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THE FUNCTIONAL RESPONSE OF IMMOBILIZED MICROBIAL COMMUNITIES TO INCREASE LOADING RATES OF THE PESTICIDES CHLORPYRIFOS AND BIFENTHRIN

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Abstract

Several technologies have been developed to remediate contaminated terrestrial or aquatic environments; in contrast, few have been tested to prevent the pollution of aquatic ecosystems. Permeable reactive biobarriers (PRBs) are one of several emerging ecotechnologies that could prevent the contamination of surface waters by the arrival of agrochemical compounds carried by drainage and runoff from agricultural lands. A PRB functions as a biofilm reactor. Its usefulness depends on the capacity of the microbial communities (MC) immobilized in the biobarrier to resist and recover their degrading capacity after environmental disturbances, such as the presence of several types of pollutants and the increase of their loading rates to the biobarrier. This work evaluates the functional response of two taxonomically different MCs acclimated on diazinon to the increasing supply of two widely used pesticides, chlorpyrifos and bifenthrin. A D-Stat is an unsteady-state continuous culture technique used to provoke a continuous environmental disturbance in PRBs through the gradual increase in pesticide concentration. The results showed that regardless of the taxonomic structure of the immobilized MCs, a fast, functional adjustment occurred when continuously increasing loading rates (B_V) of pesticides were supplied to the biofilm reactor, observing synchrony between pesticide loading (B_V) and removal rates (R_V).

Key words: D-Stat, diazinon, ethyl chlorpyrifos, functional convergence, gradient feeding

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1. Introduction

There is an increasing interest in developing and optimizing bioremediation processes to deal with environments contaminated with toxic compounds (Abraham and Silambarasan, 2018). The methodology for the restoration of aquatic ecosystems has mostly focused on groundwater. In the case of surface water bodies affected by non-point source pollution, their remediation firstly requires actions to prevent their contamination by runoffs and subsurface drainage tile lines. As an alternative for retaining and degrading agricultural wastewater pollutants, some types of biological barriers have been studied, such as

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constructed wetlands (García-García et al., 2015) or variants of surface or subsurface barriers (Hassanpour et al., 2019).

In this context, permeable reactive biobarriers are reactors in which physical, physicochemical, and biological processes occur. The biological process involves microbial communities (MCs) attached to a support material, forming a biofilm that must operate indefinitely in changing climatic conditions and variations in the flow rate and composition of agricultural wastewater (Cabrera-Orozco et al., 2017; Ordaz-Guillén et al., 2014). The biodiversity found in a microbial community (MC) maintained in a stable environment, where the rates of nutrient supply and consumption remain unchanged implies that the MC functions in a concerted manner, with each taxonomic group performing specific functions. However, when an environmental disturbance occurs, such as the arrival of nutrients different from those used by the microbial consortium in a steady-state condition, it is expected that the relative abundance of specific microbial populations varies, displaying functional plasticity to face such environmental changes.

According to some authors, after an environmental disturbance, the functional recovery of a MC depends on the presence of inorganic nutrients, co-substrates, or toxic compounds, but fundamentally on the lapse of time needed for some microbial populations to grow enough to carry out a detectable degradation of xenobiotics (Wiggings et al., 1987).

It is well known that a MC subjected to an environmental change, after a considerable period, tends to acclimatize to the new environment (Nava-Arenas et al., 2012). Thus, its taxonomic structure and functional behavior eventually must change to cope with new environmental pressures. The potential of a MC to recover from disturbances depends on its functional and taxonomic structure. The MC recovery is reflected in the population growth and re-colonization of the surviving organisms (Pesce et al., 2017), and its functional stability to degrade the xenobiotic stressors (Griffiths et al., 2004).

In a biological reactor, the environment in which a microbial population develops can be modified by manipulating its operating conditions. Microorganisms respond to environmental changes by adjusting their physiological state. This response is kinetically measurable by estimating physiological state parameters (specific reaction rates and conversion efficiencies). When working with communities integrated by a great diversity of microbial species, the response to environmental disturbances is related to their taxonomical structure. Throughout the MC acclimatization, a biunivocal relationship occurs between the environment and microorganisms. An environmental change affects the community members' physiological state and their mutual interactions, which alter the environment they inhabit until the community finally reaches stability in the new environment. However, when a microbial community is exposed to a continuous environmental perturbation, its structure and dynamic behavior must respond rapidly to avoid collapsing. The celerity with which the MC responds reflects its plasticity. One way to quantify the MC plasticity through a transient environmental disturbance is by estimating the degree of synchrony in its functional response (R_V) to an induced environmental stimulus, such as the continuous change in the loading rate (B_V) of a new anthropogenic stressor.

The techniques used for studies in suspended cell cultures (Ochoa-Estopier and Guillouet, 2014) can also be applied to study the dynamics of microbial communities immobilized in biofilm reactors. A usual strategy to study the physiology of microbial populations consists of the establishment of constant environmental conditions leading to a steady-state condition. For each equilibrium condition, a specific kinetic behavior is obtained, reflecting the microbial population's physiological state. Unfortunately, this strategy is time-consuming, and the observed kinetic responses correspond to different taxonomic structures attained after the MC acclimatization to every steady-state condition. As an alternative, the procurement of quantitative data of microbial metabolism as a response to diverse environmental conditions can be carried out in a single experiment by disturbing a steady-state condition, applying a sudden or gradual change of environmental variables. A sudden change disturbs the equilibrium generating a transition from a physiological state (*I*) to a new state (*II*). In contrast, a gradual change in environmental conditions causes a controlled shift and a transient state continuum.

