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RESEARCH ARTICLE

Integrated methodology to evaluate bioremediation potential of creosote-contaminated soils

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Abstract

*Integrated methodology including chemical analyses as well as biological and ecotoxicological testing of soil was used to evaluate bioremediation potential of an aged, highly contaminated soil from a wood preservation plant. The soils contaminated with coal tar creosote originated from two sites (*Site I*: 8000 mg/kg and* Site II*: 133,800 mg/kg). A toxicity test-battery able to detect different effects using a variety of endpoints was developed and applied for the ecotoxicological assessment of creosote in soils:* Azotobacter agile *and* Pseudomonas fluorescens *dehydrogenase enzyme activity test,* Vibrio fischeri *bioluminescence test,* Sinapis alba *root and shoot elongation test and* Folsomia candida *mortality test. The biodegradation and its enhancement were investigated in small-scale bioreactors in short-term laboratory experiments. The joint evaluation of chemical, biological and ecotoxicological results made possible to compare different technologies, and investigate their applicability for remediation of soils contaminated with coal tar. The most sensitive direct contact tests that also correlated well with the creosote-reduction were* Vibrio fischeri *bioluminescence test, followed by* Folsomia candida *mortality test and* Sinapis alba *shoot elongation test.*

Keywords

creosote · ecotoxicology · integrated methodology · soil bioremediation · toxicity test-battery · lab-scale experiment

Acknowledgement

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1 Introduction

Many sites have been polluted by coal tar creosote as a result of wood-preserving activities worldwide. Chemical, biological and thermal treatment technologies for creosote-contaminated soils including thermal desorption, solvent extraction, landfarming, solid and slurry phase bioremediation are accepted by the United States Environmental Protection Agency (USEPA) [1]. Bioremediation, based upon biodegradation of pollutants can be effective and low-cost treatment technology of creosote-contaminated soil, providing microbes capable of degrading the constituents of coal tar creosote. Microorganisms capable of degrading creosote-components (e.g. polycyclic aromatic hydrocarbons (PAHs), pentachlorophenol (PCP)) in coal tar contaminated soil have been reported [2–5].

Biodegradation of polycyclic aromatic hydrocarbons in coal tar contaminated soils from wood treatment facilities and the enhancement of bioremediation were also investigated and evaluated in the few past years [6–9].

One of the aims of this work was to assess, at lab-scale levels, the feasibility of bioremediation technologies of historically, highly contaminated soils from a wood treatment facility. Feasibility studies are essential and can have an enormous impact on the cost of full-scale remediation [10].

Coal tar creosote is toxic, and the Environmental Protection Agency of United States has determined that coal tar creosote is probably a human carcinogen [11]. This black viscous fluid is a mixture of exceedingly complex constituents; thus it is not possible to represent the chemical formula and structure of these materials. In consequence of this complexity of the coal tar creosote, and the potential biotransformation of constituents, monitoring and evaluation of bioremediation, and characterization of the contaminated soil require high quality methodology.

Traditionally, chemical analyses are used in monitoring of soil remediation processes. Assessment of contaminated soil based on chemical analysis is not feasible, because chemical methods alone do not give information about the interaction of chemicals, do not consider the partition and mobility of pollutants, and do not indicate the biotransformation and biodegradation of contaminants in the soil. Thus the chemical parameters do not pro-

vide sufficient basis for evaluating the real risk potential due to missing information about biodegradation, partition, toxic and related harmful effects.

Biological and ecotoxicological characterization of contaminated soil gives additional important information to the results of chemical analyses. Only a limited number of compounds of creosote can be analysed by chemical analyses, so the bioassays can add and provide valuable and complementary information.

The results of biological and ecotoxicological methods show the effects of all contaminants and integrate interactions between contaminants and toxic contaminant and matrix.

Ecotoxicity tests measure the effects of the bioavailable ratio of the contaminants, the chemically not measurable or not measured toxicants, and the intermediary metabolites. For contaminated soil assessments ecotoxicological and biological methods are currently used during bioremediation [10, 12–16]. Most of the ecotoxicity testing methods, even in case of solid phase samples, are applied to aqueous phase or extracts, which differ from whole soil considerably. The soil assessment with elutriate testing can lead to an underestimation of total soil toxicity. For these reasons the direct contact testing of whole soil has got increasing importance recently [16–21]. Results of biological tests show the degradative activity, the adaptation and/or the adaptive potential of the soil microorganisms in connection with the biodegradation process.

The toxicity tests, which can assess and monitor the bioremediation has only been recently developed, and the knowledge on the toxic effect of coal tar contaminated soils is still limited. Single species and bacterial bioassays have been mostly used for the characterization of the toxic effect of creosote contaminated soils [22–25]. To get a full picture of the quality of the environment and a realistic view about the risk of the soil pollutant, however, a battery of the toxicity tests representing different trophic levels of testorganisms is necessary.

The arguments mentioned above stress the importance of application of an integrated methodology in all phases of soil remediation: site assessment, selection and design of the technology, technology monitoring, and after-monitoring of the site. Detailed monitoring and final evaluation of remediation efficiency are important for process control, as well as for ensuring environmental safety.

The main objective of the present work was to develop and apply a complex chemical-biological-ecotoxicological methodology to follow and evaluate the bioremediation of creosote-contaminated soils and to design a direct contact ecotoxicological test-battery representing different trophic levels. Biodegradation experiments were performed with creosote-contaminated soils in solid and slurry phase bioreactors modelling bioventing and slurry phase biotreatment.

