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ORIGINAL ARTICLE

Performance of a continuous stirred tank bioreactor employing an immobilized actinobacteria mixed culture for the removal of organophosphorus pesticides

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Abstract

In this study, we evaluated polyurethane foam (PF), volcanic rock (VR), and a modified plastic cap (MPC) as supports for the immobilization of organophosphorus (OP) pesticide-degrading actinobacterial strains. The colonization and activity of four streptomycetes were favoured by PF, which was selected as the carrier to use in a continuous stirred tank bioreactor (CSTR) that can be operated at increasing inflows of a pesticide mixture that contains the insecticides chlorpyrifos (CP) and diazinon (DZ). Our results demonstrate that the CSTR can be operated at flow rates of 10 and 40 mL h^{-1} with greater than 85% removal of the pesticides in the short term. A significant decrease in the efficiency of CP removal was observed at the highest inflows into the reactor. The CP and DZ loading rates in the bioreactor ranged from 0.44 to 1.68 mg L^{-1} h^{-1} and from 0.50 to 2.17 mg L^{-1} h^{-1}, respectively. Although the treated wastewater exhibited moderate toxicity for *Raphanus sativus*, a bioreactor inoculated with a mixed culture formed by *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 may provide an effective biotechnological strategy for the reduction of OP pesticide residues produced during agronomic and manufacturing practices and therefore prevent environmental pesticidal pollution.

Keywords Polyurethane · Continuous flow · Removal · Toxicity · Chlorpyrifos · Diazinon

Introduction

The presence of pesticides in the environment and in foods has been frequently confirmed; therefore, these compounds have been recognized as potential hazards to the environment and to human health (Carvalho 2017). Pesticides can enter the environment after direct application to soils or because of improperly stored or untreated wastewater containing high loads of an individual pesticide or a mixture of pesticides from agricultural industries (Karas et al. 2011). Moreover, pollution caused by improperly stored and obsolete pesticides has also been reported (Błaszak et al. 2011).

The organophosphorus (OP) pesticides belong to a class of very toxic compounds that are widely used in global agriculture. These are inhibitors of the enzyme acetylcholinesterase, which is responsible for synaptic activity in the nervous system. In addition, this kind of compound is associated with the risk of cancer development (Adeyinka and Pierre 2020). Chlorpyrifos (CP) and diazinon (DZ) are insecticides that are commonly used for the control of pests that are encountered in vegetable and fruit farming and in the livestock industry, respectively. In the environment, CP exhibits a short to moderate persistence (11–141 days) and reduced mobility in soil due to its low solubility (1.4 mg L^{-1}) and high organic carbon partitioning coefficient (K_{oc} values 360–31,000) (Christensen et al. 2009). DZ is mobile and moderately persistent due to its high solubility in water (60 mg L^{-1}) and low K_{oc}

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(40–432) (Aggarwal et al. 2013). For both CP and DZ, removal can occur by a combination of processes, including chemical hydrolysis, photolysis and biodegradation, with 3,5,6-trichloro-2-pyridinol (TCP) and 2-isopropyl-6-methyl-4-pyrimidinol (IMHP) as the main degradation by-products, respectively (Dębski et al. 2007; Solomon et al. 2014). Degradation of CP and DZ has been reported amongst diverse types of bacteria (Cycón et al. 2017; Dar et al. 2019). Specifically, actinobacteria from the *Streptomyces* genus are able to remove both insecticides and other OP pesticides as single strains and as mixed cultures (Briceño et al. 2012, 2016a, b), rendering these species promising tools for decontamination processes.

Removal of pesticides from wastewater is of great importance, especially for the production of drinking water and the preservation of aquatic ecosystems. Therefore, efforts have been made to identify an appropriate bioreactor configuration to avoid and reduce the volume of wastewater containing pesticides prior to its final disposal (Gonzalez-Cuna et al. 2016). Bioreactor configurations can include the use of immobilized microorganisms (Yadav et al. 2014; Pradeep and Malavalli 2016), and the support material for cell immobilization is one of the main features that affects bioreactor configuration and performance (Cabrera-Orozco et al. 2017). In this context, an evaluation of different supports must be conducted to obtain optimal colonization and activity of the microorganisms (Tarjányi-Szikora et al. 2013) and therefore an appropriate reactor design. Continuous stirred tank bioreactors (CSTR) have been widely adopted throughout the chemical and bioprocessing sectors owing to their simplicity (Abbott et al. 2013). In this context, it is a system that has proven successful for the continuous production of bioethanol using a biocatalyst *Clostridium ljungdahlii* (Acharya et al. 2017), bioremediation technology for effectively treating organic compounds such as hydrocarbons (Moscoso et al. 2012) and other applications.

Although removing OP pesticides from liquid samples using different types of bacteria has been studied extensively for bioremediation purposes, few studies have done so using bioreactor systems. A bacterial consortium immobilized in alginate beads and rock (tezontle) in a packed flow bioreactor demonstrated up to 75% efficiency for the removal of two OP pesticides (Yañez-Ocampo et al. 2011). On the other hand, Yadav et al. (2014) reported a 91% removal efficiency for CP in an aerobic packed-bed bioreactor using *Pseudomonas* sp. immobilized on polyurethane foam (PF) pieces. Recently, the bioremoval of profenofos and quinalphos from an aqueous solution was evaluated with a *Kosakonia oryzae* strain VITPSCQ3 biofilm in a circulating vertical-flow packed-bed biofilm bioreactor using charcoal, gravel and mushroom as biofilm carriers. The results showed an optimal biodegradation capacity (92–96%) for both pesticides within 120 min reaction time (Dash and Osborne 2020).

Although CP and DZ are both amongst the most popular and widely used OP pesticides (Adeyinka and Pierre 2020), they contaminate various components of ecosystems such as soil, sediments, water, air, and food, as well as human fluids such as blood, breast milk, and urine (Dar et al. 2020). For economic reasons, developing countries are still using it. Specifically, in Chile both compounds are regularly used for fruit growing over an area of 300,000 ha (Apey 2019). In this region, there is a high probability of finding wastewater containing CP and/or DZ. There is no information whatsoever concerning the removal of a pesticide mixture containing both CP and DZ from wastewater using a bioreactor, making it imperative to develop methods to minimize, or even avoid, its release into the environment.

Actinobacteria have been studied for pesticide bioremediation purposes (Alvarez et al. 2019), but few studies exist on their use as immobilized cells in the field of pesticide wastewater treatment with bioreactors (Saez et al. 2015). Within this framework and taking into consideration the ability of *Streptomyces* strains to remove OP pesticides, the goal of this study is to select an appropriate support for cell immobilization of *Streptomyces* spp. to evaluate the performance of a small-scale laboratory continuous stirred bioreactor inoculated with a defined mixed culture consisting of four *Streptomyces* strains under increasing inlet flows of pesticides.

