degraded 20 mg/l of BTEX after only six hours (Figure 5;

T75). The results indicated that, the two strains not only are able to grow together, but they are more efficient in BTEX degradation together, than alone. When strain BFB1_13 was inoculated alone in the test medium a slow degradation of BTEX-compounds was observed. In general, a long lag phase, 168 hours of incubation, was needed for the strain to start the degradation of BTEX. On the other hand, strain BFHA4_7 was capable of degrading only toluene, *m*- and *p*-xylene from the BTEX-compounds, however it degraded very fast, after 48 hours. In co-cultures the strains degraded efficiently all the six BTEX compounds after an incubation time as short as 6 hours. At the end of the experiment both strains could be re-isolated in high numbers from the test solutions (10^8 cells/ml order of magnitude (Figure 6 B and C). The T-RFLP method proved to be useful for the detection of strains; the detected ratio between strains BFB1_13 and BFHA4_7 was 2:1 (Figure 6 A).
The co-cultivability, as well as the mutualistic relationship between the two strains, BFB1_13 and BFHA4_7, during BTEX-degradation has been confirmed. All the above mentioned indicate that, strains *V. paradoxus* BFB1_13 and *P. veronii* BFHA4_7 are biocompatible and are able to exert their useful metabolic capabilities in the presence of the other.

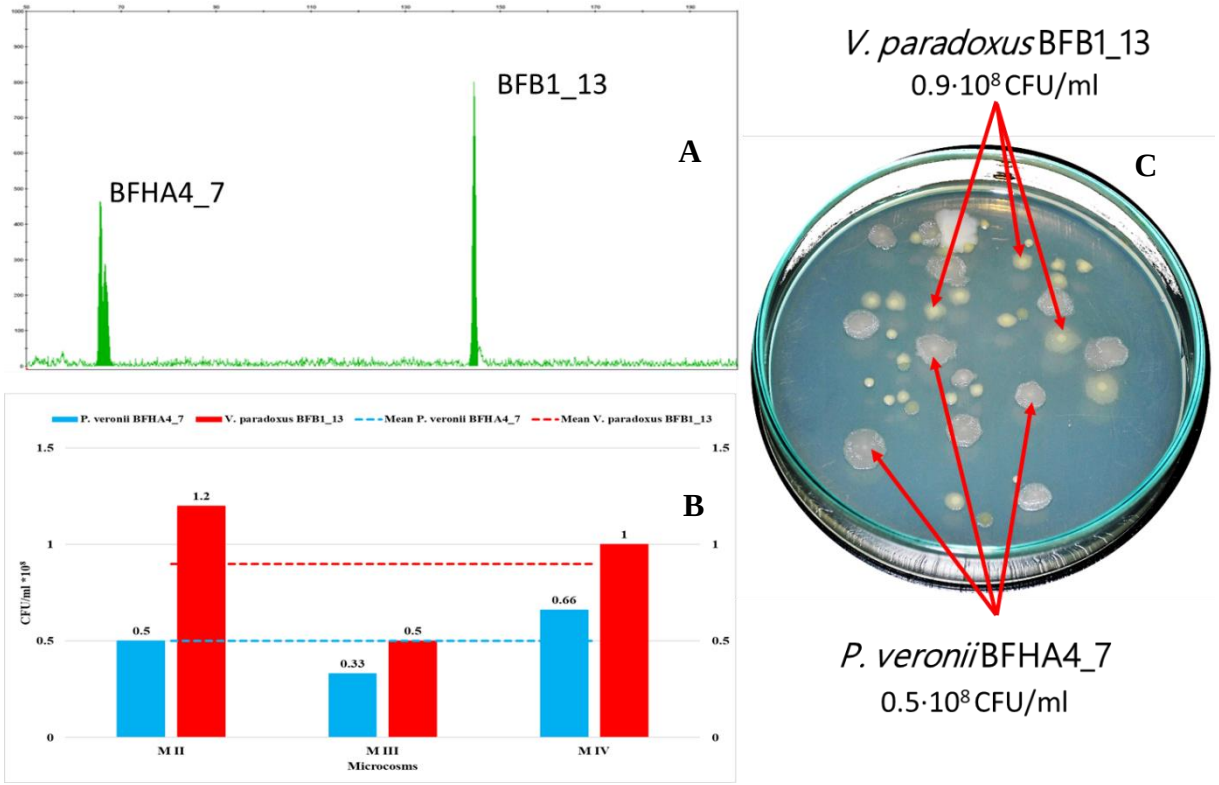

Figure 6. The number (B) and relative abundance by using T-RFLP (A) of *V. paradoxus* BFB1_13 and *P. veronii* BFHA4_7 bacterial cells (A, C) after 8 days of incubation in co-cultures in a BTEX-amended test media.