This paper presents the results and evaluation of the integrated methodology developed and used in this lab-scale feasibility study. Physico-chemical analyses of soil characteristics, determination of extractable petroleum hydrocarbon content and several biological parameters were also evaluated, like the concentration of aerobic heterotrophic cells and creosote-degrading cells and soil respirometry. Five contact ecotoxicity tests with 3 microbial, 1 plant and 1 animal testorganisms were modified and applied for direct soil investigation in addition to the chemical analyses. A toxicity test-battery was developed with regard to the usefulness of monitoring or assessing the bioremediation process.

2 Materials and Methods

2.1 Experimental Setup of Bioremediation Experiments

The representative soil samples originated from different points of a heterogeneously contaminated actual site of a wood preservation plant. The initial coal tar concentration was: 8000 mg/kg (*Site I)* and 133,800 mg/kg (*Site II*). We carried out technological experiments with coal tar oil contaminated soils in solid phase and slurry phase bioreactors.

Solid Phase Bioremediation (Modelling Bioventing)

Self-designed flow-through system with small-scale (1 dm^3 of volume) static reactors (modelling bioventing) was used in solid phase laboratory experiments for 4 weeks. The contaminated soil samples (500 g) were intensively aerated and the CO_2 production of the soil microflora was continuously measured. Optimal humidity (10–15 % $^{w}/_{w}$) was maintained throughout the whole experiment. We evaluated the efficiency of bioventing by comparison of the results of the integrated methodology before treatment and after bioventing.

Slurry Phase Bioremediation

Biodegradation experiments in slurry phase were carried out in small scale (1dm^3 of volume with 500 g of soil) stirred, slightly aerated reactors for 10 weeks. The soils were supplemented with nutrients and inoculated with indigenous, adapted microflora or with H10CS commercial inoculate. The H10CS is a proprietary blend of microaerophilic bacteria and micronutrients [26]. The granulated commercially available inoculate was resuspended in mineral salt medium before adding to the slurry phase soil. The creosote degrading inoculate was prepared in our lab by the propagation of the selected and isolated microorganisms of indigenous microflora. A control experiment without inoculation was used for studying the effects of inoculation. The soil samples were taken and analysed by the integrated methodology after 1 week, 3, 6, 8 and 10 weeks.

2.2 The Integrated Methodology for Soil Characterization and Evaluation of Bioremediation

We developed a complex methodology including specific combinations of the methods in all phases of remediation depending on the aim of testing. The applied test-set includes standardized methods as well as newly developed and modified ones.

Chemical Analyses

Extractable organic material content was measured after hexane-acetone (2:1) extraction by gravimetry [27]. The so-called Extractable Petroleum Hydrocarbon (EPH) content was analysed from the same extract by gas chromatography with flame ionization detector (GC-FID) according to the Hungarian Standard [27].

Biological Methods

The concentration of cultivable aerobic heterotrophic bacterial cells in the soil is proportional with the microbial activity. *Aerobic heterotrophic bacterial cell concentration* was determined by colony counting after cultivation of microorganisms occurring in soil suspensions in water on Peptone-Glucose-Meat extract (PGM) agar plates in Petri-dishes. After colony counting the result was given as Colony Forming Unit (CFU/g soil).

The *cell concentration of the pollutant-degrading microbes* in a soil can be measured by any growth- or respiration test applying the contaminant as the only carbon source in the test system containing whole soil. We developed a relatively simple test for measuring the concentration of hydrocarbon (or any organic xenobiotic compound) degrading cells in soil. The *population density of the creosote-degrading cells* was measured after cultivation in tubes of liquid nutrient medium. For growing the creosote-degrading cells a dilution series of contaminated soils were used in 3 replicates, containing coal tar creosote as the only carbon source supplemented with inorganic salts, trace elements and with an artificial electron acceptor of the 2-(p-iodophenyl)–3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). After one-week incubation the Most Probable Number (MPN) was calculated from the red colour (+/-) in the tubes by using probability tables [28].

The basal respiration of the soil during bioventing was determined by measuring of the CO_2 *production* of the soil microflora. The produced CO_2 was absorbed in NaOH and determined by HCl titration.

Toxicity Test-battery

For direct contact ecotoxicity testing testorganisms of three different trophic levels were used. The interactive ecotoxicity tests ensure the contact between the soil and the testorganism, showing the actual toxicity and ensuring higher environmental reality. These are self-developed tests based on similar Hungarian, German and European standard methods for wastewaters or hazardous waste materials. The sensitivity of different testorganisms to coal tar creosote was investigated in a preliminary study. *Azotobacter agile* [29] and *Pseudomonas fluorescens* dehydrogenase enzyme activity test [30], *Vibrio fischeri* (named *Photobacterium phosphoreum* previously) bioluminescence test [31], *Sinapis alba* root and shoot elongation test [32] and *Collembola* (*Folsomia candida)* [33] mortality test were modified for soil and applied in all experiments. In all ecotoxicological methods artificial OECD soil [34] was also used as a reference soil and for dilution of the contaminated soils. This standard reference soil was spiked with different concentrations of coal tar to study the sensitivity of the testorganisms.

In this work *Azotobacter agile* and *Pseudomonas fluorescens* typical soil-living bacteria were used as testorganisms in bacterial dehydrogenase enzyme inhibition tests.

