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Development of analytical methodologies to assess recalcitrant pesticide bioremediation in biobeds at laboratory scale

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ABSTRACT:

To assess recalcitrant pesticide bioremediation it is necessary to gradually increase the complexity of the biological system used in order to design an effective biobed assembly. Each step towards this effective biobed design needs a suitable, validated analytical methodology that allows a correct evaluation of the dissipation and bioconvertion. Low recovery yielding methods could give a false idea of a successful biodegradation process. To address this situation, different methods were developed and validated for the simultaneous determination of endosulfan, its main three metabolites, and chlorpyrifos in increasingly complex matrices where the bioconvertor basidiomycete Abortiporus biennis could grow. The matrices were culture media, bran, and finally a laboratory biomix composed of bran, peat and soil. The methodology for the analysis of the first evaluated matrix has already been reported. The methodologies developed for the other two systems are presented in this work. The targeted analytes were extracted from fungi growing over bran in semisolid media YNB (Yeast Nitrogen Based) with acetonitrile using shaker assisted extraction, The salting-out step was performed with $MgSO_4$ and NaCl, and the extracts analyzed by GC-ECD. The best methodology was fully validated for all the evaluated analytes at 1 and 25 mg kg^{-1} yielding recoveries between 72 and 109% and RSDs <11% in all cases. The application of this methodology proved that A. biennis is able to dissipate 94 % of endosulfan and 87 % of chlorpyrifos after 90 days. Having assessed that A. biennis growing over bran can metabolize the studied pesticides, the next step faced was the development and validation of an analytical procedure to evaluate the analytes in a laboratory scale biobed composed of 50 % of bran, 25 % of peat and 25 % of soil together with fungal micelium. From the different procedures assayed, only ultrasound assisted extraction with ethyl acetate allowed recoveries between 80 %-110 % with RSDs <18 %.

Linearity, recovery, precision, matrix effect and LODs/LOQs of each method were studied for all the analytes: endosulfan isomers (α & β) and its metabolites (endosulfan sulfate, ether and diol) as well as for chlorpyrifos. In the first laboratory evaluation of these biobeds endosulfan was bioconverted up to 87 % and chlorpyrifos more than 79 % after 27 days.

Keywords: Basidiomycete; Recalcitrant pesticides; Bioremediation; Method validation

1. Introduction

Environmental pollution caused by hazardous wastes containing recalcitrant xenobiotic chemicals has become a major problem that threatens the sustainability of the ecosystems as well as human health. Unlike the naturally occurring organic compounds that are readily degraded upon introduction into the environment, some of these synthetic chemicals are extremely resistant to biodegradation by native microorganisms. Particularly, surface and ground waters are exposed to diffuse pollution due to pesticides via percolation, runoff, drainage and drift (i.e. movement of airborne spray droplets) as well as spills during equipment washing. These critical points are the main sources of soil and water contamination, contributing significantly to the deterioration of natural water sources [1, 2-5]. This is an issue of global strategic importance as groundwater represents about 98 % of the available fresh water of our planet [6]. Several field surveys and measurement campaigns on a catchment scale have demonstrated that 40–90 % of surface water contamination by pesticides is attributable to direct losses of the active ingredients [1, 3-5]. To protect ground and surface water quality, practical solutions to minimize the pesticide entrance into hydrographic networks are continuously investigated. One of the possible strategies to minimize it is to degrade the pesticides before releasing the machinery washings and container triple rinses to the environment.

As the degradation of recalcitrant pesticides occurs slowly under natural conditions, the process has to be enhanced.

A newly explored route to diminish pesticide spillage into waters or in places where it can lixiviate to ground water is the biobeds technology. Biobeds are environmentally friendly solutions for the remediation of impacted zones as the pesticides convertor and degrading agents are saprophytes microorganisms. This methodology has been used in

Europe for several years and it is now being evaluated in South America as a friendly alternative for remediation of recalcitrant compounds [7, 8]. Ligninolitic fungi are biodegradetors of particular interest because they have shown to degrade and mineralize a large variety of recalcitrant compounds due to the nonspecificity of their enzyme machinery [9, 10]. White rot fungi produce a number of extracellular oxidative enzymes including laccases, lignin peroxidases and manganese peroxidases, which are normally involved in the breakdown of the plant structural material lignin [11] and are also responsible for the degradation of xenobiotics.

