J Incl Phenom Macrocycl Chem (2011) 70:307–313
DOI 10.1007/s10847-010-9855-8

ORIGINAL ARTICLE

Application of cyclodextrins in environmental bioassays for soil

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Received: 20 June 2010 / Accepted: 31 August 2010 / Published online: 14 September 2010

Abstract Based on our former experience on contaminant solubilisation and mobilisation in the process of soil remediation we used cyclodextrins as additives in environmental bioassays, for improving solubility and bioavailability of the contaminant in soil and as a consequence sensitivity of the bioassay. In this article we introduce the findings on the application of RAMEB (randomly methylated β-cyclodextrin) for testing PCP (pentachlorophenol) in soil, in three bioassays: bacterial luminescence-inhibition test with *Vibrio fischeri*, protozoon growth inhibition test with *Tetrahymena pyriformis*, and Ames mutagenicity test. We applied RAMEB which has a high solubilising capacity on many typical soil contaminant and PCP, because contradictory results were published for its toxicity and mutagenicity. The RAMEB-aided Ames test, gave a sudden and expressed increase in the mutagenicity of PCP, however, Ames mutagenicity was negative without cyclodextrin (CD). Based on these results we tried to apply RAMEB for increasing sensitivity of other bioassays, such as acute toxicity tests with different test organisms. According to our results the effect of RAMEB on bioavailability and toxic effect depends not only on the K_{ow} value (octanol–water partition coefficient) of the chemical substances, but also on the test organism, the water-content of the test-matrix and the applied concentration of RAMEB, as well as its ratio to PCP. We collected all the characteristics of the bioassays applied for PCP and some other contaminants and showed the measured effect data in comparison with each other. We found that in the complex system of soil and soil suspension, used in the bioassays, the interactions between soil solid, water and gaseous phases, as well as between the test organism and RAMEB result in K_{ow} dependent partition of the contaminant between solid and water phases of soil, RAMEB, and the test organism. The conclusion is that RAMEB undoubtedly has an influence on the fate and behaviour of the contaminant in soil and soil suspensions, and the direction of the RAMEB-induced changes depends on the effective concentration of the RAMEB in the bioassay, the time of contact, the type of test organism, and the characteristics of the RAMEB–contaminant complex. In those cases, when RAMEB increased the effect of a contaminated environmental sample, this CD-induced increase can be considered as a "realistic worse case" situation, which can be very useful in risk assessment, resulting in a moderate overestimate in the value of environmental risk.

Keywords Cyclodextrins in bioassays · Environmental bioassay · Toxicity · Mutagenicity · Bioavailability of contaminants · Mobility of contaminants · Risk assessment · Contaminated soil · Randomly methylated beta-cyclodextrin · Pentachlorophenol

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Introduction

Measuring toxicity and other harmful effects of chemical substances with bioassays is an essential and well developed tool for risk assessment. The application of bioassays for environmental samples especially for soil and other solid-phase containing environmental samples is far behind

the general testing of pure chemical substances, because of the low reliability and sensitivity due to strong interactions, matrix effects and non-equilibrium state. Based on the experience on contaminant solubilisation and mobilisation in the process of soil remediation we made many trials for the application of cyclodextrins as an additive in environmental bioassays, for improving solubility and bioavailability of the contaminant in soil and as a consequence sensitivity of the bioassay. Using cyclodextrins in direct contact soil tests the adverse effects of the contaminated environmental sample can be measured without traditional extraction of contaminants with organic solvents.

Rising of harmful effects is highly influenced by the bioavailability of the contaminant in the environmental compartments. In the assessment of adverse effects a conservative approach is applied, which requires the over estimation of bioavailability in a moderate and realistic way. The traditional chemical analyses aim to achieve total extraction when determining contaminant concentration in environmental samples and the effective (able to interact with living organisms) concentration is highly over estimated. Most of the organic solvents cannot be used in bioassays, due to their toxicity on test organisms. Another possibility is using water extracts in bioassays, but it often gives false negative result for hydrophobic hazardous components with low solubility and high K_{ow}, due to low solubility and to the dilution by water extraction.

