

Title: “Anaerobic PAHs biodegradation in thermophilic environments”

Permit 8106.

Summary

Polycyclic aromatic hydrocarbons (PAHs) are a group of widely distributed pollutants composed of two or more fused aromatic rings, which produce hazardous effects on human health. These substances are natural components of fossil raw materials such as coal and petroleum and they are also produced during the incomplete combustion of such organic matter. In PAH-contaminated sites, oxygen is rapidly depleted. Thus, microorganisms able to use these compounds as a carbon source in the absence of molecular oxygen are crucial for their consumption. Slow bacterial rates in such conditions, together with the low solubility and bioavailability of PAHs have impaired elucidating the metabolic pathways involved in the anaerobic biodegradation of these compounds, especially for those composed of three or more aromatic rings. This project aims at studying the mechanisms for the anaerobic degradation of PAHs by thermophilic microorganisms from petroleum-containing hot springs at Yellowstone National Park. The increased PAHs solubility and bacterial growth rates at high temperatures provide an excellent opportunity for overcoming previous limitation in the study of PAHs biodegradation.

Activities performed in 2019

A visit to Yellowstone National Park was performed between July, 25th and August, 2nd, 2019. During this time, we obtained mixed water/sediment samples from 3 locations:

- Calcite Springs (547701 UTM_East_NAD_83_Zone_12 4972676 UTM_North_NAD_83_Zone_12). 7 x 100 ml flasks.
- Tower Bridge Hot Springs (547005 UTM_East_NAD_83_Zone_12 4974281 UTM_North_NAD_83_Zone_12). 2 x 100 mL flasks.
- Rainbow Springs Mirror Plateau (557801 UTM_East_NAD_83_Zone_12 4957463 UTM_North_NAD_83_Zone_12). 5 x 100 ml flasks.

At each site, 3 liters of water were also filtrated using 2 µm membrane filters contained in plastic filter-holders attached to 50 ml syringes. The filters were collected in plastic tubes. Further field procedures included measurement of temperature and pH at the different locations using an outdoor thermometer and pH paper, respectively.

The collected samples were sent to the University of Duisburg-Essen, Essen, Germany. Upon reception of the samples, these were stored at room temperature until further analysis.

Conducted researched and preliminary results

- 1) The water phase of each sample was subjected to the following analysis:
 - Ion chromatography, for evaluating the concentration of different anions and cations.

Anions:

Sample	Chloride µM	Nitrite µM	Nitrate µM	Phosphate µM	Sulfate µM
MQ	1.17	n.a.	n.a.	n.a.	n.a.
Calcite_Springs_1	78.29	n.a.	205.54	169.21	409.75
Calcite_Springs_2	82.66	n.a.	n.a.	199.14	462.27
Calcite_Springs_3.1	124.99	n.a.	129.81	193.82	136.52
Calcite_Springs_4	121.96	n.a.	240.91	188.39	125.81
Calcite_Springs_5	125.95	n.a.	136.61	244.60	172.57
Calcite_Springs_6	133.16	n.a.	n.a.	142.84	90.17
Calcite_Springs_7	135.64	n.a.	n.a.	150.74	96.76

Tower_Bridge_8	152.24	n.a.	153.40	177.63	116.09
Tower_Bridge_11	83.77	n.a.	65.24	171.08	124.33
Rainbow_Springs_1	62.53	n.a.	n.a.	172.81	40.77
Ranbow_Springs_2	55.56	n.a.	n.a.	70.34	29.39
Rainbow_Springs_3	58.80	n.a.	n.a.	111.81	12.56
Rainbow_Springs_4	60.94	n.a.	n.a.	121.04	17.03
Rainbow_Springs_5	60.56	n.a.	44.80	128.18	13.29

Cations:

Sample	Sodium μM	Ammonium μM	Potassium μM	Magnesium μM	Calcium μM
Calcite_Springs_1	1543.56	376.20	298.34	560.71	901.10
Calcite_Springs_2	1151.33	379.54	322.03	620.39	944.09
Calcite_Springs_3.1	1498.19	215.43	181.12	202.16	186.63
Calcite_Springs_4	1832.52	25.00	271.70	213.59	151.54
Calcite_Springs_5	1551.35	52.60	242.61	338.67	267.39
Calcite_Springs_6	1839.78	24.60	231.07	62.31	108.37
Calcite_Springs_7	1820.36	23.92	175.18	88.19	176.79
Tower_Bridge_8	1612.60	98.57	260.64	160.01	507.30
Tower_Bridge_11	1348.19	68.78	218.77	107.22	304.63
Rainbow_Springs_1	1255.60	25.10	198.12	123.95	124.19
Ranbow_Springs_2	1300.24	72.69	249.86	109.87	120.09
Rainbow_Springs_3	1090.76	66.67	112.43	85.92	87.56
Rainbow_Springs_4	1244.72	26.81	233.60	124.53	140.74
Rainbow_Springs_5	1274.87	28.40	182.31	84.76	81.38

- Ferrozine assay (Stookey, 1970), for total iron determination.