Materials and methods

Chemicals, microorganisms and culture media

Analytical-grade CP and DZ and their respective metabolites TCP and IMHP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other analytical-grade reagents and solvents were obtained from commercial manufacturers. Stock solutions (5000 mg L^{-1}) of CP and DZ in methanol were sterilized by filtration through 0.22-µm pore-size membranes and used for contamination of liquid media.

The microorganisms used for the study were four *Streptomyces* sp. strains (AC5, AC9, GA11 and ISP13). These strains were previously isolated from agricultural soils where pesticides had been applied. In addition, these strains were selected due to the absence of inhibition amongst them and their abilities to increase the degradation of OP pesticides when they are cultured together (Briceño et al. 2012, 2016a, b). The four strains produce the enzyme OP hydrolase, which degrades OP pesticides. Moreover, the AC5 strain has the ability to remove the main CP metabolite from liquid samples (Briceño et al. 2016b).

The microorganisms were routinely cultured in starch–casein culture medium containing 10 g of soluble starch, 0.5 g of K_2HPO_4 and 1.0 g of casein in 1000 mL of

distilled water, pH 7.0; and in liquid medium containing 4.0 g of glucose, 0.5 g of L-asparagine, 0.5 g of K_2HPO_4, 0.20 g of $MgSO_4 \cdot 7H_2O$ and 0.01 g of $FeSO_4 \cdot 7H_2O$ in 1000 mL of distilled water, pH 7.0.

Supports for cell immobilization

The immobilization of *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13 was evaluated using three supports: PF, volcanic rock (VR) and a modified plastic cap (MPC). Commercial PF (Scotch Brite 3 M Chile S.A) was cut into 1-cm squares prior to use. The VR was obtained from an area near the Llaima volcano in the Araucanía Region, Chile. The material was sieved to obtain stones of approximately 5.0 mm that were then washed with distilled water and dried prior to use. The MPC consisted of a disposable juice cap from which the bottom was removed to obtain a cylinder of 2.5 cm diameter. Then, two rods obtained from cotton sticks were inserted in the cylinder, which was perforated to keep the sticks in a fixed position (Fig. 1). This last support was constructed with recycled materials, simulating the typical moving-bed biofilm carriers used in bioreactors.

The specific surface areas, pore diameters and total pore volumes for the supports were quantified using a Nova 1000e Surface Area & Pore Size Analyzer Quantachrome Instrument. In addition, the adsorption capacity was determined using methylene blue, which was measured with a Mecasys Optizen Pop spectrophotometer at 660 nm. For this measurement, the supports were placed in solutions containing methylene blue at a concentration of 5.0 mg L^{-1} and afterwards were shaken for 1 h. After the adsorption process, the content of methylene blue in the liquid phase was determined, and adsorption was expressed as mg methylene blue g^{-1} support (Tarjányi-Szikora et al. 2013).

Streptomyces spp. cell immobilization

Five pieces of VR, five pieces of PF, and one MPC were placed in Erlenmeyer flasks containing 30, 50 and 100 mL of starch–casein medium, respectively. Each support was added in said amounts and to different media volumes to ensure cell immobilization and an adequate agitation. According to our observations, the use of higher numbers of supports favoured friction amongst them (VR and MPC) or the absorption of the liquid medium (PF), therefore interfering with the formation of biomass on them. After sterilization at 121 °C for 20 min, the medium containing each support was inoculated with spores and mycelia of each individual *Streptomyces* sp. strain AC5, AC9, GA11 and ISP13 maintained in slants. To promote cell adhesion onto the support, incubation on a rotary shaker (30 °C and 120 rpm) was performed for 20 days. Then, the culture medium was removed and each support washed with a sterile solution of 0.85% NaCl to remove non-adherent cells.

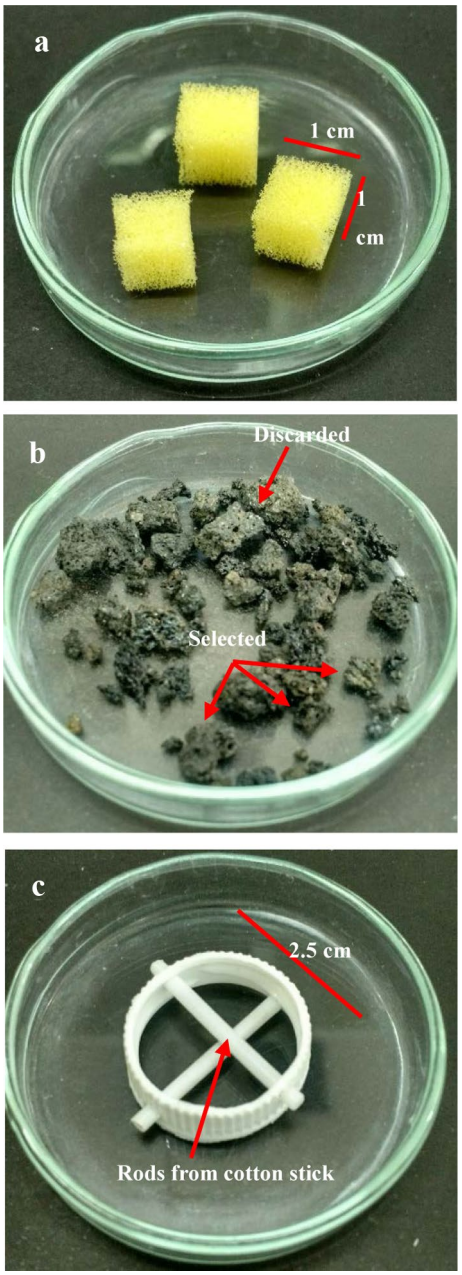

Fig. 1 General view of the supports used for cell immobilization. **a** Commercial polyurethane foam (PF) cut into 1-cm squares; **b** volcanic rocks (VR), arrows indicate the sizes that were discarded and selected (~5.0 mm); and **c** modified plastic cap (MPC) constructed with disposable juice caps (2.5 cm in diameter) and two rods obtained from cotton sticks

Colonization by the *Streptomyces* sp. strains was evaluated by measuring dehydrogenase and acid phosphatase enzyme activity according to Alef (1995) and Tabatabai and Bremmer (1969), respectively. Dehydrogenase activity was expressed as µg of triphenyl formazan produced g^{-1} h^{-1}

after quantification at 546 nm, and acid phosphatase was expressed as µg of *p*-nitrophenol produced g^{-1} h^{-1} after quantification at 400 nm. Colony-forming units (CFU) were also determined after removing the immobilized biomass on the supports by vortexing a tube containing phosphate-buffered saline pH 7.4 for 10 min. Then, serial dilutions were prepared after taking an aliquot which was seeded in plates containing starch–casein agar medium. After incubating for 7 days at 30 °C, colonies were counted, and the results were expressed as CFU g^{-1} of support. Finally, immobilized biomass on the supports was observed with a scanning electron microscope (SU 3500 Hitachi-Japan) to confirm the immobilization of *Streptomyces* sp. strains.