Direct contact toxicity testing avoids the problems of extraction and increases environmental reality by ensuring the interaction between test organims and soil contaminant [1], but the matrix effect of the soil and the low absorption of the hydrophobic contaminants by test organisms, decreases the sensitivity of the bioassays [2]. In recent years it has been proven that there is a correlation between the contaminant concentration extracted by a CD solution, usually HPβCD (hydroxy-propyl β-cyclodextrin) and the availability of the same contaminants for the biota [3]. Numerous scientists tried to measure this relationship in different ways: they compared the bioassay results to the amount of CD-extracts in case of biodegradation tests [4–8] or bioaccumulation tests [9–11]. Molnár et al. [12] published a comprehensive comparative study on three chemical extraction methods and four bioassays for biodegradation and found HPβCD a good simulator of bioavailability of complex hydrocarbon mixtures.

As cyclodextrins are well-known and widely used solubilising agents for high K_{ow} organic compounds; they are water-soluble and have high solubilising/mobilising effect on a lot of typical soil contaminants, such as volatile hydrocarbon contaminants of soils [13]. In toxicity testing CDs were used in aqueous solution of a contaminant (for example: benzo(a)pyrene, pyrene, phenantrene, diuron, 2,4-dichlorophenol) and compared to the contaminant solution without CD [9, 14]. The results indicated that CDs can cover the effective or harmful part of the molecule and decrease the toxicity. In direct contact soil bioassays the situation is different: contaminant connected to the soils organic material can be solubilised by CDs to intensify the harmful effect. Molnár et al. [15] used this bioavailability enhancing effect in remediation technologies: a month after addition of CD to aged transformer oil contaminated soil, the toxicity increased in the contaminated soil, but in time the microbiological degradation was intensified, the transformer oil concentration and toxicity were reduced. Behind these results we proved, that the strongly bound organic contaminant can be released from soil organic matter with CD, and it is able to increase water solubility, extractability and biological availability of the contaminants in real contaminated soil.

As a model system we applied PCP contaminated soil, because PCP is a typical contaminant in our environment, and PCP is able to form inclusion complexes: solubility of PCP in water can be increased 3.6 times and its log K_{ow} decreased almost two orders of magnitude by RAMEB [16]. Due to decreased K_{ow} the PCP more likely will be released from the organic matter of the soil and the harmful effect can be measured with bioassays. Three different bioassays were applied to PCP: bacterial *Vibrio fischeri* luminescence-inhibition test, protozoa *Tetrahymena pyriformis* growth inhibition test, and Ames mutagenicity test.

Materials and methods

Contaminated soils

The brown forest soil was artificially spiked with 12.5, 25, 50, 100, and 200 mg PCP/kg air dried soil and left to mature for 2 weeks. The spiked brown forest soil originates from the Buda mountains, and has 4.18% humus content and the physico-chemical characteristics of silt loam.

Pre-treatment with RAMEB

RAMEB was used for the soil pre-treatment, assuming that the mobilising effect would appear in the soil. RAMEB was added to the soil 24 h before testing. For the pre-treatment we used 1.0, 2.5, 5, and 10% (related to the air-dried soil) of RAMEB in powder form and mixed thoroughly.

Randomly methylated β-cyclodextrin (RAMEB) is a product of Wacker Chemie (Munich, Germany), and PCP was purchased from Reanal (Budapest, Hungary; purity 98%).

Direct contact testing of soil

The application of whole soil, without extraction simulates a realistic interaction between test organisms and contaminated soil during biotesting [1].

Testing mutagenicity with Ames test

The mutagenic effect of PCP-contaminated soils is measured with three of the frequently used Salmonella strains: TA 1535, TA 1537, and TA 1538, which all are different histidine auxotroph mutants. These mutants normally cannot grow without histidine, if the mutants are exposed to mutagenic effect the histidine auxotrophy most likely disappears, and the strains can grow without external histidine supplement [17]. In our previous trials the TA 1538 was the most sensitive strain, suggesting that much of the mutagenic activity in PCP contaminated soil is frame shift activity [18]. In the bioassay the test organism was spread on the surface of the histidin-free agar surface containing contaminated soil (0.1 g soil/10 ml agar) and after 48 h the revertant colonies were counted [16]. The mutagenic effect was confirmed in case the following two requirements were fulfilled:1) the number of revertants counted in the Petri dish containing the contaminated soil sample is more than double the spontaneous revertant number in the blind sample;2) the revertant number is proportional with the contaminant concentration.