Description	Fe (II) cc (mM)	Total Fe cc (mM)
Calcite_Springs_1	0.524	1.170
Calcite_Springs_2	1.855	3.574
Calcite_Springs_3.1	0.032	0.515
Calcite_Springs_4	0.000	0.359
Calcite_Springs_5	0.005	0.185
Calcite_Springs_6	0.005	0.296
Calcite_Springs_7	0.238	0.670
Tower_Bridge_8	0.034	0.452
Tower_Bridge_11	0.076	0.426
Rainbow_Springs_1	0.087	0.522
Ranbow_Springs_2	0.054	0.800
Rainbow_Springs_3	0.042	0.270
Rainbow_Springs_4	0.653	1.115
Rainbow_Springs_5	0.025	0.570

- Spectrophotometric determination of hydrogen sulfide (Cline, 1969).

Description	Sulfide cc (mM)
Calcite_Springs_1	0.001
Calcite_Springs_2	0.001
Calcite_Springs_3.1	0.008
Calcite_Springs_4	0.000
Calcite_Springs_5	0.004
Calcite_Springs_6	0.002
Calcite_Springs_7	0.006
Tower_Bridge_8	0.230
Tower_Bridge_11	0.195

Rainbow_Springs_1	0.004
Ranbow_Springs_2	0.014
Rainbow_Springs_3	0.042
Rainbow_Springs_4	0.002
Rainbow_Springs_5	0.046

- 2) A fraction of the sediment samples (~ 5 mL) was transferred into anoxic mineral media containing different individual PAHs (naphthalene, phenanthrene and acenaphthylene) as sole carbon source and sulfate as electron acceptor, in order to enrich anaerobic PAHs-degrading microorganisms.

Cell growth in these media is being monitored by measuring sulfate consumption (ion chromatography) as well as cell abundance (DAPI-staining and cell counting). Once growth is detected, aliquots will be transferred into fresh medium at 10 % dilutions. These steps will be repeated until highly enriched or pure cultures are obtained. If such cultures are successfully obtained, the microorganisms will be thoroughly characterized, including their physiology and biochemistry.

- 3) Sediments were also subjected to DNA extraction using a standard extraction protocol previously described by (Inskeep *et al.*, 2013). Samples were treated with 1 ml of Buffer A (200 mM Tris, pH 8; 50 mM EDTA; 200 mM NaCl; 2 mM sodium citrate; 10 mM CaCl₂) and 1 mg/ml lysozyme (37 °C, 1.5 h). Then, 1 mg/ml Proteinase K and 0.3 % w/v SDS was added followed by incubation at 37 °C for 0.5 h. Finally, DNA was extracted with phenol-chloroform, and precipitated in ethanol. Alternatively, DNA was extracted from some of the samples using the PowerSoil DNA extraction kit (MoBio).

The isolated DNA was amplified by PCR with universal primers targeting the conservative regions of the 16S rRNA genes of bacteria and archaea (Takahashi *et al.*, 2014), followed by tag-amplicon library preparation and sequencing using the MiSeq Illumina platform. The obtained data is currently under analysis in order to determine the microbial composition of the described hot springs.

On the other hand, DNA samples were subjected to paired-end Illumina HiSeq sequencing, for shotgun-DNA sequencing. The obtained sequences will be used for the reconstruction and annotation of microbial genomes for evaluating the metabolic capacities of the organisms inhabiting these hot springs, including the presence of hydrocarbon-degradation genes.

Future work

The laboratory and data analysis work will be continued at the University Duisburg-Essen. However, the fieldwork required for this project has come to an end and no further samples will be collected from Yellowstone National Park.

Bibliography

Cline, J. D (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnology and Oceanography* 14: 454-458.

Inskeep, W. P., Jay, Z. J., Herrgard, M. J., Kozubal, M. A., Rusch, D. B., et al. (2013) Phylogenetic and Functional Analysis of Metagenome Sequence from High-Temperature Archaeal Habitats Demonstrate Linkages between Metabolic Potential and Geochemistry. *Front Microbiol* 4: 95.

Stookey, L. L., 1970. Ferrozine-a new spectrophotometric reagent for iron. *Anal. Chem.* 42, 779-781.

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M. (2014). Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. *PLoS ONE* 9: e105592.