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AN APPROACH TO BIOREMEDIATION OF MINERAL OIL POLLUTED SOIL

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Summary: Mineral oil spill, especially waste-crankcase oil affects the soil which remains bereft of fertility for a long time. In the present study, an approach to soil bioremediation, based on application of mixed algal culture was described. Cyanobacteria and algae release oxygen in the soil and increase the rate of abiotic processes of oxidation which destroy the pollutant to harmless products. The released oxygen is also of use to the roots of higher plants in the polluted soil. The algal extracellular organic substances are a useful substrate for the soil bacteria and fungi which metabolize the pollutant.
Seed germination, plant growth and development in oil polluted soil, watered with mixed algal culture, were quantified using *Tribulus terrestris* as an experimental plant. The plant growth parameters were very low at 5 % of heavy diesel fuel in the polluted soil, whereas up to 1 % of waste-crankcase oil was overcome. In case of higher pollutant concentrations the remediation required addition of sand, soil or compost.

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INTRODUCTION

The spill of mineral oil products in the environment is against the low, and in some cases it is regarded as a criminal act. The aftermaths for the soil fertility are really heavy. Longer chain hydrocarbons remain in the soil for a long period. The vegetation is extremely scarce in the polluted soil or it is totally absent. According to Harmsen (2004) biodegradation of hydrocarbons is a slow process. Even under laboratory conditions the process takes long time (Sabaté et al., 2004). The higher concentration of the pollutant increases the negative effect on plant growth and development (Vwioko and Fashemi, 2005).

Bioremediation of the oil pollution comes down to presence and functioning of the ω-oxidation enzyme system which can oxidize hydrocarbons to alcohols:

$$RCH_2CH_3 \rightarrow RCH_2CH_2OH$$

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The oxidation of unsaturated hydrocarbons flows significantly easier:

$$RCH_2CH=CHCH_3 \rightarrow R.CH_2CH.OH.CH_2CH_3$$

The next oxidation step from alcohol to aldehyde and acid takes place probably in all organisms. Higher plants do not have the ω-oxidation enzyme system. They cannot metabolize alkanes and often cannot withstand the pollution. Some microorganisms, such as bacteria and fungi, are in possession of the ω-oxidation enzyme system. Although slowly, they can metabolize hydrocarbons (Basuki et al., 2011; Onifade and Abubakar, 2007; Ekundayo et al., 2012).

The approach to soil bioremediation presented in this study is based on the application of a mixed algal culture. This experiment was based on the fact that microalgae release oxygen, which is beneficial for the roots of higher plants and for the aerobic bacterial microflora. Also, the rate of all abiotic oxidative processes is enhanced by the higher oxygen concentration in the soil and this contributes to the degradation, at least of unsaturated hydrocarbons.

As it is well known, microalgae excrete organic substances which are a substrate for heterotrophic microorganisms – bacteria and fungi. The bacteria, which accompany microalgae, possess active oxidases, which metabolize some oil hydrocarbons. Under conditions of high light intensity combined with CO_2 deficiency, soil algae excrete glycolic acid (Kambourova et al., 2006) which is quickly metabolized by the aerobic bacteria and facilitates the rate of the biological processes in the soil. The nitrogen-fixing cyanobacteria *Nostoc* and *Anabaena* present in the soil can improve both soil fertility and plant growth. In a previous study, the green alga *Scenedesmus* was found to demonstrate significantly higher resistance to oil pollution compared to other photosynthetic microorganisms, namely *Chlorella* and the cyanobacterium *Spirulina* (Petkov et al., 1992). Algae from different taxa were recently isolated from oil polluted soil and recognized microscopically as cyanobacteria *Nostoc* sp., two different *Phormidium* sp, green algae *Klebsormidium* sp. and *Chlamydomonas* sp. (Iliev et al., 2011).

The aim of this work was to study the germination, growth and development of higher plants in polluted soil in the presence of microalgae and cyanobacteria. As for the choice of a proper higher plant species, it ought to be able to withstand the impact of the pollutants. Preliminary experience, based on observation mostly, showed that puncturevine, *Tribulus terrestris*, could be the apt one. The plant has often been found around railways where the concentration of coal dust and crude oil is higher as a rule. The creosote-treated railway sleepers represent another source of pollution. On the other hand, the ground alongside railways is a place where the vegetation is relatively scarce due to the lower soil humidity, usually higher temperature due to the heated railway track stones, the dark color of the rails and railway sleepers. The usual natural habitats of puncturevine are desert soils and salty sands (Petkov, 2010; 2011). Herewith, the task comes down to studying the germination of puncturevine seeds in oil polluted soils and their growth and development to mature plants. The application of photosynthesising

microorganisms has been accepted as a main point contributing both to plant growth and soil remediation. The return of the indigenous flora on the bare spots would be a token for remediation of affected soils and a proof for usefulness of the method.

