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Screening method for detection of hydrocarbon-oxidizing bacteria in oil-contaminated water and soil specimens

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ABSTRACT

Toxic action of crude oil on the living world and ecosystems in general is a global problem of both aquatic and terrestrial environments. Bearing in mind the possibility of biodegradation of this toxicant, the procedures of determining counts and activity of cultivable microorganisms, and especially of bacteria responsible for degradation processes, are of great significance. The aim of this work was to study the possibility of modifying some solid media by adding triphenyltetrazolium chloride reagent as an indicator of the dehydrogenase activity, to develop a simple screening method for a simultaneous assessment of the count and activity of cultivable hydrocarbon-oxidizing bacteria in the oil-contaminated environments. The modified method appeared to be rapid and very suitable for the intended purposes.

Contamination and degradation of the environment caused by oil hydrocarbons and their derivatives represent a great problem because of the toxic action on the living world and functioning of ecosystems (Jacobuci et al., 2001). However, like many other pollutants, oil undergoes degradation, which may take place by biotic and abiotic processes. For removal of oil contamination recently has been used various remediation techniques that are, to a smaller or greater extent, based on biodegradation. The biodegradation efficiency relies upon microorganisms, first of all on bacteria that are adapted to grow in an oil-contaminated environment, using oil as a source of carbon and energy (Wyszkowska and Kucharski, 2001; Atlas, 1981; Ron and Rosenberg, 2002). Hydrocarbon-oxidizing microorganisms that are capable of degrading hydrocarbons originated from oil are widespread in the ecosystems. However, because of the complex nature of oil and its derivatives as contaminants, the estimation of the count and activity of this group of microorganisms is not always an easy task (Atlas, 1981; Li et al., 2000). Microbiological assessment of the bioremediation potential of water and soil contaminated with oil assumes the application of diverse methods, among which the determination of the counts of those microorganisms that are capable of degrading oil components and the determination of dehydrogenase activity (DHA) of microorganisms have a dominant role.

The determination of the count of hydrocarbon-degrading bacteria usually assumes their cultivation on a medium that contains crude oil or refined oil derivatives as a selective medium (Walker et al., 1976). Many researchers dealing with oil-contaminated environments determine the count of hydrocarbon-oxidizing bacteria in liquid media as a most probable number (for instance Gunkel and Trekel, 1967), whereas the others use solid agarised media (e.g. Walker et al., 1976; Adebusoye et al., 2006). The majority of media are of mineral composition, without readily uptakable organic matter (Gunkel and Trekel, 1967; Adebusoye et al., 2006). On the other hand, some media contain small amounts of organic matter such as peptones and yeast extract (Walker et al., 1976). Irrespective of the basic composition of the medium, the main source of carbon is always a mixture of alkanes (oil) (e.g. Delille et al., 2002 use Arabian light crude oil; Walker et al. (1976) use 0.5% oil, etc.), or one particular hydrocarbon (for example, Gunkel and Trekel (1967) use *n*-hexadecane). The application of oil powder from paraffinic oil base blurs significantly the medium, hindering thus the possibility of discerning small and colorless colonies. Because of that, some authors add 0.003% phenol red to the medium to enhance the contrast between the colonies and medium and thus facilitate counting (Walker et al., 1976).

Oxidation of organic matter by microorganisms assumes the involvement of dehydrogenase enzymatic system, so that its activity is a good indicator of the microbiological action in the contaminated environment, as well as of the potential and dynamics of bioremediation. The DHA is measured by colorimetric method using triphenyltetrazolium chloride (TTC) as electron acceptor, the reduction of which

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Table 1
The list of analyzed samples

Origin of specimens	Designation	Samples[a]
Water supply network	–	Tap water
Ground water wells with horizontal drains	BHD	BHD-2, BHD-6_1; BHD-6_2; BHD-7; BHD-8; BHD-9_1; BHD-9_2
Danube water on the location close to the wells and the Refinery	Danube BHD	Danube BHD-2_1; Danube BHD-2_2; Danube BHD-7_1; Danube BHD-7_2
Ground water from piezometers	PJC	PJC-5_1; PJC-5_2; PJC-6_1; PJC-6_2; PJC-8_1; PJC-8_2
Ground water from shallow piezometers	PPJC	PPJC-1_2; PPJC-1_3; PPJC-1_5; PPJC-Mp-6
Soil samples from bioreactor surface	SB	SB-1; SB-2; SB-3; SB-4; SB-5; SB-6; SB-7; SB-8
Soil samples from bioreactor depth	BB	BB-1; BB-2; BB-3; BB-4; BB-5; BB-6; BB-7; BB-8