Challenging targets to assess biobeds suitability are organochlorine (OC) agrochemicals. They are persistent compounds which have been accumulating in the biosphere after decades of massive application in agriculture. Among them, Endosulfan, is an actually banned OC which had been used in many countries until recent years, leaving highly contaminated zones as an unsolved problem. In addition, there are also huge amounts of already synthesized endosulfan that have to be stored, waiting for a final destination. At the same time, massive use of highly toxic endosulfan, has been substituted by chlorpyrifos situation that renders an even more complicated scenario.

The proof of the efficacy of a bioremediation process is sustained on validated, highly sensitive analytical methods that assess the efficiency of the whole process. Agrochemical biodegradation has to be assayed in the laboratory, with microorganisms growing in conventional culture media, in order to select the microorganisms capable of dissipating them. Once the microorganisms have been selected and the efficiency of the transformation process evaluated, trial biobeds made of soil, peat and bran are assayed at a lab scale. Dissipation kinetics within the bioreactor is established, and the residual water that lixiviates through it is evaluated for pesticide non detection [12].

In a previous communication our group described ligninolitic fungi capable of degrading recalcitrant pesticides using endosulfan as a model compound in culture media, highlighting the importance of having analytical methods that can assure the effective dissipation of the pesticides into harmless products [13]. This work presents the advances in the development of a bioreactor using endosulfan and chlorpyrifos as model compounds, based in the Swedish biobed design [12] and native Basidiomycetes fungi as bioconvertors.

For each of the three different complex matrices employed: culture media, bran and the biobmix where the fungal mycelium grows and the biotransformation is performed, different methodologies were developed. Linearity, recovery, precision, matrix effect and LODs/LOQs, were determined for each of the endosulfan isomers (α & β) and some of its metabolites (endosulfan sulfate, endosulfan ether and diol) as well as for chlorpyrifos. Although chloryrifos metabolites, particularly TCP (3,5,6-trichloropyridinol) [14], is toxicologically important from an environmental point of view [15], only the dissipation of the parent compound as a model has been followed in this study. At this point, our interest was focused in the improvement of the biobed design and future work will need to study TCP degradation in the final biobed.

2. Materials and methods

2.1. Standards and reagents

Analytical grade organic solvents, pesticide residues free were purchased from Merck (Darmstandt, Germany). Pesticide standards and the internal standard were from Dr. Ehrenstorfer (Augsburg, Germany, 99 %). The culture media were provided by Difco.

Bran and land peat were commercially available. Magnesium Sulfate and Sodium Sulfate p.p.a from Sigma-Aldrich (St.Louis, MO, USA); Sodium Chloride from Merck (Darmstandt, Germany), Silica: MN Kiesel 60 from J.T. Baker (State of Mexico).

Stock solutions were prepared from the standard substances at 1000 and 2000 mg L^{-1} in ethyl acetate. Working standard mixtures were prepared by appropriately diluting the stock solutions with ethyl acetate. All solutions were stored at 4°C.

2.2 Apparatus and experimental conditions.

Gas chromatographic (GC) analyses were performed using a Shimadzu GC 17A equipped with an ECD detector and a PTV injector using internal standard method. All compounds were resolved on a capillary column Mega 68 (30m, 0.32 mm ID, 0.25 µm film thickness) Mega Legnano (Italy). The experimental conditions were as follow: PTV, 60ºC (0.3min), then 5ºC min^{-1} to 280ºC (40min). Oven temperature, 100°C (3min), 100-180ºC at 10°C min^{-1}, 180°C (15min), then 180-270°C at 5°C min^{-1}, 270ºC (10 min). Detector temperature, 280°C.

Orbital shaker: SL1 Stuart (Staffordshire,UK). Ultrasonic bath: Elma®Transsonic T460/H. Centrifuge IEC: HNS-II (U.S.A.).

