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RESEARCH ARTICLE

Comparative evaluation of microbial and chemical methods for assessing 4-chlorophenol biodegradation in soil

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Abstract

Reliable methods for assessing soil microflora and it's activity are a prerequisite for successful technology planning and sustainable bioremediation of contaminated sites. The main objectives were to evaluate several microbiological soil-testing methods for characterizing the 4-chlorophenol biodegradation in the soil microcosm and to find the most appropriate methodology for testing biodegradation potential.

The activity of the soil microflora were characterized by contaminant degrading cell concentration, dehydrogenase enzyme activity, three types of soil respiration and substrate utilization of the microbial community. The contaminant concentrations were measured by exhaustive extraction and by non-exhaustive cyclodextrin extraction.

Most of the applied biological methods were found to be reliable indicators of chlorophenol biodegradation in soil, and can be useful as a pre-implementation methodology to support technology selection and design. The microbial community analyses by BIOLOG EcoPlateTM provided very good results and can be suitable for use in biodegradation assessment and evaluation in soil.

Keywords

4-chlorophenol · integrated methodology · soil microbial community · BIOLOG EcoPlate™ · non-exhaustive cyclodextrin extraction

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Abbrevations

4-CP 4-chlorophenol
UV-VIS ultraviolet-visible spectrophotometry
GC-FID gas chromatometry with flame ionization detection
GC-MS gas chromatometry with mass spectrometry
REP GC randomly methylated beta-cyclodextrin extracted 4-CP content measured with GC-FID (Table 3)
HEP GC hydroxypropyl beta-cyclodextrin extracted 4-CP content measured with GC-FID (Table 4)
CP chlorophenol
GN Gram-negative
PCR-DGGE polymerase chain reaction and denaturing gradient gel electrophoresis
PCP pentachlorophenol
SIR substrate induced soil respiration in closed bottle
PICT pollution-induced community tolerance
ERA environmental risk assessment
RAMEB randomly methylated beta-cyclodextrin
HPBCD hydroxypropyl beta-cyclodextrin
EC electric conductivity
RAMEB-GC chemical analysis by gas chromatography using flame ionisation detector after randomly methylated beta-cyclodextrin extraction
HPBCD-GC chemical analysis by gas chromatography using flame ionisation detector after hydroxypropyl beta-cyclodextrin extraction
RAMEB-SP chemical analysis by UV-VIS spectrophotometry after randomly methylated beta-cyclodextrin extraction
HPBCD-SP chemical analysis by UV-VIS spectrophotometry after hydroxypropyl beta-cyclodextrin extraction
CP-CFU chlorophenol degrading cell concentration
TPF triphenyl formazan
DEH dehydrogenase enzyme activity
TTC 2,3,5-triphenyl-tetrazolium-chloride
RES-CB soil respiration in closed bottle
RI respiration index
RES-AER soil respiration in dynamic aerated reactors
AWCD average well color development
H' Shannon diversity index

C absorbance value in the control cells
R absorbance value in wells containing carbon sources
OD optical density

1 Introduction

Halogenated hydrocarbons are very important group of organic soil contaminants because of their widespread utilization. Chlorophenols have been commonly applied as dyestuffs, synthetic intermediates in polymers, disinfectants and pesticides, contaminating large areas. 4-chlorophenols (4-CP) were used as intermediates for higher chlorophenols, polymers, dyes and pesticides or as disinfectants. These halogenated chemicals represent a hazard for soil microbial communities, and may influence the turnover of nutrients and soil fertility [1–4]. Their typical feature is that they are mostly persistent, are able to adsorb strongly to the solid soil matrix and are highly toxic, carcinogenic and mutagenic to most living organisms. Since they are not easily biodegradable they persist in the soil for a long time therefore they give rise to serious environmental risk [5–8].

In situ environment-friendly technologies based on biodegradation have become recently a promising approach for remediation of soil contaminated by organic pollutants considering their low cost. Decisions to apply bioremediation at a given contaminated field sites require a comprehensive characterization of the site-specific biodegradation processes. At the same time the cost effective site characterization is a very important step toward the efficient management of contaminated land and its sustainable remediation. Therefore cost- efficient pre-implementation methods providing specific information are necessary throughout the site characterization process, in order to take correct decisions and to ensure the optimum performance of the remediation actions. A complex approach, biological and testing paired to the chemical analysis should be applied for the characterization of processes in the contaminated soil [9–11].

The direct chemical measurement of contaminants residues in the soil or groundwater determines overall losses and thus provides equivocal evidence for biodegradation [12, 13]. A variety of quantitative and qualitative techniques are available to measure organic contaminant losses in the environment (soil, sediment, groundwater etc.). The choice of a particular method usually depends on the type of the contamination and soil and on the information required. The analyses of the organic contaminants usually require extraction with organic solvents from the matrix, separation of the components and means of detection. Organic solvents of different polarities (hexane, acetone, n-pentane, dichloromethane) are used depending on the chemical nature of the contaminants to be analysed in soil. The applicability of non-exhaustive extraction using cyclodextrin solutions to predict the biologically available fraction of different hydrocarbons has been recently demonstrated [14–16, 18].

During the preparation of soil bioremediation, the microcosms testing of the pollutant biodegradation can help to select and design the best possible remedial technology, as well as the reliable methods for monitoring and assessing soil microflora and its activity [19, 20].