Testing acute toxicity with bacterium

Effect of soils on *Vibrio fischeri*, the widely used laboratory test organism was determined by the modified bioluminescence inhibition test, using solid soil and direct contact [1]. Contaminated soil (25 mg) was added to the cell suspension (250 µl). The light emission of the test-bacterium was measured after 30 min exposure time with Lumac Biocounter M1500P. The light emission of contaminated soil exposed bacteria was compared with control soil to calculate inhibition percents.

Testing acute toxicity with protozoon

Tetrahymena pyriformis growth inhibition test characterises the toxic effect of the contaminated soils on a protozoon. Contaminated soil (1.5 g) was added to the cell suspension (30 ml). The measured endpoint, the number of cells is determined after 96 h exposures time according to [19] by counting in Bürker-chamber, and the results are compared to control soil to calculate growth inhibition percents.

Evaluation of the tests

The test results were presented on a dose–response curve, where the measured inhibition% or the number of revertants (the endpoint of the applied biotests) is plotted against PCP concentration. During testing the PCP and RAMEB concentrations of the soil are diluted by the test suspension of bacteria or protozoa. In the Ames mutagenicity test the dilution ratio is 1:100, in the luminescence inhibition test it is 1:10 and in the growth inhibition test the dilution ratio is 1:20. This way we apply two contaminant concentrations: the original contaminant concentration in the soil and the actual concentration—as a result of the dilution—in the test-vessel. When analysing soil, we use the "concentration in the soil to be analysed", but in this case, when we evaluate the sensitivity of the method, we use the "actual contaminant concentration in the test-vessel". In Figs. 1, 2, and 3 the PCP and RAMEB concentrations are the concentrations formed in the test-vessel to get the real ratio of PCP and RAMEB, for the comparison of different bioassays. CD application in different concentrations and the contaminant concentrations in soils provide various CD–PCP molar ratios which significantly influence the inhibitory effects and inclusion forming. The same soil samples pretreated or not with RAMEB were used in the various bioassays. The different conditions (dilution rate, CD–PCP ratio) in the biotests resulted in different final PCP and RAMEB concentrations in the final test-suspensions.

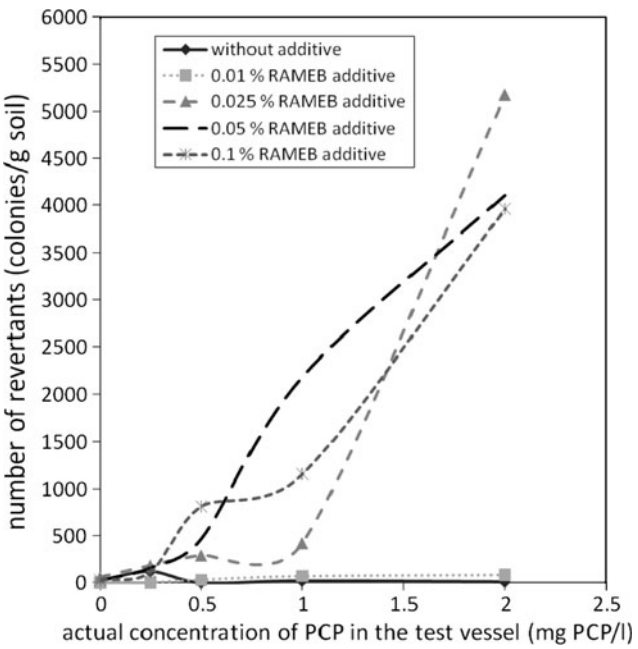

Fig. 1 Mutagenic effect of soils after RAMEB addition–frame shift mutation (*Salmonella typhymurium* TA 1538)