2.3 Microbiological matrix preparation for validation study

2.3.1 Matrix A (Bran)

The prepared inoculum was added to a mixture of 5 mL of semisolid medium YNB (Yeast Nitrogen Base) and 2.2 g of bran which was previously homogenized in a water bath for 90 min at 45°C. Then, it was incubated at 28±2°C for 20 days.

Inoculum: A portion of 1 cm diameter of the external growth of preinoculum was added to 10 mL of Malt Extract medium and incubated at 28±2 °C for 10 days.

Preinoculum: Abortiporus biennis was cultured in solid media Potato Dextrose Agar (PDA) at 28±2 °C for 5 days.

2.3.2 Matrix B (Biobed)

The laboratory scale biobed was prepared by adding 20 times matrix A to 1 kg of a mixture containing 50 % bran, 25 % soil and 25 % peat and incubated for 30 days at room temperature.

2.4 Recovery tests

2.4.1 Recovery tests for matrix A

For recovery studies the matrix was prepared spiking at two levels: 1 and 25 mg kg^{-1} with chlorpyrifos, endosulfan and its metabolites. Levels 1 and 25 mg kg^{-1} were prepared in quintuplicate by adding 0.3 and 3.5 mL respectively of a solution containing 100 mg L^{-1} of endosulfan α and β, endosulfan ether, endosulfan diol, endosulfan sulfate and chlorpyrifos to matrix A.

2.4.2 Recovery tests for matrix B

For recovery studies the matrix was prepared spiking at three levels: 1, 25 and 50 mg kg^{-1} with endosulfan and its metabolites and 1, 30 and 60 mg kg^{-1} with chlorpyrifos. Level 1 mg kg^{-1} was prepared in quintuplicate by adding 0.05 mL of a solution containing 200 mg L^{-1} of chlorpyrifos, endosulfan α and β, endosulfan ether, endosulfan diol and endosulfan sulfate to grown fungi in 10 g of matrix B (laboratory

biobed); level 25 and 50 mg L^{-1} for endosulfan and its metabolites were prepared by adding 0.12 mL and 0.25 mL respectively of a 2025 mg L^{-1} solution and level 25 and 60 mg L^{-1} for chlorpryrifos were prepared by adding 0.12 mL and 0.30 mL respectively of a 2020 mg L^{-1} solution.

2.5 Extraction and clean up methods comparison

2.5.1 Bran (Matrix A)

The evaluated methods were:

1. Extraction with ethyl acetate and homogenization in Stomacher® and clean up using open column packed with silica and sodium sulfate.
2. Extraction with ethyl acetate and homogenization in orbital shaker for 24 h and clean up using open column packed with silica and sodium sulfate.
3. Extraction with acetonitrile and water, homogenization in orbital shaker for 2 h and salting out with NaCl and $MgSO_4$.

The comparison of the recovery results obtained with the three extraction methods tested was performed spiking at level 50 mg L^{-1} of endosulfan and 60 mg L^{-1} of chlorpyrifos.

2.5.1.1. General aspects for all the extraction procedures for Matrix A:

The internal standard (IS) used was methyl bromophos, the final aliquot was evaporated with a gentle stream of nitrogen, redissolved with IS and diluted to volume with ethyl acetate to obtain a final vial concentration of 1 mg L^{-1} of the IS and the evaluated. The chromatographic analysis was performed in a GC-ECD under the conditions explained in “Apparatus and experimental conditions”.

Extraction and clean up methods for matrix A are summarized in table 1 so that they can be easily compared.

Table 1.

2.5.2 Laboratory scale Biomix (Matrix B)

The compared methods were:

1. Extraction with acetonitrile and water, homogenization in orbital shaker and salting-out with magnesium sulfate and sodium chloride.
2. Extraction with ethyl acetate and homogenization in ultrasonic bath (ultras) for 15 min.
3. Extraction with acetonitrile and homogenization in ultrasonic bath for 15 min.
4. Extraction with ethyl acetate and homogenization in shaker with iron pellets assistance.
5. Extraction with ethyl acetate and homogenization in ultrasonic bath (3 cycles of 15 min. each one).
6. Extraction with ethyl acetate in soxhlet apparatus.