The most extensively used biological methods for assessing the effects of contaminants on the metabolic activity in the soil, for characterization of biodegradation of the contaminants and for monitoring the feasibility of bioremediation are: soil microbial counts; soil respiration; and dehydrogenase activity [13, 21, 22]. As a microbiological endpoint reproduction /growth of a microbial culture, the number of cells or their measurable structural and functional compartments or products are suitable (cell number, growth curve, nitrogen- content, chlorophyll-content, light emission, etc.), when directly associated with biodegradation. The quantitative determination of soil microflora (cell concentration) may closely relate to the biodegradation of a contaminant, if this contaminant is the only substrate for energy production or biosynthesis.

Measuring soil respiration is one of the oldest and still the most frequently used method for quantifying microbial activities in soils [12, 13, 23, 24]. Endpoints to measure in this case can be the depletion of O_2, production of CO_2, any actor of the energy production and respiration chain in the cells.

The measurement of one or more enzyme activities in the respiratory chain can be used as an index for the total oxidative activities of the cell; therefore dehydrogenase enzyme activity has been used as a measure for overall microbial activity [23].

The BIOLOG microplates used in numerous studies are also powerful tools for characterizing microbial communities in soil. The estimation of microbial functional diversity by carbon source use profiles has been reported to be a sensitive approach to detect modifications due to soil management [25]. Principles, advantages and weaknesses of this BIOLOG technique have been collected in a paper [26]. BIOLOG microplates can be useful in studies on pollution-induced community tolerance (PICT) of polluted soils as a tool in environmental risk assessment (ERA) [26, 27].

The phytotoxicity of triclosan using plant growth, soil microbial activity with soil respiration and phosphatase activity tests and soil microbial functional diversity using the BIOLOG ECO plates were studied [28]. Researchers found that triclosan inhibited plant growth in soil and soil respiration in soil treated with more than 10 mg kg^{-1} triclosan during the first 4 days of incubation, but recovered after longer incubation. Phosphatase activity was also inhibited for all the triclosan contaminated soils. They evinced that triclosan treatment increased the utilisation of carbon sources and had no adverse effect on diversity of soils.

Chlorophenol-contaminated soils were studied during full-scale bioremediation by composting [29]. The microbial status of soils were followed by 1) conventional enumeration of microbes on selective and general media, 2) assessing microbial activity by soil respiration and 3) determining community structure with BIOLOG GN microplates utilizing sole carbon source. Researchers also followed the utilization of ammonium, nitrate and soluble phosphor. It was found that the best indicator of

Tab. 1. Physico-chemical characteristics of the soil

Sample	pH_{H2O}	$K_{(A)}$	EC mS/cm	Total salt m/m%	$CaCO_3$ m/m%	C m/m%	Total N m/m%	NH_4-N mg/kg	NO_3-N mg/kg	P mg/kg
Test soil	**8.19**	**25**	**204**	**<0.02**	**5.00**	**0.661**	**0.063**	**4.44**	**8.44**	**432**

EC=electric conductivity

the actual chlorophenol degradation efficiency was the number of microbes growing on plates with 2 mM pentachlorophenol as the sole carbon sole.

Tab. 2. Physico-chemical characteristics of the soil

	Mechanical composition		
Sample	sand % (w/w)	silt % (w/w)	clay % (w/w)
Test soil	**85.47**	**6.61**	**7.92**

The impact of three different chlorophenolic compounds (2-chlorophenol, 2,4,6- trichlorophenol and pentachlorophenol) on granitic and calcareous soils were tested by a group of researchers [4]. They studied the effect of different contaminant concentrations on soil microbial activity using manometric respirometry (such as cumulative respiration and substrate induced respiration) and polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) analysis. They concluded that the presence of the chlorophenols did not affect the global activity of soils, however increased concentration of the pollutants caused decrease of activity and changes of population.

The aim of our study was to compile a chemical-biological test-battery supporting the cost-effective assessment of contaminated sites as well as planning and monitoring the biodegradation based bioremediation technologies. The main objectives of this present research were a) to evaluate several biological soil testing methods for characterizing the biodegradation processes in 4-chlorophenol contaminated soils; b) to test the usefulness of cyclodextrin-extraction technique for simulating the "biological extraction", and bioavailable fraction of the pollutant; c) to compare and evaluate the applied chemical and biological test methods with each other; to find the most appropriate analytical test-battery for measuring biodegradation potential and to follow its possible enhancement in contaminated soil.

2 Materials and methods

Small-scale 10-weeks-long laboratory experiments in soil microcosms were performed to study the impact of 4-CP contamination on biological and microbiological community activity of soil and to evaluate the parallel applied biological and chemical methodologies. Biological and chemical methods were used for measuring microbial activity, and for the characterization of the biodegradation processes and the bioavailability of the contaminants in the soil.

2.1 Materials

4-chlorophenol (≥98% of purity, CAS number: 106-48-9) was purchased from Sigma–Aldrich Ltd. Stock solutions of 4-chlorophenol were prepared in distilled water. For the non-exhaustive cyclodextrin extraction, randomly methylated beta-cyclodextrin (RAMEB) and hydroxypropyl beta-cyclodextrin (HPBCD) (both Wacker Chemie, Munich, Germany) solutions were applied. In the experiment a test soil was used and artificially contaminated. The main physico-chemical characteristics of the soil is shown in Table 1–2. These measurements were done by the Institute for Soil Sciences and Agricultural Chemistry, Centre for Agricultural Research, Hungarian Academy of Sciences. According the results, the soil applied in this experiment was classified as loamy sand.