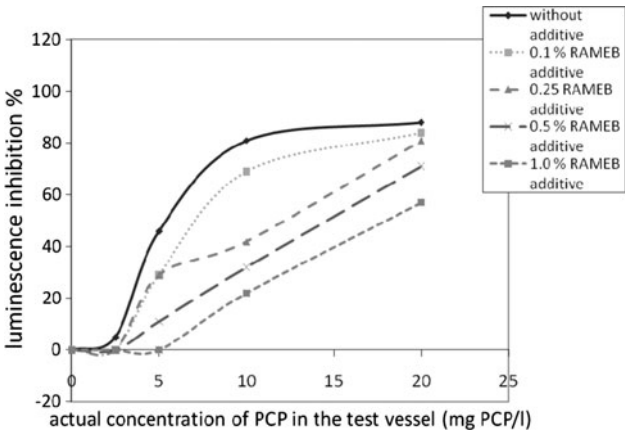

Fig. 2 Acute toxic effect of soils on *Vibrio fisheri* with and without RAMEB

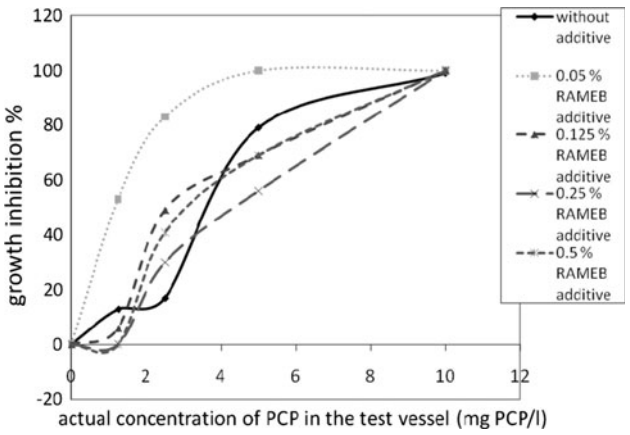

Fig. 3 Acute toxic effect of soils with and without RAMEB on the growth inhibition of *Tetrahymena pyriformis*

Results

The mutagenicity and toxicity of contaminated soil is measured by direct contact bioassays, which means that the surface of the test organism's cells are in direct connection with contaminated soil in a soil suspension.

Mutagenicity of PCP with RAMEB-aided Ames test

The testing of mutagenicity of PCP is reasoned by the contradictory results of the literature: the mutagenicity of PCP is proved in animal tests [20, 21], but Ames mutagenicity is reported both as positive [22] and negative [23]. In our experiments RAMEB addition to PCP-contaminated soil lead to measurable mutagenicity as Fig. 1 shows: the number of revertants increased significantly on the effect of 0.025, 0.05, and 0.1% RAMEB and was proportional with PCP concentration. Above a certain limit level, which does not show any effect (0.01%), higher RAMEB concentrations caused sudden increase in the number of revertants related to lower PCP concentration: 0.025, 0.05, and 0.1% RAMEB at 1, 0.5, and 0.025 mg PCP/l test suspension concentrations, respectively. It suggests that the equilibrium between the complex association of PCP with RAMEB and the dissociation of the formed complex is shifted towards the association, and the PCP included in the complex is that exerts mutagenicity.

Acute toxicity of PCP on *Vibrio fisheri*, the luminescent bacterium

Addition of RAMEB in the range of 1–10% related to the soil, which is 0.1–1.0% related to the test-suspension decreased the inhibition of *Vibrio fisheri* luminescence on the effect of PCP. The decrease is proportional with the RAMEB concentration as it is shown in Fig. 2. It means that the RAMEB–PCP complex is less toxic, than the free or soil bound PCP.

Acute toxic effect of PCP on the growth of *Tetrahymena pyriformis*

0.125–0.5% RAMEB concentrations lowered the toxicity of PCP under 1.5, and above 3.0 mg/l, but increased it between 1.5 and 3.0 mg/l PCP. 0.05% RAMEB increased the effect at all PCP concentrations. As *Tetrahymaena* is an animal cell with a phospholipid-containing cell membrane there is one more participant playing role in the binding of PCP, and influencing the partition of PCP between solid soil particles, liquid soil moisture or pore water, RAMEB (forming a complex with it) and the test organism (binding to its membrane-receptors).