The test ran in growing dilution of the suspension of the contaminated soil. An alternative electron acceptor, the TTC (2,3,5-triphenyl-tetrazolium-chlorid) was added to the test-medium. The stock solution (TTC and inoculum of the test bacteria) was injected into the tubes that contained the dilution series of the contaminated soils. The serial dilutions were incubated in the dark at 28 ± 2 °C for 72 hours. TTC is reduced by microbial activity to red-coloured formasan. If the respiration of the testorganism is not inhibited, a pink colour appears, colour intensity is proportional with the respiration rate. Semi-quantitative result can be obtained by visual evaluation, quantitative result by measuring the colour-intensity as primary endpoint by a simple spectrophotometer, after solvent extraction of the 1,3,5-triphenyl-formasane (TPF). The tested soil should be sterile. Dehydrogenase enzyme inhibition test can be used for general testing of contaminated soil and sediment during assessment and remediation.

Vibrio fischeri (NRRL B-111 77) is a marine-living bacterium very commonly used for ecotoxicity testing. This bacterium is not a soil-living one, but similar bacteria are members of the soil microflora. It is a well-known, standardized testorganism of marine origin, easily grown in laboratory. As an adverse effect of the contaminant a decrease in the intensity of the luminescence can be measured. To ensure the direct contact between soil and bacteria, a soil suspension is added to the media containing testorganism. Soil samples were suspended in 2% NaCl solution. A dilution series was prepared from the contaminated soils. After measurement of the reference luminescence intensity, dilution series of contaminated soils were added to the test medium. The luminescence intensity was repeatedly measured after 30 minutes exposure time. The inhibition of the light production of bacteria, caused by the contaminated soil was measured by a simple luminometer (Lumac Biocounter M 1500 L). This test is generally used in our practice for soil characterization, for site and technology monitoring.

Direct contact *plant tests* are interestingly less popular for testing soils as an individual habitat; because some of the plants are not sensitive enough, some others are too sensitive. Plants are used mainly for the testing of the extracts of dangerous wastes: generally germination or root elongation test is applied. Plant tests have increasing importance in the assessment of contaminated land and soils, their result play an important role in risk assessment and in the creation of quality criteria. Their response, as representatives of one of the most important trophic level (producers) in soil, is crucial. If we have to calculate the predicted no effect concentration, which does not effect soil ecosystem, we have to use testorganisms of minimum three

trophic levels, including plants and extrapolate from the results of the single species to the whole ecosystem. Bioaccumulation and food chain effects are also based on plant behaviour, so plant tests have growing importance in ecological and human risk assessment. A large number of plants were examined in our laboratory for testing contaminated soil before, during and after remediation based on growth inhibition.

In this research the widely used white mustard *(Sinapis alba)* was applied as test-plant. A dilution series was prepared from the tested soil with sand or standard soil (e.g. OECD artificial soil). The 20 seeds per dilution were seeded in Petri-dish directly into the soil to ensure the interaction between plant root and soil. The test dishes were kept in the dark at 20±2 °C for 72 hours. Plant growth was determined by measuring root and shoot elongation.

Tests using soil living animals apply generally a direct contact between soil and testorganism. Microarthropods as e.g. springtails are said to have an important function regarding the maintenance of soil functions. Due to their short life cycles, high number of species and their high density, the important requirements for using them as indicator organisms are fulfilled. The existing and standardized *Folsomia candida* (*Collembola*) and *Eisenia foetida* (earthworm) are the most popular testorganisms. We carried out mortality test with the Collembolans, commonly known as springtails. Springtails are the most numerous and widely occurring insects in terrestrial ecosystems. Ten *F. candida* specimens of twenty-days-old springtails from a synchronized culture were transferred into the test flasks, containing different dilutions of contaminated soil and reference OECD soil. Test flasks were incubated at 20±2 °C in the dark for 7 days. At the end of the incubation period, each soil in the test flasks was flooded with distilled water and the floating, living animals were evaluated by counting.

Statistical Evaluation of the Toxicity Tests

In all ecotoxicological methods a dilution series of contaminated soil was tested. The endpoints used for the bacterial, plant and animal tests were ED_{20} (LD_{20}) or ED_{50} (LD_{50}) values, soil effect doses that caused 20 % and 50 % inhibition (lethality). The concentrations of coal tar creosote that caused 50 % inhibition or lethality (EC_{50}, LC_{50}) were determined in case of the preliminary sensitivity-tests. Dose Response Analysis (inhibition percent values of different dilutions) by ORIGIN 6.0 software was applied to determine ED (LD) and EC (LC) values. For better interpretation in case of *Vibrio fischeri* test the inhibition of samples is given in Cu-equivalent in addition to ED_{20}, ED_{50} values. We have been working on a modified application of the *Vibrio fischeri* bioluminescence test for years. According to our method inhibition is given also in Cu-equivalent (ΣCu20 and ΣCu 50 [mg Cu/kg soil]), interpolating the measured results onto a Cu-calibration curve. Cu-equivalent values are the Cu concentrations, which would cause the same toxicity as detected in the samples analysed. These values can be compared with the effect based on soil quality guidelines. ($\Sigma Cu20 = ED_{20Cu} / ED_{20sample} * 10^6$, $\Sigma Cu50 = ED_{50Cu} / ED_{50sample} * 10^6$)

On the bases of Cu-equivalent values we characterized the samples as: "non toxic", "slightly toxic", "toxic" and "very toxic".

Data evaluation of the experiment series was processed by correlation analyses using StatSoft® Statistica 6 program.

3 Results and Discussion

In this study different biotechnologies for the treatment of coal tar contaminated soil and the integrated monitoring of these technologies are discussed.

