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Enzymatic degradation of organophosphorus insecticides decreases toxicity in planarians and enhances survival

Laetitia Poirier¹, Lucile Brun¹, Pauline Jacquet¹, Catherine Lepolard¹, Nicholas Armstrong¹, Cédric Torre¹, David Daudé², Eric Ghigo¹ & Eric Chabrière¹

Organophosphorus insecticides (OPs) are toxic compounds used for agricultural purposes and responsible for severe types of contamination worldwide. OPs may also induce chronic deleterious effects and developmental disruption. Finding remediation strategies is a major concern to diminish their impact on environment and human health. Enzymes have emerged as a promising eco-friendly route for decontaminating OPs. The enzyme *SsoPox* from the archaea *Sulfolobus solfataricus* has been particularly studied, considering both its tremendous stability and phosphotriesterase activity. However, the toxicity of the degradation products generated through enzyme hydrolysis has been poorly investigated. To address both neurotoxicity and developmental perturbation, freshwater planarians from Platyhelminthes were considered to evaluate the impact of OP and degradation product exposure. Planarians have a large proportion of stem cells that give them an unconventional capacity for regeneration. OPs were found to be highly toxic to planarians and enzyme decontamination drastically enhanced survival rate. Although not completely innocuous, the degradation products were found to be less toxic than insecticides and reduced poisoning effects by increasing NOEC values by up to eight-fold. *SsoPox* also limited detrimental consequences on planarian mobility and enabled them to recover a non-exposed type regeneration process suggesting that enzymatic decontamination is a promising alternative to bioremediation.

Organophosphorus pesticides (OPs) are highly toxic chemicals that have been widely developed for agricultural purposes since the 1950s¹. Their widespread use has led to serious contamination worldwide and constitutes a major health and environmental issue². The acute toxicity of OPs is due to their capacity to inhibit acetylcholinesterase, a key enzyme involved in the overall regulation of the central and peripheral nervous system. OPs disrupt nervous signals by deactivating AChE through phosphorylation and irreversible binding to the catalytic serine causing acetylcholine accumulation and cholinergic overstimulation³. Exposure to high doses of OPs is associated with many symptoms including salivation, diaphoresis, emesis, miosis, and tremors that may cause paralysis and death^{4,5}. Furthermore, low dose chronic exposure to OPs may have deleterious effects including impaired neurobehavior performance and may negatively affect human fetal and childhood health^{6,7}. Some reports have also pointed out the possible link between low level chronic exposure to OPs and health problems^{8,9}.

Considering the high toxicity of OPs and their intensive use, finding efficient methods of decontamination is a major concern. Numerous approaches, including chemical, physical and biological methods, have been considered for developing decontamination strategies against OPs. However, these methods usually involve harsh conditions, are not compatible with the decontamination of people, and cannot be considered for large-scale environmental remediation¹⁰. Recently, enzymes have emerged as a promising alternative to smoothly and quickly decontaminate OPs. Of these, the phosphotriesterase-like lactonase *SsoPox* from the archaea *Sulfolobus solfataricus* is an appealing candidate for remediation. This enzyme has been engineered and proven to be highly efficient

