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Estimation of the acute cesium toxicity by the microbial assay for risk assessment (MARA) test

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Abstract. The microbial assay for risk assessment (MARA) test was used for acute cesium toxicity evaluation in water solutions. The test contained 11 different microorganisms with a wide spectrum of sensitivity. The resistance of microorganisms to cesium was characterized as follows: microbial toxic concentration (MTC), half maximal inhibitory concentration (IC50), maximal inhibitory concentration (IC100). The sensitivity to cesium was characterized by the lowest observed effect level (LOEL). High levels of sensitivity in the range 3.1–6.3 mM were shown by the following microorganisms: *Serratia rubidaea* > *Pseudomonas aurantiaca, Delftia acidovorans, Citrobacter freundii, Staphylococcus warneri*. Lower levels of sensitivity (up to 16 mM) were noted for *Comamonas testosteroni, Microbacterium* species, *Kurthia gibsonii, Pichia anomala,* whereas that in the range 24–31 mM for *Brevundimonas diminuta* > *Enterococcus casseliflavus*. High resistance to Cs^+ was found for *E. casseliflavus* (MTC 86.9 g/l) > the yeast – *P. anomala* (MTC 19.3 g/l) > *K. gibsoni* (MTC 17.4 g/l) > *B. diminuta* (MTC 13.4 g/l). The phenomenon of resistance of enterococcus and yeast strains was discussed.

Key words: cesium toxicity • microorganisms' resistance to cesium • resistance of *Enterococcus casseliflavus* to cesium • *Microbacterium* species • *Brevundimonas diminuta* • *Citrobacter freudii* • *Comamonas testosteroni* • *Enterococcus casseliflavus* • *Delftia acidovorans* • *Kurthia gibsonii* • *Staphylococcus warneri* • *Pseudomonas aurantiaca* • *Serratia rubidaea* • *Pichia anomala*

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Introduction

The presence of cesium-137 in the environment is anthropogenic as a byproduct of the nuclear fission and an effect of nuclear reactor incidents. Radiocesium has not diminished yet in the natural environment after the Chernobyl disaster, while a new source appeared recently due to the Fukushima Dai-ichi nuclear disasters in Japan in 2011. Thus, the environmental pollution with radioactive cesium still poses a problem. Hence, the more necessary are the current investigations of the biological resistance to Cs. In such investigations, radiocesium is often replaced with stabile cesium because within the biological cycle of contaminated ecosystems, equilibrium of radiocesium with stable cesium is observed [21]. Cesium-137 may exert chemotoxicity and, additionally, radiolytic effects. The last effects are not specific to Cs but to the characteristic of the radioactive decay which is beta decay with gamma ray in the case of Cs-137. So, this paper is focused on the toxicity of stable Cs^+ towards microorganisms in general. Such investigations require toxicity tests targeted to a group of microorganisms. One of these tests is the acute toxicity test – MARA.

Table 1. Microorganisms applied in the MARA test

Strain no.	Strain name	Phylogenetic classification
1	*Microbacterium species*	Gram +
2	*Brevundimonas diminuta*	Gram – (α-proteobacteria)
3	*Citrobacter freudii*	Gram – (γ-proteobacteria)
4	*Comamonas testosteroni*	Gram – (β-proteobacteria)
5	*Enterococcus casseliflavus*	Gram +
6	*Delftia acidovorans*	Gram – (β-proteobacteria)
7	*Kurthia gibsonii*	Gram +
8	*Staphylococcus warneri*	Gram +
9	*Pseudomonas aurantiaca*	Gram – (γ-proteobacteria)
10	*Serratia rubidaea*	Gram – (γ-proteobacteria)
11	*Pichia anomala*	Yeast (saccharomycetaceae)

Materials and methods

MARA (microbial assay for risk assessment) test is a test for acute toxicity evaluation in water solutions or for toxicity screening of toxic samples derived from environment. The test was developed by J. Gabrielson's team in 2002, as a simple and quick method of bioindication of toxicity. The test is carried out with 11 different lyophilized microorganisms, with a wide spectrum of sensitivity, on a transparent well plate. The microorganisms with their phylogenetic classification are listed in Table 1.