MATERIALS AND METHODS

Analysis of oil pollutants

Soil from spills of mineral oil products was used throughout the experiments. The soil samples were extracted 3 times with hexane at a proportion of 1:20 (w/v). The extracts were evaporated *in vacuo* and estimated gravimetrically. The samples were purified using thin layer chromatography (TLC) on silica gel plates and hexane as a mobile phase. A gas chromatograph (Hewlett Packard 6890 System) equipped with a 5973 mass-selective detector (splitless mode) was used for analysis of the pollutants. The analysis was carried out in an HP-5MS capillary column (60 m × 0.25 mm I.D, film thickness, 0.25 μm) and He was used as a carrier gas. The MS detection was full scan, (m/z) 40 - 750. The ion source was set at 230ºC and the ionization voltage was 70 eV. Identification was based on comparing the mass spectra of the chromatographic peaks to those reported in the NIST08 and Wiley libraries, using authentic standards, GC retention times and interpretation of mass-fragmentation patterns.

Microalgal cultures

Photosynthesizing microorganisms were isolated from small stones on the surface of the polluted ground. The algae were maintained and prepared for watering in 5-fold diluted nutrition medium described by Petkov (1995) at uninterrupted illumination with luminescent lamps, and bubbling with 3 cm^3 s^{-1} air, enriched with 0.5 % CO_2.

Growth of *Scenedesmus* sp. and mixed algal culture in a medium with hydrocarbons was carried out at 75 μmol m^{-2} s^{-1} light intensity and temperature of 30 °C in glass tubes. The nutrition medium of Zehnder (Staub, 1961) was used for the growth experiment. The algal density was measured spectrophotometricaly.

Planting the seeds of *Tribulus terrestris*

Seeds of puncturevine, *Tribulus terrestris* L., were collected from non-polluted soil near the town of Pazardzhik and the Black Sea coast. Two types of polluted soil, with heavy diesel fuel and waste-crankcase oil, were used in the experiments. Seeds and plants were sown in transparent plastic pots as was described elsewhere (Petkov, 2010; 2011). Young plants of *T. terrestris* or their seeds were watered up once after sowing with the microalgal suspension. Seeds of the same origin sown in sand were used as a control, irrigated with the mixed algal culture too.

As part of the work, polluted soils were sought for, and no experimental pollution was observed.

RESULTS AND DISCUSSION

A mixed culture of photosynthesizing microorganisms, together with their concomitant bacteria, was isolated from small stones on the surface of the polluted soil. The microscopic observation showed that cyanobacterium *Nostoc* sp. and green alga *Scenedesmus* sp. were

the predominating photosynthesizing organisms which corresponded with previously described results (Iliev et al., 2011).

The green alga *Scenedesmus* sp. as well as the mixed algal culture were grown for 10 days in the laboratory at three different concentrations of the pollutants in the medium (50, 150, 200 mg dm^{-3}) using extracted oils from the polluted soil.

The growth curves of *Scenedesmus* sp. (Fig. 1) showed that this alga overcame the influence of oil hydrocarbons in the medium. The mixed culture showed resistance to the petroleum in the nutrition medium (Fig. 2). The pollutants applied at three different concentrations did not affect the growth of the culture. The alga *Scenedesmus* sp. predominated in the mixed culture on the 10th day of cultivation.

Two types of soil polluted with mineral oil products were used throughout the experiments. According to the gravimetric analysis, preparative TLC and GC/MS of the pollutants, the different soils were contaminated to a different degree with heavy diesel fuel and oil from internal combustion engine.

Two parallel experiments were carried out with soil polluted with heavy diesel fuel that continued 100 days, knowing that the plant usually gives ripe seeds 60 days after sowing.

In the first experiment seeds of *T. terrestris*, preliminary characterized as having high germination, were sown as previously described (Petkov, 2010; 2011) and watered with the mixed algal culture. The final concentration of the suspension was 0.2 g dm^{-3} as total dry weight of algal biomass. The first and the only seed germinated on the 40th day after sowing. We did not observe germination of other seeds of *T. terrestris* to the 100th day, the end of the experiment.