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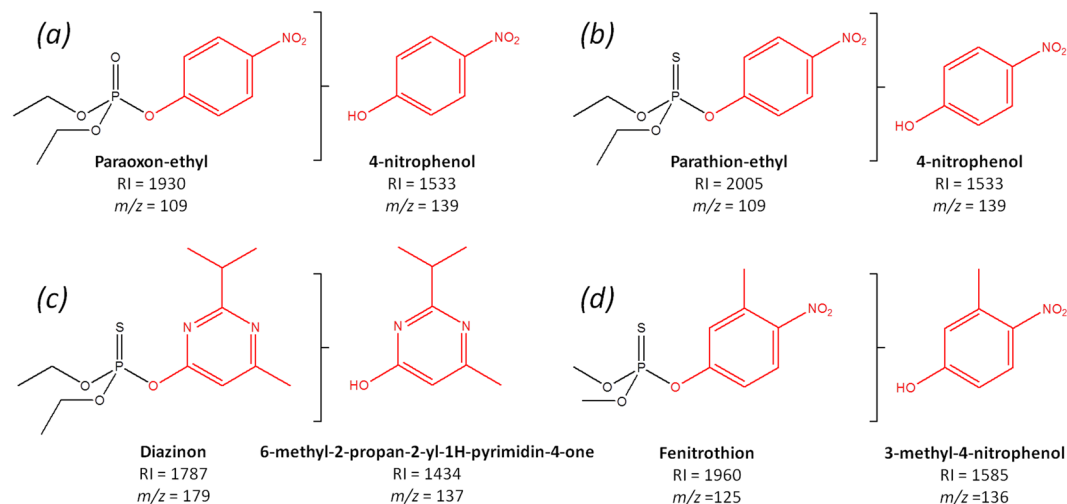


Figure 1. GC/MS characterization of the enzymatically-generated degradation products. RI is the experimental Kovats retention index of the molecule and m/z is the mass-to-charge ratio of the base peak fragment detected for each molecule. The leaving group and the corresponding degradation product are emphasized in red. (a) Paraoxon-ethyl (CAS 311-45-5) led to 4-nitrophenol (CAS 100-02-7); (b) Parathion-ethyl (CAS 56-38-2) led to 4-nitrophenol (CAS 100-02-7); (c) Diazinon (CAS 333-41-5) led to 6-methyl-2-propan-2-yl-1H-pyrimidin-4-one (CAS 2814-20-2); (d) Fenitrothion (CAS 122-14-5) led to 3-methyl-4-nitrophenol (CAS 58864-63-4).

for degrading a broad panel of OPs^{11–14}. This enzyme displays a phosphotriesterase activity that enable to degrade OPs through an hydrolysis reaction leading to two degradation products, a phosphodiester and an alcohol. Due to its extremophilic origin, *SsoPox* is also tremendously robust, including resistance to solvents and detergents¹⁵ and long-term storage or activity over a wide range of temperatures¹⁶. The enzyme is, moreover, highly convenient for immobilization and could be used in filtration devices to treat effluents soiled with OPs^{16–18}. Recently, *SsoPox* was engineered and variants with drastically enhanced phosphotriesterase activity were generated (manuscript in revision). Of these, variant *SsoPox*- α sD6 was of utmost interest and it is this which we considered in this study.

Although enzyme decontamination would decrease the neurotoxicity of OPs, little is known about the toxicity of the generated degradation products and little research effort has been dedicated to their fate. This may require further consideration, as these molecules may affect the environment and non-targeted organisms¹⁹. The lack of a convenient and easy-to-handle *in vivo* model that could be used to assess both neurotoxicity and developmental perturbation may explain the low levels of attention that have been given this topic. Freshwater planarians, belonging to Platyhelminthes, have a large proportion of stem cells that give them an unconventional capacity for regeneration and are, subsequently, highly interesting for developmental studies^{20,21}. Indeed, planarians appear to be particularly relevant for investigating developmental disruption in addition to classic toxicological investigations. Recently, planarians have emerged as an alternative animal model for toxicology and neurotoxicology^{22,23}. *Dugesia japonica*²⁴, *D. gonocephala*²⁵, *D. dorotocephala*²⁶, and *D. tigrina*²⁷ have been shown to be negatively affected by OP exposure. Cholinesterase activity was demonstrated in planarians and their sensitivity to OPs has been highlighted albeit lower than *Homo sapiens* AChE (*Hs*-AChE)²⁴. Moreover, evolutionary considerations have suggested that AChE from Platyhelminthes, including planarian and *Schistosoma*, are probably early cholinesterase related to other lower vertebrates such as the medaka *Oryzias latipes* and the hagfish *Myxine glutinosa*^{28–30}. Such evolutionary trajectory may explain the difference in sensitivity to OP exposure with higher vertebrates for which a gene duplication event lead to a divergence between AChE and butyrylcholinesterase³¹.