Characteristic of microorganisms used in the MARA test

1. *Microbacterium* sp. – genus of gram-positive bacteria in family *Microbacteriaceae*. Thermoduric, saprophytic, nonmotile, found chiefly in dairy products, and human gastrointestinal tract. Widespread in natural environment [8].
2. *Brevundimonas diminuta* – non-lactose-fermenting environmental gram-negative bacilli assigned to genus *Pseudomonas*. Commonly used as test organism in water filters efficiency tests [6].
3. *Citrobacter freundii* – aerobic gram-negative bacilli assigned to family of *Enterobacteriaceae*. Citrobacter freundii are long rod-shaped bacteria typically 1–5 μm in length. Most *C. freundii* cells are surrounded by many flagella used to move about, but a few are non-motile. Its habitat includes the environment (soil, water, sewage), food, and the intestinal tracts of animals and humans [19]. *C. freundii* can be also opportunistic pathogen and may cause a wide variety of nosocomial infections of the respiratory tract, urinary tract, and blood [20]. It could be also useful in bioaccumulation and bioremediation of heavy metals, especially copper [16].
4. *Comamonas testosteroni* – gram-negative aerobic soil bacterium, formerly named *Pseudomonas testosteroni*. Noticed in different environments both soil and aquatic, especially muds, swamps, in plant and animal tissues. Also found in very polluted environments. *C. testosteroni* can assimilate testosterone, 4-chloronitrobenzene, and use phenylacetate and maleate as carbon sources [8].
5. *Enterococcus casseliflavus* – gram-positive anaerobic cocoids mostly paired, family *Enterococcaceae*, type *Firmicutes*. Slightly pathogenic, can be assumed as Vancomycin-Resistant Enterocossus but vancomycin resistance phenotype in *E. casseliflavus* is suppressed by VANC1 and VANC2 chromosomal genes which makes them low resistant to vancomycin and this resistance is not inherited [4]. These heat-durable microorganisms show wide tolerance to the temperature, growth in range 10–45°C, and ability to survive heating at 60°C for 30 min.
6. *Delftia acidovorans* – gram-negative aerobic, motile β-proteobacteria living in soil and water. Growth optimum temperature is 30–37°C, in the presence of 0.5 or 1.5% NaCl. Converts sulfur to sulfates. Very rarely causes infections but often pollutes water [20]. *D. acidovorans* is known (both with *Cupriavidus metallidurans*) for its ability to produce gold nuggets. These bacteria dissolve gold into nanoparticles, which migrate in soil and rocks and create gold ore [14].
7. *Kurthia gibsonii* – gram-positive bacilli, aerobic, common in sewage, meat, animal originated products and soils contaminated with animal feces. Optimal growth temperature is 42°C [11].
8. *Staphylococcus warneri* – gram-positive bacteria coagulase-negative, mostly aerobic. It is a commensal organism found in skin flora on humans and animals. Not pathogenic but in some cases can cause sepsis and endocarditis [12]. (Commensalism is a relation between two organisms where one organism benefits without affecting the other).
9. *Pseudomonas aurantiaca* – gram-negative bacilli belonging to γ-proteobacteria. Lives in different enviromnents, more likely in soils. It produces 2,4-diacetylofluoroglucylmethane – antibiotic active against gram-positive organisms. This makes *P. aurantiaca* helpful in plant preservation against pathogenic microorganisms [5].
10. *Serratia rubidaea* – gram-negative bacilli in family *Enterobacteriaceae*, γ-proteobacteria. Anaerobic bacteria present in water, soil and alimentary products. It produces a characteristic red dye, prodigiosin. *S. rubidaea* is a motile opportunistic pathogen, rarely causing infections in humans (pneumonia and infections of the urinary tract [18].
11. *Pichia anomala* – yeast species assigned to family *Saccharomycetaceae*. It lives in industrial sewages, soils, tree resins, cereal grains, and sweet waters. It grows in wide range of environmental conditions, could be applied in biotechnology due to its

antibacterial properties. It fights aflatoxins. Recent experiments on living pistachio trees showed 97% reduction of pathogenic fungi (*Aspergillus* sp.) in result of spraying [7].

Procedure of measurements

The microorganisms are incubated with subsequent dilutions of the examined toxic agent (element or chemical compound) in the presence of tetrazolium salts. The growth of microorganisms induces reduction of tetrazolium salts to insoluble forms which change color and precipitate at the bottom of the well. Incubation time takes 18 h after which the plate is subject to image analysis, where the amount of precipitated salts is compared to the control, and the growth rate is calculated. Scan of one of the samples shown in Fig. 1.

This method can be useful in the evaluation of the reactions of ecosystems to the toxicity of many substances. Each substance shows in this test a specific "fingerprint", an image of plate with different responses of microorganisms, unique and feasible to compare with other images. For the purpose of the test, the microbial toxic concentration value has been introduced. MTC is calculated by MARA software in the plate images from standard optical scanner with two sources of light, placed at the bottom and above the plate. The scanned image is processed in the software and the MTC values are given for each microorganism and a mean for all microorganisms. As a result of calculation, the software gives a matrix of results for growth or growth inhibition of microorganisms depending on the concentration in comparison to the nontoxic control. MTC value is defined with the following equation:

$$\mathrm{MTC} = c_{\min} \times d^{(p_{\mathrm{tot}} - p_{\mathrm{o}}) - 1}$$

where: $c_{\min}$ – lowest concentration in the test, d – dillution coefficient, p_{tot} – sum of growth size in 6 wells (all concentrations of the investigated sample) for individual strains, p_{o} – growth size in control wells.

