MONITORING ASSAYS FOR MTBE TOXICITY AND GENOTOXICITY

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This research is focused on determination of toxicity, genotoxicity and discovery of MTBE-degrading bacterial strains. Toxicity was detected using bacterial luminescent method Microtox while genotoxicity was monitored by Ames test with *Salmonella typhimurium* his$^-$ (TA 98, TA 100, YG 1041 and YG 1042) as indicator strains. Potential MTBE-degrading strains were tested by Bioscreen®. This method monitors the growth of bacterial strains in the presence of MTBE as the sole source of carbon and under different nutritional limits. MTBE is utilise via cometabolim of organic substrates (alkanes, acetone, cyclohexane, toluene or lactate), respectively. Two strains of *Pseudomonas* spp. and soil isolates were employed for measurement of specific growth rates - μ. Oxygen consumption of the tested strains was measured by OxiTop®.

1. INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is a synthetic compound that was developed as a solution to a technology-derived problem created by air pollution from vehicle emissions. Since 1979, MTBE has been used in the United States as an octane-enhancing replacement for lead, mainly in mid- and high-grade gasoline at concentrations higher than 8 percent (by volume). MTBE is very easily soluble in water, it can move rapidly through soils and aquifers. MTBE has been detected in urban air, surface water, drain water and ground water. While the half-lives of MTBE in the atmosphere and surface water are relatively short (on the order of days), a long half-life (on the order of years) has been estimated in subsurface system. The mobility of MTBE in the subsurface is due in the part to high aqueous solubility, low octanol water partition coefficient and chemical structure that is relatively resistant to microbial attack (1). Apart from influence on taste and odour of water, MTBE has a potential toxic effect not only on microorganisms but, more importantly, it was considered as a potential human carcinogen by US Environmental Protection Agency (USEPA). One of the potential methods used for removing of MTBE from soils and aquifers is microbial decomposition.

The major aromatic components of gasoline, benzene, toluene, ethylbenzene, and xylene (BTEX) degrade relatively quickly under aerobic and anaerobic conditions. When MTBE containing gasoline is released, BTEX plumes may still stabilize on average within 100 m of the source of contamination but MTBE may continue to migrate. While much is known about the biodegradation of many gasoline components under both ærobic and anaerobic conditions, the biodegradation of MTBE is not fully understood. However, recent studies have reported the ability of several bacterial and fungal cultures from various environmental sources to degrade MTBE under aerobic or anaerobic conditions either as the sole source of carbon and energy or cometabolically (2).

2. MATERIALS AND METHODS

2.1 Bacterial bioluminescence toxicity test

For the toxicity detection of pollutants in groundwater bacterial bioluminescence toxicity test (Microtox®) was carried out. The principle of the method is based on the measurement of the decrease in bioluminescence versus concentration of a particular toxicant during a certain period of time. The toxicity of each toxicant was expressed as EC50. It is concentration of toxicant, which reduces the luminescence by 50% of tested strain under defined conditions. The lower is EC50 the higher is the toxicity. As an indicating organism, bacterial strain *Vibrio fischeri* (ISO 11348-2) was

used. All determinations were carried out in saline solution (2% NaCl, pH 7.0) in order to simulate the natural seawater conditions. During each experiment, two parallel detections were performed. $ZnSO_4$ (30 mg/l) was used as a model toxicant to attest appropriate sensitivity of a particular bacterial suspension. Measurements were performed at 15°C.

2.2 Ames test

The Ames *Salmonella*/microsome mutagenicity assay (*Salmonella* test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several histidine dependent *Salmonella* strains each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*$^+$) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. The preincubation assay (modification of the standard plate incorporation assay) was used for monitoring genotoxicity of MTBE. The testedr strains were exposed to MTBE for a short period (40 min) in a small volume (0,5 ml0 of either buffer or S-9 mix, prior to plating on glucose agar minimal medium suplement with a trace amount of histidine. MTBE was dissolved in DMSO (organic solvents) and then the concentration gradient was prepared.

There were used YG1041, YG1042, TA98 and TA100 strains. Strains YG1041 and YG1042 are constructed for detection of the same mutation type as strains TA 98 and TA 100 respectively. YG strains were selected from TA strains and have enlarged enzymatic equipment enabling them to metabolise tested substances in a different way than TA strains and can be more sensitive. Due to the differences in enzymatic equipment between prokaryotic and eukaryotic organisms, some substances do not have any mutagenic effect on bacterial detection systems and show mutagenicity after metabolic activation in mammalian organisms. Thus a test based on metabolic activation *in vitro*, which uses liver homogenate S9 prepared from either rat or hamster liver was developed The exogenous enzymatic activation fraction has to be added to bacteria together with tested substances (3).