[a] The numbers next to designation represent different sites, and the second numbers (in the subscript) represent different time of sampling.

by dehydrogenase gives the red triphenylformazan. The DHA is calculated from the value of the absorbance at 485 nm (Page et al., 1982).

It is important to point out that both the method of determining bacteria counts in solid media and the method of measuring DHA have certain advantages and shortcomings. The main advantage of the enumeration method is in the fact that it provides information about the passive bioremediation potential and proportion of hydrocarbon-oxidizing bacteria in the heterotrophic bacteria count, whereas the main shortcoming is the presence of uncultivable bacteria in the investigated environments (Balba et al., 1998). The determination of DHA takes also into account the uncultivable bacteria, but it has many shortcomings too. Thus, the DHA depends on the total metabolic activity of the microorganisms in the medium and it is not always in agreement with total number of viable microorganisms (Page et al., 1982). Besides, several other factors such as nitrates, nitrites and iron (III), functioning as alternative electron acceptors, may influence the activity of this enzyme (Balba et al., 1998). The aim of this study was to combine the cultivation methods (the determination of the count of hydrocarbon-oxidizing bacteria on solid nutritive media) and biochemical methods (detection of DHA of microorganisms) to work out a rapid and simple procedure for assessment of the bacterial count and detection of hydrocarbon-oxidizing activity of grown colonies from the oil-contaminated environments and thus estimate the capacity of bioremediation.

Counts of hydrocarbon-oxidizing bacteria were determined in water and soil samples (Table 1). Groundwater samples were taken from the wells with horizontal drains of the water supply system of the city of Novi Sad, which are situated close to the Oil Refinery, destroyed by bombing in 1999. Water samples were also taken from shallow and deep piezometers from the Oil Refinery area in different time intervals. In addition, water samples were taken from the Danube water on the locations close to the wells and Refinery and the water supply network of Novi Sad. The soil samples were taken from a laboratory bioreactor for in situ bioremediation monitoring of the oil-contaminated soil taken from the polluted Refinery area (Novi Sad). The soil in bioreactor was treated by aerated water or water with Mg_2O_2 addition several times, in order to stimulate oxidative processes of native microflora. Samples were taken from the surface layer and layers at particular depths monthly, during eight months of the setup operation.

Bacterial counts were determined by cultivation on agarised media using the spread plate method. The inocula of 0.5 ml and 0.1 ml of water samples were plated in triplicate, while the wet soil samples were previously 10 fold diluted in physiological saline from 10^{-1} to 10^{-5} and 0.1 ml of appropriate dilutions were plated in triplicate (10^{-3}–10^{-5}). The plates were incubated for 5–7 days at 26–28 °C. The grown colonies were enumerated and the average counts being calculated per ml of water or per gram of absolutely dry soil.

Table 2
Counts of hydrocarbon-oxidizing bacteria in water samples, grown on different nutritive media

Sample	Bacterial count (CFU/ml)[a]			
	MS	MST	T	TT
Tap water	20	20	0	0
BHD-2[b]	33	21	7	5
BHD-6_1	1.50×10^2	2.00×10^2	1.40×10^2	0
BHD-6_2	2.3×10^2	50	0	0
BHD-7_1	23	5	0	0
BHD-8	50	7	5	0
BHD-9_1	4.5×10^2	80	1.5×10^2	0
BHD-9_2	70	50	0	0
Danube BHD-2_1[c]	2.78×10^3	3.90×10^2	7.90×10^2	0
Danube BHD-2_2	2.20×10^3	6.00×10^2	9.00×10^2	0
Danube BHD-7_1	6.30×10^3	2.41×10^2	3.70×10^2	60
Danube BHD-7_2	4.00×10^3	1.40×10^2	1.60×10^2	0
PJC-5_1[d]	2.17×10^3	4.30×10^2	0	0
PJC-5_2	4.20×10^2	0	6	0
PJC-6_1	3.00×10^2	4.60×10^2	0	0
PJC-6_2	70	1.30×10^2	30	0
PJC-8_1	1.14×10^3	3.40×10^2	40	0
PJC-8_2	90	60	30	0
PPJC-12	50	10	40	0
PPJC-13	7.80×10^2	3.00×10^2	1.30×10^2	40
PPJC-15	5.50×10^2	5.40×10^2	40	40
PPJC-Mp-6	70	40	2	0