2.5.2.1 General aspects for all the extraction procedures for Matrix B:

Sample humidity was adjusted with a solution of NaCl (10 %). The internal standard (IS) used was methyl bromophos. The final aliquot was evaporated with a gentle stream of nitrogen and redissolved with a solution of the IS in ethyl acetate and diluted to volume with ethyl acetate in a 25 mL volumetric flask obtaining a final vial

concentration of 1 mg L^{-1} of the IS. The chromatographic analysis was performed in a GC-ECD under the conditions explained in "Apparatus and experimental conditions". Extraction methods for matrix B are summarized in table 2 so that they can be easily compared.

Table 2.

(*) Addition of 1 iron pellet per g of sample.

(**) 3 cycles of 15 min each one and manual agitation between cycles.

2.6 Sample preparation for endosulfan/its metabolites and chlorpyrifos analysis.

2.6.1 Final method for Bran (Matrix A)

Matrix A was finally analyzed using method 3 summarized under "Extraction and clean up methods comparison, Bran (Matrix A)":

The whole flask of rice straw with the fungi grown in semisolid media was extracted with 15 mL acetonitrile and 15 mL water in an orbital shaker apparatus for two hours. Then, 20g of $MgSO_4$ and 2g NaCl were added and placed in an orbital shaker for 5 hours. Finally it was centrifuged 10 min at 3000 rpm and an aliquot of 0.5 mL of the extract was evaporated with a gentle stream of nitrogen, redissolved with IS and diluted to volume with ethyl acetate to obtain a final vial concentration of 1 mg L^{-1} of the IS and analytes evaluated. The chromatographic analysis was performed in a GC-ECD under the conditions described in "Apparatus and experimental conditions".

2.6.2 Final Method for the laboratory scale Biomix (Matrix B)

Matrix B was finally analyzed using method 5 summarized in "Laboratory scale Biomix analysis (Matrix B)":

10 g of biobed mix and inoculum (Microbiological matrix preparation for validation study: Matrix B (Biobed)) were placed in a 250 mL Erlenmeyer flask and 10 mL solution of 10 % NaCl and 50 mL of ethyl acetate were added and extracted in an ultrasonic bath (3 cycles of 15 min each one). The final aliquot (0.5 mL) was evaporated with a gentle stream of nitrogen and redissolved with a solution of the IS in ethyl acetate and diluted to volume with ethyl acetate in a 25 mL volumetric flask obtaining a final vial concentration of 1 mg L^{-1} of the IS and analytes evaluated. The chromatographic analysis was performed in a GC-ECD under the conditions explained in "Apparatus and experimental conditions".

2.7. Methods validation

2.7.1 Matrix A

The following parameters were evaluated for the analytical method validation: linearity, recoveries, repeatability (RSDr), within-laboratory reproducibility (RSDwR), LOQs and LODs. All the tests were performed at two levels, five replicates in three different days. Solvent and matrix matched calibration curves were compared and matrix effects were quantified. Matrix effects were evaluated at two different amounts of matrix 0.5 and 0.02 g mL^{-1} of extract which correspond to the dilutions needed to cover the wide range of concentrations 1 to 25 mg L^{-1}.

2.7.2 Matrix B

The parameters evaluated for the analytical method validation exposed in the item "Methods validation, Matrix A" were evaluated in the same conditions. Matrix

effects were evaluated at three different amount of matrix 0.2, 0.07 and 0.03 g mL^{-1} of extract which corresponds to the dilutions needed to cover the wide range of concentrations 1 to 50 and 60 mg L^{-1} for endosulfan and chlorpyrifos respectively.

The different parameters evaluated were calculated as explained below:

RSDr presented are an estimation of the precision of the method. They were calculated as the relative standard deviation (coefficient of variation) of the five replicates from the recovery test measurement of each analyte, obtained using the same method on the same sample in a single laboratory over a short period of time, during which differences in the materials and equipment used and the analysts involved did not occur.