Fourth, the abovementioned BTEX-amended microcosm experiments were repeated, but this time three bacterial strains were inoculated simultaneously in the test solutions in the same bacterial cell numbers: *V. paradoxus* BFB1_13, *P. veronii* BFHA4_7 and *S. pasteurii* DSM 33. The biodegradation of BTEX-compounds by the consortium showed the same kinetics even in the presence of strain DSM 33. Complete BTEX degradation occurred and at the end of the experiment strains BFB1_13 and BFHA4_7 could be re-isolated in high numbers. However, no *S. pasteurii* DSM 33 cells could be re-isolated using R2A plates or Caso Agar plates supplemented with urea. These results indicated that the presence of *S. pasteurii* strain DSM 33 did not inhibit the BTEX-biodegradation capability of the two other bacteria in a mineral salts solution, where BTEX were the sole source of carbon and energy. In addition, these results also shed light on the fact, that *S. pasteurii* DSM 33 was not able to survive in the BTEX-amended test solutions, where no urea and easily metabolizable carbon sources were present, but simple aromatic BTEX-compounds were the single carbon and energy sources.

The social behavior analysis of the three selected organisms was continued in a **fifth** experiment, when the impact of the petroleum-hydrocarbon degrading and biofilm forming organisms was determined on the calcium-carbonate precipitation capacity and porous-matrix bio-cementing ability of *S. pasteurii* strain DSM 33. Briefly, in a lab-scale bio-cementing model system (Figure 7), molds were filled with quartz sand (350 ml of sand of 0-4 mm size, the pore volume of the system was 150 ml) and inoculated simultaneously in equal bacterial cell numbers with strains BFB1_13, BFHA4_7 and DSM 33 (50 ml of each, 48 hours old active cultures). A mold inoculated only with *S. pasteurii* DSM 33 was also started as the positive control. After bacterial inoculations, by the aid of silicon tubing and a peristaltic pump the molds were fed sequentially with the appropriate solutions needed for the microbially induced calcium-carbonate precipitation to happen. After two weeks of calcium-carbonate precipitation (in total 14 MICP feeds) the sand particles had been cemented in both molds. After disassembly of the molds, visually, no remarkable difference was observed between the cemented objects by the three membered consortium or by the *S. pasteurii* DSM 33 alone. Rigid structures, “bio-bricks”, were obtained from both molds (Figure 8). These findings suggested that *V. paradoxus* strain BFB1_13 and *P. veronii* strain BFHA4_7 did not impact negatively the growth and the MICP properties of *S. pasteurii* DSM 33.