It also gives MTC values for each microorganism, and mean MTC value for the test with information whether the sample was nontoxic, low toxic or toxic. When the mean MTC for the plate is lower than 20%, the sample is assumed as nontoxic.

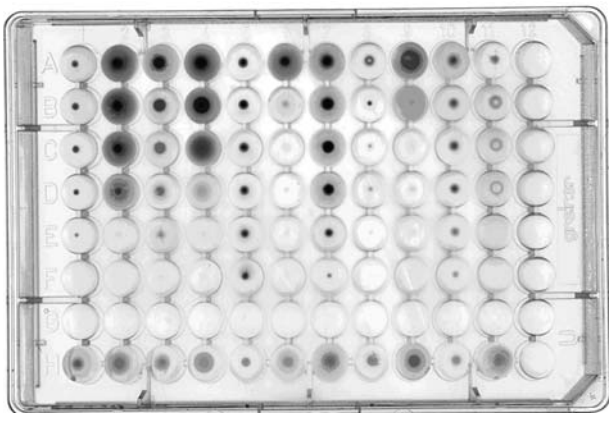

Fig. 1. Original sample scan – toxicity fingerprint.

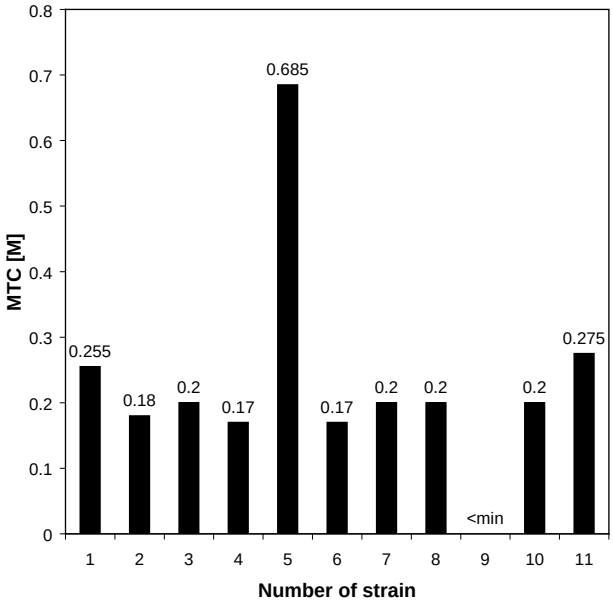

Fig. 2. Mean MTC values in MARA test, CsCl exposition ranged between 0.31 and 10 M.

Results and discussion

In this work the toxicity of cesium chloride was investigated. CsCl concentrations ranged between 0.0016 M and 10 M. Concentrations 5 and 10 M showed absolute toxicity to most organisms, 100% of growth inhibition except *E. casseliflavus* (no. 5), where MTC value was the highest (Fig. 2).

The most sensitive microorganisms in the test – no. 6 (*D. acidovorans*) and no. 9 (*P. aurantiaca*) started to grow when the range of concentrations was between 0.0063 M and 0.2 M, while the most durable *E. casseliflavus* showed no significant differences in growth in this range (Fig. 3).

Different ranges of toxicity showed that the response measured by the test may vary depending on

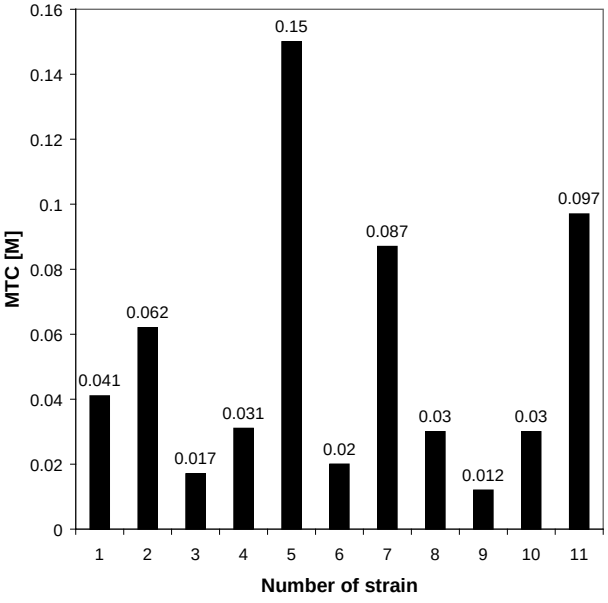

Fig. 3. Mean MTC values in MARA test, CsCl exposition ranged between 0.0063 and 0.2 M.