Before starting of the experiments we determined the main characteristics of the less contaminated dark clay soil (*Site I*), and the highly contaminated soil (*Site II*), black muddy filling with gravels. *Table 1* shows the main characteristics of the soils before treatments.

Tab. 1. The characteristics of contaminated soils before treatment

	Characteristics of the soils	*Site I*	*Site II*
Physical-chemical	pH_{KCl}	6.96	6.82
	NO_2–NO_3–N [mg/kg soil]	0.10	0.80
	P_2O_5 [mg/kg soil]	77.3	108.7
	Humus content [%]	1.31	3.26
	$CaCO_3$ [%]	5.0	0.8
Chemical	Extract-content [mg/kg soil]	20,016	165,349
	EPH-content [mg/kg soil]	8000	133,800
Biological	Aerobic heterotrophic cells [CFU/g soil] · 10^7	18.2	6.09
	Coal tar-degrading cells [cell/g soil] · 10^4	46.0	4600
Ecotoxicological	*Vibrio fisheri* luminescence-inhibition ED_{50} [g soil]	0.0074	0.0077
	Vibrio fisheri luminescence-inhibition Cu_{50} [mg Cu / kg soil]	457 toxic	439 toxic
	Azotobacter agile enzyme inhibition ED_{50} [g soil]	0.11	> 0.50
	Pseudomonas fluorescens enzyme inhibition ED_{50} [g soil]	0.11	0.45
	Sinapis alba root elongation inhibition ED_{50} [g soil]	1.3	0.12
	Sinapis alba shoot elongation inhibition ED_{50} [g soil]	0.70	0.15
	Folsomia candida mortality LD_{50} [g soil]	0.75	< 0.02

The results gave information on the presence of viable creosote degrading cells even at high creosote concentration and at high toxicity of the soil. The initial nutrient supply was very low, for this reason both soils were amended with inorganic nutrients ($(NH_4)_2SO_4$, KNO_3, KH_2PO_4) to reach a final C:N:P ratio of about 100:10:1.

The lab-scale experiments were carried out in solid and slurry

phase, modelling bioventing and slurry phase biotreatment. The technology monitoring applied an integrated chemical-biological-ecotoxicological methodology.

3.1 Solid Phase Biodegradation Experiments

The contaminated soil was intensively aerated for 4 weeks in the self-designed reactors. The CO_2 production during bioremediation was determined (Fig. 1).

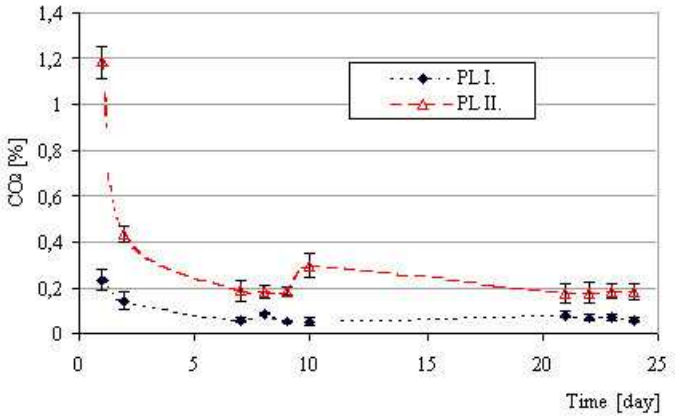

Fig. 1. CO_2 production during bioventing. Values are the means of three replicates. Error bars represent standard deviations.

In aerated solid phase reactors the microbial activity determined by CO_2 production of microorganisms was continuously higher at the higher coal tar contamination (*Site II*). The significantly higher CO_2 production indicated that the presence of high concentration of creosote in the soil did not prevent microbial degradation of contaminants. The percentage removal of the extractable petroleum hydrocarbons was also higher in case of soil from *Site II*.

The bioventing technology was not efficient in case of soil contaminated by 8000 mg/kg coal tar oil (*Site I*); only 13 % decrease was measured after 4 weeks. Poorly available and degradable fraction of the contaminants may be the explanation for the low microbial activity in biodegradation. Considerable toxicity-reduction of the soil originated from *Site I* was only shown by plant (*Sinapis alba* root elongation) test. In case of highly contaminated soil the bioventing was more efficient, more than 50% of the coal tar creosote has been removed during the 4 weeks bioremediation process, and significant decrease in soil toxicity in case of plant and animal tests was also observed.

Both treated contaminated soils (*Site I* and *Site II*) were found to be more toxic than at the beginning as determined by the bioluminescence test. The reason can be 1. increasing mobility and availability, 2. the selective biodegradation of hydrocarbon mixtures and 3. possible, but not identified toxic metabolites. Increase in toxicity determined by luminescence test was much higher in case of low contaminated soil (*Site I*). To take these results into consideration, we carried out lab-scale slurry-phase biotreatment for the remediation of these soils.