[a]The average count of three replicates.
[b]BHD — ground water wells with horizontal drains.
[c]Danube BHD — Danube water on the location close to the wells and the Refinery.
[d]PJC — samples from deep piezometers.
[e]PPJC — samples from shallow piezometers.

Two solid media were used: Tauson (T) medium ($(NH_4)_2SO_4$ 1.0 g/l; $CaSO_4$ 0.5 g/l; $MgSO_4\times7H_2O$ 0.3 g/l; $FeSO_4\times7H_2O$ 0.005 g/l; K_2HPO_4 0.15 g/l; KH_2PO_4 0.15 g/l) (Tauson, 1928) and modified MSWY medium (MS) (NaCl 5.0 g/l; KCl 0.75 g/l; $MgSO_4\times7H_2O$ 7.0 g/l; yeast extract 1.0 g/l; Proteose peptone 1.0 g/l; K_2HPO_4 0.3 g/l; KH_2PO_4 0.3 g/l; 20.0 g/l agar) (Walker et al., 1976). Tauson medium is basically a pure mineral medium, whereas MS contains also organic matter (peptones) which, in view of many authors, facilitates the metabolism of hydrocarbons by bacteria. As the MSWY medium was originally used for detecting hydrocarbon-oxidizing bacteria in sea water, it was modified in the

Table 3
Counts of hydrocarbon-oxidizing bacteria in soil samples from the bioreactor, grown on different nutritive media

Sample	Bacterial count (CFU/ml)[a]			
	MS	MST	T	TT
SB-1[b]	1.50×10^7	0	2.94×10^6	0
SB-2	5.40×10^6	4.41×10^5	3.67×10^5	0
SB-3	1.06×10^6	6.08×10^5	0	0
SB-4	1.20×10^8	4.29×10^7	0	0
SB-5	1.37×10^9	4.20×10^8	2.07×10^8	1.39×10^7
SB-6	9.52×10^8	1.76×10^8	1.03×10^9	0
SB-7	3.04×10^7	2.47×10^7	5.40×10^6	3.80×10^2
SB-8	6.85×10^7	1.30×10^7	2.31×10^7	3.00×10^2
BB-1[c]	6.32×10^6	6.32×10^6	1.80×10^6	0
BB-2	3.66×10^6	2.67×10^5	2.63×10^5	0
BB-3	5.9×10^6	3.30×10^6	0	0
BB-4	1.24×10^7	2.3×10^6	0	0
BB-5	1.71×10^7	4.77×10^7	3.79×10^7	1.19×10^2
BB-6	1.78×10^6	1.34×10^7	1.64×10^6	0
BB-7	4.00×10^6	2.00×10^5	1.30×10^5	0
BB-8	4.16×10^5	4.10×10^6	3.00×10^6	7.9×10^6

[a] The average count of three replicates.
[b] SB — soil samples from bioreactor surface.
[c] BB — soil samples from bioreactor depth.