Removal of pesticides by an immobilized *Streptomyces* sp. mixed culture

To determine which type of support favoured the removal of CP and DZ (Saez et al. 2012), a study was conducted using mixed cultures of the four *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13. For this purpose, four VR, four PF or four MPC pieces containing each member of the immobilized strains were added in separate studies to flasks containing previously established volumes of 30, 50 and 100 mL of liquid medium supplemented with 50 mg L^{-1} CP or DZ. Cultures were incubated at 30 °C and 120 rpm for 72 h, and then 2.5 mL of supernatant was sampled and analysed by HPLC to determine the concentrations of CP and DZ and their respective metabolites TCP and IMHP. Removal was estimated by comparing concentrations in the samples and controls. Each assay was performed in triplicate.

Removal of pesticides in a continuous stirred tank bioreactor (CSTR)

The experimental setup for a continuous reactor operated with PF with immobilized *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13 consisted of a 1-L glass bottle (internal diameter = 8.5 cm, length = 20 cm, 800 mL working volume and 1000 mL total volume, Fig. 2). The reactor was continuously fed with an artificial pesticide solution consisting of liquid medium containing a pesticide mixture of CP and DZ, each added at a concentration of 50 mg L^{-1}. The bioreactor was inoculated, inside a laminar flow chamber, with 20 pieces of PF for each *Streptomyces* sp. strain, for a total of 80 foams (10^{10} CFU g^{-1} support). For purposes of aeration and immobilized support movement inside the reactors, the setup included magnetically stirred reactors (130 rpm) with and without (abiotic control) immobilized microorganisms. The continuous bioreactor feeding process was conducted using an intelligent flow rate peristaltic pump (FPP-Lab V3) at increasing flow rates of 10, 20, 30, 40 and 50 mL h^{-1} and therefore five hydraulic retention times of 80, 40, 26.6, 20 and 16 h^{-1}, where hydraulic retention time = void volume (L)/flow rate (mL h^{-1}). The bioreactor was operated at room temperature (20–25 °C) to avoid energy consumption during its operation and at an initial medium pH of 7.0–7.2. The treated effluent was sampled at different times to analyse concentrations of pesticides and pesticide metabolites by HPLC, the biomass by dry weight released at 105 °C, and the toxicity of the effluent.

The removal and overall performance of the bioreactor was evaluated at various loading rates in terms of removal efficiency and volumetric removal rates using the following formulas:

$$\%\ \text{removal efficiency} = \frac{C_0 - C_f}{C_0} \times 100 \quad (1)$$

$$\text{volumetric removal rates} = \frac{F(C_0 - C_f)}{V}, \quad (2)$$

where C_0 and C_f are the pesticide concentrations in the liquid entering and leaving the bioreactor, respectively, F is the volumetric flow rate and V is the hold-up volume of the bioreactor.

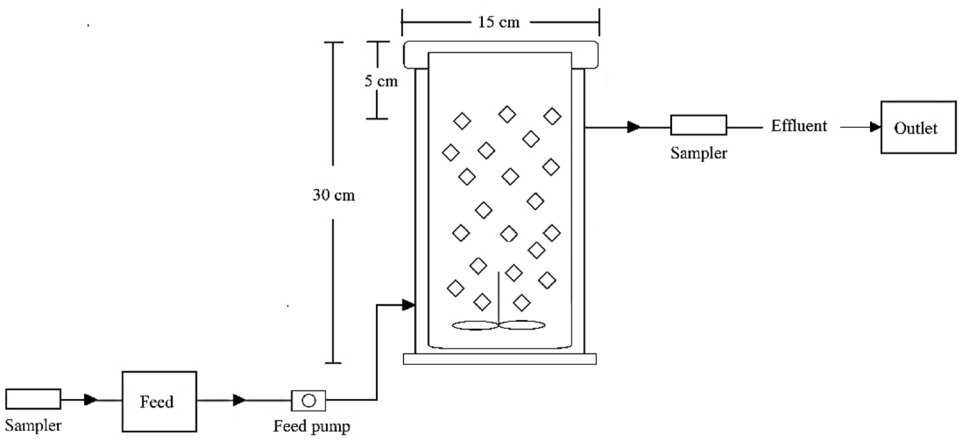

Fig. 2 Schematic diagram of continuous stirred tank bioreactor (CSTR) system for the removal of a mixture formed by the insecticides chlorpyrifos (CP) and diazinon (DZ) by a mixed culture of *Streptomyces* spp. immobilized on polyurethane foam (PF) pieces

Pesticide analysis

The extraction of pesticides from liquid samples was performed using dichloromethane and ethyl acetate, following Briceño et al. (2016b). Analyses were performed using a Shimadzu LC-20AT liquid chromatograph equipped with a diode array detector. A Purospher Star RP-18e column (Merck®, film thickness 5 µm, 150 × 4.6 mm) was used for the separation of compounds. The chromatographic conditions were as follows: oven temperature, 35 °C; mobile phase at 25%, 0.1% phosphoric acid–75% acetonitrile at a flow rate of 1 mL min^{-1}. The retention times for CP and DZ were 8.10 ($\lambda = 289$) and 6.10 ($\lambda = 247$) min, respectively, and for TCP and IMPH, 2.49 min ($\lambda = 298$) and 1.96 min ($\lambda = 267$), respectively. Calibration was performed using standard linear curves for each compound with concentration values ranging from 0.01 to 10 mg L^{-1}.

Phytotoxicity testing of treated bioreactor effluent

Toxicity of treated synthetic wastewater in the CSTR was evaluated using seeds of *Raphanus sativus* var. Sparkler (radish). Seeds were grown in Petri dishes containing sterile filter paper (Whatman No. 1) that was moistened with 2 mL of treated synthetic wastewater for 24 h. Thirty radish seeds were placed in each plate and incubated in the dark for 5 days at 20 °C and 70% relative humidity. Two treatments using liquid medium without pesticides were used as controls. Numbers of germinated seeds were determined at the end of the incubation period, and the length of each root was measured using a millimetre scale. The relative germination percentage, the relative growth of radicles, and the germination index were calculated for each treatment using the following equations, following Tiquia et al. (1996):

$$\text{Relative germination (\%)} = \frac{n^{\circ}\text{ seeds in the treatment}}{n^{\circ}\text{ seeds in the control}} \times 100 \quad (3)$$

$$\text{Relative growth of radicles (\%)} = \frac{\text{radicle elongation in the treatment}}{\text{radicle elongation in the control}} \times 100 \quad (4)$$

$$\text{Germination index} = \frac{(\text{RGP} \times \text{RGR})}{100}. \quad (5)$$

Statistical analysis

Determinations were carried out in triplicate and the results were expressed as average values. One-way ANOVA (Tukey test, $p \leq 0.05$) was employed to compare treatments. Statistical analyses were performed using SPSS statistical software version 17.