2.2 Experimental setup of microcosms

Ten-week-long laboratory microcosm experiments were carried out with artificially contaminated soil. 10-10 kg test soils were contaminated by three different concentrations 10, 100 and 1000 mg kg^{-1} and placed in covered 15 dm^3 volumetric, static reactors. Microcosms were incubated under dark, aerobic conditions at 25 ± 5°C. Monitoring of the biodegradation started after two days adaptation period following spiking the soil. The uncontaminated test soil was used as a control and treated the same way as the contaminated soils. The soils were amended with inorganic nutrients in order to have a beneficial effect on the biodegradation of contaminants. 100-100 ml nutrient solution was applied once in a fortnight. The nutrient solution consisted $NaNO_3$ (23.5 mM), $MnCl_2$ (0.79 mM), K_2HPO_4 (5.75 mM), $MgSO_4$ (0.42 mM) and $ZnSO_4$ (0.12 mM).

2.3 Chemical methods

Solvent extractable 4-CP was measured by gas chromatography – mass spectrometry analysis (GC MS) after exhausting dichloromethane extraction according to the Hungarian Standard (HS 21470–97:2004).

As a chemical model for the estimation of contaminant bioavailability and biodegradability aqueous cyclodextrin solutions (10%) were applied for the ultrasonic extraction of the contaminant from soil. Cyclodextrin derivatives of high solubility and high solubilising potential such as randomly methylated beta-cyclodextrin (RAMEB) and hydroxypropyl beta-cyclodextrin (HPBCD) were used and compared to other chemical and biological methods. The cyclodextrin extracts were transferred into methanol after solid-phase extraction and analysed by 1) gas chromatography using flame ionisation detector

(RAMEB-GC; HPBCD-GC) and by 2) UV-VIS spectrophotometry (RAMEB-SP; HPBCD-SP). The aim of using two different analytical techniques was to compare the detection limits and reliability of the methods.

The results of the non-exhaustive cyclodextrin extraction (RAMEB-GC; HPBCD-GC, RAMEB-SP; HPBCD-SP) were compared with the results of the exhaustive extraction by dichloromethane (GC MS) and with the results of the biological tests characterizing the bioavailability and biodegradation of the contaminants.

2.4 Biological methods

Chlorophenol degrading cell concentration (CP-CFU) is a value of the specific cells and shows the number of the soil microorganisms, which are able to degrade the contamination of 4-CP. There are two purposes of this test: to prove the 4-CP degrading ability of the microorganisms living in the contaminated soils and to determine the concentration of the microbes that are able to degrade the contaminant. With this cell concentration we can deduce the degrading activity of the soil, which is very important information during planning the remediation. The number of specialized microbes was determined on minimal medium agar [29], which contained 100 ppm 4-CP as sole carbon source and the following nutrients per liter: 2.1g K_2HPO_4; 0.4 g KH_2PO_4; 0.5 g NH_4NO_3; 0.2g $MgSO_4 \cdot 7\ H_2O$; 0.023 g $CaCl_2 \cdot 6\ H_2O$ and 0.002 g $FeCl_3 \cdot 6\ H_2O$. 1 g of soil was shaken with 9 ml of water for 30 minutes at 150 rpm, and then a 10-fold serial dilution was made. 100–100 μl of the 3rd, 4th and 5th member of this serial dilution was added to the minimal medium agar and were kept for 6 days at 30 °C. For the interpretation of the results the CP-CFU/g wet soil was calculated by averaging the three parallels.

The assay of dehydrogenase can be used for characterising activity of soil microbes and the possible inhibitory effect of the contaminant on the soil microorganisms [30]. Almost all microorganisms are able to reduce 2,3,5-triphenyl-tetrazolium-chloride (TTC) as an artificial electron acceptor to triphenyl formazan (TPF), which can be colorimetrically measured. *Dehydrogenase enzyme activity* (DEH) based on the estimation of the TTC reduction rate to TPF was measured and used as an index for overall microbial activity of the soil [31]. Contaminated soil (20 g) was incubated for 24 h at 28 °C in 10 ml TTC-tris-HCl buffer solution. TPF concentration was measured by colorimetry after extraction with acetone at 546 nm. For the interpretation of the DEH μg TPF/g soil value was calculated.

Soil respiration can be determined by measuring CO_2 production or O_2 consumption in flow-through reactors with continuous aeration and as a consequence in constant oxygen- supply or in closed bottle, with continuously decreasing oxygen supply. *Soil respiration in closed bottle* (RES CB) was characterized and evaluated by the Oxitop Sensomat System. A vial containing NaOH is placed in each vessel to trap CO_2. So if oxygen is consumed in the closed vessels at a constant temperature, negative pressure develops.

Pressure difference (decrease) due to microbial activity in closed 500-ml vessels filled with 100 g of moist soil was measured for 5 days at 25 °C and read remotely. For the interpretation of the results the linear part of the respiration curve (pressure–time) was used from which the respiration index (RI) was determined by linear regression.

During the measurement of *glucose induced soil respiration in closed bottle* (SIR), the respiration response of microorganisms was studied using an easily degrading substrate, similarly to Torstensson [32]. This response is proportional with that part of the soil microflora, which are able to utilize the easily degradable substrate. 5 ml of 200 g l^{-1} glucose solution was given to the 100 g of moist soil in the previously described closed-bottle system. For the interpretation of the results the linear part of the respiration curve (pressure-time) was used from which the respiration index (RI) was determined by linear regression.