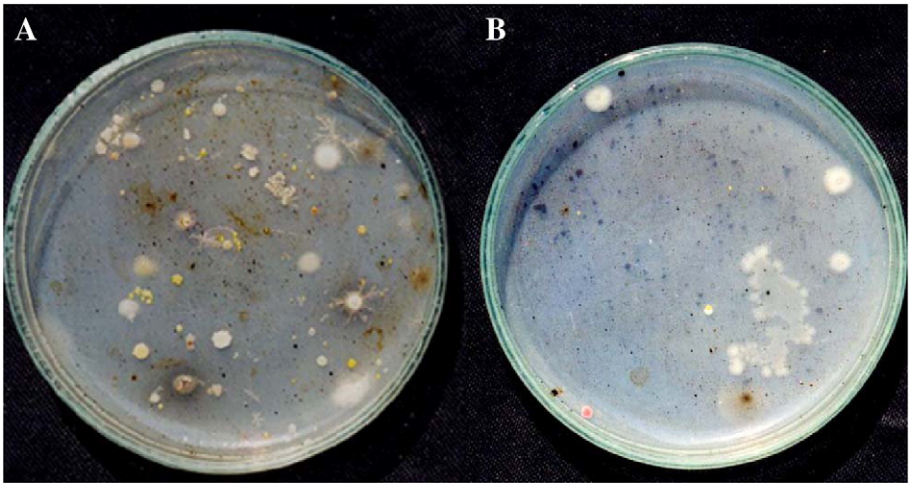

Fig. 1. Colonies of hydrocarbon-oxidizing bacteria on MS (A) and T medium (B).

study by reducing the content of NaCl. Oil powder from paraffinic base oil was used as the main carbon source in both media. The oil powder was obtained by mixing 20 g of oil and 20 g of silica gel in 60 ml of diethylether. After the homogenization and evaporation of diethylether, the oil powder was added to the medium (10 g/l). The media, sterilized in an autoclave, were used either as such or in a modified form. Modification of the medium, i.e. the combination of cultivation and enzymatic methods, consisted in adding TTC to the basic media (MS and T). After sterilization, TTC reagent (10 ml of 1% solution/l), sterilized by filtration, was added to one part of MS and T media (MST and TT, respectively). Each sample was inoculated in triplicate on MS, T, MST and TT media.

After the incubation on the MS and T media, all grown colonies were counted, whereas on the MST and TT only red colonies and/or colonies with red centers were enumerated.

The significance of difference in the counts of hydrocarbon-oxidizing bacteria on the media with and without TTC was subjected to nonparametric Kruskal–Wallis Test using Statistica 7.1 (StatSoft Inc., 2005). Differences were considered significant if $p<0.05$.

Counts of hydrocarbon-oxidizing bacteria in water and soil samples are presented in Tables 2 and 3. Depending on the medium type, the counts were in the span from 0 to 6.3×10^3/ml in water samples and from 0 to 1.03×10^9/g absolutely dry soil.

The counts of hydrocarbon-oxidizing bacteria obtained on MS medium were larger compared to those obtained on T medium for water samples ($p=0.0054$), but not for soil samples ($p=0.2079$). The presence of nutrients in MS medium yielded the growth of a larger number of bacteria from water samples compared to that on T medium. Kersten (1964) thinks that hydrocarbon-oxidizing bacteria can be generally classified into two groups. Those bacteria that grow on mineral media with hydrocarbons and do not use other organic compounds belong to one group. According to this author, the majority of hydrocarbon-oxidizing bacteria make the other group of more widespread bacteria, which grow on both mineral media with hydrocarbons and on media with other organic compounds.

With the soil samples, no statistically significant difference was found between the counts on MS and MST media ($p=1$) and T and TT ($p=0.0928$), whereas the difference between the counts of colonies on MST and TT was significant ($p=0.0013$). A similar conclusion is also valid for the counts of hydrocarbon-oxidizing bacteria in water samples — the difference between the values on MS and MST was not statistically significant ($p=0.8220$), whereas the difference between T and TT, as well as MST and TT was statistically significant ($p=0.0277$ and <0.0001, respectively). A lower count of bacteria on the media with TTC addition cannot be explained by the 'toxic' action of the reagent on the bacteria growth. Bauer et al. (1991) reported that tetrazolium salts were toxic to bacteria only at high concentrations or during the incubation at a high temperature, which was not the case in our study. Kluber et al. (1995) found that among the hydrocarbon-oxidizing bacteria isolated from soil, even 90% showed the ability of reducing TTC. Also, many other authors have successfully used TTC for different purposes — for detecting bacterial biofilm on medical biomaterials (Rozalska et al., 1998), establishing the germicide effect of chemical agents (Hurwitz and McCarthy, 1986), detection of the