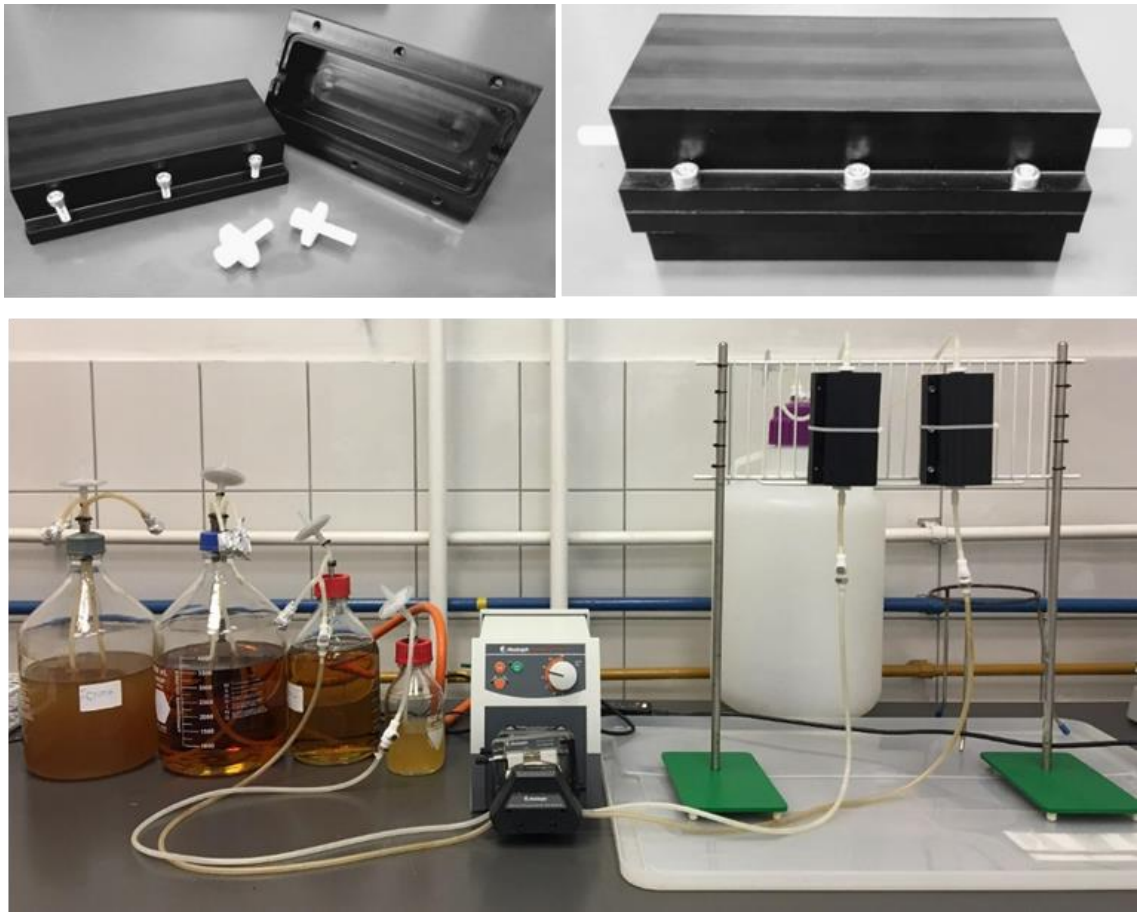

Figure 7. Lab-scale bio-cementing model system. Molds used for the bio-cementing of the porous matrix are shown in the upper part of the figure.

Figure 8. The bio-cemented porous matrix obtained by using the engineered consortium (K) or the *S. pasteurii* DSM 33 alone (DSM 33).

Consequently, based on all the aforementioned, it can be speculated that the three bacteria are biocompatible, substantially do not inhibit each other's growth and metabolic activity, and an engineered consortium consisted of *V. paradoxus* strain BFB1_13, *P. veronii* strain BFHA4_7 and *S. pasteurii* strain DSM 33 may be used in the development of durable mineral-biobarriers applicable in the containment and decontamination of BTEX contaminated groundwaters during passive *in situ* bioremediation.

Laboratory scale MICP-biobarrier systems- Endurance tests

The engineered bacterial consortium, containing all the three investigated strains BFB1_13, BFHA4_7 and DSM 33, were used to form lab-scale calcium carbonate-biofilm based bio-barrier systems. The work was done in three replicates. 10 ml graduated glass pipettes were used as columns and very coarse sand as porous media. After sterilization, columns were inoculated separately with actively growing cultures of strains DSM 33 (SPBB type columns), BFB1_13 (BB), BFHA4_7 (BB) and a consortium made of the three, in 1:1 ratio (MBB). A set of columns was not inoculated but underwent the same treatments, these were the negative control columns (NBB). MICP-solutions were added at the top of the columns in the right sequence. After given time periods the fluid retention capacity (frc) of the porous matrix was determined. In the case of biofilm formation and/or calcium-carbonate precipitation the frc of the porous matrix is expected to increase.
Results indicated that, by using the engineered bacterial consortium it is possible to develop MICP-biofilm based biobarriers. As compared to NBB, by the end of the experiment (after 10 feeds), the fluid retention capacity of SPBB and MBB type columns increased by 95 and 65%, respectively. Inside the columns, the development of a rigid structure was observed due to the adhesion of the sand grains. In the case of BB columns only a 37% increase was observed, no adhesion of sand particles was recorded. The increased frc was most probably attributed to the EPS-matrix/biofilm produced by bacteria. The slight difference in frc between SPBB and MBB columns might be attributed to the previously observed slight antagonistic effect of strain BFHA4_7 on strain DSM 33.
During the endurance tests it was found that pH 4 slightly dissolved the precipitated calcium-carbonate crystals, which influenced the frc in not an expected manner. The frc of the columns increased rather than decreased. It can be assumed that the partly dissolved calcium-carbonate crystals clogged the column. Higher pH levels did not have any effect on the columns.
During the desiccation test (incubation of columns at 60, 80 and 100 ºC for 24 hours each) the frc of SPBB columns further increased by an additional 20%, while in the case of BB columns it decreased