Results and discussion

Characterization of supports

The VR, PF and MPC used in this study were analysed prior to colonization with *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13. Table 1 describes the supports and shows that PF was the support that exhibited the highest values of specific surface area, pore volume, and pore diameter. These properties are related to the capacity of the support to adsorb methylene blue. In this context, PF adsorbed the highest amount of methylene blue (21.44 mg g^{-1} support), probably due to the formation of a hydrophobic ionic pair (Baldez et al. 2008), whilst the lowest adsorption was observed with VR, which showed the lowest specific surface area, pore volume and diameter. In this study, methylene blue was used as an indicator of cell immobilization, as the surface charge of bacteria is usually negative; therefore, if more positively charged methylene blue is adsorbed on a support, attachment of fewer bacteria could be expected (Tarjányi-Szikora et al. 2013). Based on the extent of adsorption of methylene blue on the evaluated supports, the theoretical quantity of bacterial cell attachment would be expected to occur in the order: PF < MPC < VR. However, all three supports are expected to be acceptable for cell immobilization.

Table 1 Characteristics of the polyurethane foam (PF), volcanic rock (VR) and modified plastic caps (MPCs) used as supports for cell immobilization

Support	Specific surface area (m^2 g^{-1})	Pore volume (cc g^{-1})	Pore diameter (nm)	Adsorption on methylene blue (mg g^{-1} support)
VR	0.65 ± 0.00	0.003 ± 0.000	2.13 ± 0.10	10.92 ± 0.93
PF	6.20 ± 0.03	0.012 ± 0.000	2.89 ± 0.03	21.44 ± 2.06
MPC	2.70 ± 0.01	0.004 ± 0.000	2.15 ± 0.03	18.22 ± 0.58

The average values and the standard error are presented ($n = 3$)

Streptomyces spp. immobilization

To select the support that favours microbial activity and colonization by *Streptomyces* sp. strains AC5, AC9, GA11 and ISP13, dehydrogenase and acid phosphatase enzyme activity, as well as CFUs, were evaluated at 20 days of incubation. This time was selected after monitoring the time required (more than 10 days) to observe biomass immobilization. Table 2 shows that all of the studied strains were more active when they were immobilized on PF. Dehydrogenase activity, which provides an indication of the overall activity of live microorganisms (Alef 1995), ranged from 2.93 to 411.13 µg of triphenyl formazan g^{-1} h^{-1}. The microbial activity observed on VR (1.02–6.50 µg triphenyl formazan g^{-1} h^{-1}) and on MPC (0.07–0.42 µg triphenyl formazan g^{-1} h^{-1}) was lower than that obtained on PF. A similar trend was observed when acid phosphatase activity was evaluated. On PF, the activity was between 28.54 and 200.64 µg of *p*-nitrophenol g^{-1} h^{-1}, whilst on VR and MPC the values did not exceed 0.80 µg of *p*-nitrophenol g^{-1} h^{-1}. In this study, acid phosphatase activity was evaluated, since it was previously shown that these strains are positive for the activity of this enzyme, indicating colonization by the strains of different substrates (Briceño et al. 2017). As observed with enzyme activity, the CFU counts showed that the attached biomass by support type exhibited the following order: PF > VR > MPC. Finally, Fig. 3 shows that extensive immobilization was visible on all three supports. Moreover, scanning electron microscope observations demonstrated that porous structures and the surface of the PF were well colonized by the *Streptomyces* spp. hyphae. In contrast, in both VR and MPC, the *Streptomyces* spp. strains were attached and formed a biofilm on the surfaces of the supports, probably due to the limitation of pore structures in the matrix that are capable of entrapment. In this context, porous structures present in the PF allow greater diffusion of oxygen, favouring the development of these aerobic microorganisms (Ory et al. 2016). Previous evaluations have shown that PF can immobilize up to 75% of fungal cells; therefore, this support is considered as a recyclable and low-cost support for immobilization (Castro et al. 2017).

Removal of pesticides by immobilized *Streptomyces* spp. mixed culture

In the present study, three immobilized supports were evaluated to determine the best support for the removal of CP and DZ. Table 3 shows that removal rates of the pesticides were significantly different ($p \leq 0.05$). CP removal was close to 100% using the *Streptomyces* spp. mixed culture immobilized on PF after 72 h of incubation. In previous studies, however, 50 mg L^{-1} CP required more than 100 h to result in 100% removal from a liquid medium (Briceño et al. 2016b). Therefore, immobilization accelerated the removal of CP, perhaps because of the high density of cells immobilized in/on the support (Tallur et al. 2015). In this context, PF could be a suitable carrier for the entrapment of *Streptomyces* strains, because cells near the surface have high metabolic activity, whereas cells located inside the support are protected against environmental factors (Dzionek et al. 2016). When MPCs were used for *Streptomyces* immobilization, more than 80% removal was observed for both pesticides. In the current study, the MPCs were used as an alternative to the reutilization of materials that are highly resistant to natural degradation. However, despite demonstrating adequate removal of both contaminants, manipulation of this type of support was difficult, because the biomass was easily detached from the support when placed in the liquid medium. This could be explained by a surface charge effect that is influenced by the ionic strength and the pH (7.0–7.2) of the bulk liquid (Horemans et al. 2016). Both VR and PF produced the lowest values of metabolite production (TCP and IMHP) with the immobilized strains of *Streptomyces* spp. at the evaluated times, probably because they were quickly converted to other metabolites, considering the ability of these streptomycetes to metabolize these compounds (Briceño et al. 2016a, b). Although VR has been demonstrated to be a good support for cell immobilization and for

Table 2 Dehydrogenase and acid phosphatase enzyme activity and colony-forming units (CFU) for *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13 immobilized on polyurethane foam (PF), volcanic rock (VR) and modified plastic caps (MPCs), after 20 days of incubation in liquid culture

Strain	Dehydrogenase activity (µg triphenyl formazan g^{-1} h^{-1})			Acid phosphatase activity (µg *p*-nitrophenol g^{-1} h^{-1})			Immobilized biomass (log CFU g^{-1} support)		
	VR	PF	MPC	VR	PF	MPC	VR	PF	MPC
AC5	1.02 ± 0.00	2.93 ± 0.00	0.07 ± 0.00	0.77 ± 0.18	29.03 ± 0.13	0.17 ± 0.01	12.63 ± 0.14	15.94 ± 0.69	9.94 ± 0.30
AC9	1.93 ± 0.01	128.90 ± 0.01	0.42 ± 0.00	0.34 ± 0.00	127.03 ± 0.28	0.70 ± 0.10	14.02 ± 0.07	17.24 ± 0.14	9.00 ± 0.04
GA11	4.00 ± 0.00	411.13 ± 0.08	0.12 ± 0.00	0.36 ± 0.00	200.64 ± 0.06	0.56 ± 0.12	13.97 ± 0.37	16.31 ± 0.07	9.38 ± 0.22
ISP13	6.50 ± 0.02	19.53 ± 0.00	0.15 ± 0.00	0.80 ± 0.06	28.54 ± 0.01	0.22 ± 0.00	12.95 ± 0.73	16.37 ± 0.17	7.90 ± 0.72