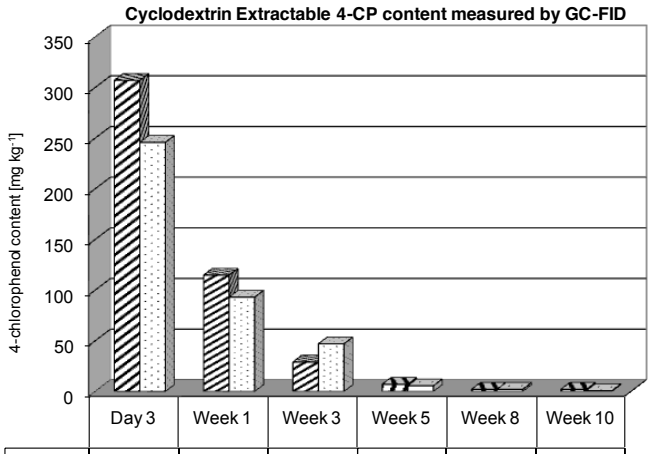


Fig. 1. 4-CP content in the 1000 ppm contaminated soil extracted by cyclodextrin solutions and measured by GC-FID

The *soil respiration*, CO_2 production of microbes in *dynamic aerated reactors* (RES AER) indicates the aerobic biodegradability of the contaminating substances, the adaptive potential and the activity of soil microorganisms. Aerated reactors can be used by measuring CO_2 production for the characterization of microbial activities, for testing biodegradability, bioavailability or toxic effect of organic contaminants. Also the effect of technological parameters and additives such as aeration rate, effect of additives, etc. can be investigated [33–35]. Self-designed flow-through column-reactors of 1 dm^3 volume – each filled with 500 g of contaminated soil were used – at 25±5°C. The four reactors filled with the control (uncontaminated) and contaminated soils were aerated for 4 hours with an aeration rate of 10 L air h^{-1}. Two flow-through traps filled with NaOH ensured the CO_2-free atmospheric air. The CO_2-free air was sucked through the soil columns by a vacuum produced by a water-jet pump.

The CO_2 produced by the soil microorganisms was trapped in 1 N NaOH solution and measured by HCl-titration. Optimal moisture content (10–15% by weight) was maintained throughout the whole experiment. For the interpretation of the results the produced amount of CO_2 [mmol] was calculated.

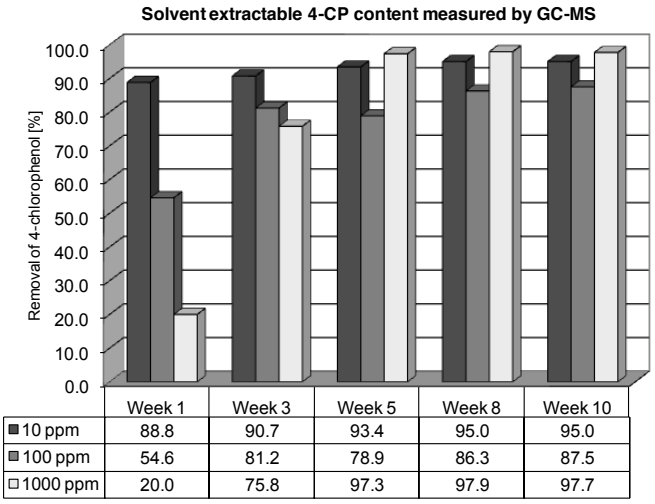
BIOLOG ECO plates were used to study the substrate utilization patterns of 4-CP contaminated soils microbial community. The Biolog EcoPlates contain 31 different, ecologically relevant carbon substrates (hydrocarbons, amino acids, other nitrogen-compounds, lipids, biological polymerics etc.) for soil microbial community analysis. Incubating environmental samples on these multiwell microplates, results in characteristic reaction patterns, called metabolic fingerprints. If certain microbes of the environmental samples can utilize the carbon source the respiration of these microbes reduces the tetrazolium dye, so the content of the wells becomes purple according to the rate of utilisation. 10 g soil was shaken in 90 ml 0.85% NaCl solution for 30 minutes at 150 rpm. After 10 minutes standing, 1 ml of this suspension was taken to 9 ml 0.85% NaCl solution. 125 μl of this suspension was pipetted into the wells, and then the plate was incubated at 25 ± 5°C in the dark. The metabolic fingerprints on the plates were analysed with DIALAB EL800 Microplate Reader at every 24 hours for 7 days at 490 nm. Two types of data corrections were applied by the OD values before the evaluation: with the control well value (there is only water in this well) and with the initial-value (measured after filling up the wells with the suspension) of each well. For the interpretation of the results average well color development (AWCD) was calculated for six groups of carbon sources (carbohydrates, carboxylic acids, amino acids, polymers, amines, miscellaneous) with the following equation: $AWCD = \sum(C - R)/31$, where C is absorbance value in the control cells and R is absorbance value in each carbon source well [36]. Shannon diversity index for studying soil microbial functional diversity was also calculated using the equation: $H' = -\sum P_i \ln(P_i)$, where P_i can be calculated by subtracting the control absorbance from each substrate absorbance and then dividing the value by the total color change for all 31 substrates [28].

3 Results and discussion

3.1 Evaluation of the results of the chemical analyses

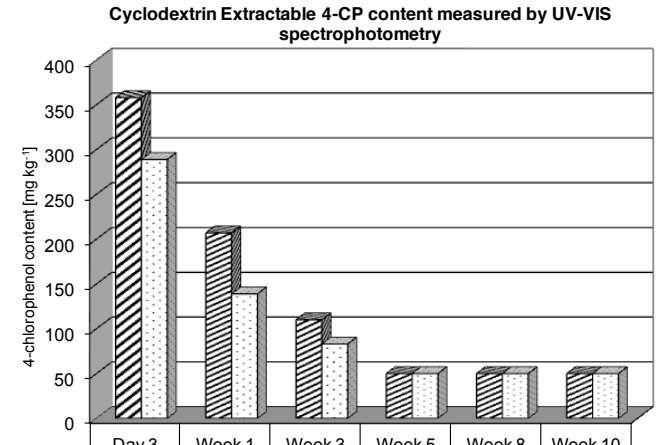
The removal percentage of the 4-CP was calculated from the total content of the contaminant extracted by dichloromethane compared to the 4-CP content measured at the start of the experiment. The results of the solvent extractable 4-CP showed decreasing tendency (Figure 2), due to the activity of the microbes. The percentage contaminant removal showed clearly (Figure 2) that in the 10 ppm 4-CP contaminated soil microbes transformed a large amount of the contaminant by the end of the first week. Microbes in soils contaminated with 100 ppm and 1000 ppm biodegraded the biggest part of the 4-CP for the 3rd week.