Microbial communities are dynamical systems intertwined by metabolic links (Gonze et al., 2018), and their metabolic functions are encoded in their aggregate genomes depending on their biodiversity (Knowles et al., 2017). Although the taxonomic structure of a MC varies according to environmental changes, its functional structure, reflected in its dynamic behavior, could remain stable thanks to the biodiversity and redundancy of microbial species. However, a rapid fine-tuning of key microbial populations could also contribute to dynamic stability. Thus, one of the purposes of this work was to evaluate the dynamics of change in microbial communities by estimating their functional response (R_V) to the continuous change in the loading rate (B_V) of the ethyl chlorpyrifos (EC) and bifenthrin (BF). The second purpose was to compare two microbial communities' structural and kinetic behavior of different origins, acclimatization history, and taxonomic structure (C_X and C_T). The comparison was made by exposing two biofilm reactors operating in a continuous D-Stat system (Kasemets et al., 2003) to the same transient environmental pressures.

2. Material and methods

2.1. Chemicals

Commercial formulations of the insecticides: Horta 25, containing diazinon (DZ) (25%), Tyson 2E, containing ethyl chlorpyrifos (26.2%); both from

Agroquímica Tridente, SA de CV, Mexico, and Akaris, containing bifenthrin (12.15%), from Agricultura Nacional, SA de CV, Mexico. The commercial formulations also include diluents, wetting, and dispersing agents as adjuvants. Analytical standards of diazinon (DZ), ethyl chlorpyrifos (EC), 3,5,6-trichloro-2-pyridinol (99.5%) (TCP), and 2-isopropyl-6-methyl-pyrimidin-4-ol (99%) (IMP), with a purity higher than 99%, were supplied by Chen-Service USA. The analytical standard of bifenthrin (BF) (99%) was purchased from Sigma-Aldrich, USA. Absolv-grade acetonitrile was from Tedia, USA.

These three insecticides are widely used for flower production in Tenancingo, Mexico, the area where soil samples were collected to isolate microbial communities. Besides, they were detected in the drains of greenhouses used for flower cultivation. As observed in Fig. 1, EC and DZ are structurally similar insecticides. Both are organophosphorothionate compounds, and the main difference is the presence of chlorine substituents in the heterocyclic ring of EC. On the contrary, the fluorinated insecticide, BF, is a member of the pyrethroid family of chemicals showing wide chemical structural differences with EC and DZ.

2.2. Culture medium

The salts medium (MS) contains, in g L^{-1}, NaH_2PO_4, 0.1; K_2HPO_4, 0.4; $MgSO_4 7H_2O$, 0.1; $FeCl_3 6H_2O$, 5 x 10^{-5}; $ZnSO_4 7H_20$, 4.5 x 10^{-4}; $Na_2MoO_4 2H_2O$, 2.5 x 10^{-4}; $MnSO_4 H_2O$, 5.0 x 10^{-4}, and as a carbon and nitrogen source, DZ, EC, or BF at concentrations of 50 mg L^{-1} for microbial enrichment, and kinetic studies in biofilm reactors.

2.3. Microbial communities

Two MCs were implanted in biofilm reactors of identical construction, configuration, and operating conditions. One MC, denominated C_X, was obtained from samples of lacustrine sediments collected near agricultural areas at Xochimilco, Mexico City. This MC was acclimatized for an extended period exclusively on DZ. The other MC, denominated C_T, came from soil samples collected near greenhouses, where pesticide mixtures are applied for flower production (Tenancingo, State of Mexico). Specific microbial communities were enriched by successive transfers and acclimated separately on DZ, EC, and BF.

2.4. Microbial enrichment of the C_T community by successive transfers (repeated batch culture)

Mineral medium containing 50 mg L^{-1} of one of the pesticides probed, fragments of porous volcanic stone to support biofilm formation, and the corresponding soil sample were placed in Erlenmeyer flasks. They were incubated for seven days at room temperature (24±2°C) in a rotatory shaker. A flask containing the sterile medium was also maintained as an abiotic control. Once observed a change in the UV region's absorption spectrum, the flasks' exhausted medium was decanted and substituted by a fresh medium, incubating them under the same conditions. The procedure was repeated at least seven times. After the last replacement, the microbial communities were transferred to packed bed columns to be acclimated separately on DZ, EC, and BF.

2.5. Integration of the microbial community C_T

Specific microbial communities separately enriched and acclimated on DZ, EC, and BF on packed bed columns were used to integrate the C_T community. After batch growing on DZ, the integrated community C_T and the community C_X were used to inoculate two packed bed biofilm reactors (PBRs), which simulate permeable reactive biobarriers.

2.6. Packed bed biofilm reactors

Square-section PBRs were constructed of Plexiglas plates, with dimensions 13.8 x 13.8 x 8.3 cm and nominal volumes of 1580 cm^3. The reactors, PBR-C_X (reactor containing the microbial community C_X) and PBR-C_T (reactor containing the microbial community C_T), were packed with 511 g and 491 g of porous rock fragments, respectively. The liquid volumes (V_L) in each reactor were respectively 370 and 355 cm^3. These values were used to compute the loading and removal rates of pesticides; ($B_V = F\ S_{IN}/V_L$),-and ($R_V = F\ (S_{IN} - S_{OUT})/V_L$), respectively. The terms S_{IN} and S_{OUT} refer to the input and output concentrations of the pesticide supplied to PBRs, and the term F refers to the liquid flow rate used to feed the PBRs containing the microbial communities C_X or C_T.