2.3 Measurement of the growth inhibition by Bioscreen®

Bioscreen® served as a kinetic measurement of the growth of tested bacterial strains, it provides measuring of optical density (OD_{400nm}, 22ºC) in each well in regular intervals for several days. The growth rates (μ) of bacterial strains (*Pseudomonas* sp. P2, *Pseudomonas* sp. PC12B and isolates from contaminated soil label 1A, 2A, 3A, 4A and 6A) in the presence different concentration of MTBE as the sole source of carbon and via cometabolim of organic substrates (n-alkanes, acetone, cyclohexane, toluene or lactate, max concentration in a sample was 1% v/v) was monitored. It was used a mineral medium ABC (10ml of a solution **A**, 0,1ml of a solution **B** and of a solution 0,1ml **C** is added to 100 destilated H_2O). **A**: K_2HPO_4 7g/200ml, KH_2PO_4 3g/200ml, NH_4Cl 1g/200ml and NaCl 1g/200ml. **B**: Na_2SO_4 4,2g/30ml. **C:** $MgCl_2.6\ H_2O$ 4,5mg/30ml. OD_{400nm} of the samples with and without toxicants (MTBE), and time necessary to reach maximum OD (specific growth rate μ) were compared.

2.4 Oxygen consumption

Biochemical oxygen demand (BOD) measurement with the OxiTop® measuring system is based on pressure measurement (difference measurement). The measuring is made by pressure measurement via piezoresistive electronic pressure sensors. System does not use mercury (Hg) and was developed by Merck company (4).

3. RESULTS AND DISCUSION

3.1 Measurement of bioluminiscence

Toxicity of MTBE was measured by Microtox®. It was found a value EC50 33 mg/l. This concentration value is quite low, which means that MTBE is very toxic for tested strain. The result corresponds to the previously published data. (5).

3.2 Measurement of genotoxicity

Four bacterial strains were tested for mutagenicity screening of MTBE. The highest concentration of MTBE 3mg/plate was chosen according to the latest issued publications [6]. It was used preincubation assay, because MTBE is a volatile compound. The results, summarized in TABLE 1, show that maximal used concentration of MTBE is toxic for strains YG1042, TA98 and TA100. Lower concentration of MTBE had not effect; MTBE is not mutagenic for tested strains. MTBE is not mutagenic for strains TA98 and TA100 with S9 activation. These dates are consistent with previous reports (6).

TABLE 1. Summary of influence of concentration of MTBE on strains YG1041, YG1042, TA98 and TA100 with and without S9 activation.

Tested strain	Concentration of MTBE 3mg/plate	Concentration of MTBE 1.5mg/plate	Concentration of MTBE 1.5mg/plate + S9
YG1041	o	o	/
YG1042	Toxic	o	/
TA98	Toxic	o	o
TA100	Toxic	o	o

O - it means, that tested concentration of MTBE is not mutagenic

3.3 Measurement of the growth inhibition by Bioscreen®

The influence of MTBE on growth of tested bacterial strains was measured. The TABLE 2 summarized the results. Maximal concentration of primary source of carbon and energy was 1% (v/v) and an addition of MTBE was maximal 0,5%. Bacterial strains PC12B and P2 provide the best growth rate with MTBE as a sole source of carbon. The bacterial strain PC12B was able to utilize n-alkanes (Al), cyklohexane (C), lactate (L) and toluene (T). The growth rates were comparable (date not shown).

TABLE 2. Summary of influence of different source of carbon (n-alkanes (Al) and lactate (L)) presence of MTBE on growth of tested strains (soil isolates: 1A, 2A, 3A and 4A; PC12B).

Tested strains	Source of carbon and energy	μ (h^{-1})	Source of carbon and energy	μ 1(h^{-1})	μ 2 (h^{-1})
1A	1% Al	0.014	1% L	0.031	0.024
1A	1% Al + 0,05% MTBE	0.016	1% L + 0,05% MTBE	0.031	0.029
1A	1% Al + 0,5% MTBE	-	1% L + 0,5% MTBE	0.031	0.025
1A	0,5% MTBE	0.029			
2A	1% Al	-	1% L	0.079	-
2A	1% Al + 0,05% MTBE	0.026	1% L + 0,05% MTBE	0.065	0.026
2A	1% Al + 0,5% MTBE	0.036	1% L + 0,5% MTBE	0.050	0.027
2A	0,5% MTBE	0.034			
3A	1% Al	0.013	1% L	0.032	0.013
3A	1% Al + 0,05% MTBE	0.018	1% L + 0,05% MTBE	0.032	0.020
3A	1% Al + 0,5% MTBE	0.017	1% L + 0,5% MTBE	0.032	0.026
3A	0,5% MTBE	0.011			
4A	1% Al	0.025	1% L	-	0.011
4A	1% Al + 0,05% MTBE	0.021	1% L + 0,05% MTBE	0.067	0.013
4A	1% Al + 0,5% MTBE	0.022	1% L + 0,5% MTBE	0.067	0.016
4A	0,5% MTBE	0.017			