Previous research carried out by diesel and transformer oil contaminated soil [19] has revealed that extraction by cyclodextrin can be useful for assessing the biologically available fraction of the contaminants and predicting the biodegradation of the pollutant in the soil. The cyclodextrin extractable 4-CP content was determined from just the 1000 ppm contaminated soil. After extraction by aqueous cyclodextrin solutions (10%), both chemical methods (GC and SP) gave similar results until week 3 (Figure 1–3). The RAMEB and HPBCD extractable 4-CP content decreased continuously during the experiment. After that there was a detection limit of 50 ppm by UV-VIS spectrophotometry.



	Week 1	Week 3	Week 5	Week 8	Week 10
10 ppm	88.8	90.7	93.4	95.0	95.0
100 ppm	54.6	81.2	78.9	86.3	87.5
1000 ppm	20.0	75.8	97.3	97.9	97.7

Fig. 2. Percentage of the removal of the 4-CP content in soils



	Day 3	Week 1	Week 3	Week 5	Week 8	Week 10
RAMEB	358	207	110	50	50	50
HPBCD	289	139	83	50	50	50

Fig. 3. 4-CP content in the 1000 ppm contaminated soil extracted by cyclodextrin solutions and measured by UV-VIS spectrophotometry

Percentage values for the organic solvent extractable and cyclodextrins extractable 4-CP content were also determined (Table 3–4).

Periodic changes can be observed in the percentage rate of the biologically available fraction (defined as cyclodextrin extractable) as a result of the integration of different processes: mobilization and biodegradation of the contaminant fraction

Tab. 3. Biologically available fraction of 4-CP after extraction by aqueous RAMEB solution

1000 ppm contaminated soil	Start	Week 1	Week 3	Week5	Week8	Week 10
GC MS	997.00	798.00	241.00	26.70	20.90	22.90
REP GC	307.00	114.75	29.00	7.00	2.00	2.00
%	30.79	14.38	12.03	26.22	9.57	8.73

along with adaptation of the microbes to the residual fraction of biodegradation. Our results suggest that the non- exhaustive cyclodextrin extraction can be suitable also for the prediction of chlorophenols biodegradation in soil.

3.2 Evaluation of the results of the biological methods

4-chlorophenol effects on the contaminant degrading cell concentration

The highest chlorophenol degrading cell concentration was shown in 100 mg kg^{-1} 4-CP contaminated soil until week 5, later on reasonably in the 1000 ppm 4-CP contaminated soil (Table 5). 10 ppm 4-CP in soil lets the cells immediately and intensively work on 4-CP degradation. 100 ppm 4-CP contamination in soil needed a short adaptation period, but after this no inhibition of soil microflora and high degrading cell concentration was measured. The microflora of the 1000 ppm 4-CP contaminated soil was toxic and for this reason the number of the degrading cells started to increase only after a longer adaptation period. Maximum cell concentration in highly contaminated soil was measured in week 5. Chlorophenol degrading cell concentration of the 1000 ppm contaminated soil decreases slightly between week 5 and week 8, due to the decreased amount of the biologically available fraction (see Table 3 and Table 4).

However the maximum cell concentration of the 1000 ppm 4-CP contaminated soil could not reach the maximum cell concentration measured during the experiment in the 100 ppm 4-CP contaminated soil.

Effects on dehydrogenase enzyme activity

Immediately after contamination, in the 10 ppm contaminated soil, microbes were able to show the highest activity (Table 6). The results show that 10 ppm 4-CP intensified the microorganisms resulted in higher dehydrogenase activity than the control. 1000 ppm has slight impact on total dehydrogenase activity, but lower DEH was observed at the beginning, but higher after the 3rd week, which suggests that the microflora could be adapted to the contaminant and taking into consideration the increased 4-CP-degrading cell concentration, they could not only be adapted but they also learned to utilise the 4-CP. Main effect of 1000 ppm 4-CP in soil is inhibition, 10 weeks was not enough for an efficient adaptation for tolerating and utilizing 4-CP in soil at such a high concentration.

Effects on three types of soil respiration

At the beginning, the 100 ppm contaminated soil had the highest microbial activity because they could adapt and utilize well the contaminant as substrate (Table 7). The microbes in soil contaminated with 10 ppm had already utilized much of the 4-CP until week 3. The results showed a decreasing tendency of the microbial activity (respiration) of the soil in all cases, not only in the contaminated but also in the control soil. It is because the tested soil contains dominantly aerobic microorganisms, for which the decreasing redoxpotential in the closed bottle is not favourable. In spite of the general decreasing respiration rate, the 10 and 100 ppm 4-CP contaminated soil showed an increase compared to the control. This unambiguously proves the anoxic character of the ongoing respiration.

With the addition of glucose, the biological activity of the soils increased: at the beginning, the 100 ppm contaminated soil had the most active microflora, and then in week 5, the 1000 ppm 4-CP contaminated soil took over the leader position (Table 8). Adding glucose solution to the microflora of the 4-CP contaminated soil is interesting. On the one hand, it can activate the microbes which are able to utilize the easily degradable glucose (see the control) but on the other hand, the microbes in the contaminated soils showed much more increased respiration, forecasting a cometabolic pathway in 4-CP degradation.