Here, we investigated the toxicity of four widely used insecticides, paraoxon-ethyl, parathion-ethyl, diazinon and fenitrothion, which have been widely used in agriculture, as well as their respective degradation products generated by enzymatic hydrolysis with *SsoPox*- α sD6. Mortality, behavior and regeneration were assessed in *Schmidtea mediterranea*. OPs were shown to be toxic to planarians and to affect both mobility and regeneration capacity while the use of a decontaminating enzyme drastically decreased the deleterious effects of OPs. The global toxicity of both pesticides and their corresponding degradation products were thus assessed and the results underlined the strong potential of *SsoPox* for bioremediation.

Results

GC/MS analysis of enzymatically-generated degradation products. *SsoPox* has been previously reported to efficiently degrade a broad spectrum of organophosphorus insecticides. Here, the cleavage site for each substrate was unambiguously assigned. The leaving groups generated through enzyme hydrolysis were characterized by GC/MS and were consistent with the phosphotriesterase mechanism of *SsoPox*. Paraoxon-ethyl and parathion-ethyl hydrolysis led to 4-nitrophenol while diazinon and fenitrothion led to 6-methyl-2-propan-2-yl-1H-pyrimidin-4-one and 3-methyl-4-nitrophenol respectively (Fig. 1).

Phylogeny. Phylogenetic analysis was performed to evaluate the evolutionary trajectory of putative planarian AChE as compared to characterized AChE from other phyla (Fig. 2). AChE sequences from *Schmidtea*