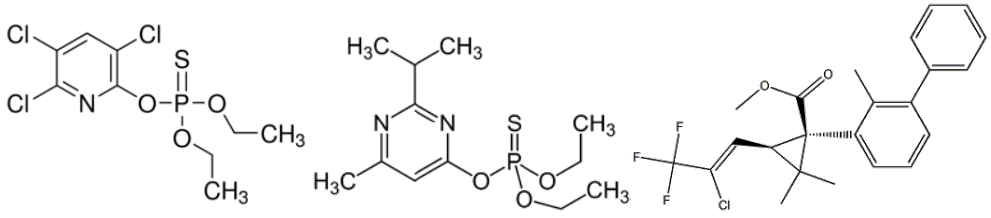

Fig. 1. Chemical structures of diazinon, ethyl chlorpyrifos, and bifenthrin

Both reactors have three transversely positioned vertical ducts of rigid plastic mesh for aeration and liquid sampling. These ducts act as airlift devices, allowing the axial and radial circulation of oxygenated liquid through the packed bed zone. The reactors were loaded with porous volcanic stone fragments. The determinations of their equivalent mean diameter ϕ_P = 5.8 ± 0.71 mm, and particle density ρ_P = 1.93 g cm^{-3}, were made following to the procedure described by Gómez-De Jesús et al., (2009). In both reactors, the air was injected through porous diffusers.

2.7. Continuous operation of PBRs

Initially, mineral salts medium (MS) containing DZ (S_{IN} = 50 mg L^{-1}) was fed at equal liquid flow rates (F_X *or* F_T) to each bioreactor using a variable speed double-head peristaltic pump as shown in the experimental set-up (Fig. 2). The operational conditions tested in both reactors continuously operated are shown in Table 1.

2.8. Method used for the gradual increase in the loading rate of substrates

A method known as D-stat, initially developed for homogeneous (well mixed) bioreactors, was adopted to feed the packed bed biofilm reactors (PBRs) containing the microbial communities C_X and C_T. With the D-stat method, the dilution rate in the homogeneous reactor is kept constant. Once reached the steady-state, an environmental variable (e.g., temperature, pH, substrate, or toxic agent) is gradually changed. Thus, the environmental impact on the microbial kinetics can be measured (Adamberg et al., 2015). In this work, the gradual increase in EC and BF loading rate was achieved by maintaining a constant dilution rate and increasing the compounds' concentration in the medium supplied to the PBRs.

A two-head peristaltic pump and serial tanks were used to implement the D-Stat procedure. The liquid medium was transferred to the gradient tank G of volume V_G from the reservoir tank R, which contains a substrate concentration SR, at a flow rate F_1. The flow rate F_2 from the G tank is bifurcated in F_X and F_T streams entering the packed bed biofilm reactors (PBRs) containing, respectively, the microbial communities C_X and C_T (Fig. 2).

To achieve a gradual increase in the substrate concentration supplied to bioreactors, several techniques can be used. A modification of the method described by Salazar-Huerta et al., (2019) was used to reach a transient change in substrate concentration $S_G(t)$. The continuous variation in S_G modifies the substrate loading rates B_V to the PBRs containing the C_X and C_T microbial communities (Eqs. 1, 2).

$$B_{V,X} = F_X S_G(t)/V_{LX} \quad (1)$$

and

$$B_{V,T} = F_T S_G(t)/V_{LT} \quad (2)$$

V_{LX} and V_{LT} are the liquid volumes of packed bed biofilm-reactors holding the microbial communities C_X or C_T.

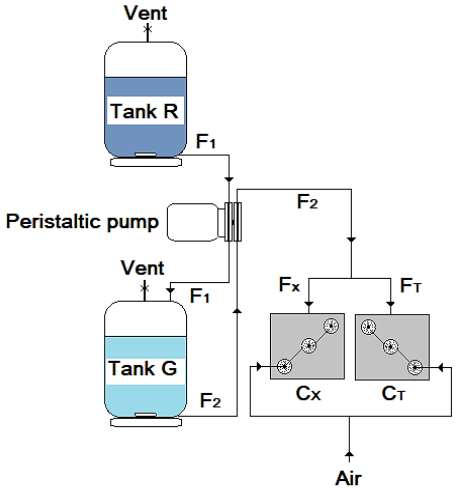

Fig. 2. Experimental set-up of the D-Stat system for the gradual increase of substrate loading rate to the PBRs containing the microbial communities Cx and CT. Tank R, medium reservoir tank; tank G to generate a gradient concentration of pesticides $S_G(t)$; F1; flow rate of the medium from R to G tank; F2, flow rate of the medium from G tank, branching into Fx and FT streams feeding the reactors containing the Cx and CT communities

2.9. Analysis of the microbial diversity of MCs extracted from the packed bed biofilm reactors (PBRs)

Throughout the study, the biofilms of both PBRs were sampled. Initially, the continuously operated PBRs fed with DZ, holding the C_X and C_T microbial communities, were sampled in the steady-state condition; these samples were denominated C_{X0} and C_{T0}. Then, PBRs were supplied with a constant concentration of DZ and a gradient supply of EC.

All samples were used to extract the bacterial genomic DNA and prepare 16S rDNA libraries according to the process described by González-Cuna et al. (2016). The DNA sequencing and analysis for microbial diversity were made using Ion 318 v2 Chips and Ion Torrent PGM System, employing the corresponding software, as described by García-Mena et al. (2016). The results were analyzed to compare the taxonomic structure of the microbial communities C_X and C_T.

2.10. Isolation and identification of predominant cultivable microorganisms

The isolation of predominant cultivable microorganisms in the PBRs was made according to the procedures described by González-Cuna et al. (2016).