Table 2. MTC, IC50, IC100, and LOEL values aggregated to all MARA plates, CsCl exposition ranged between 0.0016 and 10 M

Strain no.	MTC (M)	IC50 (M)	IC100 (M)	LOEL (M)	SD	
					IC50	IC100
1	0.0536 (9.024 g/l)	0.045	0.617	0.016	0.0205	0.236
2	0.0794 (13.368 g/l)	0.095	0.366	0.024	0.0164	0.139
3	0.0288 (4.849 g/l)	0.025	0.424	0.0063	0.0058	0.208
4	0.0328 (5.522 g/l)	0.032	0.345	0.016	0.0125	0.135
5	0.5163 (86.924 g/l)	0.742	2.290	0.031	0.1280	1.030
6	0.0215 (3.620 g/l)	0.0313	0.135	0.0063	0.0037	0.102
7	0.1032 (17.375 g/l)	0.102	0.739	0.016	0.013	0.218
8	0.0303 (5.101 g/l)	0.049	0.231	0.0063	0.010	0.165
9	0.0136 (2.290 g/l)	0.0169	0.085	0.0063	0.0014	0.012
10	0.0342 (5.758 g/l)	0.025	0.610	0.0031	0.0102	0.417
11	0.1148 (19.328 g/l)	0.137	0.950	0.016	0.042	0.309

the highest concentration, that starts the subsequent dilutions. Some microorganisms are very sensitive at low concentrations (*D. acidovorans, P. aurantiaca*) while other are stable even at very high concentrations (*E. casseliflavus)*. The analysis of all investigated ranges of concentrations showed that the mean MTC for *P. aurantiaca* was 0.0136 M, for *D. acidovorans* – 0.0215 M. The most durable *E. casseliflavus* showed MTC 0.5163 M. Nevertheless, these values were not obtained on one test plate, so there were additional calculations done. Apart from MTC, there were other parameters calculated, like IC50, IC100 and LOEL, based on growth rates from different plates, with the use of logarithmic curves method. IC50 and IC100 were also completed with standard deviation calculation. Specific values of these parameters for each strain are showed in Table 2.

These results confirmed that *E. casseliflavus* has wide tolerance to the high CsCl concentrations (IC50 – 0.71 M) while the *P. aurantiaca* was more sensitive (IC50 – 0.017 M), for *D. acidovorans* – IC50 was 0.027 M. It also confirmed that MTC values supported by additional calculations like IC50 and LOEL can give comprehensive information about the fate of toxic compounds in biota, the potential impact and risk of pollution to the ecosystem, not only to single organisms.

The highest resistance to the Cs^+ in the MARA test was recorded for the *E. casseliflavus* strain, in which following values were reached: MTC – 0.51 (equivalent to 86 000 mg/l), IC50 – 0.74, and IC100 – 2.29. This enterococcus is an important hospital pathogen and is intrinsically resistant to low levels of vancomycin and has been found to colonize the human intestinal tract [3, 9, 10, 17]. According to other reports, *E. casseliflavus* was sensitive to gentamicin, vancomycin and chloramphenicol and resistant to cefazolin, ofloxacin, gatifloxacin and ciprofloxacin [15]. The knowledge about their resistance to several antimicrobial agents can be supplemented with information about resistance to relatively high cesium concentrations. For the first time, such high resistance of *Enterococcus casseliflavus* to Cs^+ has been shown. This special resistance of *Enterococcus casseliflavus* to Cs^+ shows that it is worth to undertake further investigations to explain this observation.

The next most resistant organism in the test was *Pichia anomala*: a yeast assigned to the family *Saccharomycetaceae*. It's MTC value reached 0.12 – equivalent to 19 000 mg/l, IC50 value – 0.14, and IC100 – 0.95. Toxicity screening of several yeast strains has shown that cesium and lithium (at concentrations up to 80 mM in MYGP agar) were the only toxic alkali metals of group I over this concentration range. Minimum inhibitory concentration (MIC) for *Saccharomyces cerevisiae* was 48 mM [13], while *P. anomala* in our tests showed higher sensitivity to Cs^+, where LOEL (equivalent to MIC) was 16 mM, respectively. Accumulation of Cs^+ in the yeast was metabolically-dependent [2]. Perkins and Gaad [13] concluded that the vacuole may play an important role in yeast intracellular Cs^+ compartmentation and detoxification.

It is very important to get knowledge of the MARA results, since cesium is one in a series of hazardous substances. Stable (non-radioactive) cesium has been found in at least 8 of the 1636 National Priority List (NPL) sites identified by the Environmental Protection Agency (EPA). Radioactive cesium has been found in at least 23 of the 1636 NPL sites identified by the EPA [1].

Abbreviations

EC50 – half maximal effective concentration
IC50 – half maximal inhibitory concentration
IC100 – maximal inhibitory concentration
LOEL – the lowest observed effect level
MARA – microbial assay for risk assessment
MIC – minimal inhibitory concentration
MTC – microbial toxic concentration
SD – standard deviation

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