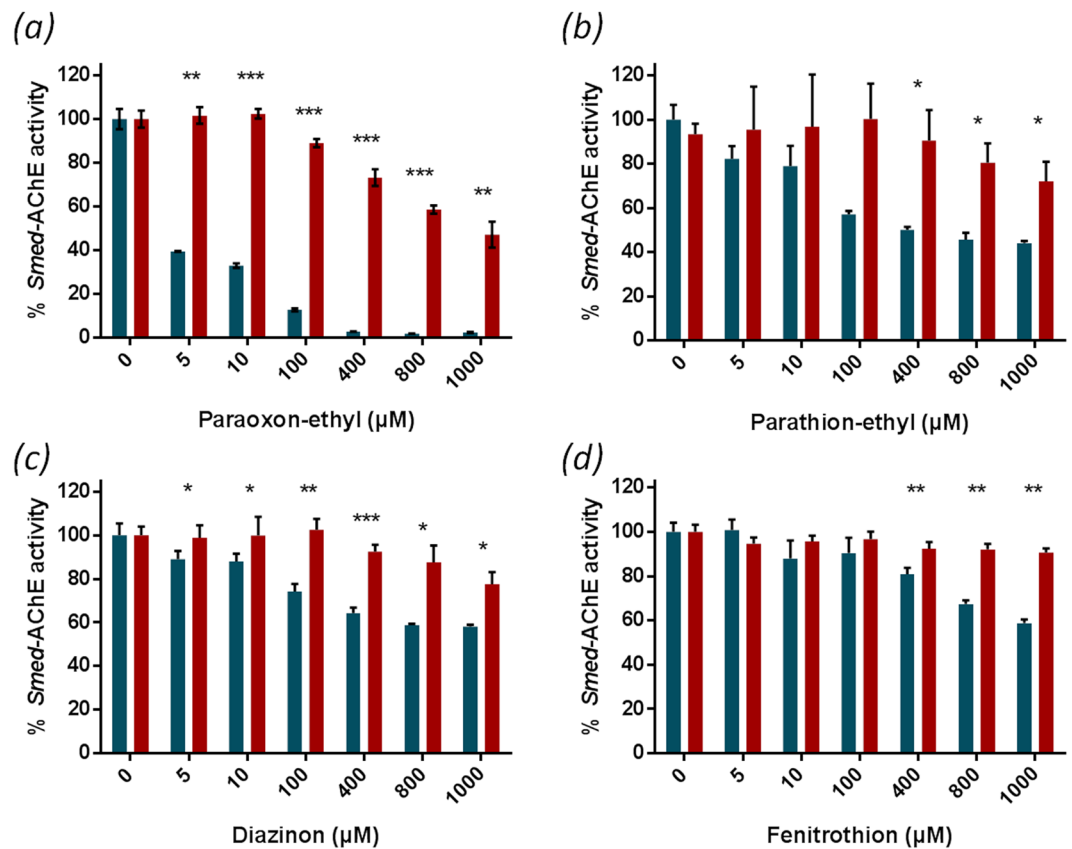


Figure 3. Characterization of Smed-AChE activity in planarian homogenates. Evaluation of Smed-AChE inhibition by insecticides and the associated enzymatically-generated degradation products in planarian homogenates. Blue and red bars represent the inhibition of Smed-AChE by insecticides and their degradation products respectively for paraoxon-ethyl (a), parathion-ethyl (b), diazinon (c) and fenitrothion (d). The values represent the mean \pm SD (standard deviation) of three replicates. Black stars (*) indicate a significant difference between insecticide and degradation product for a given concentration (one star to indicate $p < 0.05$; two stars to indicate $p < 0.005$; three stars to indicate $p < 0.0005$ according to Student's *t*-test).

NOEC values were increased by four and five-fold for fenitrothion and paraoxon-ethyl respectively and by up to eight-fold for both parathion-ethyl and diazinon (Supplementary Table 1).

Effect of OPs on mobility. The impact of insecticides and their associated degradation products on planarian behavior was evaluated (Fig. 5a–e). Planarians were incubated in aqueous solutions containing lethal or sub-lethal concentrations of insecticides. For all insecticides, disruption of mobility was observed. The modifications of mobility were classified into three types: “mobile at normal speed” when planarians moved normally; “immobile” when planarians were prostrate and “non-normally mobile” when planarians were neither normally mobile nor immobile; (Supplementary Figure 2, Supplementary Video 1). Direct visual evaluation of mobility was performed by several observers blinded to the treatment. These observations were reproducible and did not affect the results. The highest concentrations of insecticides led to complete immobility of planarians, followed by death. Low concentrations altered mobility by significantly slowing down the planarian and no or low modification was observed for the lowest 10 μM concentration. After enzymatic degradation of insecticides, planarian mobility was drastically enhanced. Planarians recovered normal behavior for concentrations up to 50 μM , 100 μM and 200 μM for paraoxon-ethyl, diazinon and fenitrothion respectively. For the highest concentrations, enzymatic degradation improved both mobility and survival rate although no complete remission was observed. Mortality was still observed after degradation by the enzyme at concentrations of 200 μM and 600 μM of paraoxon-ethyl and fenitrothion respectively. Ten worms were used for each concentration and the phenotype presented in Fig. 5 was reproducible and was shared by a minimum of 80% of the population. As pesticides were initially prepared in ethanol before dilution in water to reach final concentration, control solutions with the final ethanol concentrations for each condition were performed. We found no effect of ethanol up to the maximum concentration assayed of 0.4%.

Effect on planarian regeneration. Planarians have the capacity to regenerate an entire worm from a tissue fragment. Under normal conditions, regeneration is complete 14–15 days after amputation. The effect of sublethal or partially lethal concentration exposure to insecticides and their degradation products, on planarian regeneration was monitored over 15 days (Fig. 6a–f). Head and tail regeneration were followed from tail and head