RSDwR presented are within laboratory reproducibility. They were calculated as the relative standard deviation (coefficient of variation) of all the replicates from the recovery test measurement of each analyte obtained using the same method in a single laboratory, by different analysts, and over a period in which differences in the materials and equipment occurred.

LOQs limits of quantitation (quantification) presented are the lowest concentration of the analyte that has been validated with acceptable accuracy (recoveries between 70-120% and RSDs < 20%) by applying the complete analytical method as recommended by DG-SANCO [16].

LODs limits of detection presented are the lowest concentration of the analyte that presents a response with a signal to noise ratio equal or above 3 by applying the complete analytical method.

3. Results and Discussion

The global strategy followed to accomplish the biobed design at laboratory scale is based in the developing of fit for purpose analytical methodologies for very complex and heterogeneous matrices, in which the pesticides and their metabolites have to be determined.

The Swedish biobed is composed of a mixture of peat, straw and soil (1:2:1). As the selected fungi did not grow properly over straw to yield enough biomass, we looked for other fungi grow promoting substrates. It has been reported that straw can be replaced by other agricultural byproducts with equal or superior biotransformation capacities [17].

Looking for straw alternative substrates, good biomass amounts were obtained with the fungi growing over cereal bran, which is a more nutritive substratum. Therefore, the bioconversion of the targeted pesticides by fungi growing over bran was assayed and the best analytical methodology for this matrix was selected and validated. The pesticides to evaluate the performance of the bioreactor were chosen based on their use in agriculture their persistence and their toxicity [18-20].

3.1. Methods comparison

3.1.1. Bran (Matrix A)

Three methods were evaluated aiming to find a good methodology to analyze chlorpyrifos, endosulfan and its metabolites in the microbiological media prepared in the laboratory with bran as the major component of the system. The combination of different extraction parameters (solvents, homogenization methods and different shaking systems to assist the extraction) and clean up strategies allowed the selection of

a method with recoveries between 70-120 % for endosulfan, its studied metabolites and chlorpyrifos. Method 3 presents the best recoveries of the analytes as shown in Table 3. The other methodologies evaluated did not accomplish DG-SANCO guidelines criteria for pesticide residue analysis [16], obtaining recoveries under our laboratory conditions < 70 % or > 200 % with RSDs between 1 and 10 %.

Table 3.

3.1.2. Laboratory scale biomix (Matrix B)

As for matrix A, different methods were tested. This matrix required six method comparisons due to its increased chemical complexity as soil, peat and bran were together in the biomix (Table 4).

Method 5 presented the best results probably due to the formation of a biphasic system through water addition and ultrasound assisted extraction which also made the extraction time shorter. Also, ethyl acetate proved to have better extractability of the analytes than acetonitrile (used in Methods 1 and 3). Traditional methods such as soxhlet (Method 6) provide low recoveries which would lead to a overestimation of the bioconversion. Performing a second extraction (Method 1) gave a large load of co-extractives and unacceptable recoveries above 120 %.

Table 4.

3.2. Method validation.

3.2.1. Matrix A

The selected method yielded good results for endosulfan, its studied metabolites and chlorpyrifos (Table 5); recoveries were between 72-109 % at all concentration levels studied. The method was precise: its repeatability (RSDr) was below 11% and its within-laboratory reproducibility (RSDwR) is below 17 % for all analytes at all the evaluated levels, according to DG-SANCO guidelines criteria for pesticide residue analysis [16]. The determined LOQs and LODs were adequate for the purpose of the method: the quantification of chlorpyrifos and endosulfán, residues as well as its metabolites in a fungi growing in cereal bran in semisolid media. The values for LOQs and LODs are 1 mg kg^{-1} and 0.3 mg kg^{-1} respectively. It was not possible to perform the sub sampling and sample comminution steps prior to solvent addition in order to achieve good accessibility of the solvent to the analytes because the fungi grows heterogeneously.

Table 5.