Microbes in soils can be activated by aeration, so this method shows not only the present condition of the soils but also the possible consequences of the aeration, changes in environmental parameters and the decreasing concentration of the contaminant. As the microflora utilized the nutrients from soil and no additional supply was ensured, after an initial enhancement, the intensity of the respiration decreased continually in both the control and treated soils (Table 9).

CO_2 production was higher in 10 and 100 ppm 4-CP contaminated soils than in the control, and 1000 ppm was also not much under the control. It means that most of the activity has been kept also in the soil with high contamination.

4-chlorophenol effects on microbial community–level substrate utilization and Shannon-diversity

AWCD value is used as an indicator of general microbial activity; it indicates the overall color development of the Biolog microplate [37]. Shannon-index shows the microbial diversity of the examined soils. The value of this index usually falls between 1.5 and 3.5 and it's widely used for comparing diversity between various habitats [39]. The index assumes that 1) individuals are sampled randomly from an independently large population and 2) all the species are represented in the sample [38].

At the beginning (Start) 1000 mg kg^{-1} contaminated soil had the lowest AWCD value, but by the end of the experiment the microflora of this soil could combat the disadvantage and showing the highest value (Table 10, Figure 4).

Tab. 4. Biologically available fraction of 4-CP after extraction by aqueous HPBCD solution

1000 ppm contaminated soil	Start	Week 1	Week 3	Week5	Week8	Week 10
GC MS	997.00	798.00	241.00	26.70	20.90	22.90
HEP GC	245.75	93.00	47.00	5.52	2.00	0.75
%	24.67	11.65	19.50	20.66	9.57	3.29

Tab. 5. Most Probable Number (MPN) of contaminant degrading cell*

10^5 cells g^{-1} soil	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	28	40	25	12	13	16
10 mg kg^{-1}	192	152	82	24	57	16
100 mg kg^{-1}	408	547	169	94	100	28
1000 mg kg^{-1}	4	15	36	369	142	157

* MPN number was determined statistically using probability tables

Tab. 6. Dehydrogenase enzyme activity

10^5 cells g^{-1} soil	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	9	8	7	10	10	13
10 mg kg^{-1}	15	14	14	8	8	17
100 mg kg^{-1}	7	7	10	8	11	12
1000 mg kg^{-1}	5	1	1	1	2	4

Tab. 7. Soil respiration in closed bottle

hPa* 1000 min^{-1}	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	5.0	4.1	3.4	2.9	2.8	2.4
10 mg kg^{-1}	4.5	4.8	2.8	2.7	1.6	1.6
100 mg kg^{-1}	6.2	3.6	2.4	2.4	2.2	2.3
1000 mg kg^{-1}	3.0	2.6	2.8	2.0	1.6	1.3

Tab. 8. Glucose induced soil respiration in closed bottle

hPa* 1000 min^{-1}	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	61.1	73.8	37.8	37.2	38.3	55.0
10 mg kg^{-1}	61.6	79.5	64.6	37.2	54.7	57.3
100 mg kg^{-1}	113.2	114.1	38.2	36.2	35.0	65.5
1000 mg kg^{-1}	5.4	6.5	36.7	123.8	63.0	53.9

Tab. 9. Soil respiration in dynamic aerated reactor

Produced CO_2 in mmol	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	0.277	0.403	0.254	0.263	0.171	0.228
10 mg kg^{-1}	0.398	0.468	0.289	0.289	0.210	0.193
100 mg kg^{-1}	0.306	0.439	0.237	0.237	0.237	0.228
1000 mg kg^{-1}	0.249	0.249	0.175	0.184	0.223	0.214

The Shannon indexes of 0, 10, 100 mg kg^{-1} contaminated soils mainly moved together. For the week 10, the 10 ppm contaminated soil had the lowest and the 1000 ppm contaminated soil had the highest Shannon index value (Table 11, Figure 5). The Shannon indexes of 1000 mg kg^{-1} contaminated soil showed increasing tendency similarly to the AWCD values of this highly contaminated soil.

Figure 4 clearly demonstrates that the AWCD values of 0, 10 and 100 ppm contaminated soils decreased, while 1000 ppm contaminated soils increased during the examined time compared to the Start point.

At week 3, a significant drop can be noticed. Presumably, after consumption of more easily available contaminant fractions an adaptation period was necessary for new microbial community.

Tab. 10. AWCD values after 120 h incubation*

AWCD values	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	1.07(±9.9)	1.02(±7.1)	0.83(±3.3)	0.77(±8.0)	1.04(±3.6)	0.59(±3.7)
10 mg kg^{-1}	0.94(±6.6)	0.98(±9.6)	0.92(±5.6)	1.09(±2.1)	0.90(±8.1)	0.37(±2.1)
100 mg kg^{-1}	1.2(±5.1)	1.13(±9.4)	0.91(±8.7)	0.90(±0.5)	1.14(±3.8)	0.64(±28)
1000 mg kg^{-1}	0.54(±2.0)	1.03(±4.2)	0.96(±8.0)	1.27(±3.9)	1.27(±4.5)	1.21(±2.4)