Table 1. Operational conditions of PBR-C_X and PBRC_T throughout the D-Stat experiments. Diazinon (DZ), ethyl chlorpyrifos (EC), and bifenthrin (BF) concentrations in reservoir tank (R)

Pesticide supplied	*DZ concentration in R tank (mg L^{-1})*	*EC concentration in R tank (mg L^{-1})*	*BF concentration in R tank (mg L^{-1})*	*Initial liquid volumes in R and G tanks $V_{R0}=V_{G0}$ (L)*	*Flow rates in R and G tanks $F_1 = F_2$ (L h^{-1})*	*Flow rates in PBRs (L h^{-1})*	*Liquid vol. in PBR-C_X (L)*	*Liquid vol. in PBR-C_T (L)*	*D-Stat duration (h)*
DZ	49.1±1.6	0	0	4.0	0.090±0.001	0.046±0.001	0.37	0.32	-
DZ + EC	49.5±2.2	53.7±4.5	0	4.0	0.089±0.001	0.045±0.001	0.37	0.32	25
DZ + EC + BF	50.7±3.1	53.4±1.8	49.2±1.5	4.0	0.088±0.002	0.046±0.001	0.37	0.32	75

2.11. Quantification of pesticides and intermediates by Gas Chromatograph - Mass Spectrometry (*GC-MS*)

To the aqueous samples collected from PBRs, a change of solvent was made, eliminating water and transferring the components of interest to acetonitrile. To 1.5 mL samples, an equal volume of acetonitrile is added, together with one gram of a mixture of salts whose composition is: anhydrous $MgSO_4$, 61.60%; dibasic sodium citrate, 7.70%; tribasic sodium citrate, 15.35%; and NaCl, 15.35% (Thermo Fisher Scientific, USA). The combination of salts modifies the density and viscosity of the aqueous phase which becomes immiscible with acetonitrile. Liquid-liquid extraction is carried out by vigorous stirring. To separate the organic phase from the aqueous phase and acetonitrile the mixture is centrifuged at 3000 rpm for 5 minutes. The acetonitrile supernatant, which contains the pesticides and their metabolites is filtered in acrodisc-syringe-filters (0.2 μm pore size); finally, it is placed in two mL glass vials for chromatographic analysis.

An Agilent 7820A chromatograph was routinely used. The coupled detector was a MSD 5977E quadrupole. The column used was a VF 1701ms 30 m x 0.25 mm id x 0.25 m film. The hydrogen flow rate was 35 cm s^{-1} and an oven ramp from 70°C to 240°C.

3. Results and discussion

The functional parameters used to compare microbial communities' kinetic behavior were the volumetric biodegradation rates of each pesticide (Eqs. 3, 4).

$$R_{V,X} = F_X\,[S_G(t)\text{-}s_X(t)]/V_{LX} \qquad (4)$$

$$R_{V,T} = F_T\,[S_G(t)\text{-}s_T(t)]/V_{LT} \qquad (5)$$

where s_X and s_T are the compound concentrations in the outflowing liquid from PBR-C_X and PBR-C_T, respectively.

To accomplish this objective, the MCs were implanted in identical PBRs. The first one, denominated C_X, resulted from two-year acclimatization on a commercial formulation of diazinon. The other denominated C_T resulted from the mixture of three communities, separately acclimatized in liquid media containing only one pesticide and showing the capacity to degrade DZ, EC, or BF.

These communities had important differences in their taxonomic structure. In the C_X community, the phylum *Chlorobi* predominates, as well as the families *Sphingomonadaceae* and *Rhodocyclaceae* (Table 2). In contrast, in the C_T community, the most abundant taxonomic groups corresponded to the phylum *Cyanobacteria* and the *Solibacter* and *Rhizobial* orders (Table 3).

The change in environmental conditions was attained by the sequential and additive supply of ethyl chlorpyrifos and bifenthrin to aerobic biofilm reactors (PRBs) running at the same operational conditions. Here, the dynamic responses of MCs to gradual environmental changes were compared.

3.1. Kinetic behavior of C_X and C_T communities during the gradual loading rate of ethyl chlorpyrifos

As observed in Figs. 3A and 3B, a similar kinetic response to the increasing EC concentration in the medium inflowing to bioreactors, was observed in C_X and C_T communities. When the PBRs' input and output concentrations of EC were correlated in terms of the transient removal rates $R_{V,EC}(t)$ as a function of the gradual increase in the volumetric loading rates of EC $B_{V,EC}(t)$, it was evidenced that both communities showed similar behavior and an equal delay in their EC's removal rates of (Fig. 4).

3.2. Kinetic behavior of C_X and C_T communities during the gradual loading rate of bifenthrin

To reach a new stable state, both reactors were operated for almost three months at a constant loading rate of a mixture of DZ and EC. Subsequently, a new state transition was induced in C_X and C_T communities by the gradual increase in the loading rate of BF. Figs. 5A and 5B show a similar kinetic response of C_X and C_T communities to the increasing BF concentration in the medium inflowing to bioreactors. By correlating the input and output results in terms of the BF removal rates $R_{V,BF}(t)$ as a function of the gradual increase in the BF volumetric loading rates $B_{V,BF}(t)$, a quasi-identical kinetic pattern was observed for both communities (Fig. 6). In this case, no removal delay in their BF removal capacity was found by the gradual increase in the volumetric loading rate $B_{V,BF}(t)$. The BF entering the reactor was immediately removed without detectable accumulation.