The fungal mycelium and the components of bran are responsible for marked matrix effects when the amount of matrix injected is higher, being very important for experiments were the initial endosulfan concentration was 1 mg/kg and at 25 mg kg^{-1} (Table 6). Matrix-matched calibration curves were used for quantitation at all concentration levels because the influence of the amount of matrix on the analyte response was very heterogeneous. The recommendation is to perform the quantitation step using matrix matched calibration curves when assessing pesticide dissipation and metabolites generation with a blank extract prepared in the same way as the samples.

Table 6.

One of the advantages of this method is that as the sample contained (fungi + semisolid culture medium + bran) is completely extracted, no sub sampling is necessary, so all problems related to representativeness are avoided. Also this characteristic improves the results in terms of repeatability and reproducibility.

3.2.2. Matrix B

The results obtained with the best method for chlorpyrifos, endosulfan, and the different metabolites assayed are presented in Table 7. Recoveries were between 71-110 % at all concentration levels studied. The method was precise: its repeatability (RSDr) was below 17 % and its within-laboratory reproducibility (RSDwR) is below 18 % for all analytes at all the evaluated levels, according to DG-SANCO guidelines criteria for pesticide residue analysis [16]. The determined LOQs and LODs were adequate for the purpose of the method: the quantification of endosulfan, chlorpyrifos residues and its metabolites for a fungi growing in a laboratory biobed. The values for LOQs and LODs are 1 mg kg^{-1} and 0.3 mg kg^{-1} respectively.

Table 7.

Matrix effects vary with the different analytes (Table 8), for example, endosulfan sulfate showed the highest matrix effect at all concentrations but for endosulfan ether it was zero. The recommendation is to always quantify with matrix matched calibration curves when assessing endosulfan, its metabolites or chlorpyrifos dissipation. These curves are generated with a blank extract prepared in the same way as the samples injecting the same amount of matrix co-extractives.

Table 8.

Ultrasound assisted extraction allowed better solvent accessibility to the analytes. The method has few steps and is easy to perform in the laboratory considering the complexity of the matrix composed of a microbiological organism grown in a biobed degrading recalcitrant pesticides.

A global strategy to study bioremediation of contaminants at laboratory scale, based on the precise knowledge of the concentration of pesticides present in the biobed is presented. Two very employed; semipersistent to persistent pesticides were used as models. The extraction and clean up methodologies used for the sample preparation of matrix A and B are adequate for the extraction of chlorpyrifos, endosulfan and its metabolites as it was shown during the validation steps. These methods allow the study of the proposed bioreactor and provide tools for studying biotransformation processes of endosulfan, its metabolites and chlorpyrifos by GC-ECD. Exploratory trials of endosulfan and chlorpyrifos dissipation at laboratory scale biobeds yielded 87 % for endosulfan and 79% for chlorpyrifos degaradation after 27 days. Figure 1 shows the chromatogram of the residual pesticides in the biobed after 9 and 27 days of bioconversion respectively, proving the viability of the proposed bioreactor to degrade the agrochemicals under study.

Further work is in progress seeking to optimize the conditions of the bioreactor. The results obtained are a step forward in the search of an environmentally friendly tool to diminish the impact of recalcitrant compounds in affected areas.

4. Acknowledgements

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Captions for Figures and Tables

Table 1. Extraction and clean up methods comparison for matrix A.

Table 2. Extraction methods comparison for matrix B.

Table 3. Recoveries (Rec (%)) comparison for the evaluated methods to determine endosulfan and chlorpyrifos and their respective RSDs (%) in matrix A.

Table 4. Recoveries (%) and their respective RSDs (%) for the 6 evaluated methods in matrix B.

Table 5. Recoveries (%), repeatability (RSDs %) and within-laboratory reproducibility (RSDwR %) of the evaluated pesticides in matrix A.

Table 6. Matrix effect for chlorpyrifos, endosulfan and its metabolites at 1 and 25 (mg kg^{-1}) by GC-ECD in matrix A.