* Standard deviation [%] can be found in the brackets

Tab. 11. Shannon diversity index values after 120 h incubation*

Shannon index values	Start	Week 1	Week 3	Week5	Week8	Week 10
0 mg kg^{-1}	3.28(±0.5)	3.17(±2.2)	3.12(±3.2)	3.17(±2.9)	3.23(±0.5)	3.08(±1.0)
10 mg kg^{-1}	3.20(±0.5)	3.25(±1.2)	3.09(±2.2)	3.20(±1.9)	3.13(±2.4)	2.85(±4.6)
100 mg kg^{-1}	3.28(±0.8)	3.28(±1.0)	3.23(±0.7)	3.22(±1.1)	3.27(±0.9)	3.13(±3.5)
1000 mg kg^{-1}	2.85(±3.3)	3.16(±0.4)	3.14(±0.9)	3.26(±0.5)	3.27(±0.6)	3.24(±0.5)

* Standard deviation [%] can be found in the brackets

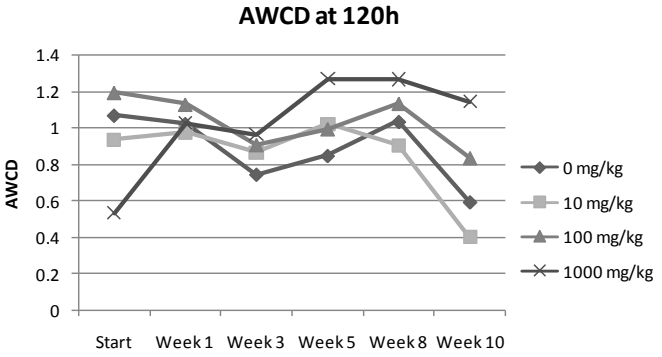


Fig. 4. AWCD values after 120 h incubation

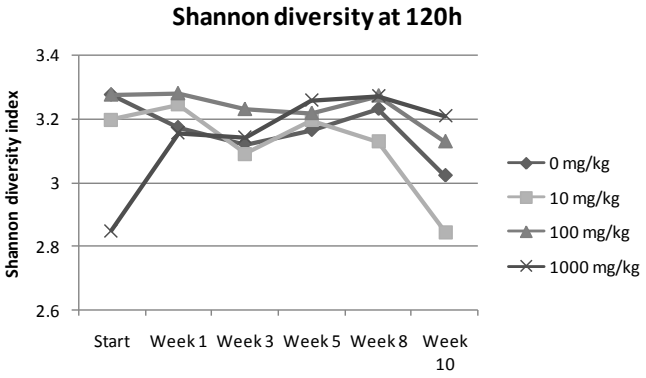


Fig. 5. Shannon diversity index after 120 h incubation

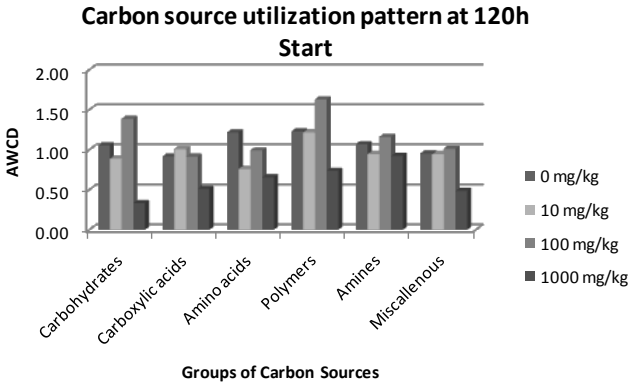


Fig. 6. Utilization of different carbon sources after 120 h incubation for start sample

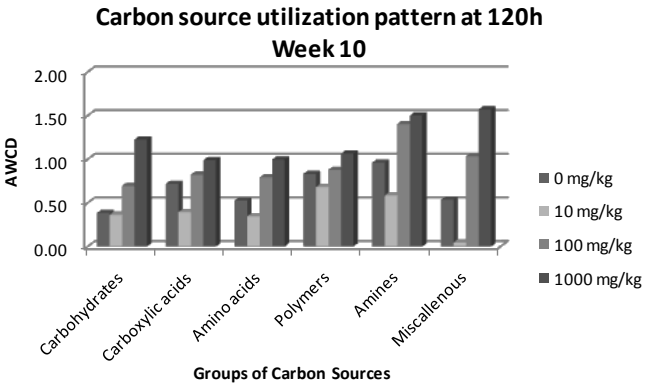


Fig. 7. Utilization of different carbon sources after 120 h incubation for 10 weeks sample

The 31 substrates which can be found in the Biolog Eco microplates were classified into six main groups as follows: carbohydrates, carboxylic acids, amino acids, polymers, amines and miscanellous. The microflora in the control soil showed a common substrate utilisation. The 10 ppm contamination resulted the same or in some cases lower AWCD values at start point as the control. At the beginning (Start) the microflora of 100 ppm contaminated soil showed mainly better utilization pattern as the control soil. For week 10, the AWCD values of the 0, 10 and 100 ppm contaminated soils decreased compared to the beginning (Start). There were significant changes in the substrate-utilisation of the 1000 ppm contaminated soil during the examined period. At first sampling (Start), the microflora of 1000 mg kg^{-1} 4-CP contaminated soil was able the least of all to uti-

lize any of the groups of carbon sources but by the end of the experiment this soil showed the highest values of all the others (Figure 6–7).

Correlation analysis of chemical and biological methods

The results of the chemical and the biological methods were compared to each other by correlation analyses using the StatSoft® Statistica 7.1 program. In the control soil, a good correlation was found between the contaminant degrading cell concentration and the soil respiration in dynamic aerated reactor. In the 10 ppm contaminated soil, significant correlation was obtained between the contaminant degrading cell concentration and the soil respiration in closed bottle ($r = 0.89$; $p = 0.017$), such as the contaminant degrading cell concentration and the soil respiration in dynamic aerated reactor ($r = 0.85$; $p = 0.031$). In the 100 ppm contaminated soil, significant good correlation was obtained between the contaminant degrading cell concentration and the soil respiration methods ($r > 0.85$; $p = 0.006 - 0.034$).