Tab. 2. The characteristics of contaminated soil before and after 4 weeks bioventing

	Characteristics of the soils	*Site I* before	*Site I* after	*Site II* before	*Site II* after
Chemical	Extract-content [mg/kg soil]	20.016	17.706	165.349	119.786
	EPH-content [mg/kg soil]	8000	6986	133.800	65.311
Biological	Aerobic heterotrophic cells [CFU/g soil] *10^7	18.2	3.97	6.09	7.07
	Coal tar-degrading cells [cell/g soil] *10^4	46.0	46.5	4600	46.5
Ecotoxicological	*Vibrio fisheri* luminescence-inhibition ED_{50} [g soil]	0.0074	0.0026	0.0077	0.0065
	Vibrio fisheri luminescence-inhibition Cu_{50} [mg Cu/kg soil]	457 toxic	1508 very toxic	439 toxic	713 toxic
	Azotobacter agile enzyme inhibition ED_{50} [g soil]	0.11	0.11	> 0.50	0.45
	Pseudomonas fluorescens enzyme inhibition ED_{50} [g soil]	0.11	0.11	0.45	0.11
	Sinapis alba root elongation inhibition ED_{50} [g soil]	1.3	4.5	0.12	> 5.0
	Sinapis alba shoot elongation inhibition ED_{50} [g soil]	0.70	1.91	0.15	1.70
	Folsomia candida mortality LD_{50} [g soil]	0.75	0.75	< 0.02	0.72

3.2 Slurry Phase Biodegradation Experiments

In the slurry phase treatment the effect of the inoculants was compared during 10 weeks. The mixed slurry phase reactors (500 g) were supplemented with nutrients and inoculated with indigenous, adapted microflora (indigenous) or with H10CS commercial inoculate (H10CS). A control reactor without inoculation (-) was used to compare the effects of the two inoculates. Soil samples were taken from the reactors and analysed after 1 week, 3, 6, 8 and 10 weeks.

The changes in extractable petroleum hydrocarbons (EPH) content are shown in Figs. 2–3.

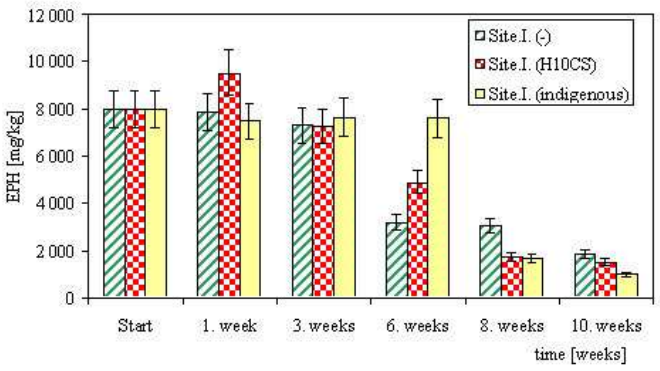

Fig. 2. Changes in EPH-content of low contaminated soil during slurry phase treatment. Error bars represent standard deviations.

The measured contaminant content is the resultant of two contrary procedures: an increase due to mobilization and a decrease due to biodegradation.

The time shift in mobilization and following biodegradation resulted in periodic changes in the measured contaminant content.

The decrease in EPH content at lower contamination level (*Site I*) started after 3 weeks, whereas at high contamination level (*Site II*) it was observed later, after 6 weeks. In stirred slurry phase reactors the final degradation rate was 48 % (no inoculation), 56 % (H10CS), 63 % (indigenous) in case of highly contaminated soil, respectively. The degradation rate after 10 weeks was higher in case of lower coal tar oil contamination (*Site I*): 77 % (no inoculation), 81 % (H10CS), 87 % (indigenous), respectively.

The augmentation with microbes increased the degradation rate in contaminated soils, the inoculate containing indigenous microbes were more effective at the end of the experiment.

Table 3 shows the coal tar oil-degrading cell concentration during slurry-phase treatment.

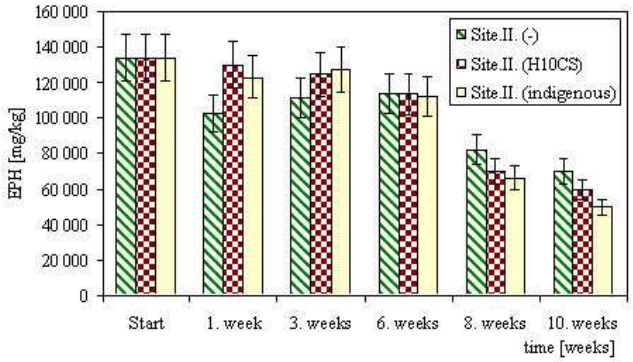

Fig. 3. Changes in EPH-content of highly contaminated soil during slurry phase treatment
Error bars represent standard deviations

The positive effect of inoculation was marked during the first period in case of lower coal tar contamination. The adaptation period was longer at high contamination level in agreement with the data of chemical analyses. The number of oil-degrading cells decreased with the consumption of the contaminant.

Ecotoxicity testing gives refined information on the changes in soil quality. The results of the direct contact toxicity tests performed on coal tar contaminated soils from slurry-phase bioreactors are presented in the following figures (Figs. 4-10).

In general, increasing ED_{50} and LD_{50} values indicated decreased soil toxicity by the end of the study. Reductions in toxicity of soils coming from *Site II* were considerable less - due to the extremely high creosote contamination here - than in case of soils originated from *Site I*.