remarkably by 42% (BFHA4_7) and 52% (BFB1_13), reaching almost the initial values. After desiccation no significant increase in frc was observed in the case of MBB columns. These findings may indicate that high temperatures favored the MICP process in the case of SPBB columns. This is in accordance with the fact that the urease is the most active at 60 ºC. In the case of BB columns, the remarkable reduction of the frc was due to the desiccation of the EPS/biofilm matrix.
In the case of SPBB and MBB columns a stable, rigid structure was obtained inside of the columns, which seemed resistant to low pH and high temperatures. High volumetric flow rates did not affect the fluid retention capacity of the MICP columns at all. The sand grains as it was expected cemented together and did not allow washout of the filling material. The sand grains were so cemented that their removal from the SPBB and MBB columns was not possible at the end of the experiment.

Laboratory scale MICP-biobarrier systems – Investigating the reactive nature of the mineral-biobarriers

During the second stage of the research (01.12.2019-30.11.2020) the engineered microbial consortium was used for the development of porous biofilm based semipermeable reactive barriers. The aim of this research phase was to assess the reactive and semipermeable nature of the developed biobarriers. A biobarrier consisted of a syringe, packed as a porous matrix with very coarse sand (grain size diameter 0-4 mm). A headspace was left above the packing for medium so that the rate of the flow through could be measured. After sterilization, by using a series of sterilizing agents, syringes, containing the porous matrix, were inoculated with bacteria and fed with the appropriate nutrient media for microorganisms to develop biofilms and/or exert MICP properties. To determine the hydraulic conductivities at given time intervals the columns were filled with known volumes of fresh media, and the time required for the media to percolate through the column was recorded. The rate of flow (ml/sec) is a measure of the fluid retaining capacity of the mineral-biobarrier system. Syringes were closed with a rubber stoppers (Figure 9).

To assess the reactive nature of the mineral-biobarriers the following “biobarrier microcosms” were set up in syringes in triplicates:

a) Columns containing uninoculated sterile porous media – NBB (negative-biobarriers)
b) Columns containing porous media inoculated solely with *S. pasteurii* DSM 33– SPBB
c) Columns containing porous media inoculated with the engineered consortium without the ureolytic component – BB (biobarriers).
d) Porous media containing columns inoculated with the engineered bacterial consortium – MBB (mineral-biobarriers).

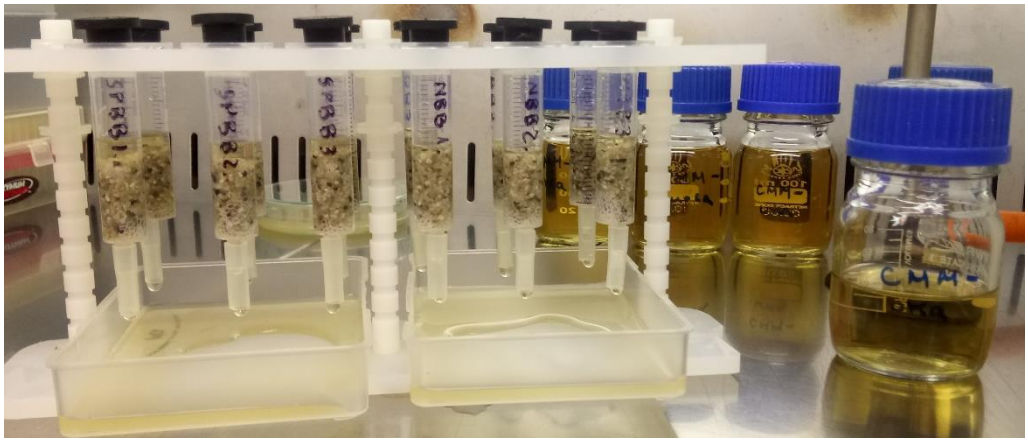

Figure 9. Lab-scale mineral-biobarrier systems used for investigating the reactive nature of the bio-cemented porous matrix.