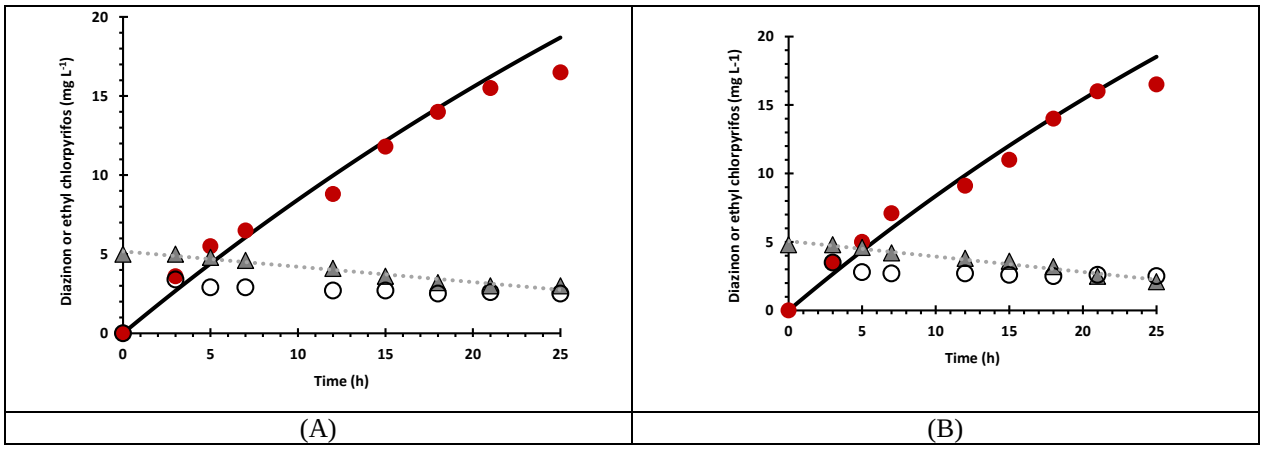

Fig. 3. Transient variation of DZ and EC concentrations in PBRs containing microbial communities C_X (A) and C_T (B) after increasing the EC loading rate, using a D-Stat technique. Tanks R and G contain constant DZ concentrations (49.1 ± 1.6 mg L^{-1}), the concentration of EC in tank R (53.7±4.5 mg L^{-1}). Red filled circles, experimental EC concentrations in G tank. Void circles, observed EC concentrations in PBRs. Gray filled triangles, observed DZ concentrations in PBRs. Dotted lines represent the trend of experimental values of DZ in PBRs. The solid line represents EC concentration change in the G tank [$S_G(t)$]

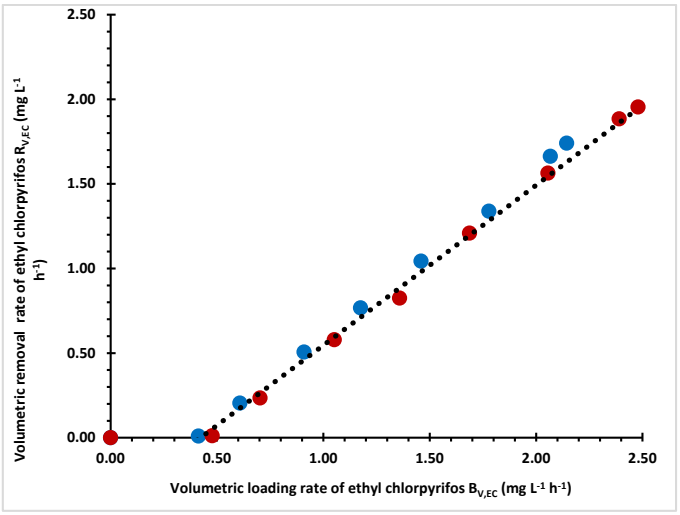

Fig. 4. Transient behavior in the removal rate of EC ($R_{V,EC}$) during the gradual increase in the EC loading rate ($B_{V,EC}$) using the D-Stat technique on microbial communities C_X (blue filled circles) and C_T (red filled circles). The dotted line represents the trend of experimental removal rates of EC, $R_{V,EC} = f(B_{V,EC})$ as a function of the corresponding loading rates