Table 7. Recoveries (%) and RSDs (%) of the evaluated pesticides for the analytical method developed in matrix B; levels 1, 25 and 50 mg kg^{-1} for endosulfan and metabolites and levels 1, 25 and 60 mg kg^{-1} for chlorpyrifos

Table 8. Matrix effects for endosulfan, chlorpyrifos and its metabolites at 1, 25, 50 mg kg^{-1} by GC-ECD in matrix B. 1, 25 and 50 mg kg^{-1} for endosulfan and it metabolites and 1, 25 and 60 mg kg^{-1} for chlorpyrifos.

Figures:

Figure 1. GC-ECD chromatogram of the chlorpyrifos and endosulfan residues in labs biobeds after 9 and 27 days, showing their degradation. Black trace: residues after 9 days; purple trace: residues after 27 days.

Table 1.

	Method 1	Method 2	Method 3
Sample (g)	15	15	15
Solvent, volume (mL)	AcOEt, 40	AcOEt, 40	MeCN, 15
Water addition (mL)	-	-	15
Agitation/time (h)	Stomacher/0.08	Shaker/24	Shaker/2
Clean up: $NaSO_4$/Silica column (g)	20 / 30	20 /30	-
Elution: solvent, volume (mL)	AcOEt, 100	AcOEt, 100	-
Salting out: NaCl/$MgSO_4$ (g)	-	-	2/20
Agitation/time(h)	-	-	Shaker/5
Centrifugation (min)/(rpm)	-	-	10/3000
Final aliquot (mL)	0.1	0.1	0.5

Table 2.

	Method1	Method2	Method3	Method4	Method5	Method 6
Sample (g)	15	15	15	10	10	10
Humidity (%)	40	40	40	70	70	40
Solvent (mL)	MeCN(30)	AcOEt(30)	MeCN(30)	AcOEt(50)	AcOEt(50)	AcOEt(250)
Water (mL)	15	-	-	*	-	-
Extraction	shaker	ultras	ultras	shaker	ultras	soxhlet
Time (h)	2	0.15	0.15	5	0.45**	2.30
$MgSO_4$/NaCl	20(g)/2(g)	-	-	-	-	-
Solvent (mL)	MeCN(30)	-	-	-	-	AcOEt(50)
Shake (h)	5	-	-	-	-	-
Centrifugation (min/rpm)		10/3000	-	10/3000	10/3000	-
Aliquot (mL)	0.5	0.5	0.5	0.5	0.5	0.5

Table 3.

	Endosulfan α			Endosulfan β			Chlorpyrifos		
	Rec	RSDr	RSDwR	Rec	RSDr	RSDwR	Rec	RSDr	RSDwR
		(%)	(%)	(%)	(%)	(%)		(%)	(%)
Method 1	41	3	6	43	4	9	201	20	10
Method 2	63	2	3	65	1	1	205	12	6
Method 3	83	3	4	91	3	4	76	8	10

Table 4.

		Endosulfan α			Endosulfan β			Chlorpyrifos		
	Rec (%)	RSDr (%)	RSDwR (%)		Rec (%)	RSDr (%)	RSDwR (%)	Rec (%)	RSDr (%)	RSDwR (%)
Method 1	170	-	-		169	-	-	220	-	-
Method 2	111	1	1		115	2	1	172	0	0
Method 3	55	1	2		62	1	2	60	2	3
Method 4	122	8	7		119	10	8	116	8	7
Method 5	94	10	11		92	10	11	75	5	7
Method 6	67	10	15		65	10	16	60	8	13

Table 5.

	1 (mg kg^{-1})			25 (mg kg^{-1})		
	Rec(%)	RSDr(%)	RSDwR(%)	Rec(%)	RSDr(%)	RSDwR(%)
Endosulfan ether	83	3	3	86	3	8
Endosulfan alcohol	78	11	16	106	8	14
Endosulfan α	80	3	11	89	5	10
Endosulfan β	80	4	14	96	7	8
Endosulfan sulfate	90	3	12	81	1	17
Chlorpyrifos	72	5	13	109	7	9

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Table 6.