After one week of MICP experiments (in total five MICP feeds) the hydraulic conductivity of the SPBB and MBB columns decreased by around 50%. After the porous-matrix cementing phase of the study, the content of the syringes were transferred to 100 ml vials, which contained mineral salts solution and BTEX-

compounds as sole source of carbon and energy (8 mg l^{-1}) (Figure 10) – inoculation of BTEX-amended mineral salts solution with the engineered consortium from the MICP experiment. Subsequently, the hermetically closed vials were put into the incubator (145 rpm, 28 °C). After every two days the concentration of the remaining BTEX in the vials was determined by using GC-MS. At this phase of the study the aim was to determine if the BTEX-degradation capacity of the consortium was still active even after calcium-carbonate precipitation occurred in the syringes during the MICP phase. If the biofilm forming and/or petroleum-hydrocarbon (BTEX) degrading members of the engineered consortium got trapped inside of the calcium-carbonate crystals the concentration of the BTEX-compounds will not decrease with time of incubation. It was expected that the concentration of BTEX will significantly decrease owing to the activity of hydrocarbon degrading member of the engineered consortium.
Unfortunately, based on GC-MS results, in the case of those vials, which were inoculated with the content of MBB syringes no biodegradation of BTEX-compounds was detected after two weeks of incubation. In the meantime, during the incubation the sand particles attached to the bottom of the vials (Figure 10). These observations suggest that *S. pasteurii* DSM 33 indirectly, through the formation of calcium-carbonate crystals, inhibited the biodegradation of BTEX by strains BFB1_13 and BFHA4_7. The hydrocarbon degrading and biofilm forming members of the engineered consortium most probably got trapped inside of the calcium-carbonate crystals. On the basis of three independent experiments, it can be stated, that after calcium-carbonate precipitation, the developed mineral-biobarriers did not retain their previously assumed petroleum-hydrocarbon (BTEX) biodegradation capabilities, the hydrocarbon degrading members of the engineered bacterial consortium lost their function.

Based on the above mentioned the followings are suggested:

- to create a durable semipermeable, reactive mineral-biobarrier aimed in this study first the bio-cementing of the porous matrix should take place by using solely the *S. pasteurii* strain DSM 33
- Once the stable in structure porous matrix is obtained it can be inoculated with the petroleum-hydrocarbon degrading bacterial culture containing strains V. *paradoxus* BFB1_13 and *P. veronii* BFHA4_7.

Figure 10. Porous matrices (quartz sand) from the syringes transferred to crimp sealed serum bottles containing mineral salts solution supplemented with BTEX-compounds as sole source of carbon and energy. NBB-negative biobarrier without bacteria, MBB-mineral biobarrier containing all the three bacteria, SPBB-biobarrier containing only S. pasteurii DSM 33. In the case of sand particles originating from the MBB and SPBB syringes the calcium-carbonate precipitation and bio-cementing continued in the vials during the biodegradation experiments too. The sand particles adhered to the bottom of the vials, and after the bottles were laid on their sides they remained sticked to the bottom of the vials.

PUBLICATIONS APPEARED DURING THIS RESEARCH PROJECT

Scientific Articles in Highly Impacted Journals

1. **Benedek, T.**, Szentgyörgyi, F., Szabó, I., Farkas, M., Duran, R., Kriszt, B., Táncsics, A. (2020) Aerobic and oxygen-limited naphthalene-amended enrichments induced the dominance of *Pseudomonas* spp. from a groundwater bacterial biofilm. *Applied Microbiology and Biotechnology* 104: 6023-6043 I.F. 3.65 Q1

2. **Benedek, T.**, Szentgyörgyi, F., Gergőcs, V., Menashe, O., Figueroa-Gonzlez, A., Probst, A., Lauchnor, E., Kriszt, B., Táncsics, A. (2020) A comprehensive study to assess the applicability of *Variovorax paradoxus* strain BFB1_13 in the bioremediation of BTEX-contaminated sites. Prediction of BTEX metabolic pathways from whole-genome sequence analysis. *Environmental Pollution* I.F. 6.792 Q1, ***Ready for publication***.

Conference Participations

1. **Benedek T,** Szentgyörgyi F, Táncsics A, Kriszt B (2020) Engineering a bacterial consortium for the complete and rapid biodegradation of all the six BTEX-compounds. Annual Congress of the Hungarian Society of Microbiology, XIV. Fermentation Colloquium, October 14-16, Kecskemét, Hungary.

2. Szentgyörgyi F, Táncsics A, Tóth E, Kriszt B, **Benedek T** (2020) Engineering a bacterial consortium for bio-cementing porous matrix applicable in bioremediation. Annual Congress of the Hungarian Society of Microbiology, XIV. Fermentation Colloquium, October 14-16, Kecskemét, Hungary.

3. Szentgyörgyi F, Kriszt B, **Benedek T** (2020) Investigation of ureolytic calcium-carbonate precipitating ability of biofilm-bacteria for innovative biotechnological purposes XIV. Szent-Györgyi Albert Conference, April 24-25 – Conference cancelled due to Covid-19 pandemic

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