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Exploring the potential enzymatic bioremediation of vermicompost through pesticide-detoxifying carboxylesterases

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ABSTRACT

Vermicompost is a known biofertilizer of potential use in soil bioremediation. This study was undertaken to explore the capacity of grape marc-derived vermicompost to inactivate methyl carbamate (MC) and organophosphorus (OP) pesticides via exploring the carboxylesterase (CE) activity level and its response to pesticide exposure. We first optimized the method for enzyme activity assay comparing the CE activity in two contrasting homogenization procedures (30-min mixing and mortar grinding). Thereafter, we assessed the sensitivity of the enzyme by both *in vitro* and vermicompost incubation trials with selected pesticides. The main findings can be summarized as follows: i) grinding the vermicompost in water (2% w/v) yielded maximum enzyme activity; ii) at concentrations around 10^{-4} M, highly toxic oxygen-analog metabolites of OPs strongly inhibited the CE activity (76–93% inhibition), but MC did not inhibit the enzyme activity; iii) liquid vermicompost was able to degrade chlorpyrifos and inactivate its highly toxic metabolite chlorpyrifos-oxon. Our results suggest that liquid vermicompost is the most appropriate preparation to increase the enzymatic potential of vermicompost in pesticide-contaminated soils.

1. Introduction

Vermicompost is a finely divided peatlike material produced from organic wastes in a mesophilic bio-oxidative process in which microorganisms and earthworms cooperate in a functional role (Domínguez et al., 2017). This material is an excellent fertilizer which ultimately improves soil quality and plant growth due to characteristics such as the low C:N ratio, high content of humic substances, high moisture-holding capacity and high porosity (Bhat et al., 2018). In the last decade, vermicompost technology has expanded to seek for its soil bioremediation potential. The addition of vermicompost to soils contaminated by organic pollutants can be considered of potential value for bioremediation via biostimulation (i.e., the addition of organic matter and nutrients that stimulates potential native microbial degraders) and bioaugmentation (i.e., the inoculation of contaminated soils with non-native microorganisms with the potential capacity to break down pollutants) (Megharaj et al., 2011; Morillo and Villaverde, 2017). Indeed, some studies have demonstrated that vermicompost accelerates biodegradation of environmental contaminants. For example, addition of olive cake-derived vermicompost to soils contaminated by imidacloprid favored dissipation of the neonicotinoid insecticide, increased soil microbial activity and abundance of bacterial communities (Castillo Diaz et al., 2017). Likewise, this type of vermicompost mixed with olive tree prunings and soil significantly increased the degradation rates of the pharmaceuticals diclofenac and ibuprofen, and the personal care product triclosan respect to those in both soil and sterilized mixture (Delgado-Moreno et al., 2019). Furthermore, vermicompost is also able to immobilize pesticides because of its high organic matter content. Winery-distillery vermicomposts reduced the availability of diuron in sandy loam soil with a low organic carbon content, thus decreasing the leaching potential of this systemic herbicide (Fernández-Bayo et al., 2008, 2015). The high organic carbon and lignin content of these vermicomposts account for its high pesticide sorption capacity (Fernández-Bayo et al., 2009). Despite these studies, a more detailed examination of the role of extracellular enzymes in contaminant degradation is needed to clarify the precise molecular mechanisms underlying vermicompost-assisted bioremediation.

Oxidoreductase and hydrolase enzymes are able to breakdown a wide range of environmental contaminants (Gianfreda and Rao, 2008). Indeed, organic contaminant such as polycyclic aromatic hydrocarbons, phenols, amines, organochlorine and organophosphorus pesticides, and dyes can be degraded by laccases, peroxidases, tyrosinase and esterases, among other enzymes (Rao et al., 2014; Gianfreda et al., 2016). Within the group of pollutant-detoxifying enzymes, carboxylesterases (CEs) are

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serine hydrolases of promising application in the bioremediation of pesticide-contaminated soils. In animals, these esterases hydrolyze synthetic pyrethroids and stoichiometrically bind methyl carbamate (MC) and organophosphorus (OP) pesticides (Sogorb and Vilanova, 2002; Hatfield et al., 2016). The latter two groups of pesticides display a high affinity for the active site of CEs; it is considered a non-catalytic mechanism of pesticide inactivation whereby the pesticide is no longer available to interact with other molecular targets. Although most studies dealing with this "pesticide-enzyme" interaction have been performed in a wide range of organisms (Wheelock et al., 2008), the capacity of soil CE to irreversibly bind OP pesticides has been comparatively less studied despite first study was published in the 1970s (Satyanarayana and Getzin, 1973).Carboxylesterase activity has been also described in several vermicomposts including those derived from winemaking by-products (Domínguez et al., 2017) and spent coffee grounds (Sanchez-Hernandez and Domínguez, 2017). However, the role of these detoxifying enzymes in inactivating both OP and MC pesticides has not been investigated in detail.

With the purpose of increasing the knowledge on the potential role of vermicompost as a remediator substrate for agricultural soils, the hypothesize underlying the present study is that CE activity in grape marc-derived vermicompost inactivates MC and OP pesticides. Therefore, the primary aim was to examine the sensitivity of vermicompost CE activity to selected pesticides, and to evaluate to what extent the esterase activity acts as a molecular scavenger mechanism for pesticides, thus protecting vermicompost microbial activity. However, we first optimized the measurement of vermicompost CE activity, which involved two contrasting homogenization procedures using water. We expect that our findings will improve the current understanding of the potential role of vermicompost in enzymatic bioremediation, thus increasing the environmental applications of vermicompost beyond its well-known function as a soil biofertilizer.