The average values and the standard error are presented ($n = 3$)

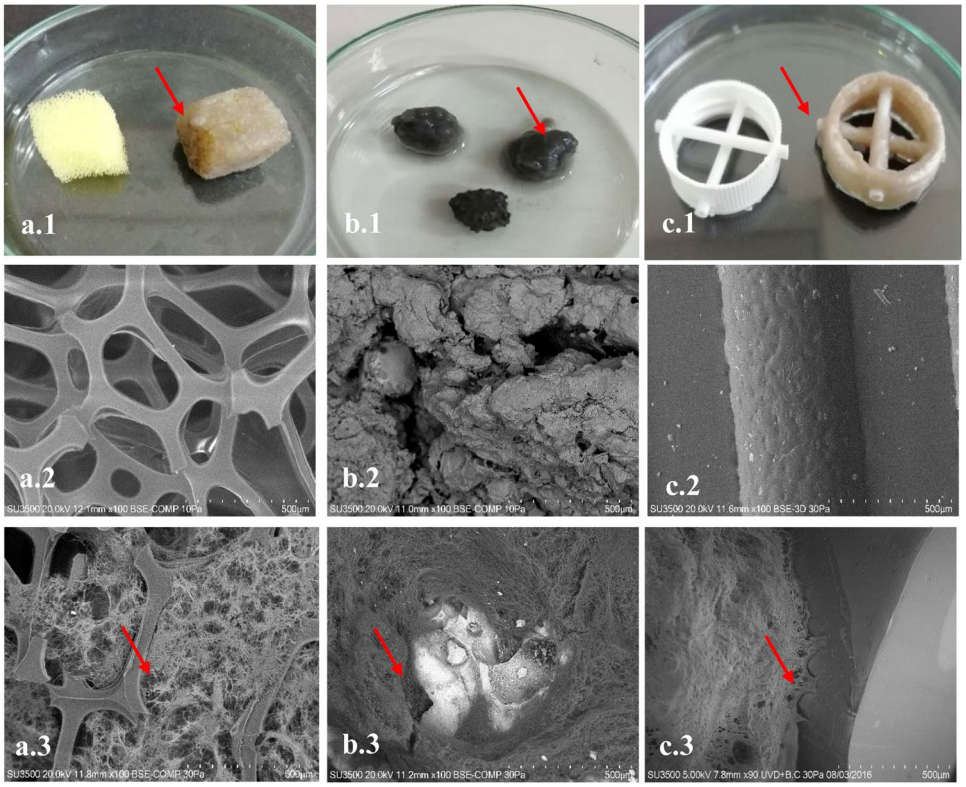

Fig. 3 View of polyurethane foam (PF, **a**), volcanic rock (VR, **b**) and modified plastic caps (MPCs, **c**) evaluated as supports for cell immobilization. Observation obtained using a photographic camera of biomass immobilized on PF (**a.1**), VR (**b.1**) and MPCs (**c.1**), and observation by scanning electron microscopy of PF, VR and MPC free of biomass (**b.1**, **b.2** and **b.3**, respectively) and with immobilized biomass (**c.1**, **c.2** and **c.3**, respectively) of *Streptomyces* strains. Red arrow indicates immobilized biomass on/in the supports

Table 3 Chlorpyrifos (CP) and diazinon (DZ) removal, and 3,5,6-trichloro-2-pyridinol (TCP) and 2-isopropyl-6-methyl-4-pyrimidinol (IMHP) concentration with *Streptomyces* mixed-culture immobilized on polyurethane foam (PF), volcanic rock (VR) and modified plastic caps (MPCs), after 72 h of incubation

Support	CP removal (%)	TCP (mg L^{-1})	DZ removal (%)	IMHP (mg L^{-1})
PF	99.58 ± 2.30 a	n.d	63.69 ± 1.52 b	0.79 ± 0.05 b
VR	91.92 ± 1.08 b	n.d	64.74 ± 2.40 b	0.77 ± 0.02 b
MPC	83.53 ± 1.46 c	0.08 ± 0.02	85.19 ± 1.69 a	4.69 ± 0.33 a

The average values and the standard error are presented ($n = 3$)

The analyses were done in the same column between carriers. The values with different letters indicate significant differences ($p \leq 0.05$, Tukey test)

applications in bioremediation systems (Yañez-Ocampo et al. 2009; Dzionek et al. 2016), PF produced better results for *Streptomyces* spp. colonization and CP removal; therefore, PF was selected for further removal studies using a CSTR. The use of PF as a support for cell immobilization has proven effective in the removal of different contaminants such as petroleum oil, chloroaromatic compounds and OP pesticides (Yadav et al. 2014; Mulla et al. 2016; Alessandrello et al. 2017).

Removal of a pesticide mixture using a continuous stirred tank bioreactor (CSTR)

The performance of a bioreactor for the treatment of wastewater greatly depends on the flow and therefore the hydraulic retention time, the composition of inflowing medium, the microbial cell concentration, and its catabolic capabilities (Gonzalez-Cuna et al. 2016; Grandclément et al. 2017). In this study, the CSTR was operated at flow rates of 10–50 mL h^{-1}, corresponding to a hydraulic retention time of 80–16 h. Figure 4 shows the variation in removal efficiency (%) and biomass released for the different flow rates over time (0–120 h). Flow rate significantly influenced CP removal ($p \leq 0.05$), which was on average greater than 90% when the synthetic wastewater outflowed at 10–40 mL h^{-1}. When the flow rate was increased to 50 mL h^{-1}, the removal efficiency of CP decreased after 10 h, reaching efficiency levels of 52% and 35% at 12 h and 24 h, respectively. These results may be so due to increased flows that can create an unstable condition in