Discussion

Understanding the importance of the ratio of the partners playing role in RAMEB-amended bioassays, we made a comparison with our present and some former research results. Table 1 shows results for phenantrene (polycyclic aromatic hydrocarbon) and cypermethrin (insecticide) contaminated as well as transformer oil (a mixed petroleum hydrocarbon) contaminated soils. The bioassay conditions, including contact time, soil–water ratio, and the applied RAMEB concentration as well as the K_{ow} of the contaminant and its modified value in 10% RAMEB solution are shown.

Five different bioassays in Table 1 all proved the influence of RAMEB on mobility of the contaminants in soil. The results clearly show that RAMEB concentration and the RAMEB ratio to the contaminating chemical substance and their interactions are not the only parameters, which influence the effect of contaminants in soil. The test

Table 1 Effect of RAMEB addition on toxicity and mutagenicity test results of different contaminants

Bioassay			Contaminant			RAMEB final concentration in the bioassay	Result	Reference
Assay name	Contact time	Soil: water ratio	Contaminant name, and concentration in soil	log K_{ow} in water	log K_{ow} in 10% RAMEB solution			
Vibrio fischeri luminescence inhibition	0.5 h	1:10	PCP 12.5, 25, 50, 100, and 200 mg/kg air dried soil	pH 6: 2.98 pH 8: 1.98	pH 6: 1.70 pH 8: 0.32	0.1–1%	Decreased toxicity	Fig. 2 in this paper
Tetrahymena pyriformis growth inhibition test	96 h	1:20				0.05	0.05% increased toxicity	Fig. 3 in this paper
						0.1–0.5%	0.1–0.5% Decreased toxicity	Fig. 3 in this paper
Ames mutagenicity test	72 h	1:100				0.01–0.1%	Increased mutagenic effect	[17]
Azomonas agilis dehydrogenaze activity test	72 h	1:5	Transformer oil 5000, and 10000 mg/kg air dried soil	5.1	3.7	0.2%	Increased toxicity	[24]
Vibrio fischeri luminescence inhibition	0.5 h	1:10				0.1%	Decreased toxicity	[24]
Folsomia candida mortality test	7 days	5:1				1%	Decreased toxicity	[24]
Azomonas agilis dehydrogenaze activity test	72 h	1:5	Phenantrene 100, and 200 mg/kg air dried soil	4.65	2.92	0.2%	Increased toxicity	[24]
Vibrio fischeri luminescence inhibition	0.5 h	1:10				0.1%	Decreased toxicity	[24]
Folsomia candida mortality test	7 days	5:1				1%	Decreased toxicity	[24]
Azomonas agilis dehydrogenaze activity test	72 h	1:5	Cypermethrin 900 mg/kg air dried soil	4.65	3.26	0.2%	Increased toxicity	Unpublished
Vibrio fischeri luminescence inhibition	0.5 h	1:10				0.1%	Decreased toxicity	Unpublished
Folsomia candida mortality test	7 days	5:1				1%	Decreased toxicity	Unpublished

organism itself is an important factor, the DNA, the cell membrane, the receptors, the enzyme system, the growth, the age, etc. of the test organisms and the test-conditions, mainly the duration might be equally important. The most popular bioassay, *Vibrio fisheri* test always shows decreased sensitivity for all of the four tested contaminants in soil in the presence of RAMEB. It means that RAMEB protects this bacterium from the toxic effects, in this very short, only 30 min test.

Folsomia candida the little soil living insect is also defended by RAMEB. Its typical exposure route is inhalation/respiration: the contaminant concentration is always lower in the soil air, when RAMEB is present and encapsulates the contaminants into a molecular trap.

Azomonas agilis is a bacterial test organism, the test-endpoint is an enzyme activity and the test is 72 h long, carried out in a relative dense soil suspension, prepared in the beginning of the testing. The presence of RAMEB increased the toxicity in case of all contaminants.

Tetrahymena, a single cell animal with a cell-border of animal membranes shows an ambivalent response on PCP in the presence of RAMEB: the lowest RAMEB concentrations increased the toxicity of PCP, other RAMEB–PCP ratios showed sometimes higher, but mostly lower toxicity.