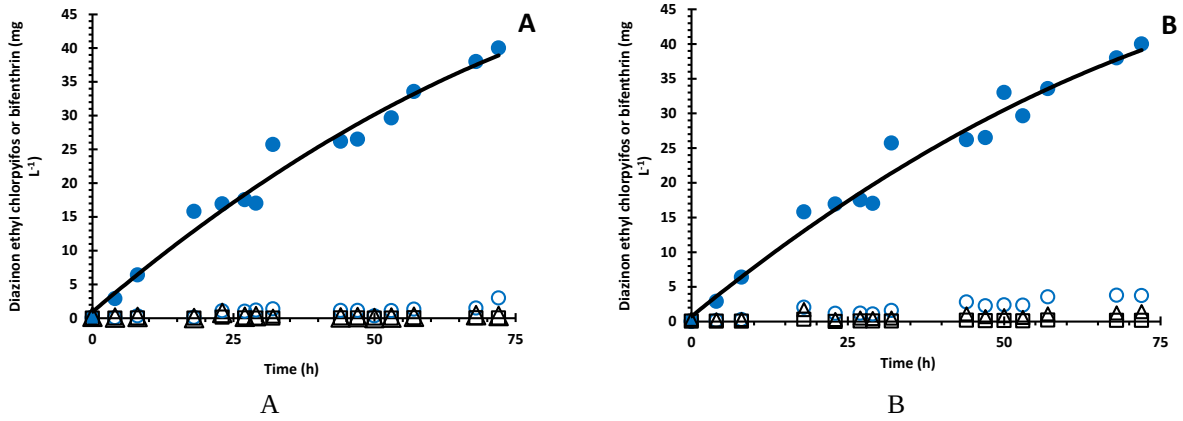

Fig. 5. Transient variation of DZ, EC, and BF concentrations in PBRs containing microbial communities C_X (A) and C_T (B) after a gradual increase in the BF loading rate, using a D-Stat technique. Tanks R and G contain constant concentrations of DZ (49.5 ± 2.2 mg L^{-1}) and EC (53.7 ± 4.5 mg L^{-1}), the concentration of BF in tank R (49.2±1.5 mg L^{-1}). Blue filled circles, observed BF concentrations in G tank. Blue void circles, observed BF concentrations in PBRs. Void triangles; observed EC concentrations in PBRs. Void squares, observed DZ concentrations in PBRs. The solid line represents BF concentration change in the G tank [$S_G(t)$]

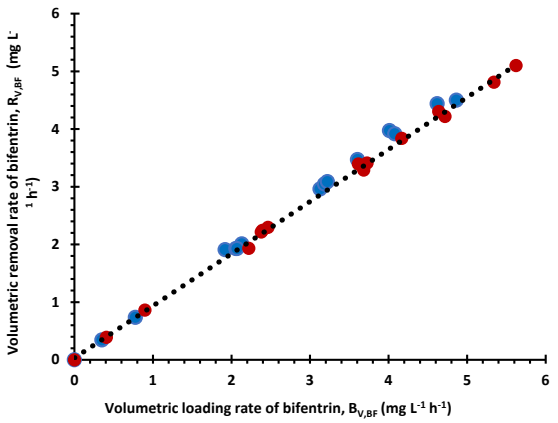

Fig. 6. Transient behavior in the removal rate of BF ($R_{V,BF}$) during the gradual increase in the BF loading rate ($B_{V,BF}$) using the D-Stat technique on microbial communities C_X (blue filled circles) and C_T (red filled circles). The dotted line represents the trend of experimental removal rates of BF, $R_{V,BF} = f(B_{V,BF})$, as a function of the corresponding loading rates

3.3. Effect of the continuous increase in ethyl chlorpyrifos loading rate on the dynamical change in the taxonomic structure of the microbial communities C_X and C_T

High-throughput genetic sequencing enlightens the structural differences of the main taxonomical groups in microbial communities caused by anthropogenic stressors (Cheung et al., 2018; Shade et al., 2013; Wells et al., 2011). In this case, after disturbing a steady-state condition of C_X and C_T communities, the change in the relative abundance of the taxonomic groups after a state transition can be observed. The perturbation of the initial steady-state conditions was provoked by the gradual increase in the volumetric loading rates B_V of EC and BF in C_X (Fig. 7) and C_T (Fig.8). communities.

Drastic changes in the original taxonomic structures of microbial communities (C_{X0} and C_{T0}) were observed after 25 hours of gradient loading of a mixture of DZ and EC. Despite their initial taxonomic differences, both communities arrived at similar structural patterns (C_{X1} and C_{T1}), showing a high prevalence of *Rhodocyclaceae*, *Cytophagaceae*, and *Sphingomonadaceae* families (Tables 2 and 3).

These families have members involved in the degradation of various xenobiotic compounds. Some aromatic compound-degrading members of the family Rhodocyclaceae have considerable potential for bioremediation of polluted environments (Oren, 2014). Toxic dioxin pollutants, pyrene, and phenanthrene are degraded by species of the family Sphingomonadaceae (Glaeser and Kämpfer, 2014, Ban et al., 2016). Zhao et al., (2019) report members of the family Cytophagaceae in microbial consortia degrading 2,3,4 trifluoroaniline.

3.4. Changes in the taxonomic structure of C_X and C_T communities after a period of acclimatization to a mixture of diazinon-ethyl chlorpyrifos

After a stabilization period of 2160 hours in a continuous steady-state culture fed with a mixture of DZ-EC, microbial communities reached stable taxonomic structures C_{X2} and C_{T2} (Figs. 7 and 8).

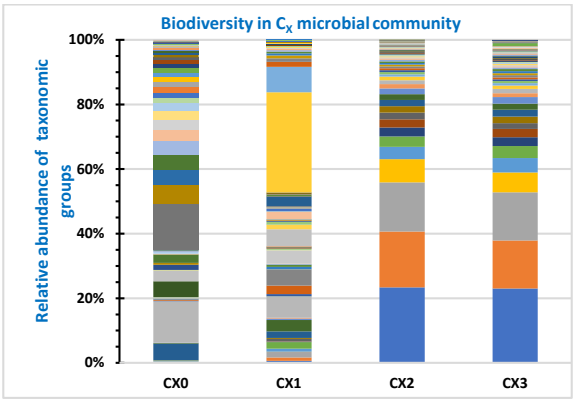

Fig. 7. Changes in biodiversity of the C_X community during the experimentation. C_{X0} original structure in steady-state culture on DZ. C_{X1}, after 25 hours of gradient loading of a mixture of DZ-EC ($B_{V,CP}$ = 0.0 to 2.38 mg L^{-1} h^{-1}), C_{X2} community stabilized in a mixture DZ-EC (after 2160) and C_{X3}, after 75 hours of gradient loading of a mixture DZ-EC-BF ($B_{V,BF}$ = 0.0 to 5.6 mg L^{-1} h^{-1})