Young plants of *T. terrestris* (14-days old) were planted in the same polluted soil (5% heavy diesel oil). A fresh diluted suspension of mixed algal culture was prepared and used for watering. The plants showed lower growth, up to 4 - 5 cm. They gave 1 - 2 flowers, as well as seeds. At the same time, the control plants grew and developed as usual, 50 - 60 cm in length,

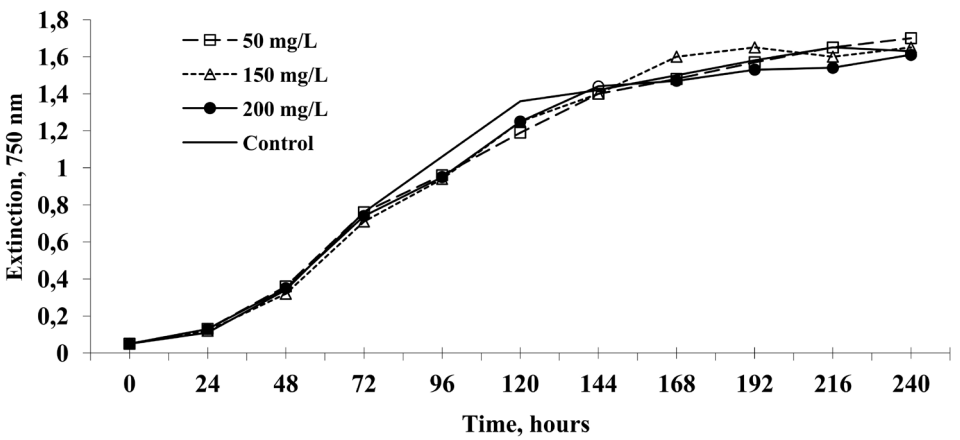

Figure 1. Growth of the green alga *Scenedesmus* sp. at three different concentrations of heavy diesel fuel in the medium (50, 150, 200 mg dm^{-3}).

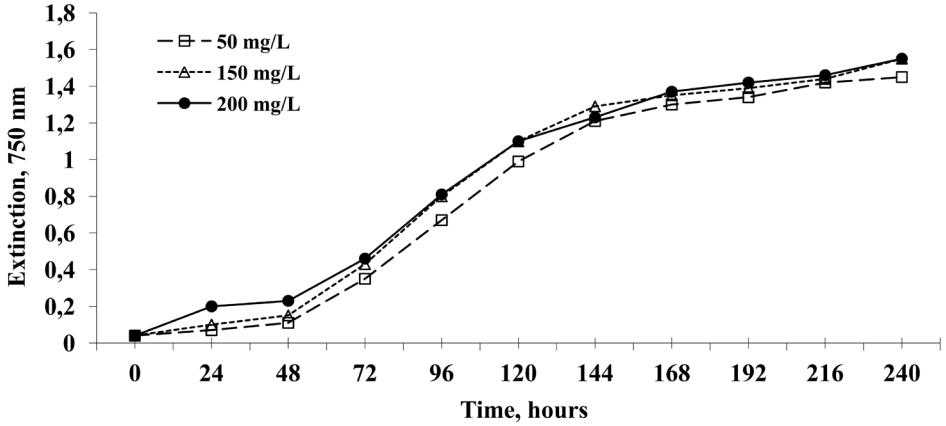

Figure 2. Growth of mixed algal culture at three different concentrations of heavy diesel fuel in the medium (50, 150, 200 mg dm^{-3}).

and gave 20 - 25 flowers per plant.

Several spots of waste-crankcase oil spills, presenting grassless bare patches, and caused by wrongdoers, had been carefully observed for 5 years. The sentence: "leaving no living blade of green" is an exact description of this case. Rain, remaining as drops on the surface, did not wet the soil enough. Canopy of snow piled slowly, and remained for a shorter time drawing the real border of the spots. No vegetation appeared during these 5 years. After removal of the upper layer (7 - 8 cm), weak vegetation scarcely grew in the following year due to the seeds of surrounding plants. The plant *Convolvulus arvensis* L. grew and gave flowers which did not open and fell down.