The soil toxicity determined by *Vibrio fisheri* bioluminescence test increased in the first period due to growing bioavailability of contaminants and not identified toxic metabolites, later

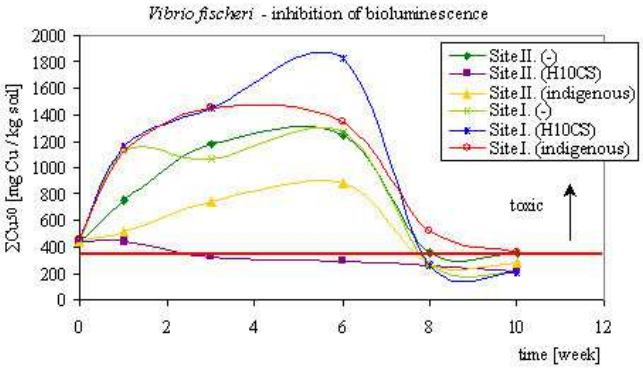

Fig. 4. Changes in the toxicity during slurry phase remediation by *Vibrio fisheri* bioluminescence test expressed in Cu-equivalent

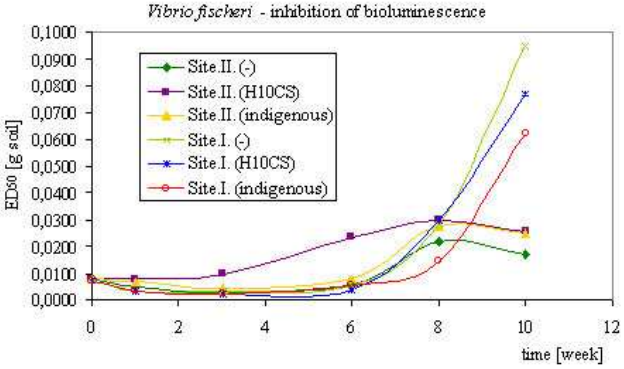

Fig. 5. Changes in the toxicity during slurry phase remediation by *Vibrio fisheri* bioluminescence test expressed in ED_{50}

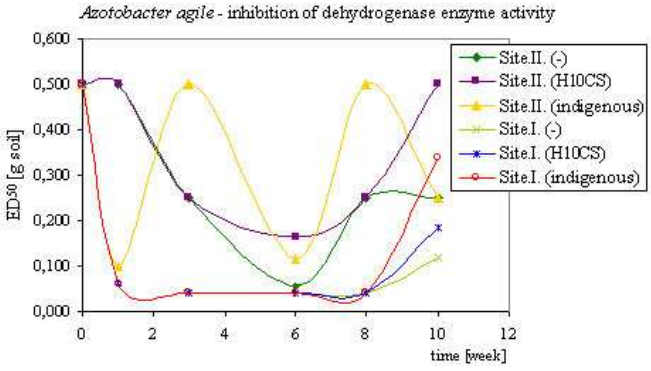

Fig. 6. Changes in the toxicity during slurry phase remediation by *Azotobacter agile* dehydrogenase enzyme activity test

decreased toxicity was found (Figs. 4–5). In case of *Sinapis alba* root and shoot elongation test continuously decreasing toxicity was observed in contaminated soils (Figs. 8–9). *Azotobacter agile* and *Pseudomonas fluorescens* dehydrogenase enzyme activity tests were less reliable than the other used toxicity methods.

We applied correlation analyses to compare the complete bioassay results with regard to creosote concentrations of soil samples. On he basis of correlation analyses carried out by StatSoft ® Statistica 6 program the bioluminescence inhibition test

Tab. 3. Concentration of creosote-degrading bacteria during the 10 weeks of slurry phase treatment. (Values are the Most Probable Number of transformer oil-degrading cells after statistical evaluation. Numbers in parentheses represent the lower and upper 95 % confidence limits.)

Coal tar oil-degrading cell concentration [*10^4cell/g soil]					
	Sampling time [week]				
Sample	1.	3.	6.	8.	10.
Site I (-)	2 (0-9)	11 (2-52)	24 (5-112)	24 (5-112)	11 (2-52)
Site I (H10CS)	15 (3-70)	750 (160-3510)	24 (5-112)	24 (5-112)	11 (2-52)
Site I (indigenous)	110 (24-515)	1100 (235-5148)	24 (5-112)	11 (2-52)	5 (1-23)
Site II (-)	230 (49-1076)	150 (32-702)	24 (5-112)	5 (1-23)	8 (2-37)
Site II (H10CS)	750 (160-3510)	110 (24-515)	240 (51-1123)	46 (10-215)	11 (2-52)
Site II (indigenous)	1100 (240-5150)	460 (98-2153)	240 (51-1123)	240 (51-1123)	11 (2-52)

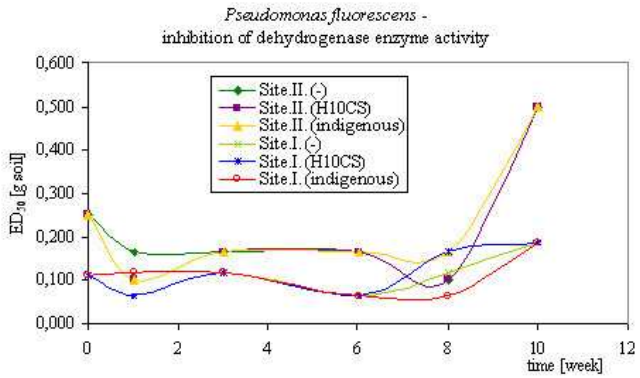

Fig. 7. Changes in the toxicity during slurry phase remediation by *Pseudomonas fluorescens* dehydrogenase enzyme activity test