The mutagenic effect of PCP in Ames test was increased by RAMEB in a very large scale. PCP without RAMEB is not mutagenic, neither the treatment with digestive enzymes nor special bile acids (generally able to increase bioavailability) did increase PCP mutagenicity [23], but RAMEB has generated mutagenicity. We guess that the direct mutagenesis of the RAMEB–PCP inclusion complex is behind the identified phenomenon: the size of the complex makes it able to bind to the DNA of the test-microbes and cause mutagenesis.

The optimal RAMEB concentration depends on the structure of the contaminant (inclusion forming ability), the concentration of the contaminant in soil, and the effect-mechanism, therefore our methodological suggestion is the use of various RAMEB (or other cyclodextrins) concentrations for soil toxicity testing, and apply the highest toxicity result in the risk assessment procedure.

In the complex and dynamic system of a direct contact biotests, the contaminant molecule is in interaction with the organic material of soil, with RAMEB and with the test organisms (their surface molecules), RAMEB is in interaction with the contaminant, with soil organic matter and the surface molecules of the test organisms, etc. Additionally, in the soil there are two physical phases: liquid and solid, and between these two phases both the contaminant and the cyclodextrin, as well as their complex are partitioning. As a result of this partition is that the test organism maybe in interaction with the dissolved free (uncomplexed) or with the complexed contaminant molecule both in the water phase, or in the solid. Additionally these processes depend on the test duration, therefore we have to ensure even in the short-term tests reaching of the equilibrium.

Conclusions

Bioassays are able to simulate and measure actual effects of the contaminants in a certain environment, but the reduced diversity, the differences between the conditions of the test methods and the real environment may result in low statistics and an under estimation of the risk of the environmental sample [25]. The main difficulty of measuring and assessing the risk of contaminated soil is the non-equilibrium state and the interactions between solid soil, soil moisture, test organism and contaminant. The estimation of all these from chemical analytical results or from bioassays on extracts is hardly possible. Extracts with organic solvents usually lead to unnecessary over estimates, water extracts to under estimate. Direct contact of the test organism with soil improves this situation, but the restricted bioavailability may cause under estimated adverse effects and risk. The effect of toxic substances is highly influenced by bioavailability, bioaccessibility and partition between soil phases. Bioavailability of organic contaminants is associated with their K_{ow}, the octanol–water partition coefficient. K_{ow} can be increased by addition of cyclodextrin: this special molecule is able to form an inclusion complex with organic pollutants, thus changing their solubility and mobility and as a result bioaccessibility and bioavailability.

According to our research on the application of RAMEB in laboratory bioassays for testing adverse effects of environmental samples we can state that it works in many cases: the CD-increased K_{ow} resulted in higher accessibility and bioavailability in the bioassay and increased the sensitivity of the test. The effect of RAMEB is concentration-dependent: it has a maximum. In some special cases, however, toxicity decreases on the effect of RAMEB; this phenomenon is mostly test-organism dependent. In case of testing the mutagenicity of PCP, the effect of RAMEB is other than solubilisation of the contaminants, the originally not measurable mutagenic effect increased with the RAMEB complexation of PCP. We assume that the size of the PCP–RAMEB complex fits better to the structure of the DNA and is able to cause direct mutagenicity.

Our practical advice in the utilisation of CDs in bioassays is, that in those cases, when the complexation of the contaminant with CD has already been proven, some CD-treated environmental samples should be tested (according to the protocol of the bioassay) to see if the effect of the sample on the test organism could be increased or not. If not, the result

of the bioassay without RAMEB is valid, if yes, we have to know, that the contaminant in soil (or sediment) can become more effective, more available for the biological system, not only in the test vessel, but also in the real environment. CD addition to the environmental sample represents a conservative estimate (meaning an effective contaminant concentration which can evolve in the environment, too) and in such a case we have to calculate with this increased adverse effect and environmental risk. This method fits well into the conservative policy of risk assessment to put into the risk assessment system an "experimental safety factor", not a theoretical one.

Acknowledgments The research was supported by the Hungarian R&D programs (MOKKA NKFP-3-00020/2005, CDFILTER TECH-08-A4/2-2008-016 and SOILUTIL. TECH-09-A4-2009-0129) and Anyos Jedlik National Research and Technology Programme (MOKKA NKFP-3-00020/2005).

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