The removed soil was a subject of laboratory study. The content of waste crankcase oil was 2% from the mass of the soil at 55% humidity. Obviously, 2% engine waste oil was a high degree of pollution. The pollutants were reduced to 1% (w/w) by mixing the soil with sand, and *T. terrestris* seeds were sown in it. The polluted soil was irrigated with the mixed algal culture as in the previous experiment with 5% heavy diesel oil.

Both control and experimental seeds began their germination on the 4th day after sowing (Table 1). The experimental

Table 1. Growth parameters of *T. terrestris* after 100 days of growth in two types of oil polluted soil irrigated with mixed algal culture.

Parameter	Heavy diesel fuel, 5 %		Engine oil, 1 %	Control
	Seeds	Plants		
Beginning of germination, day	40	-	4	4
Percentage of germination, %	3	-	40	50
Number of blossoms per plant	-	1 - 2	1	20 - 25
Length of twigs, cm	-	4 - 5	4 - 5	50 - 60

*There was no germination it both polluted soils in the case of no irrigation with the mixed algal culture.

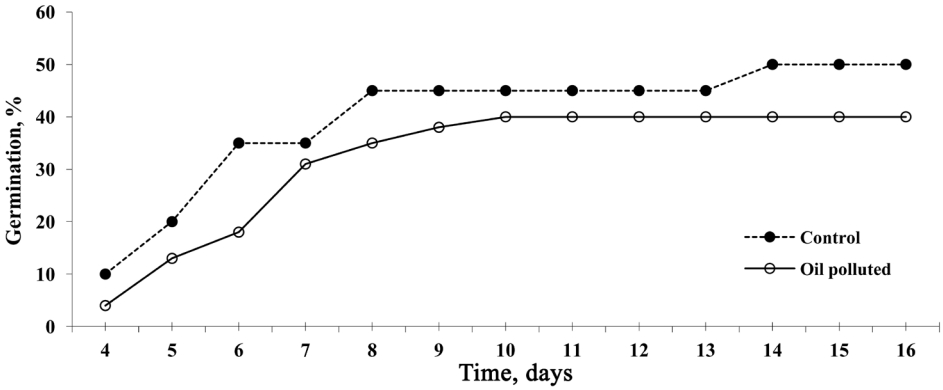

Figure 3. Germination of *T. terrestris* seeds in sand (Control) and 1% of engine oil polluted soil watered with mixed algal suspension.

plants grew up to 4-5 cm in length as in the soil polluted with heavy diesel fuel (5%, w/w). The percentage of seed germination was similar to that of the control seeds, grown in non-polluted conditions (Fig. 3).

As shown in Table 1, the heavy diesel fuel in the soil (5%, w/w) inhibited strongly seed germination. However, the experimental plants grew and developed, although weaker than the controls, in both types of contaminated soil after watering with the mixed algal suspension.

The polluted soil, being taken from the environment, contained other seeds occasionally being there. These included bindweed (*Convolvulus arvensis*), plantain (*Plantago lanceolata* L.), dandelion (*Taraxacum*) and meadow-grass (*Poa*). Bindweed slowly cropped up and developed after watering the soil with the algal suspension.

Our experiments showed that even single watering of the damaged soil with the diluted suspension of indigenous cyanobacteria and microalgae contributed to the remediation. On the field, the algal suspension has to be put in the water. The concentration of the algae must be at least 0.2 g dm^{-3} calculated as dry biomass. The alive algal culture has to be preliminary prepared with high density, for example 7-8 g dm^{-3} which can be diluted 30-40-fold on the field. In heavy cases of engine oil pollution, mixing of sand, soil or plant compost is necessary, which is in accordance with Van Gestel et al. (2003). Dilution of the oil to 1 % mends the vegetation and gradually restores soil fertility.

It is worth mentioning that plant biomass could be used for technical aims. In accordance with the legislation, a pollution index must be reached before using plant biomass as food or fodder.

CONCLUSION

The present study showed that watering with suspension of cyanobacteria and microalgae was useful and effective for the remediation of oil polluted soil. It is recommendatory, that the isolates of photosynthesizing microorganisms and their concomitant bacteria have to be taken near to the damaged places. It could definitely be said that treatment of the soil by

aid of the indigenous microflora is an environmentally friendly manner of mending and good husbandry. Growing of algae as a mixed culture is an easily doable task, thoroughly matching to a farm or a small municipality. In order to recover the vital activity in mineral oil polluted soil, as a first step it is necessary to increase the concentration of algal cells introducing them in the soil in the form of preliminary grown mixed culture.

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