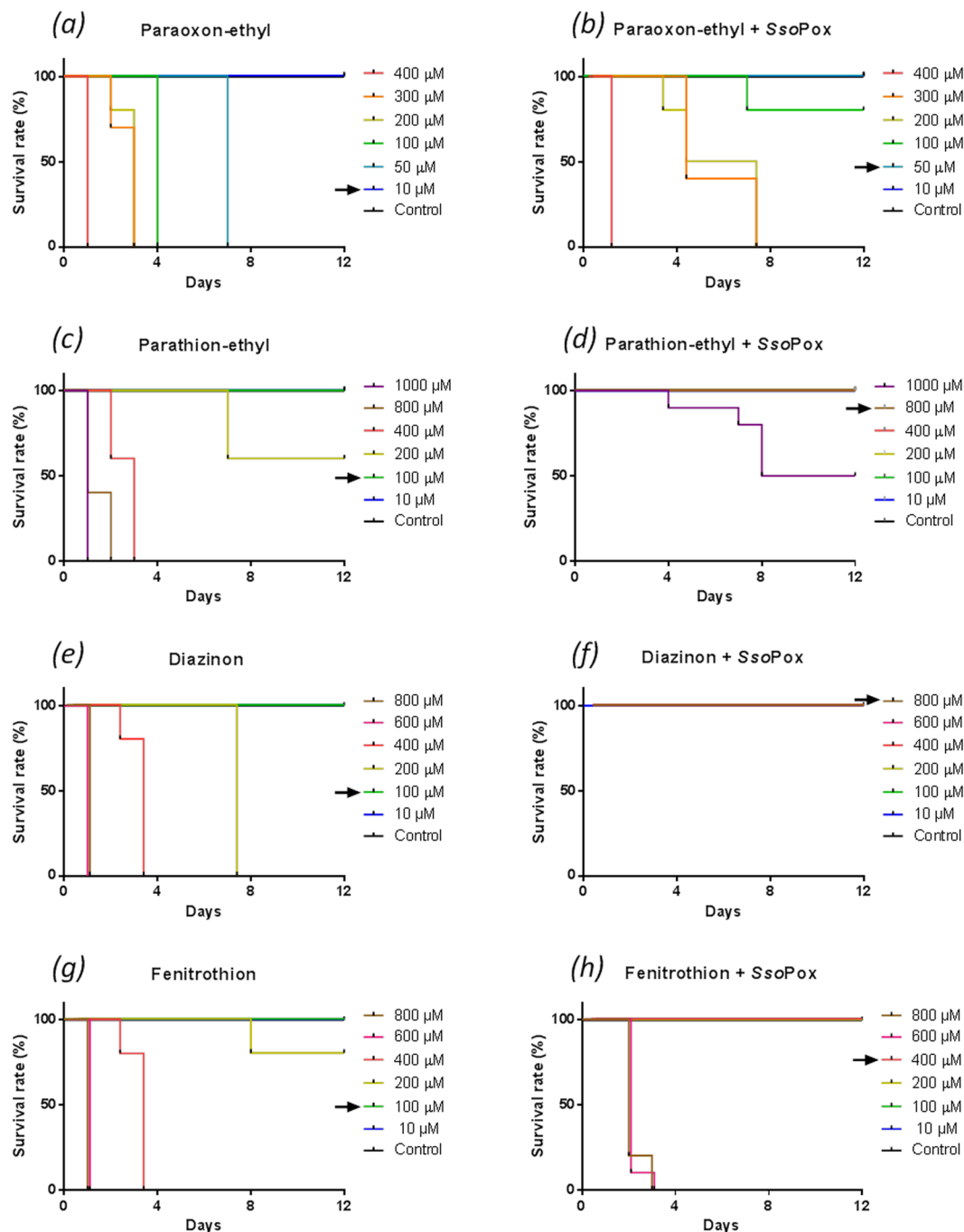


Figure 4. Enzymatic degradation of insecticides increases survival rate in planarians. The toxicity of paraoxon-ethyl, parathion-ethyl, diazinon and fenitrothion before and after degradation by *SsoPox* was evaluated for concentrations ranging from 10 μM up to 1000 μM. The curves represent the survival rates for 10 planarians exposed to pesticides and the enzymatically generated degradation products paraoxon-ethyl (a,b), parathion-ethyl (c,d), diazinon (e,f), fenitrothion (g,h). Arrows indicate NOEC values.

fragments respectively. A visual categorization was used for representing regeneration stages. These criteria were used to characterize the regeneration process and were sufficiently clear to be not dependent on the observer, three different experimentors obtaining the same results.

