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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% and 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.

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

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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 and 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 (30 m, 0.32 mm ID, 0.25 μm film thickness) Mega Legnano (Italy). The experimental conditions were as follow: PTV, 60 °C (0.3 min), then 5 °C min^{-1} to 280 °C (40 min). Oven temperature, 100 °C (3 min), 100–180 °C at 10 °C min^{-1}, 180 °C (15 min), then 180–270 °C at 5 °C min^{-1}, 270 °C (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.