2. Materials and methods

2.1. Vermicomposting grape marc feedstock

The grape marc used as feedstock was obtained from the Terras Gauda winery in O Rosal, Pontevedra (Galicia, NW Spain). The raw material was processed in pilot-scale vermireactors (6 m^2) housed in a greenhouse. The vermireactors contained the epigeic earthworm species *Eisenia andrei*, which reached a population density of about 10,000 individuals m^2. The grape marc was placed in successive layers in the vermireactors as required, for processing by the earthworms. The final vermicompost was analyzed to determine the main physicochemical properties and basal respiration (Domínguez et al., 2017), which are summarized in Table 1.

Table 1
Physicochemical properties and basal respiration of the grape marc-derived vermicompost.

Parameter	Mean ± SD (n = 5)
pH	7.1 ± 0.03
Electrical conductivity (mS cm^{-2})	0.27 ± 0.01
Basal respiration (mg O_2 kg OM^{-1} h^{-1})	68.40 ± 27.11
Organic matter (%)	74.98 ± 0.34
Total carbon (g kg^{-1} dw)	375.96 ± 1.47
Total nitrogen (g kg^{-1} dw)	29.63 ± 0.13
C/N ratio	12.68 ± 0.07
Total phosphorus (g kg^{-1} dw)	8.36 ± 0.32
Total potassium (g kg^{-1} dw)	11.40 ± 0.65
Lignin (g kg^{-1} dw)	323.54 ± 2.36
Cellulose (g kg^{-1} dw)	58.26 ± 10.48
Hemicellulose (g kg^{-1} dw)	30.56 ± 0.54

2.2. Optimizing sample preparation for carboxylesterase activity assay

Carboxylesterase activity was measured in aqueous suspensions of vermicompost prepared as for soil samples (Sanchez-Hernandez and Sandoval, 2017). The vermicompost samples were first air-dried to a water content of 55.4 ± 0.20% (w/w), then we tested two methods of preparing the aqueous suspensions of vermicompost. The first consisted of agitating (orbital shaking at 30 rpm in an Elmi® Intelli-mixer RM-2L apparatus) 1 g of vermicompost in 50 ml of distilled water (dH_2O) for 30 min. The second procedure involved grinding 1 g vermicompost in 5 ml of dH_2O using a ceramic mortar; then the volume of the extract was adjusted to 50 ml with dH_2O. Aliquots (10 ml) of both suspensions were sonicated (37 kHz) for 5 min in an ultrasonic bath (Elmasonic®, Germany) containing cool water to increase the extraction yield of the enzyme. The latter step should cause cell lysis and release the intracellular fraction of CEs (Gianfreda and Ruggiero, 2006). Carboxylesterase activity was measured in all four vermicompost-water suspensions (n = 6 samples per treatment). Additionally, the enzymatic assay was optimized by determining the vermicompost-to-water ratio (2.0–20% w/v), and the concentration of substrate in the reaction medium to guarantee the measurement of enzyme activity at saturating conditions. To this, the resulting dose-response curves were fitted to the hyperbolic Michaelis-Menten model to estimate the kinetic parameters V_{max} and the apparent K_m (app K_m) (Dick et al., 2011).

2.3. Carboxylesterase activity

The CE activity was measured in 96-deep well microplates, following the method previously described by our research group (Sanchez-Hernandez and Sandoval, 2017). Vermicompost-water suspensions were shaken vigorously and a volume of 5 ml of the suspension was removed with a Handystep® Brand repeating pipette. Aliquots (100 µl) of the sample were poured into 96-well deep microplates, containing 380 µl Tris-HCl 0.1 M buffer (pH = 7.0). The enzymatic reaction was initiated by adding 20 µl of the substrate 1-naphthyl butyrate (1-NB) (2 mM, final concentration). The microplates were shaken for 20 min at 25 °C using in an Elmi® Skyline DTS-2 shaker (800 rpm). The plates were centrifuged (636 × *g*, 10 °C and 5 min), and supernatants (150 µl) were transferred to clean 96-well flat bottom microplates. The product formed was revealed by adding 75 µl of 0.1% (w/v) Fast Red ITR salt dissolved in 1:1 (v/v) of SDS 5% (w/v) and Triton X-100 5% (v/v). The microplates were kept in dark for 20 min until completion of color development. Absorbance of the naphthol–Fast Red ITR complex was read at 530 nm using an Asys HiTech UVM340 microplate reader (Asys HiTech Gmbh, Eugendorf, Austria). The enzyme activity, expressed as µmol h^{-1} g^{-1} dry vermicompost, was determined from a calibration curve constructed for 1-naphthol. Standards were prerared in vermicompost-water suspensions to correct for potential sorption of the chromogenic substances by humic substances. Controls (substrate-free) and blanks (vermicompost-free) were used to correct background absorbance and non-enzymatic hydrolysis of the substrates, respectively.

2.4. Sensitivity of carboxylesterase activity to pesticides

Carboxylesterases are able to inactivate OPs, and at less extent MCs, by stoichiometrically binding of the pesticide molecule to the active site of the enzyme (Maxwell, 1992; Sogorb and Vilanova, 2002; Singh, 2014; Hatfield et al., 2016). Thus, the sensitivity of vermicompost CE activity to these types of pesticides was evaluated in four complementary trials that involved *in vitro* and vermicompost incubation trials. First, we compared pesticide-specific sensitivity of CE activity using model MC and OP pesticides. Second, we selected two potent CE-inhibitor pesticides to examine the dose-dependent relationship of the enzyme-pesticide interaction. Third, liquid and solid vermicomposts were spiked with chlorpyrifos to explore CE response and pesticide

breakdown in vermicompost. Chlorpyrifos was selected as model because of its stronger affinity by vermicompost CEs compared to the rest of tested pesticides. Finally, a fourth experiment using liquid vermicompost allowed us to assess the dissipation kinetic of chlorpyrifos and the role of CE activity as a molecular scavenger.