6A	1% Al	0.007	1% L	0.028	0.008
6A	1% Al + 0,05% MTBE	0.010	1% L + 0,05% MTBE	0.020	0.008
6A	1% Al + 0,5% MTBE	0.012	1% L + 0,5% MTBE	0.028	0.010
6A	0,5% MTBE	0.008			
PC12B	1% Al	0.039	1% L	0.027	0.009
PC12B	1% Al + 0,05% MTBE	0.045	1% L + 0,05% MTBE	0.026	0.015
PC12B	1% Al + 0,5% MTBE	0.042	1% L + 0,5% MTBE	0.014	0.025
PC12B	0,5% MTBE	0.048			

The bacterial strain P2 was able to utilize Al and aceton (Ac). The growth rate was higher with Ac (source of carbon and energy) then Al (date not shown). Soil isolates were cultivated in the presence of MTBE with Al or L as source of carbon. MTBE has not negative effect on the growth of all tested bacterial strains.

3.4 Oxygen consumption

Finally oxygen consumption of the tested strain was measured by system OxiTop®. It was tested the soil isolate 4A only. The presented data correspond with the obtained results by Bioscreen® as indicated on Fig.1.

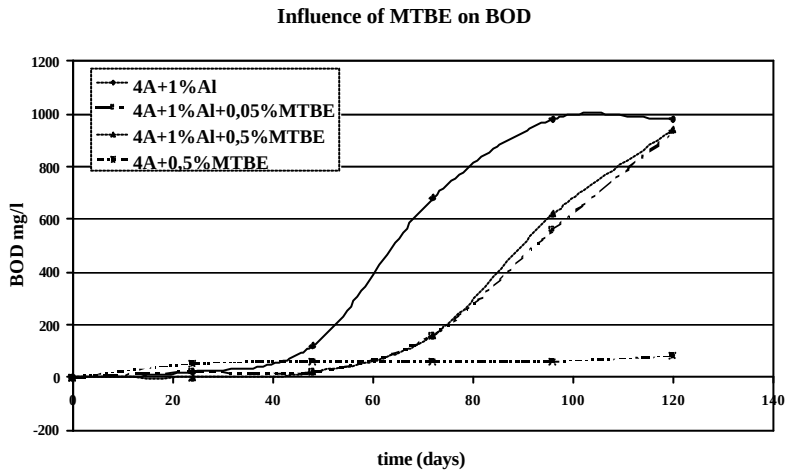

Fig.1 Influence of MTBE on BOD by soil isolate 4A.

REFERENCES:

(1), R.A. Deeb, K.M. Scow and L.Alvarez-Cohen (2000) " Aerobic MTBE biodegradation: an examination of past studies, current challenges and future research directions", *Biodegradation*, 11: 171-186.

(2) A.J. Stocking, R.A. Deeb, A.E. Flores, W. Stringfellow, J. Talley, R. Brownell and C. Kavanaugh (2000) "Biodegradation of MTBE: review from a practical perspective", *Biodegradation*, 11: 187-201.

(3) K Mortelmans, and E. Zeiger (2000) "The Ames *Salmonella*/microsome mutagenicity assay", *Mutat. Res.*, 455: 29-60.

(4) Manometric BOD Measuring Devices, *Instruction manual*, BA31107/07.00/AS/OxiTop IS-6_IS-12-1

(5) I. Werner, C.S. Koger, L.A. Deanovic and D.E. Hinton (2001) "Toxicity of methyl-*tert*-butyl ether to freshwater organisms", *Environ. Pollut.*, 111: 83-88

(6) D. Williams-Hill, C.P. Spears, S. Prakash, G.A. Olah, T. Shamma, T. Moin, L.Y. Kim and C.K. Hill (1999) “Mutagenicity studies of methyl-*tert*-butylether using the Ames tester strain TA102”, *Mut. Res.*, 446:15-21

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