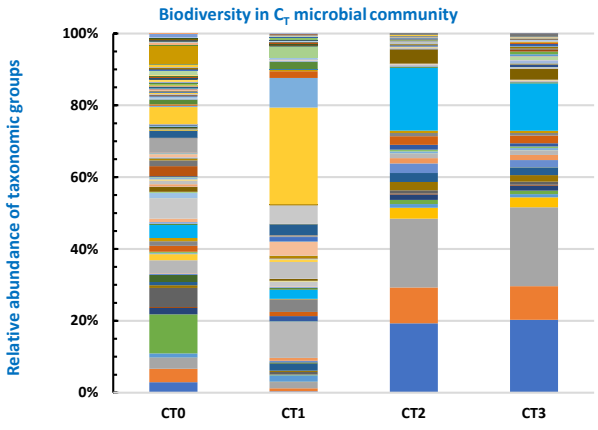

Fig. 8. Changes in biodiversity of the C_T community during the experimentation. C_{T0} original structure in steady-state culture on DZ. C_{T1}, after 25 hours of gradient loading of a mixture of DZ-EC ($B_{V,CP}$ = 0.0 to 2.8 mg L^{-1} h^{-1}), C_{T2} community stabilized in a mixture DZ-EC (after 2160), and C_{T3}, after 75 hours of gradient loading of a mixture DZ-EC-BF ($B_{V,BF}$ = 0.0 to 5.8 mg L^{-1} h^{-1})

The relative abundance of the *Sphingomonadaceae* family is similar in both structures but differ on the prevalence of the families *Bradyrhyzobiaceae* (C_{X2}) and *oc28* (C_{T2}) (Tables 2 and 3).

Table 2. Changes in the relative abundance of the main taxonomic groups in the C_X community throughout the D-Stat experiments

*M-C_X	Main taxonomic group	%
C_{X0}	p__Chlorobi; c__SJA-28; o__;f__;g__	13.7
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingobium	12.3
	p__Proteobacteria; c__Betaproteobacteria; o__Rhodocyclales; f__Rhodocyclaceae;g__	5.4
C_{X1}	p__Proteobacteria; c__Betaproteobacteria; o__Rhodocyclales; f__Rhodocyclaceae_a	30.8
	p__Bacteroidetes; c__Cytophagia; o__Cytophagales; f__Cytophagaceae	7.8
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae;g__Sphingobium	6.5
C_{X2}	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingopyxis	23.3
	p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Bradyrhizobiaceae_a	17.3
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae_a	15.1
C_{X3}	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingopyxis	22.9
	p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Bradyrhizobiaceae_a	14.8
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae_a	14.7

**Microbial community CX. Original community feed with diazinon (CX0); community after 25 hours of gradient feeding with diazinon-ethyl chlorpyrifos (CX1); community after 2160 hours of continuous feeding with diazinon-ethyl chlorpyrifos (CX2); community after 75 hours of gradient feeding with diazinon-ethyl chlorpyrifos-bifenthrin (CX3)*

Table 3. Changes in the relative abundance of the main taxonomic groups in the C_T community throughout the D-Stat experiments

*M-C_T	Main taxonomic group	%
C_{T0}	p__Cyanobacteria; c__4C0d-2; o__MLE1-12	10.4
	p__Acidobacteria; c__Solibacteres; o__Solibacterales_a	5.6
	p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales_a	5.1
C_{T1}	p__Proteobacteria; c__Betaproteobacteria; o__Rhodocyclales; f__Rhodocyclaceae_a	26.7
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingobium	9.9
	p__Bacteroidetes; c__Cytophagia; o__Cytophagales; f__Cytophagaceae	8.2
C_{T2}	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingopyxis	19.2
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae_a	19.0
	p__Chloroflexi; c__Anaerolineae; o__SBR1031; f__oc28	17.4
C_{T3}	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae_a	21.6
	p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingopyxis	20.0
	p__Chloroflexi; c__Anaerolineae; o__SBR1031; f__oc28	13.0

**Microbial community C_T. Original community feed with diazinon (C_{T0}); community after 25 hours of gradient feeding with diazinon-ethyl chlorpyrifos (C_{T1}); community after 2160 hours of continuous feeding with diazinon-ethyl chlorpyrifos (C_{T2}); community after 75 hours of gradient feeding with diazinon-ethyl chlorpyrifos-bifenthrin (C_{T3})*

The family *Bradyrhyzobiaceae* has broad participation in biogeochemical cycles. Many species form a restricted group of biological nitrogen fixers (Marcondes de Souza et al., 2014), and have members using herbicides as a nutritional source (Madureira Barroso et al., 2020). The family oc28 pertains to the class *Anaerolineae*. The *Anaerolineae* lineage of the phylum *Chloroflexi* had been identified as one of the core microbial populations in anaerobic digesters, prevailing by its cellular adhesiveness (Xia et al., 2016).

3.5. Effect of the continuous increase in bifenthrin loading rate on the dynamical change in the taxonomic structure of the microbial communities C_X and C_T

Finally, after 75 hours of gradient loading of a mixture DZ-EC-BF, the MCs reach the taxonomic structures CX_3 and CT_3. As shown in Figs. 7 and 8 and Tables 2 and 3, minor changes in the relative abundance of their main taxonomic groups, prevailing the *Sphyngomonadaceae* family.

3.6. Most abundant bacterial genera isolated from the C_X and C_T communities

Tables 4 and 5 show the most abundant cultivable species isolated from C_X and C_T communities after the gradual addition of EC and BF, respectively. Although by metagenomic analysis, the relative abundance of the genus *Pseudomonas* was low, varying from 0.39 to 3.82% in C_X and C_T communities, respectively, strains of this genera were frequently isolated. Many strains of the genus *Pseudomonas* harbor many plasmids, which constitute an important part of their transferable gene pool (Shintani et al., 2010), probably contributing to the plasticity and long-term acclimatization of microbial communities degrading xenobiotics.