2.4.1. Seeking for potential carboxylesterase inhibitors

This first experiment consisted of an *in vitro* screening trial to determine if vermicompost CE activity was sensitive to both MC and OP pesticides. Aliquots (90 µl) of vermicompost-water suspensions (2% w/v) were incubated individually for 30 min (25 °C and continuous agitation) in the presence of 10 µl of 1.1×10^{-4} M carbofuran, 3.1×10^{-4} M benomyl, 2.1×10^{-4} M ethoprophos, 1.5×10^{-4} M chlorpyrifos-oxon, 1.2×10^{-4} M paraoxon ethyl, 3.2×10^{-4} M chlorfenvinfos, 2.0×10^{-4} M coumaphos-oxon, 1.4×10^{-4} M coumaphos, 2.6×10^{-4} M chlorpyrifos, 4.6×10^{-4} M dimethoate and 4.4×10^{-4} M parathion ethyl (> 98% purity, pesticides purchased from Dr. Ehrenstorfer, Augsburg, Germany). Stock solutions (10^{-2} M) of each pesticide were prepared in ethanol. Control samples (pesticide free) were incubated with 10 µl of ethanol, and no significant change in CE activity was detected relative to vermicompost incubated with 10 µl of dH_2O.

2.4.2. In vitro dose-dependent response of carboxylesterase activity to organophosphorus

Because we found that MCs did not inhibit CE activity, the rest of experiments were performed with OPs. The second experiment examined whether CE activity responded in a dose-dependent manner to OP exposure. To this, samples of vermicompost-water suspensions (90–µl aliquots) were incubated with serial concentrations (10^{-4}–10^{-10} M) of the two potent known soil CE inhibitors: paraoxon ethyl and chlorpyrifos-oxon (Sanchez-Hernandez et al., 2014). The samples were incubated for 30 min, and the residual CE activity was assayed as described above. Inhibition kinetics were determined in four independent replicates, and the pesticide concentration that inhibited 50% of the initial enzyme activity (IC_{50}) was estimated from a non-linear regression model (*see* data analysis).

2.4.3. Testing organophosphorus inactivation by carboxylesterases

The third experiment consisted of spiking both solid vermicompost and vermicompost-liquid suspension with a low (1 µg ml^{-1} in liquid vermicompost or 1 µg g^{-1} solid vermicompost) and a high (10 µg ml^{-1} or 10 µg g^{-1}) concentration of chlorpyrifos and chlorpyrifos-oxon applied individually, and then monitoring the variation in CE activity at two sampling times (t = 4 d and 30 d). The reason for these sampling times was to explore the enzymatic response at short and long-term because of the progressive degradation of chlorpyrifos into chlorpyrifos-oxon, which may increase the inhibition degree of CE activity at longer exposure times (Sanchez-Hernandez and Sandoval, 2017). Samples of vermicompost-liquid suspensions (n = 4 replicates per treatment) were incubated in the presence of the pesticide using 50-ml centrifuge tubes, whereas incubation of solid vermicomposts (n = 4 replicates per treatment) with the pesticides were performed in Petri dishes. The pesticide solutions were directly added to the samples of vermicompost-liquid suspensions, whereas the solid vermicomposts were spiked with the aid of a pipette to distribute the pesticide on the surface of vermicompost, and then mixed with a spatula. All treatments were maintained at 20 °C in darkness. Samples of both liquid and solid vermicomposts were removed after 4 and 30 days to measure CE activity and to determine the concentration of the pesticides and the main metabolite 3,5,6-trichloro-2-pyridinol (3,5,6–TCP, > 98%, purchased from Dr. Ehrenstorfer, Augsburg, Germany).

2.4.4. Chlorpyrifos dissipation in vermicompost

We examined the degradation kinetics of chlorpyrifos in vermicompost-liquid suspensions. Samples (50 ml, n = 4) were spiked with 25 µg ml^{-1} chlorpyrifos, whereas controls received an equal volume of dH_2O. Spiked vermicomposts were incubated at 20 °C in darkness, and the concentration of chlorpyrifos and its metabolites (chlorpyrifos-oxon and 3,5,6-TCP) monitored for 39 days. The rate of chlorpyrifos dissipation was quantified using a first-order exponential decay model (*see* data analysis). At the end of the experiment, CE activity was measured to confirm the role of this enzyme as bioscavenger for OP pesticides.

2.5. Determination of pesticide residues in vermicompost

Concentrations of chlorpyrifos, chlorpyrifos-oxon and 3,5,6-TCP were determined following the QuEChERS procedure (Quick, Easy, Cheap, Effective, Rugged and Safe) outlined in AOAC official method 2007.01 (Lehotay et al., 2007), with the modifications proposed by Asensio-Ramos et al. (2010). Pesticides were analyzed using an HPLC-DAD system. The analytes were separated on an Agilent Eclipse Plus LC-18 column (0.46 × 150 mm, 5 µm particle size) at a flow rate of 0.8 ml min^{-1} and with the following solvent program: 70% acetonitrile (solvent A)/30% H_2O (solvent B) at t = 0 min, increased to 100% A in 8 min and maintained for 2 min, then decreased to 70% A in 1 min, and maintained for 4 min for equilibration. The detector was set to 290 nm (bandwidth = 8, reference wavelength = 360 nm). The average recovery rates of analytes from spiked samples was > 90%. Parathion was used as the internal standard.