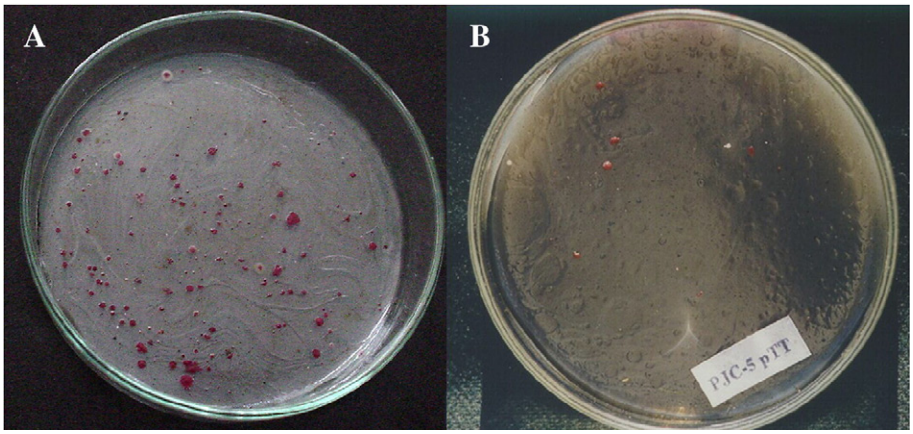

Fig. 2. Colonies of hydrocarbon-oxidizing bacteria on MST (A) and TT medium (B).

viability of *Mycobacterium tuberculosis* (Vishnevskii and Manicheva, 1992), etc. The addition of TTC to the medium with the aim of detecting hydrocarbon-oxidizing bacteria represents a novel application of this reagent, so that our first choice was MS medium with addition of TTC, since we thus could obtain simultaneously not only information on the count, but also on the presence of biochemical activity of bacterial colonies.

In order the hydrocarbon-oxidizing bacteria possessing dehydrogenase could reduce TTC to the red triphenylformazan it is necessary to ensure the presence of readily uptakable organic matter in the medium. Some authors, such as Casida (1977) and Bochner and Savageau (1977), suggested peptones as growth nutrients that act stimulatively on TTC reduction. Since MST medium contains peptones it is clear why a higher bacteria count is obtained on this medium compared to the purely mineral medium T. Moreover, the more intensive growth can also be explained by the fact that MST medium contains yeast extract, supplying bacteria with carbon and nitrogen compounds, vitamins, and other growth factors needed for bacterial reduction of TTC. Casida (1977), in studying DHA of soil, found that soil without the presence of yeast extract and glucose had a very low DHA value (0.01mg of triphenylformazan/g dry soil).

The colonies of bacteria growing on the MS and T media were colorless (nonpigmented) or differently pigmented, depending on their natural characteristics (Fig. 1A and B). Because of the specific feature of the medium, the colonies were difficult to discern. The modification of the basic media by adding TTC provided the easier discrimination of grown, red colored colonies against turbid medium (Fig. 2A and B).

In view of the fact that TTC serves to identify the DHA activity we suppose that we deal with metabolically active hydrocarbon-oxidizing bacteria. Bochner and Savagean (1977), mentioned that the colonies capable to catabolize test substrate reduce TTC and produce red formazan, whereas the colonies that insufficiently catabolize substrate remain white. The concentration of nutrients in the medium is a key factor that influences TTC reduction. On the MST and TT media the colonies of hydrocarbon-oxidizing bacteria, irrespective of their natural pigmentation, were of the same, red color. However, there also appeared colorless colonies, as well as the colonies with a red center. A question arises as to how to explain this phenomenon, especially in the case of the colonies that are partly colored red. Bochner and Savagean (1977), using the medium with TTC observed the growth of red and white or colorless colonies, and they think that this phenomenon is manifested as the competition for nutrients. The TTC reduction will be stimulated by the abundance of nutrients and inhibited in the limited presence of nutrients. These authors used peptones as nutrients for the growth of colonies, whose color depended on the peptone concentration.

Although all the four media (MS, MST, T, and TT) appeared to be suitable for practical work, all the above facts indicate that MST medium is most appropriate for detecting hydrocarbon-oxidizing bacteria. It can serve as a simple screening method for a simultaneous detection of the presence, enumeration, and determination of activity of cultivable bacteria in the natural environments.

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