In 1000 ppm contaminated soil significant correlation was obtained between the contamiant degrading cell concentration and glucose induced soil respiration in closed bottle.

Also very good correlation was found between the substrate utilization community analysis (AWCD values, Shannon-index) and the cyclodextrin extractable 4-CP content. The results of the chemical methods correlated very well with each other.

Results of correlation analyses also demonstrate that dehydrogenase enzyme activity assay is not suitable for accurately prediction of biodegradation in 4-CP contaminated soil. The r of correlation coefficients was < 0.564 for all models.

Our previous study [19] demonstrated good correlation between the chemical and most of the biological results in the case of diesel and transformer oil depending on the contaminant. All methods showed a more-or-less good correlation in diesel–oil–contaminated soil: it is a well-biodegradable, well-analysable mixture of hydrocarbons. The scale of correlation for transformer oil was generally lower, but the best ones, i.e. aerobic respiration, HPBCD-extraction and MPN, were close to each other, while RAMEB-extraction and DEH were out of the correlating range: they correlated neither with others, nor with each other. Aerobic respiration and HPBCD extraction provided the best correlation for both diesel and transformer oil.

In the present research slight correlation was found between the results of non- exhaustive extraction and most of the biological methods with the exception of microbiological community analyses by BIOLOG Eco plates where strong correlation was observed. HPBCD extraction provided better correlation with biological methods then RAMEB extraction in present research too. Presumably this method can be a better model for characterizing the bioavailability of contaminants. Further validation of this chemical model using HPBCD extraction is needed, with additional poorly available contaminants in different types of soils.

Our results suggest that a complex methodology is necessary for the assessment and evaluation of biodegradation in soil and the applied methodology has to be further validated in case of additional contaminants.

4 Conclusions

The ten-week-long laboratory microcosm experiments provided valuable information on biodegradation of 4-chlorophenol even in the highly contaminated soils, enabled to find the most appropriate test-battery for measuring biodegradation potential.

Microbiological methods were found to be reliable indicators of 4-chlorophenol biodegradation in the soil, and most of them can be useful as a pre-implementation methodology to support technology selection and design.

Glucose induced soil respiration test (SIR) in closed bottle proved to be a suitable method for characterizing hydrocarbon-degrading activity with interesting outcomes. Glucose solution activated the soil microbes which were able to utilize the easily degradable glucose but on the other hand, the microbes in the contaminated soils showed much more increased respiration, forecasting a cometabolic pathway in 4-CP degradation. Therefore this method can be taken as the measurement of the biomass of active microorganisms. The results of glucose induced soil respiration test correlated well with the chlorophenol degrading cell concentration (MPN).

AWCD value used as an indicator of general microbial activity and Shannon-index presenting the microbial diversity were the best methods by far and provided valuable information on the composition and structure of microbial communities and their potential catabolic functions with respect to 4-CP biodegradation.

Cyclodextrin extraction can be considered as the proper chemical model for the prediction of 4-CP bioavailability of the soil. Mechanism of multilevel adaptation was also presented by changes of the biologically available ratio determined by cyclodextrin-extraction of 4-CP in soil. Strong positive correlation ($R > 0.93$) was found between cyclodextrin (RAMEB, HPBCD) extractable 4-CP content and the results of the microbial community analyses carried out by BIOLOG Eco Plates. Our results have revealed that HPBCD method using hydroxypropyl beta-cyclodextrin for extraction can be a better model for characterizing the bioavailability of contaminants than the RAMEB method.

The results of this preliminary study underline the need of test-battery application to characterise biodegradation process and efficiency in contaminated soil as a dynamic system, which is able to measure responses and interactions.

Based on the present study the proposed biological test-battery for characterization of 4-CP biodegradation in soil includes 1) substrate utilization of the microbial community characterized by Biolog® System (AWCD values and Shannon-index), 2) contaminant degrading cell concentration and 3) glucose induced soil respiration in closed bottle. The application of

Tab. 12. Correlation analysis of the biological and the chemical results of the 1000 ppm contaminated soil

	CP-CFU	DEH	RES CB	SIR	RES AER	AWCD	SHANNON	GC MS	RAMEB GC	HPBCD GC	RAMEB SP	HPBCD SP
CP-CFU	1.000											
DEH	0.241	1.000										
RES CB	0.591	0.139	1.000									
SIR	0.977	0.306	0.575	1.000								
RES AER	0.541	0.433	0.174	0.671	1.000							
AWCD	0.697	0.511	0.776	0.734	0.459	1.000						
SHANNON	0.625	0.564	0.732	0.678	0.497	0.992	1.000					
GC MS	0.706	0.215	0.792	0.796	0.717	0.848	0.844	1.000				
RAMEB GC	0.581	0.487	0.715	0.667	0.666	0.931	0.958	0.923	1.000			
HPBCD GC	0.622	0.454	0.770	0.692	0.614	0.956	0.973	0.926	0.995	1.000		
RAMEB SP	0.669	0.382	0.787	0.748	0.667	0.935	0.944	0.969	0.986	0.991	1.000	
HPBCD SP	0.614	0.476	0.742	0.691	0.646	0.947	0.968	0.927	0.998	0.999	0.990	1.000

Correlation matrix between biological and chemical parameters of soil samples contaminated with 1000 ppm 4–CP.
Correlation coefficients in bold are statistically significant at $p < 0.05$.

these methods provided complementary and essential information to chemical characterization. Furthermore all these techniques are simple and non-expensive, and can be applied in the routinely monitoring of the processes performed in the study that move from lab-scale to field-scale experiments.

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