2.6. Data analysis

Differences in CE activity between treatments were determined using the non-parametric Kruskall-Wallis test for independent samples, followed by the post hoc Mann-Whitney U test. Dose-response relationships between vermicompost CE activity and the concentrations of pesticide (paraoxon ethyl and chlorpyrifos-oxon) were fitted to the non-linear regression model:

$$y = min + \frac{(max - min)}{1 + \left(\frac{x}{IC_{50}}\right)^{-Hillslope}}$$

where y is the percentage of residual CE activity relative to controls, min and max are the enzyme response to the highest and the lowest concentrations of the pesticide, respectively, x is the logarithmic form of the pesticide concentration, *Hillslope* describes the steepness of the non-linear regression, and IC_{50} is the concentration of the pesticide that causes a 50% decrease of the initial enzyme activity (Motulsky and Christopoulos, 2003).

Dissipation of chlorpyrifos in vermicompost-spiked samples was assessed by an exponential decay model (Hernández-Soriano et al., 2009):

$$C = C_0 \times e^{-kt}$$

where C is the concentration of chlorpyrifos (µg ml^{-1}) at time t after spiking, C_0 is the initial concentration of the pesticide (t = 0d), and k (d^{-1}) is the degradation rate constant.The half-life ($t_{½}$) time of chlorpyrifos was obtained from the equation:

$$t_{1/2} = \frac{\ln 2}{k}$$

3. Results and discussion

3.1. Optimizing carboxylesterase measurements in vermicompost

We measured vermicompost CE activity in aqueous suspensions prepared using two homogenization methods: 30-min mixing water-vermicompost suspensions (Deng et al., 2013), and grinding of vermicompost in water using a mortar. Results of this preliminary experiment clearly showed that CE activity was one order of magnitude higher in

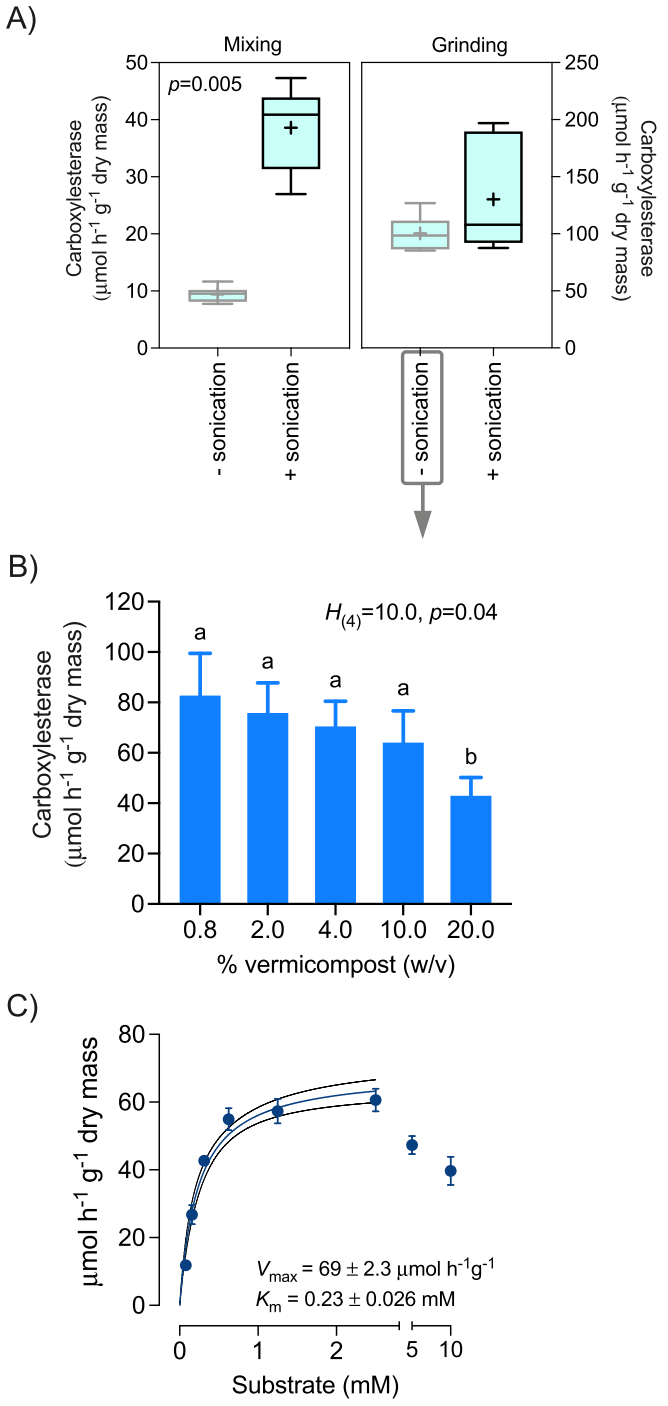

Fig. 1. Optimization of vermicompost carboxylesterase activity. A) Effect of homogenization procedure (mixing and grinding) of vermicompost, followed by a sonication step (37 kHz, 5 min), on carboxylesterase activity. Tukey box plots indicate the median, the 25th and 75th percentiles (box edges), the range (whiskers), and the mean ("+"). B) Variation in the mean (± SD, n = 4) enzyme activity with the vermicompost-to-water ratio (grinding procedure). Different letters indicate significant differences between treatments ($p < 0.05$, post hoc Mann-Whitney test). C) Effect of the substrate (1-naphthyl butyrate) concentration on carboxylesterase activity (mean ± SD, n = 4) measured in vermicompost-water suspension (2%, w/v) obtained by grinding of vermicompost. The kinetic parameters V_{max} and the app K_m were estimated from the Michaelis-Menten hyperbolic model ($r^2 = 0.94$, $p < 0.001$).