Insecticides were found to drastically alter head regeneration. Lethal concentrations were decreased down to 100 μM for diazinon and down to 50 μM for parathion-ethyl and fenitrothion as compared to whole planarians. NOEC values were decreased for parathion-ethyl, diazinon and fenitrothion down to 10 μM, 75 μM and 10 μM respectively (Supplementary Table 1). For diazinon and fenitrothion, regeneration progressively improved when the concentration was decreased, while a binary behavior was observed for paraoxon-ethyl and parathion-ethyl at threshold concentration of 50 μM. Impressively, no alteration of the regeneration was observed after the

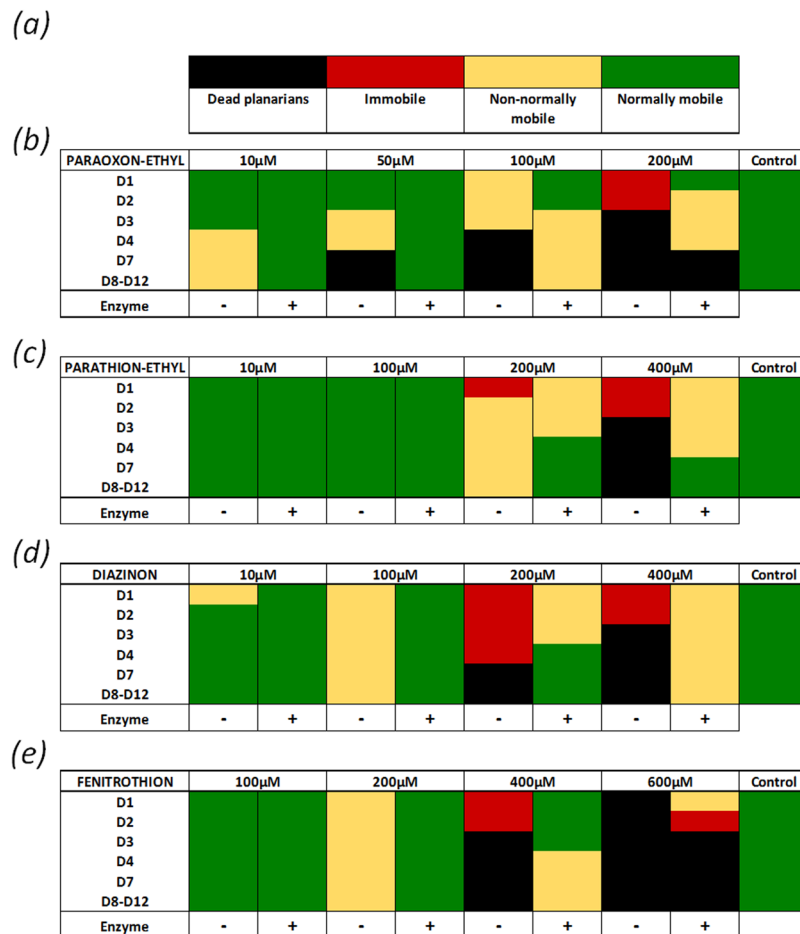


Figure 5. 12 days' incubation of planarians in insecticides and related degradation product solutions and evaluation of the impact on mobility. The average mobility of ten planarians for 12 days (D1 to D12) is presented using a color code describing normal mobility (green), non-normal mobility (yellow), immobility (red) and death (black) (a). (+) and (–) describe the solution with and without enzymatic degradation. Paraoxon-ethyl (b), parathion-ethyl (c), diazinon (d), fenitrothion (e) and the related degradation products were evaluated. As pesticides were initially solubilized in ethanol before dilution in water to reach final concentration, the control solutions represent the maximum final ethanol concentration for each insecticide.