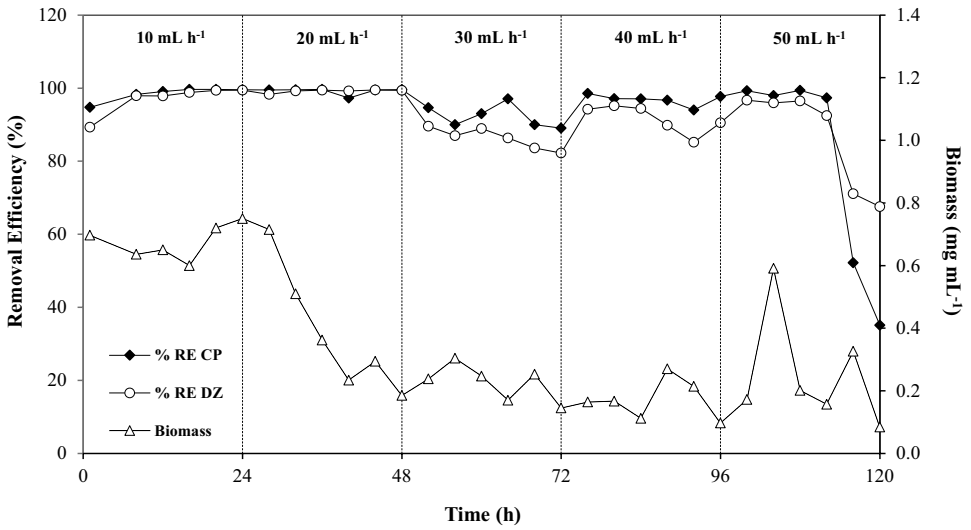

Fig. 4 Removal efficiency (RE) (%) for chlorpyrifos (CP) and diazinon (DZ) and free biomass in the continuous stirred tank bioreactor (CSTR) operated at different pesticide wastewater flow rates (10–50 mL h^{-1}) over time (0–120 h) and inoculated with an immobilized mixed culture of *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13

the bioreactor, and higher removal percentages may be obtained with longer contact time between a contaminated solution and an immobilized consortium. A similar response has been observed for other OP pesticides, where removal rates were observed of approximately 35% under a flow rate of 3.51 L h^{-1} and approximately 75% under a flow rate of 0.94 L h^{-1} (Yañez-Ocampo et al. 2011). Likewise, Yadav et al. (2014) observed that a bioreactor operated with *Pseudomonas* immobilized in PF pieces for the treatment of CP was sensitive to flow fluctuations and experienced a drop in removal efficiency when a flow rate of 40 mL h^{-1} was used. The removal efficiency for DZ was over 90% at the lowest flow rates of 10, 20, and (initially) 30 mL h^{-1}. However, at an inflow rate of 30 mL h^{-1}, the DZ removal efficiency decreased during the second half of the evaluated time, and an initial flow of 40 mL h^{-1} produced a removal efficiency between 82 and 89%. Subsequently, the removal efficiency recovered and produced values between 85 and 95%. Similar to our results, an increase in hydraulic retention time from 12 to 36 h in a moving-bed biofilm reactor resulted in an increase in diazinon removal efficiency from 76 to 98%, probably due to the longer period of contact between the pollutants and microorganisms (Azizi et al. 2019). Similarly, as observed in CP, a flow rate of 50 mL h^{-1} caused system destabilization and decreased the efficiency of DZ removal to 67 and 71%. The reduction in CP and DZ removal at the end of the process may be explained by a decline in biomass activity, which decreased to approximately 62% of the activity of the inoculum at time zero (data not shown). During the bioreactor operation, free biomass was evaluated due to the difficulty in intervening with the operation of the bioreactor. Initially, as shown in Fig. 4, biomass was more abundant when the bioreactor began operating and when the flow changed. Afterwards, the release of biomass decreased and was then maintained at a constant concentration. However, when the bioreactor was subjected to the highest flow rate, the concentration of biomass in the medium increased, probably an effect of fluid that washed the immobilized biomass from the support, such as has been reported for the removal of contaminants using packed-bed biofilm reactors or in a moving-bed biofilm reactor (Yañez-Ocampo et al. 2011; Alfonso-Gordillo et al. 2016; Azizi et al. 2019). This rapid removal of pesticides is mainly due to increased cell concentration in the immobilized support (Jesitha and Harikumar 2019).

The reactor performance was evaluated by calculating the volumetric removal rate in terms of productivity (Gonzalez-Cuna et al. 2016). Figure 5 shows the behaviour for volumetric loading rates of CP (a) and DZ (b). As observed with other pesticides, such as 2,4-D (Gonzalez-Cuna et al. 2016) and CP (Yadav et al. 2014), the bioreactor was able to support proportionally high loadings of pesticides. The CP removal efficiency (volumetric removal rate/loading rate) was calculated by considering that at a loading rate of 0.89 mg L h^{-1}, the removal efficiency is 99%, and over this loading rate, the efficiency fluctuated slightly up to a loading rate of 1.67 mg L h^{-1}; thereafter it declined by up to 75%. For DZ, at a loading rate of 1.02 mg L h^{-1}, a removal efficiency of 99% was estimated, and at higher loading rates removal efficiency estimates ranged between 86 and 95%. Therefore, the use of a CSTR with an immobilized *Streptomyces* spp. consortium for the simultaneous treatment of CP and DZ may represent an effective approach for the reduction of OP pesticide residues. According to the available information, this is the first report involving a continuous treatment using a mixed culture of actinobacteria for the simultaneous elimination of these two widely used and toxic insecticides.

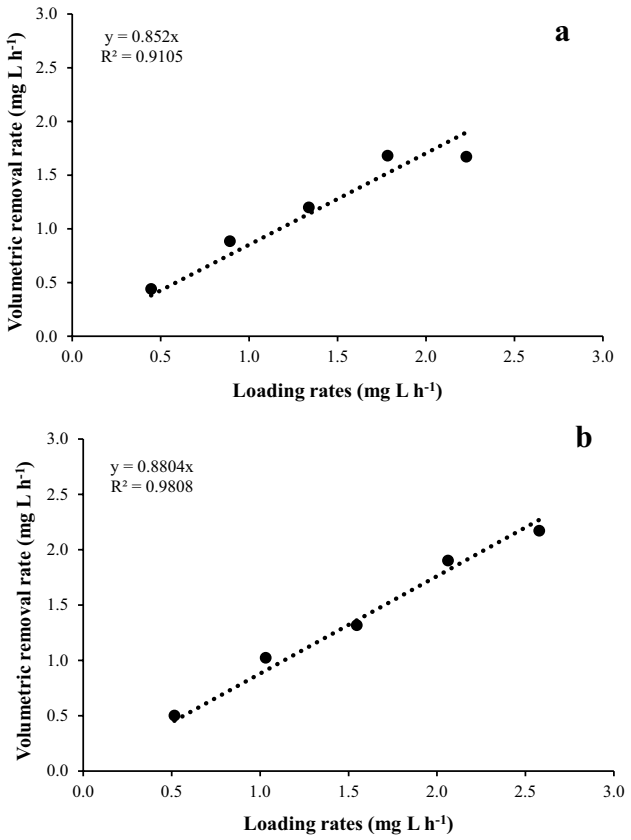

Fig. 5 Dependence of the volumetric removal rate at different loading rates for chlorpyrifos (**a**) and diazinon **b** in the continuous stirred tank bioreactor (CSTR) inoculated with an immobilized mixed culture of *Streptomyces* spp. strains AC5, AC9, GA11 and ISP13

Toxicity of treated effluent

A simple and fast phytotoxicity test was conducted to evaluate the inhibitory effect of treated synthetic wastewater on *Raphanus sativus* seed germination and growth. Table 4 shows that the relative germination rates were over 90%, and no significant differences ($p \leq 0.05$) were observed in the different flow rates evaluated. Regarding the relative growth of radicles, the values ranged between 61 and 137%, and the lowest values were obtained at flow rates of 10 and 40 mL h^{-1}. Similarly, the lowest germination index values were observed with these flow rates, suggesting that the effluent treated in the bioreactor exerted a moderate inhibitory effect on radishes, possibly due to the presence of CP and DZ residues and phytotoxic substances (Alfonso-Gordillo et al. 2016). The metabolites TCP and IMHP were not detected, which could be due to the ability of *Streptomyces* to remove these compounds in mineral salt media (Briceño et al. 2012, 2016a, b; Supreeth et al. 2016). However, the presence of other metabolites, such as CP-oxon, diethyl thiophosphate or diethyl phosphate, produced during CP metabolism is not excluded (Dar et al. 2019). Based on these results, the use of several bioindicators should be employed in OP pesticide removal processes to obtain better estimate the toxic effects of the treatment residues.