the slurry obtained after grinding vermicompost than in the regular aqueous suspension of vermicompost (Fig. 1A). Moreover, the additional step of sonication caused a significant increase ($p = 0.005$, Mann-Whitney test) of the esterase activity only in those samples prepared according to the regular procedure of mixing vermicompost with water, thus confirming that this sample preparation (i.e., 30-min mixing vermicompost with water) underestimated vermicompost CE activity. Likewise, sonication step did not alter CE activity in vermicompost-liquid suspension obtained by sample grinding ($p = 0.31$, Mann-Whitney test), so this homogenization procedure was considered as the most appropriate for measuring maximum potential CE activity in vermicompost.

We also measured the CE activity in vermicompost-liquid suspensions prepared at different ratios of vermicompost-to-water (Fig. 1B). The enzyme activity decreased gradually as the proportion of vermicompost in the aqueous suspension increased ($H_{(4)} = 10.0$, $p = 0.04$), and it was significantly lower at 20% (w/v) vermicompost than for the other proportions ($p < 0.05$, Mann-Whitney test). This concentration-dependent difference suggested that the high organic matter content of vermicompost may interfere with the interaction between the enzyme and the substrate. In fact, measurement of soil enzyme activity is significantly affected not only by the location of enzymes in the soil matrix, but also by the physicochemical properties of substrates used in the enzymatic assay that favor their binding to soil organic matter (Gianfreda et al., 2011). In light of these preliminary results, we opted to prepare sample suspensions by grinding the vermicompost in water (2% w/v).

Measurement of enzyme activities in vermicompost involves the use of traditional methods of determining soil enzyme activities. Accordingly, vermicompost sample is added to the reaction medium containing the appropriate buffer solution and substrate (and cofactors). Using this approach, various studies have investigated the dynamic of selected enzyme activities during vermicomposting (Huang et al., 2014; Usmani et al., 2018) and the subsequent maturation (earthworm-free) phases (Castillo et al., 2013; Alidadi et al., 2016). Other studies have examined feedstock-dependent microbiological and biochemical properties in relation to the capacity of vermicompost to degrade pesticides in soil (Fernández-Gómez et al., 2011). Yet, others have considered the role of specific enzymes in nutrient mineralization in vermicompost (Ghosh et al., 2018) or as complementary biological traits in assessing the quality of vermicompost (Usmani et al., 2018). However, the extremely high organic matter content of vermicompost might hinder the availability of the substrate to the active site of the enzyme as above discussed (Gianfreda et al., 2011). In addition, the active site of extracellular enzymes may also be inaccessible in this organic matter-rich environment. In our study, the procedure to prepare vermicompost-liquid suspension provided a higher enzyme activity than regular procedures, so grinding of vermicompost in water is highly recommended to measure potential enzyme activity.

We also determined the app K_m and V_{max} of vermicompost CE activity, which is needed to optimize the 1-NB concentration suitable to run the enzymatic assays at saturatingsubstrate concentrations (Fig. 1C). This is an essential step to ensure that V_{max} is being measured, which allows that differences in enzyme activity is accurately detected between contrasting treatments (Dick et al., 2011; German et al., 2011). We also observed that high substrate concentrations (> 3 mM) inhibited the enzyme activity, so we found that 2 mM was the optimum 1-NB concentration for achieving maximum hydrolytic rate avoiding enzyme inhibition by excess of substrate.

3.2. *In vitro sensitivity of vermicompost carboxylesterases to pesticides*

In vitro trials revealed that the OP pesticides parathion-ethyl, chlorpyrifos, chlorfenvinfos, coumaphos-oxon, paraoxon-ethyl and chlorpyrifos-oxon significantly inhibited CE activity (43–93% of that in controls) (Fig. 2A). However, MCs and the OPs coumaphos, dimethoate, and ethoprophos did not alter the enzyme activity at concentrations around 10^{-4} M. These findings are in agreement with data in the literature. For example, soil CE activity was more sensitive to inhibition