enzymatic decontamination of insecticides. Interestingly, no ectopic growth was observed after one regeneration cycle. Five worms were used for each concentration and the phenotype presented in Fig. 6 was reproducible and was shared by a minimum of 80% of the population. As for mobility and toxicity analyses, no effect of ethanol was observed in the range of the tested concentrations. Very similar results were obtained when studying tail regeneration (Supplementary Figure 3a–f).

Discussion

The toxicity of four widespread insecticides (paraoxon-ethyl, parathion-ethyl, diazinon, fenitrothion) and their corresponding degradation products generated through enzyme hydrolysis was addressed. The cleavage sites for each substrate were unambiguously confirmed by GC/MS analysis and were found to be in accordance with previously reported catalytic mechanisms¹³. OPs are inhibitors of acetylcholinesterase, but little is known about their toxicity after degradation. To tackle this issue, the planarian, an original flatworm from Platyhelminthes was considered, as this model can be used to evaluate both toxicity and regeneration disruption.

Acetylcholinesterase activity of *S. mediterranea* homogenates was confirmed and this activity was further shown to be susceptible to OP exposure. All four insecticides decreased *Smed*-AChE activity after incubation, although paraoxon-ethyl was the most detrimental substrate. Paraoxon-ethyl had a greater impact on *Smed*-AChE activity than parathion-ethyl, suggesting that, like *Hs*-AChE, *Smed*-AChE is significantly affected by OP in the oxon form. Indeed, many studies have demonstrated that oxon insecticides are drastically more toxic than phosphorothionates which are first activated *in vivo* during metabolism by cytochrome P450 monooxygenase^{37–39}. Phylogenetic analysis also showed that predicted AChE from planarians are clustered in a same branch of Platyhelminthes connected to the Chordata phylum, thus confirming that planarians may share an ancient common ancestor with mammals, as pointed out in a previous study²⁴.

Degradation products had a lower impact on *Smed*-AChE activity than insecticides. Degradation of parathion-ethyl, diazinon and fenitrothion led to very low *Smed*-AChE inhibition, underlining their moderate neurotoxicity. Only