Conclusion

The present study demonstrated that polyurethane foam (PF) is favoured for the immobilization of *Streptomyces* spp., which became occluded the pores of the support. The mixed culture of *Streptomyces* spp., formed by the strains AC5, AC9, GA11 and ISP13, was effective for the removal of a pesticide mixture composed of organophosphorus (OP) pesticides, chlorpyrifos (CP) and diazinon DZ, in a CSTR. The continuous bioreactor operated at flow rates of 10–40 mL h^{-1} demonstrated high levels of removal for both compounds, whilst at a flow rate of 50 mL h^{-1} removal of both pesticides declined. The bioreactor can support proportionally high loadings of pesticides. Therefore, the use of a CSTR with an immobilized *Streptomyces* mixed culture is an acceptable approach for the effective elimination of OP pesticides from wastewater. However, improved processes are required to decrease residual pesticide concentrations and to produce treated wastewaters with minimal risk to the environment.

Table 4 Relative germination percentage, relative radicle growth, and germination index of *Raphanus sativus* var. Sparkler (radish) seeds used for phytotoxicity testing of wastewater treated at different flow rates of pesticides in the continuous stirred tank bioreactor (CSTR)

Parameter	Influent flow (mL h^{-1})				
	10	20	30	40	50
Relative germination (%)	96.66 ± 1.92	93.33 ± 1.92	96.66 ± 1.92	91.11 ± 2.22	95.55 ± 1.11
Relative growth of radicle (%)	60.97* ± 1.19	136.81 ± 1.73	110.58 ± 0.80	78.14* ± 1.28	117.53 ± 2.02
Germination index (%)	58.52* ± 1.06	127.14 ± 1.43	106.99 ± 0.84	71.47* ± 1.25	112.27 ± 1.95

The asterisk indicates significant differences ($p \leq 0.05$, Tukey test)

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Abbott MSR, Harvey AP, Valente Perez G, Theodorou MK (2013) Biological processing in oscillatory baffled reactors: operation, advantages and potential. Interface Focus. https://doi.org/10.1098/rsfs.2012.0036

Acharya B, Dutta A, Basu P (2017) Ethanol production by syngas fermentation in a continuous stirred tank bioreactor using *Clostridium ljungdahlii*. Biofuels 89:1–17

Adeyinka A, Pierre L (2020) Organophosphates. [Updated 2020 Feb 18]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020. https://www.ncbi.nlm.nih.gov/books/NBK499860/

Aggarwal V, Deng X, Tuli A, Goh KS (2013) Diazinon—chemistry and environmental fate: a California perspective. Rev Environ Contam Toxicol 223:107–140

Alef K (1995) Dehydrogenase activity. In: Alef K, Nannipieri P (eds) Methods in soil microbiology and biochemistry. Academic Press Inc, San Diego, pp 214–215

Alessandrello MJ, Juárez Tomás MS, Raimondo EE, Vullo DL, Ferrero MA (2017) Petroleum oil removal by immobilized bacterial cells on polyurethane foam under different temperature conditions. Mar Pollut Bull 122:156–160

Alfonso-Gordillo G, Flores-Ortiz CM, Morales-Barrera L, Cristiani-Urbina E (2016) Biodegradation of methyl tertiary butyl ether (MTBE) by a microbial consortium in a continuous up-flow packed-bed biofilm reactor: kinetic study, metabolite identification and toxicity bioassays. PLoS ONE 11:1–21

Alvarez A, Saez JM, Davila JS, Polti MA, Benimeli CS (2019) Chapter 7. Bioremediation of pesticides and metals. In: Sanchez-Hernandez JC (ed) A discussion about different strategies using Actinobacteria. CRC Press, Boca Raton, pp 130–148

Apey A (2019) La fruticultura en Chile: tendencias productivas y su expresión territorial. Análisis realizado a partir de los Catastros Frutícolas para el período 1999-2018. Oficina de Estudios y Políticas Agrarias (ODEPA). Ministerio de Agricultura. https://www.odepa.gob.cl

Azizi A, Dargahi A, Almasi A (2019) Biological removal of diazinon in a moving bed biofilm reactor—process optimization with central composite design. Toxin Rev. https://doi.org/10.1080/15569543.2019.1675708

Baldez EE, Robaina NF, Cassella RJ (2008) Employment of polyurethane foam for the adsorption of methylene blue in aqueous medium. J Hazard Mater 159:580–586

Błaszak M, Pełech R, Graczyk P (2011) Screening of microorganisms for biodegradation of simazine pollution (Obsolete Pesticide Azotop 50 WP). Water Air Soil Pollut 220(1–4):373–385

Briceño G, Fuentes MS, Palma G, Jorquera MA, Amoroso MJ, Diez MC (2012) Chlorpyrifos biodegradation and 3,5,6-trichloro-2-pyridinol production by Actinobacteria isolated from soil. Int Biodeterior Biodegrad 73:1–7

Briceño G, Schalchli H, Mutis A, Benimeli CS, Palma G, Tortella GR, Diez MC (2016a) Use of pure and mixed culture of diazinon-degrading *Streptomyces* to remove other organophosphorus pesticides. Int Biodeterior Biodegrad 114:193–201

Briceño G, Schalchli H, Rubilar O, Tortella GR, Mutis A, Benimeli CS, Palma G, Diez MC (2016b) Increased diazinon hydrolysis to 2-isopropyl-6-methyl-4-pyrimidinol in liquid medium by a specific *Streptomyces* mixed culture. Chemosphere 156:195–203

Briceño G, Vergara K, Schalchli H, Palma G, Tortella G, Fuentes MS, Diez MC (2017) Organophosphorus pesticide mixture removal from environmental matrices by a soil *Streptomyces* mixed culture. Environ Sci Pollut Res Int 25:21296–21307