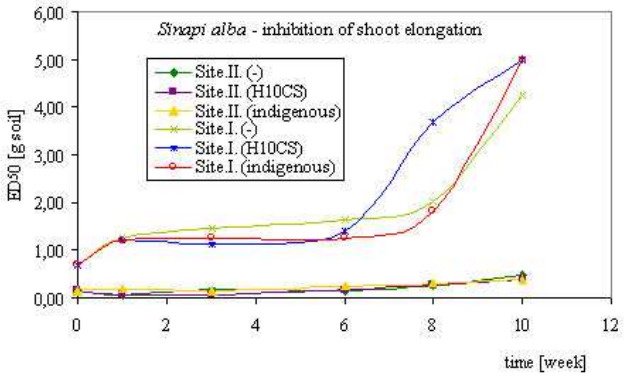

Fig. 9. Changes of the toxicity during slurry phase remediation by *Sinapis alba* shoot elongation test

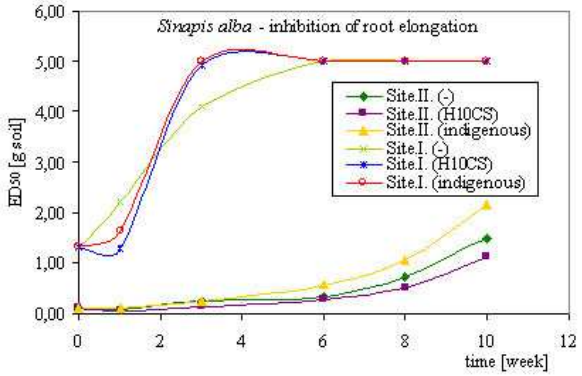

Fig. 8. Changes of the toxicity during slurry phase remediation by *Sinapis alba* root elongation test

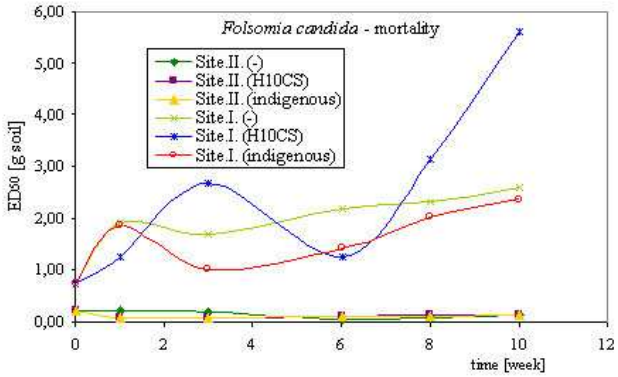

Fig. 10. Changes in the toxicity during slurry phase remediation by *Folsomia candida* mortality test

and the *Sinapis alba* plant test (inhibition of shoot elongation) showed the strongest correlation with the creosote concentrations. Correlation factors were 0.83–0.97 at $p < 0.05$ significance level. Shoot elongation associates better usually with the contaminant concentration and correlates better with other ecotoxicity test results than the root elongation, because the response of root in soil is often an abnormal elongation (but thinner in morphology) to avoid contaminated soil surface.

The following order of decreasing correlation was deduced from statistical analyses: *Sinapis alba* shoot elongation test > *Sinapis alba* root elongation test > *Vibrio fisheri* bioluminescence test > *Folsomia candida* mortality test >>*Pseudomonas fluorescens* dehydrogenase enzyme activity test > *Azotobacter agile* dehydrogenase enzyme activity test. Toxic effects of creosote contaminated soils on different testorganisms and models included in toxicity test-battery are summarized in Table 4.

Table 4 shows the EC_{50} values coming from the preliminary investigation of sensitivity and the range of ED_{50} values determined in slurry-phase biotreatment of highly contaminated soil.

The most sensitive system to creosote was bioluminescence inhibition in *Vibrio fischeri,* followed by *Folsomia candida* mortality and inhibition of shoot elongation in *Sinapis alba* plant test.

Although luminescent bacteria assay is found to be more sensitive indicator of toxicity of creosote contaminated soils than the soil-based assays, the ecological relevance of this method

Tab. 4. Toxic effects of creosote-contaminated soils on different testorganisms and end-points included in the applied ecotoxicological test-battery

Model system Testorganism	Origin	End-point Indicator	Exposure period	EC_{50} [mg/kg]	ED_{50} [mg]
Vibrio fischeri	Marine-living Bacteria	Bioluminescence	30 min	134	3–30
Azotobacter agile	Soil-living Bacteria	Dehydrogenase enzyme activity	48 h	1950	155–500
Pseudomonas fluorescens	Soil-living Bacteria	Dehydrogenase enzyme activity	48 h	3400	100–500
Sinapis alba	Terrestrial plant	Root elongation	72 h	1000	60–2150
Sinapis alba	Terrestrial plant	Shoot elongation	72 h	707	60–410
Folsomia candida	Springtails (soil-living insects)	Mortality	1 week	445	20–210

remains restricted, so combination with further soil based procedures in a battery of toxicity tests is essential. The toxicity test-set including *Vibrio fischeri* bioluminescence test, *Sinapis alba* shoot elongation test and *Folsomia candida* mortality test is proposed to assess the soil toxicity during bioremediation of creosote contaminated soils.

Table 5 shows the characteristics of soils after a 10-week slurry phase treatment.

The results presented here indicate that slurry phase biological treatment with inoculation was an effective tool for remediation of creosote-contaminated soils (*Site I, Site II*). This technology has many advantages, like homogeneity, relatively easy handling and rapid biodegradation. In case of highly contaminated soil 10 weeks of treatment were not long enough to reach the final acceptable quality, although from the results we may predict that it can be an appropriate technology.

The results of the integrated methodology proved that in case of contaminated soil coming from the loamy *Site I* slurry phase biodegradation was more efficient than bioventing. The toxicity decreased after 8–10 weeks of slurry phase treatment in low contaminated soils (*Site I*), but still high toxicity was observed after solid phase remediation. The slurry phase treatment ensures optimal environment for the biodegradation.