Some authors, testing the functional significance of microbial community composition,

concluded that microbial communities from different soil habitats are not functionally equivalent (Strickland et al., 2009). The set of results presented here indicate that regardless of the differences in the origin, acclimatization process, and taxonomic structure of two microbial communities, their dynamic response to the same induced environmental disturbances was remarkably similar, pointing to functional stability; that is, that taxa could be functionally redundant in both MCs (Beier et al., 2015).

3.7. Accumulation of intermediaries IMP and TCP during bifenthrin gradual supply to bioreactors

Using the D-Stat technique, similar behavior in the transient accumulation of the catabolic intermediary of EC metabolism, TCP, was observed during the BF increasing supply to bioreactors containing C_X and C_T communities. In both cases, a peak concentration was transiently observed at about 40 hours of operation; then, TCP returned to their basal values (≈ 4 mg L^{-1}). In the same experiment, the concentration levels of the catabolic intermediary of BF, IMP, were always below 0.04 mg L^{-1} (Fig. 9).

4. Conclusions

By determining the relative abundance of taxonomic groups in the *C_X* and *C_T* microbial communities through their metagenomic analysis, the biodiversity of culturable and non-culturable populations is reflected more accurately than by screening microbial isolates. The latter only grow under specific cultural conditions.

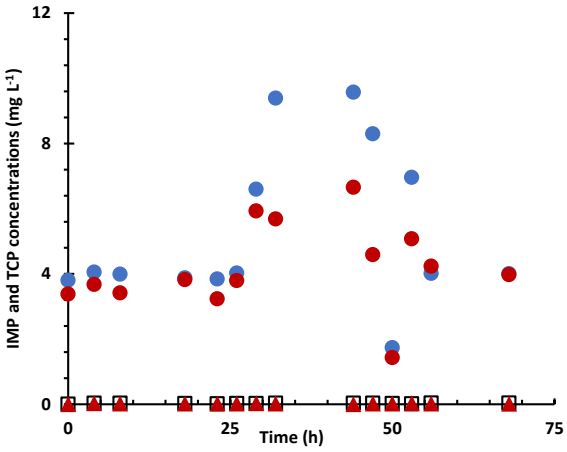

Fig. 9. Transient accumulation of the catabolic intermediaries of BF and EC; IMP and TCP, respectively, in PBRs containing C_X and C_T communities during the BF's gradual supply using the D-Stat technique on microbial communities. TCP accumulation in PBRs C_X (red filled circles) and C_T (blue filled circles). IMP accumulation in PBRs C_X (red filled triangles) and C_T (void squares)

Table 4. Most abundant cultivable bacterial strains isolated after EC addition to bioreactors containing *C_X* or *C_T* communities. Identification by similarity to the nearest microorganism

Identified microorganism	*Accession number*	*Similarity %*
C_X COMMUNITY		
Bacillus sp.	KM596856.1	100
Pseudomonas hunanensis	MG722705.1	99
Pseudomonas monteilii	CP014062.1	99
Pseudomonas nitroreducens	FJ588866.1	100
Pseudomonas putida	KF010920.1	100
Rhodococcus qingshengii	HQ202829.1	99
Uncultured Pseudomonas sp.	AB700606.1	99
C_T COMMUNITY		
Bacillus sp.	MH699239.1	99
Pseudomonas putida	MF996382.1	99
Pseudomonas sp.	KC857485.1	99
Uncultured Pseudomonas sp.	AB700606.1	99

Table 5. Most abundant cultivable bacterial strains isolated after BF addition to bioreactors containing *C_X* or *C_T* communities. Identification by similarity to the nearest microorganism

Identified microorganism	*Accession number*	*Similarity %*
C_X COMMUNITY		
Bacillus sp.	MG645166.1	99
Providencia sp.	MH718832.1	99
Pseudomonas aeruginosa	CP033832.1	99
Pseudomonas (nitroreducens)	LN995712.1	98
Pseudomonas sp.	HM755482.1	98
C_T COMMUNITY		
Chryseobacterium rhizoplanae	LN995706.1	99
Luteimonas (terrae)	MG262451.1	98
Pseudomonas (aeruginosa)	MK073021.1	98
Pseudomonas nitroreducens	FJ588866.1	99
Pseudomonas sp.	JQ394930.1	99
Uncultured Pseudomonas sp.	AB700606.1	99

The changes observed in the abundance of certain taxonomic groups may be due to their higher affinity for the new substrates, which allows them to grow and spread more rapidly, altering the microbial community's taxonomic structure.

Undoubtedly due to the C_X and C_T communities' previous acclimation processes, there were differences in their main taxonomic groups' biodiversity. The C_X community was acclimated for a long time exclusively on diazinon. In contrast, the C_T community was originated from a mixture of three communities independently acclimated to diazinon, ethyl chlorpyrifos, and bifenthrin. Despite their taxonomic differences, both MCs were functionally efficient in degrading the mixture of the three insecticides.

The quasi-identical kinetic behavior of both communities, including the transient accumulation of catabolic by-products derived from ethyl chlorpyrifos' degradation, reflected an almost immediate functional convergence, revealing their high plasticity. Thus, it was demonstrated that under fluctuating environmental situations, the catabolic versatility of a microbial consortium could ensure the long-term operation of a permeable reactive biobarrier.

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