	1 (mg kg^{-1})	25 (mg kg^{-1})
	(%)	(%)
Endosulfan ether	-49	11
Endosulfan alcohol	36	52
Endosulfan α	-39	21
Endosulfan β	-34	-1.6
Endosulfan sulfate	27	49
Chlorpyrifos	-7	25

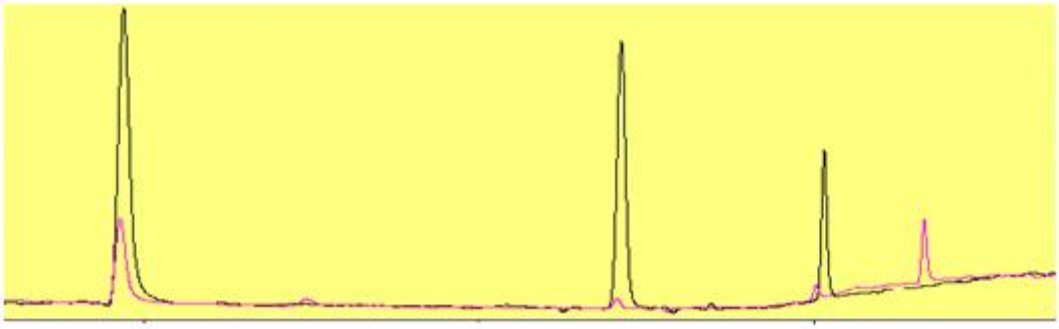
Table 7.

	1(mg kg^{-1})			25(mg kg^{-1})			50/60(mg kg^{-1})		
	Rec (%)	RSDr (%)	RSDwR (%)	Rec (%)	RSDr (%)	RSDwR (%)	Rec (%)	RSDr (%)	RSDwR (%)
Endosulfan ether	76	5	11	81	15	13	83	17	18
Endosulfan alcohol	71	8	7	90	13	17	110	15	15
Endosulfan α	80	6	10	84	13	12	94	15	16
Endosulfan β	77	5	17	87	14	12	101	18	16
Endosulfan sulfate	90	9	11	97	13	10	106	15	16
Chlorpyrifos	102	8	7	110		15	16	105	12	10

Table 8.

	1(mg kg^{-1})	25(mg kg^{-1})	50/60(mg kg^{-1})
	(%)	(%)	(%)
Endosulfan ether	0	0	0
Endosulfan alcohol	56	28	24
Endosulfan α	55	3	12
Endosulfan β	21	2	7
Endosulfan sulfate	86	53	77
Chlorpyrifos	31	29	46

672 Figure 1.



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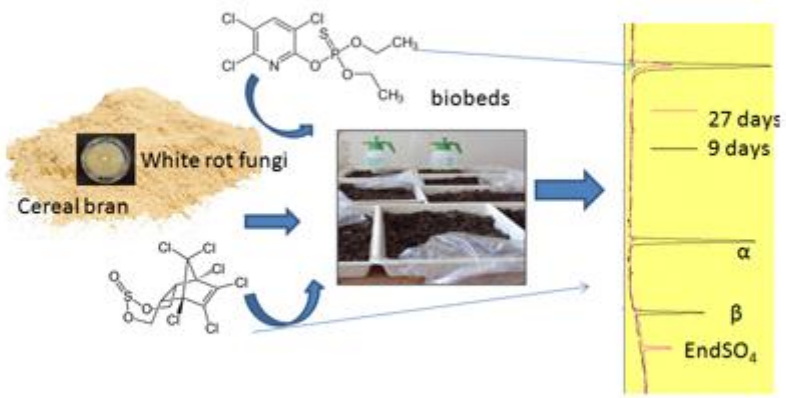
674 Highlights

675 • Xenobiotic conversion in biobeds can only be proved with validated analytical
676 methods.
677 • Cereal bran is suitable for the growth of native bioconvertor Basidiomycetes.
678 • Fit for purpose methodologies to assess biobeds performance were developed
679 • Laboratory biobeds dissipated 79 % chlorpyrifos and 80 % endosulfan after 27 days

680 Graphical abstract



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