In case of good quality humic soil both bioventing and slurry phase treatment proved to be effective. The degradation rate was almost similar in slurry phase treatment after 10 weeks to that of bioventing after 4 weeks. Although chemical analyses indicated that the applied remediation technologies were almost equally effective in case of highly contaminated soil, the results of the toxicity tests demonstrate the complexity of the "system" and show the different responses of the testorganisms.

Bioluminescence test indicates slight toxicity in slurry phase bioreactors in case of soil *Site II* after 10 weeks. In spite of this lower toxicity the soil had still a high extractable petroleum hydrocarbons (EPH) concentration after ten weeks of treatment (49,674 mg/kg – 70,108 mg/kg), demonstrating the reduced contaminant bioavailability to the *Vibrio fischeri* testorganism. On the other hand the ED_{50} values of soil-based assays (shoot elongation test and animal mortality test) indicate the toxic effect of the "residual" EPH. Although *Vibrio fischeri* was the most sensitive testorganism in our experiments, regarding the considerable differences between organisms responses to soil contamination, it is more relevant to use also soil-living testorganisms (plant and animal eg. *Collembola*), to demonstrate, that the soil is no longer toxic to the environment. These results clearly support the use of a battery of bioassays to monitor soil toxicity during soil bioremediation by demonstrating the different responses of a number of test-system.

Considering the above mentioned results both technologies are suitable for remediation of highly contaminated soil (*Site II*) and long-term treatment is required to achieve successful bioremediation and acceptable risk level. Extrapolating from the short-term laboratory tests a few months of treatment is necessary.

4 Conclusion

Short-term laboratory feasibility studies using complex methodology proved that the bioventing and the slurry phase remediation with inoculation could be suitable to treat creosote-contaminated soil. The present study has been carried out using contaminated soils from an actual site, which permitted a much closer approximation to the real working conditions encountered in the field.

In the lab-scale experiments the slurry phase biodegradation was more efficient in case of loamy soil (*Site I*), as compared to the bioventing. For remediation of loose, humic soil (*Site II*) in case of inherited contaminated sites with aged contamination, where the soil microbes had the opportunity to adapt their genetics and biochemistry to the contaminants, both technologies can be suitable.

The applied integrated methodology gave a good insight into the black box of the soil, providing detailed results not only on the quality and quantity of the contaminant and the characteristics of the soil, but also on the biological and toxicological status, and complex interactions between all of the soil compartments. The results underline the need to take ecotoxicological effects into account in order to assess remediation efficiency. Test-batteries are needed to characterize contaminated soil as a dynamic system, which are able to measure responses and interactions. Based on the present study we proposed a toxicity test-battery, which includes *Vibrio fisheri* bioluminescence test, *Sinapis alba* shoot elongation test and *Folsomia candida* mortality test. The application of the test-battery provided complementary and essential information to chemical characterization.

Tab. 5. Soil characteristics after 10 weeks of slurry phase treatment

	Characteristics after 10 weeks of slurry phase treatment	*Site I*			*Site II*		
		(-)	H10CS	Indig.	(-)	H10CS	Indig.
Chemical	Extract-content [mg/kg soil]	13,757	12,508	14,767	109,803	106,996	101,040
	EPH-content [mg/kg soil]	1845	1528	1093	70,108	59,448	49,674
Biological	Aerobic heterotrophic cells [CFU/g soil] $*10^7$	12.6	92.2	5.8	5.4	8.8	7.8
	Coal tar-degrading cells [cell/g soil] $*10^4$	11.0	11.0	5.0	8.0	11.0	11.0
Ecotoxicological	*Vibrio fisheri* lum. inh. ED_{50} [g soil]	0.095	0.077	0.062	0.0169	0.0258	0.0249
	Vibrio fisheri lum. inh. ΣCu_{50} [mg Cu/kg soil]	213 not tox	224 not tox	342 slight tox	359 toxic	214 not toxic	286 slight tox
	Azotobacter agile enz. inh. ED_{50} [g soil]	0.117	0.185	0.335	0.25	0.50	0.25
	Pseudom. fluor. enzyme inh. ED_{50} [g soil]	0.185	0.185	0.185	0.5	0.5	0.5
	Sinapis alba root elong. inh. ED_{50} [g soil]	5.0	5.0	5.0	1.5	1.12	2.15
	Sinapis alba shoot elong. inh. ED_{50} [g soil]	4.26	5.0	5.0	0.5	0.41	0.41
	Folsomia candida mortality LD_{50} [g soil]	2.60	5.59	2.37	0.14	0.12	0.12

Abbreviations

CFU	Colony Forming Unit
EC_{50}	Effect Concentration – Concentration that affects designated criterion (e.g. behavioural trait) of 50% population observed
ED_{20}, ED_{50}	Effect Dose – Dose that affects designated criterion (e.g. behavioural trait) of 20% or 50% population observed
EPH	Extractable Petroleum Hydrocarbons
GC	Gas Chromatography
INT	2-(p-iodophenyl)–3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
LD_{20}, LD_{50}	Lethal Dose – Dose that kills 20% or 50% of population observed
MPN	Most Probable Number
OECD	Organization for Economic Cooperation and Development
PAH	Polycyclic Aromatic Hydrocarbons
PCP	Pentachlorophenol
TTC	2,3,5-triphenyl-tetrazolium-chlorid
USEPA	Environmental Protection Agency of United States

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