Cabrera-Orozco A, Galíndez-Nájera SP, Ruiz-Ordaz N, Galíndez-Mayer J, Martínez-Jerónimo F (2017) Biodegradation of a commercial mixture of the herbicides atrazine and *S*-metolachlor in a multi-channel packed biofilm reactor. Environ Sci Pollut Res Int 24:25656–25665

Carvalho F (2017) Pesticides, environment, and food safety. Food Energy Secur 6:48–60

Castro CC, Nobre C, Duprez ME, De Weirelda G, Hantsona AL (2017) Screening and selection of potential carriers to immobilize *Aureobasidium pullulans* cells for fructo-oligosaccharides production. Biochem Eng J 118:82–90

Christensen K, Harper B, Luukinen B, Buhl K, Stone D (2009) Chlorpyrifos technical fact sheet; national pesticide information center, Oregon state university extension services. https://npic.orst.edu/factsheets/archive/chlorptech.html

Cycón M, Mrozik A, Piotrowska-Seget Z (2017) Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: a review. Chemosphere 172:52–71

Dar MA, Kaushik G, Villarreal-Chiu JF (2019) Pollution status and bioremediation of chlorpyrifos in environmental matrices by the application of bacterial communities: a review. J Environ Manag 239:124–136

Dar MA, Kaushik G, Villareal Chiu JF (2020) Chapter 2. Pollution status and biodegradation of organophosphate pesticides in the environment. In: Singh P, Kumar A, Borthakur A (eds) Abatement of environmental pollutants. Elsevier, Amsterdam, pp 25–66

Dash DM, Osborne DJ (2020) Rapid biodegradation and biofilm-mediated bioremoval of organophosphorus pesticides using an indigenous *Kosakonia oryzae* strain-VITPSCQ3 in a vertical-flow packed bed biofilm bioreactor. Ecotoxicol Environ Safe 192:110290

Dębski B, Kania B, Kuryl T (2007) Transformations of diazinon, an organophosphate compound in the environment and poisoning by this compound. Ekológia (Bratislava) 26:68–82

Dzionek A, Wojcieszy D, Guzik U (2016) Natural carriers in bioremediation: a review. Electron J Biotechnol 23:28–36

Gonzalez-Cuna S, Galíndez_Mayer J, Ruiz-Ordaz N, Murugesan S, Piña-Escobedo A, García-Mena J, Lima-Martinez E, Santoyo-Tepole F (2016) Aerobic biofilm reactor for treating a commercial formulation of the herbicides 2,4-D and dicamba: biodegradation kinetics and biofilm bacterial diversity. Int Biodeterior Biodegr 107:123–131

Grandclémen C, Seyssiecq I, Piram A, Wong-Wah-Chung P, Vanot G, Tiliacos N, Roche N, Doumenq P (2017) From the conventional biological wastewater treatment to hybrid processes, the evaluation of organic micropollutant removal: a review. Water Res 111:297–317

Horemans B, Albers P, Springael D (2016) The biofilm concept from a bioremediation perspective. In: Lear G (ed) Biofilms in bioremediation. Current research and emerging technologies. Caister Academic Press, Norfolk, pp 1–251

Jesitha K, Harikumar PS (2019) Development of a bioreactor system for the remediation of endosulphan. H2Open J 2:1

Karas PA, Perruchon C, Exarhou K, Ehaliotis C, Karpouzas DG (2011) Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. Biodegradation 22:215–228

Lasram MM, Dhouib IB, Annabi A, El Fazaa S, Gharbi N (2014) A review on the molecular mechanisms involved in insulin resistance induced by organophosphorus pesticides. Toxicology 322:1–13

Moscoso F, Teijiz I, Sanroman MA, Deive FJ (2012) On the suitability of a bacterial consortium to implement a continuous PAHs biodegradation process in a stirred tank bioreactor. Ind Eng Chem Res 51:15895–15900

Mulla S, Bangeppagari M, Mahadevan G, Shah Eqani S, Sajjana D, Tallur P, Megadia V, Ninnekara H (2016) Biodegradation of 3-chlorobenzoate and 3-hydroxybenzoate by polyurethane foam immobilized cells of *Bacillus* sp. OS13. J Environ Chem Eng 4:1423–1431

Ory I, Cabrera G, Ramirez M, Blandino A (2016) Immobilization of cells on polyurethane foam. In: Guisan JM (ed) Methods in biotechnology: immobilization of enzymes and cells. Humana Press Inc, Totowa

Pradeep V, Malavalli U (2016) Use of Ca-alginate immobilized *Pseudomonas aeruginosa* for repeated batch and continuous degradation of endosulfan. 3 Biotech 6:1–124

Saez JM, Benimeli CS, Amoroso MJ (2012) Lindane removal by pure and mixed cultures of immobilized Actinobacteria. Chemosphere 89:982–987

Saez JM, Aparicio JD, Amoroso MJ, Benimeli CS (2015) Effect of the acclimation of a *Streptomyces* consortium on lindane biodegradation by free and immobilized cells. Process Biochem 50:1923–1933

Solomon KR, Williams WM, Mackay D, Purdy J, Giddings JM, Giesy JP (2014) Properties and uses of chlorpyrifos in the United States. Ecological risk assessment for chlorpyrifos in terrestrial and aquatic systems in the United States. Rev Environ Contam Toxicol 231:13–34

Supreeth M, Chandrashekar MA, Sachin N, Raju NS (2016) Effect of chlorpyrifos on soil microbial diversity and its biotransformation by *Streptomyces* sp. HP-11. 3 Biotech 6:147

Tabatabai MA, Bremmer JM (1969) Use of *p*-nitrophenylphosphate to assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tallur PN, Mulla SI, Megadi VB, Talwar MP, Ninnekar HZ (2015) Biodegradation of cypermethrin by immobilized cells of *Micrococcus* sp. strain CPN 1. Braz J Microbiol 46:667–672

Tarjányi-Szikora S, Oláh J, Makó M, Palkó G, Barkács K, Záray G (2013) Comparison of different granular solids as biofilm carriers. Microchem J 107:101–107

Tiquia SM, Tam NFY, Hodgkiss IJ (1996) Effects of composting on phytotoxicity of spent pig-manure sawdust litter. Environ Pollut 93:249–256

Yadav M, Srivastva N, Sharan R, Nath S, Kumar S (2014) Biodegradation of chlorpyrifos by *Pseudomonas* sp. in a continuous packed bed bioreactor. Bioresour Technol 165:265–269

Yañez-Ocampo G, Sanchez-Salinas E, Jimenez-Tobon GA, Penninckx M, Ortiz-Hernández ML (2009) Removal of two organophosphate pesticides by a bacterial consortium immobilized in alginate or tezontle. J Hazard Mater 168:1554–1561

Yáñez-Ocampo G, Sánchez-Salinas E, Ortiz-Hernández ML (2011) Removal of methyl parathion and tetrachlorvinphos by a bacterial consortium immobilized on tezontle-packed up-flow reactor. Biodegradation 22:1203–1213