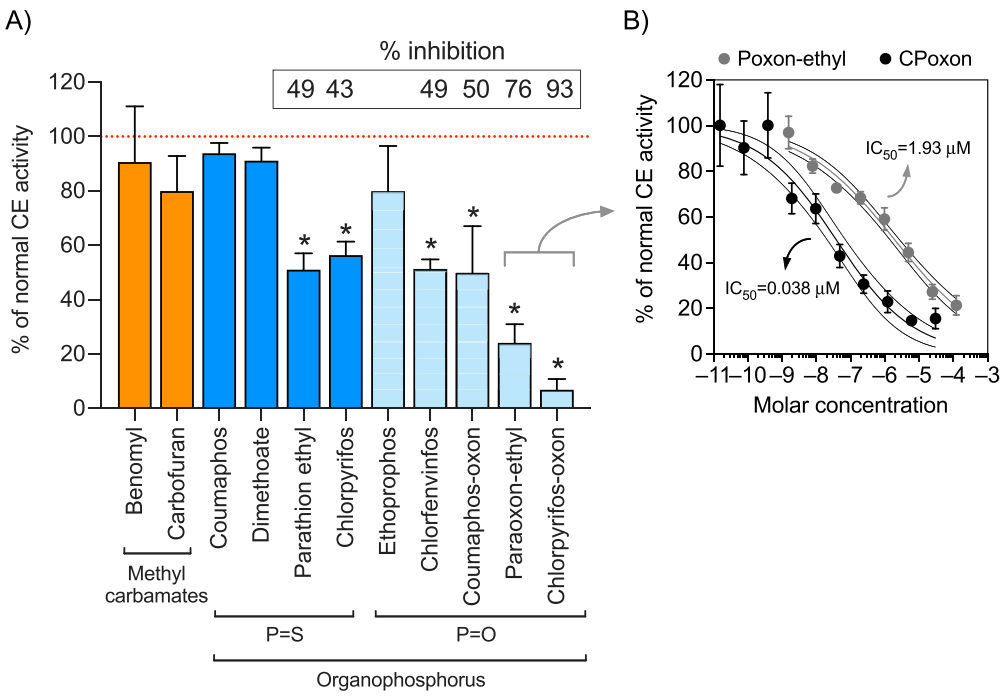

Fig. 2. Response of vermicompost carboxylesterase (CE) activity to pesticides. A) Inhibition of the enzyme activity by 10^{-4} M of selected methyl carbamates and organophosphorus pesticides [phosphorothioate-type pesticide (P═S) and oxon-analog metabolites (P═O)]. Values shown above the bars denote the percentage of enzyme inhibition relative to control activity (red dotted line). Bars represent the mean (± SD, n = 4) enzyme activity. *$p < 0.05$, Mann-Whitney test. B) Dose-response curves for vermicompost CE activity to paraoxon ethyl (Poxon-ethyl) and chlorpyrifos-oxon (CPoxon) exposure. Symbols represent the mean values and standard deviation for four independent assays, and the bands show the 95% confidence interval of the non-linear regression curves. Median inhibition concentrations (IC_{50}) were obtained from the non-linear model (*see* data analysis section for details). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by paraoxon-methyl than carbaryl (Sanchez-Hernandez et al., 2014). Likewise, the complex formed between the active site of the enzyme and MCs is less stable than that formed with OPs, so MCs are generally considered weak CE inhibitors (Sogorb and Vilanova, 2002; Wheelock et al., 2008). It is well known that phosphorothionate-type OP pesticides gain affinity for the active site of CEs after an oxidative desulfuration reaction that consists in replacing the sulfur bound to the phosphorus atom (P═S) of the OP molecule by oxygen (P═O). This bioactivation reaction occurs in organisms via cytochrome P450-dependent monooxygenases, and in the environment via oxidation reaction (Racke, 1993; Chambers et al., 2010). Accordingly, the highest percentages of CE inhibition found in our study were observed with the oxon-analog metabolites (P═O) of the OP pesticides (Fig. 2A). However, contrary to our initial expectation, CE activity was also sensitive to parathion-ethyl and chlorpyrifos. Sanchez-Hernandez and Sandoval (2017) also detected significant inhibition of soil CE activity by chlorpyrifos (IC_{50} = 121–228 µM), and it was suggested that soil phenol oxidases and peroxidases could account for chlorpyrifos bioactivation. This explanation may be feasible for vermicompost as well because of the occurrence of these oxidoreductase enzymes in vermicompost (Sanchez-Hernandez and Domínguez, 2017).

We selected the strongest CE inhibitors paraoxon-ethyl and chlorpyrifos-oxon to examine dose-dependent response of the vermicompost esterase activity. Both pesticides caused a sigmoid-type response that fitted an exponential decay model (r^2 = 0.91–0.95, $p < 0.001$). The IC_{50} values (mean ± SEM) were 1.93 ± 0.3 and 0.038 ± 0.011 µM for respectively paraoxon-ethyl and chlorpyrifos-oxon (Fig. 2B). According to these findings, chlorpyrifos-oxon was the most potent inhibitor of vermicompost CE activity.

3.3. Response of carboxylesterase activity in chlorpyrifos-spiked vermicomposts

Fig. 3 shows the response of vermicompost CE activity to chlorpyrifos and chlorpyrifos-oxon exposure. Both pesticides caused a significant inhibition of the enzyme activity in the vermicompost-liquid suspension after 4 d ($H_{(4)}$ = 17.6, p = 0.001) and 30 d ($H_{(4)}$ = 17.0, p = 0.002) of incubation, which corroborated the *in vitro* outcomes (Fig. 2A). Indeed, chlorpyrifos-oxon inhibited CE activity most strongly, although the degree of inhibition decreased after 30 d. However, the impact of both pesticides was lower in the solid vermicompost than in the liquid vermicompost, and it was not related to pesticide concentration. These variations in the CE response are probably explained by the physicochemical properties of the pesticides. The estimated values of the soil adsorption coefficient (Log K_{OC}) for chlorpyrifos and chlorpyrifos-oxon are respectively 3.83 and 2.22, whereas the estimated water solubility (25 °C) is 0.36 mg l^{-1} for chlopyrifos and 25.97 mg l^{-1} for chlorpyrifos-oxon (data taken from USEPA EPISuite™ in ChemSpider, www.chemspider.com). These data suggest a lower capacity of chlorpyrifos-oxon to bind to the organic matter respect to that of chlorpyrifos, which together to the its higher water solubility would facilitate its interaction with the active site of the enzyme. This assumption would account for the greater inhibition of CE activity by chlorpyrifos-oxon in the liquid vermicompost than in the solid form.

The measurement of chlorpyrifos, chlorpyrifos-oxon, and 3,5,6-TCP led to the following findings (Fig. 4): i) the liquid vermicompost degraded chlorpyrifos to a greater extent than the solid vermicompost because the concentration of this pesticides was significantly ($p < 0.05$) lower, irrespectively of the spiking concentrations. This was expected as chlorpyrifos is quickly hydrolyzed in water (Racke et al., 1996) and, on the other hand, the higher organic matter content of the solid vermicompost respect to liquid vermicompost would contribute to a limited availability to be degradated. ii) The main metabolite 3,5,6-TCP was preferentially detected in the liquid vermicompost spiked with chlorpyrifos-oxon. This finding can be attributed to the fact that the primary route of chlorpyrifos-oxon decomposition is hydrolysis to yield 3,5,6-TCP. The detection of low or undetectable concentrations of this metabolite in the vermicompost spiked with the low concentration of chlorpyrifos confirms the slow degradation of this molecule in the solid vermicompost. Chlorpyrifos-oxon was not detected in all samples, suggesting a rapid degradation to form 3,5,6-TCP (Racke, 1993; Racke et al., 1996) or binding to the active site of CEs as indicated by inhibition of the esterase activity (Fig. 3).

The final experiment was performed to explore the degradation kinetics of chlorpyrifos in the liquid vermicompost as well as the rate of 3,5,6-TCP formation. The chlorpyrifos degradation rate fitted a first-order model (r^2 = 0.898, $p < 0.001$) and the estimated half life time was 6.3 d (3.60–10.9 d, 95% confidence interval) (Fig. 5A). The

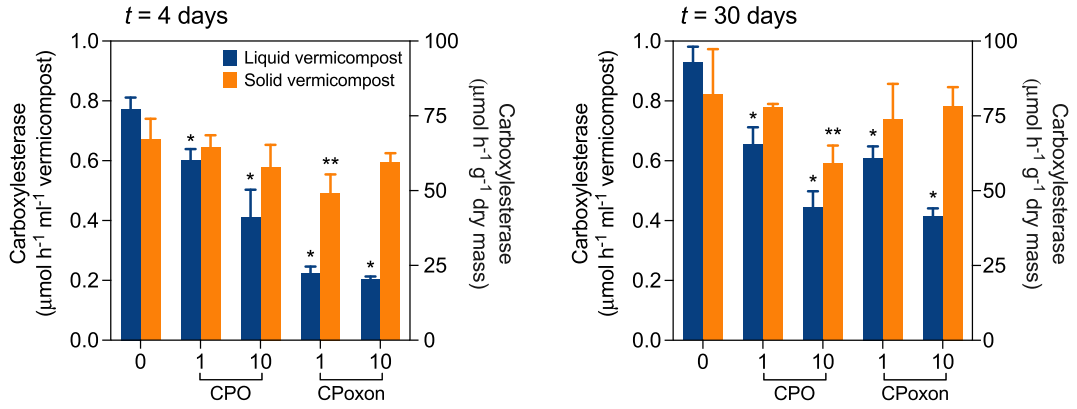

Fig. 3. Response of carboxylesterase activity in solid and liquid vermicomposts treated to low (1 μg ml^{-1} in liquid vermicompost or 1 μg g^{-1} in solid vermicompost) and high (10 μg ml^{-1} or 10 μg g^{-1}) concentrations of chlorpyrifos (CPO) or chlorpyrifos-oxon (CPoxon). Bars represent the mean enzyme activity (± SD, n = 4). *p < 0.05, Mann-Whitney test.

metabolite 3,5,6-TCP appeared in the liquid vermicompost as the parent compound was degraded. These data confirm those of the previous experiment (Fig. 4) as well as the role of CE activity in inactivating the highly toxic metabolite chlorpyrifos-oxon. Indeed, CE activity was found strongly inhibited (60% of controls) at the end of this trial (Fig. 5B). Similar results obtained by our research group for liquid vermicompost obtained from spent coffee ground-derived vermicompost (Sanchez-Hernandez and Domínguez, 2017), suggested that CE may act as bioscavenger for chlorpyrifos-oxon, thus reducing the toxicity of the pesticide to microorganisms. This enzymatic bioremediation potential seems to be more efficient with liquid vermicompost, which suggests this form of preparation (grinding of vermicompost) for field application.

4. Conclusions

The two main conclusions of this study are as follows: i) grape marc-derived vermicompost contains an important fraction of extracellular CE activity that can inactivate OP pesticides; ii) the enzymatic bioremediating capacity of vermicompost is enhanced when solid vermicompost is homogenized (grinding) in water, probably because dispersion of extracellular CE enzymes and microorganisms in the aqueous phase favors pesticide degradation or inactivation. These findings extend the possibility of the environmental use of vermicompost to the bioremediation of pesticide-contaminated soils, and suggest its application as liquid preparation.

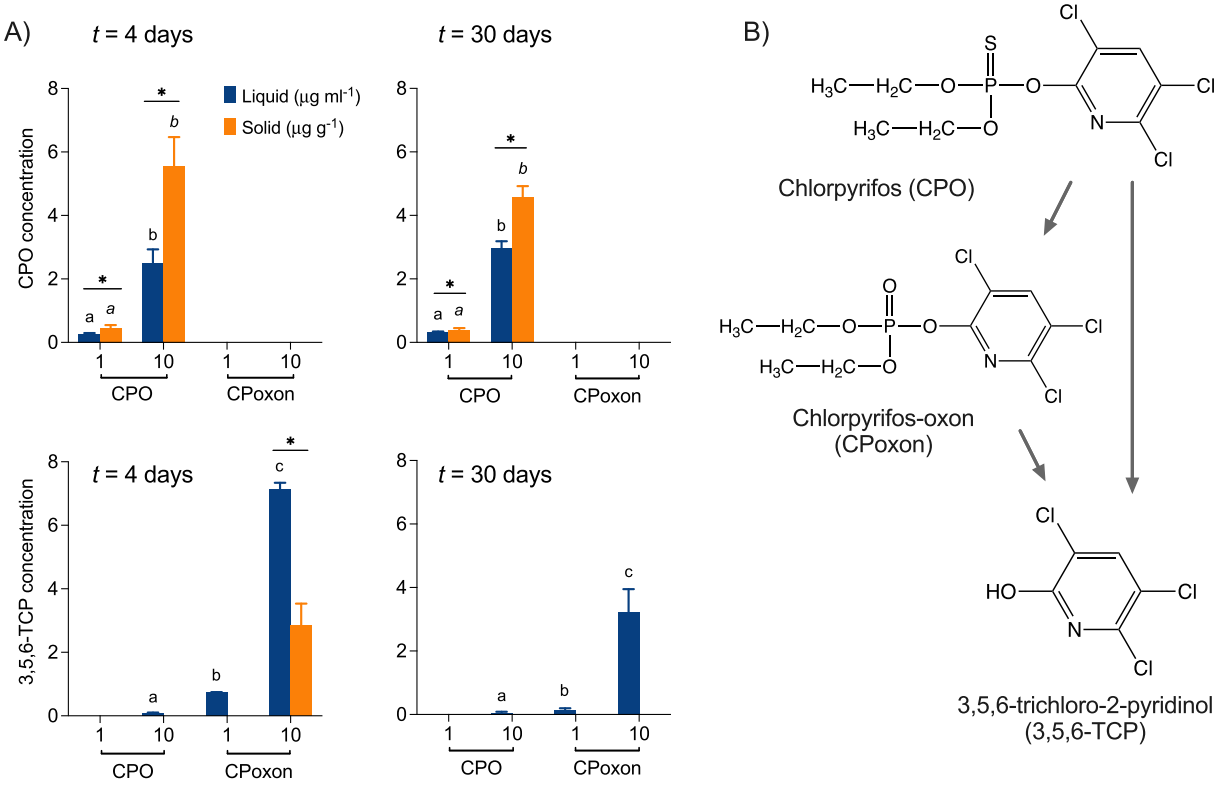

Fig. 4. A) Mean (± SD, n = 4) concentrations of chlorpyrifos (CPO) and 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) after incubation for 4 and 30 d of liquid and solid vermicomposts with 1 and 10 μg ml^{-1} (or μg g^{-1}) of CPO and chlorpyrifos-oxon (CPoxon). B) Main pathways of chlorpyrifos degradation (adapted from Racke, 1993).

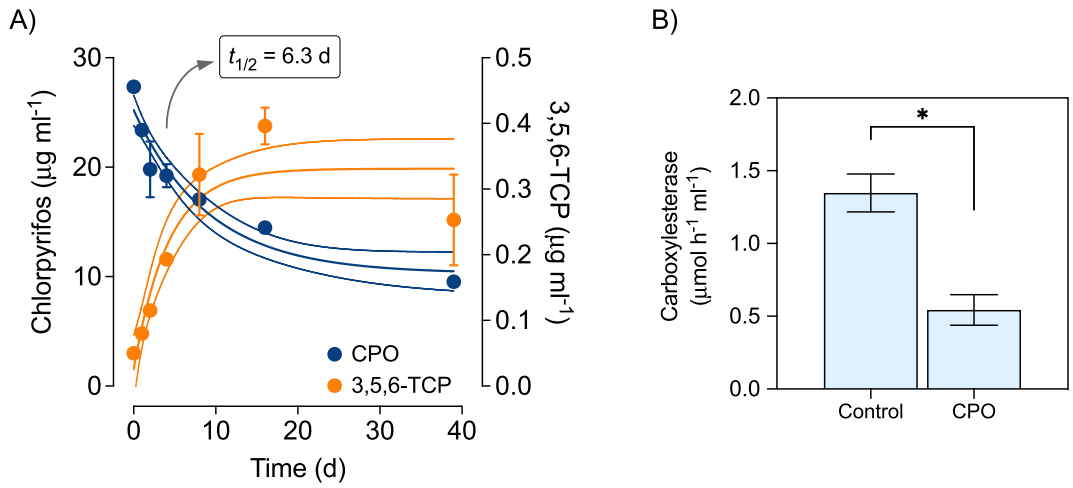

Fig. 5. A) Degradation kinetic of chlorpyrifos (CPO) and formation rate of the metabolite 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) in liquid vermicompost (2% w/v) spiked with 25 µg ml^{-1} CPO. The time for the concentration of CPO to decrease to half of the initial concentration ($t_{1/2}$) was estimated to be 6.3 d. Dotted lines denote the 95% confidence interval. B) Mean (± SD, n = 4) carboxylesterase activity in the chlorpyrifos-spiked liquid vermicompost after incubation for 39 d *$p < 0.05$, Mann-Whitney test.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109586.

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