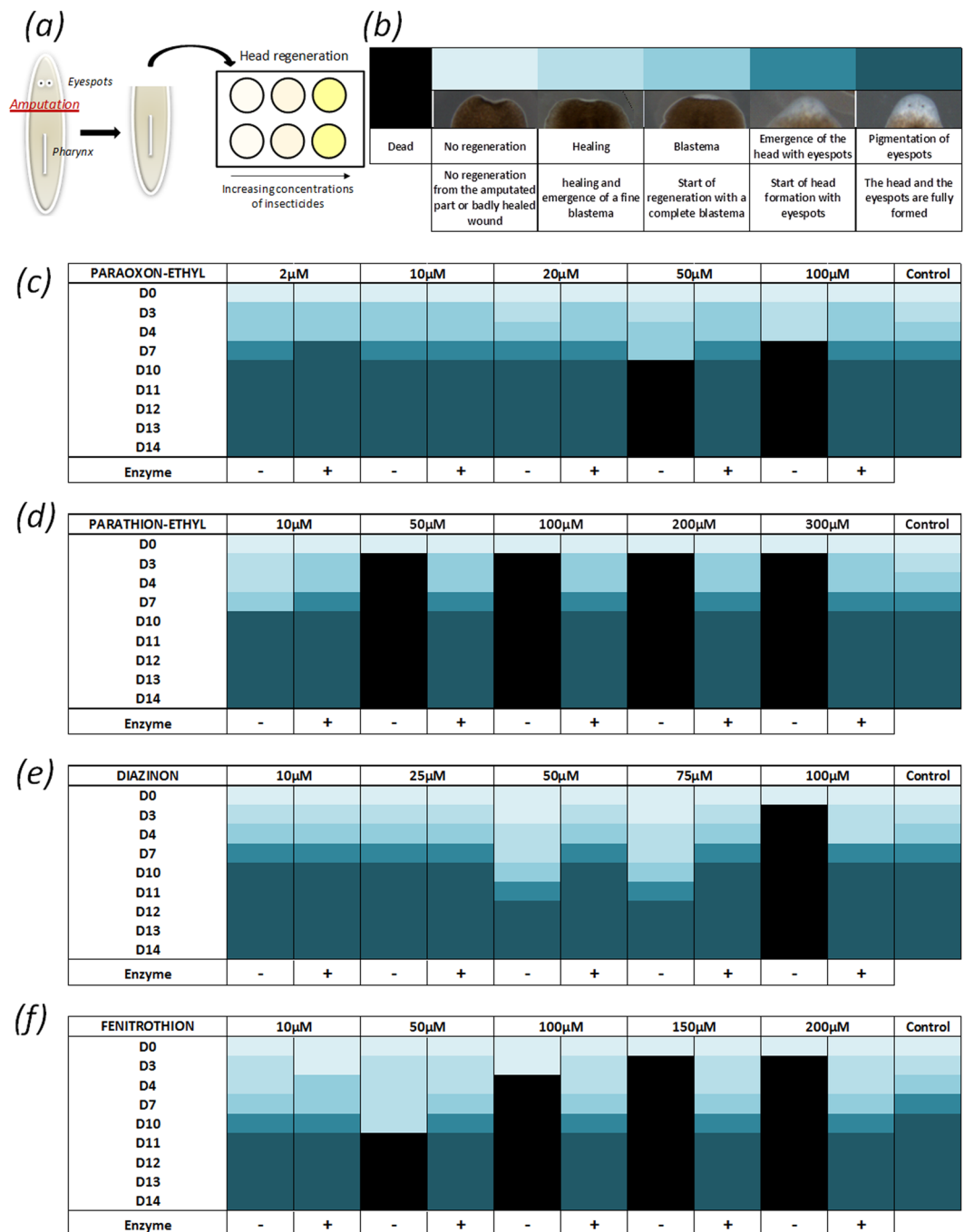


Figure 6. Evaluating the impact of insecticides and their enzymatically-generated degradation products on planarian head regeneration. Five planarians were cut above the pharynx and incubated in insecticide solutions (a). Head regeneration of planarians over 15 days (D0 to D14) is presented using a color code from light to dark blue describing initial to final regeneration stages as presented in figure caption (b). Paraoxon-ethyl (c), parathion-ethyl (d), diazinon (e), fenitrothion (f) and the related degradation products were evaluated. Dead planarians are colored black. (+) and (-) Describe the solution with and without enzymatic degradation. As pesticides were initially solubilized in ethanol before dilution in water to reach final concentration, the control solutions represent the maximum final ethanol concentration for each insecticide.

paraoxon-ethyl degradation products had a significant impact on *Smed*-AChE activity at concentration greater than 400 μM . Because paraoxon-ethyl and parathion-ethyl share one of their two degradation products, namely 4-nitrophenol, it can be safely assumed that the toxic effect observed after paraoxon-ethyl degradation is due to the phosphodiester released, while the phosphorothionate diester analogue, generated through parathion-ethyl degradation, is less toxic. It should be noted that measurements of *Smed*-AChE activity were performed with crude planarian homogenates containing other proteins and metabolites that may also interact with the compounds. Nevertheless, similar inhibitions of AChE were observed when exposing whole planarians in insecticides.

The deleterious effect of OP exposure in whole planarians was further evaluated after incubation in diluted insecticide solution. Insecticide concentrations from 10 μM up to 1 mM were assayed and were in accordance with previous studies. Concentrations up to 800 μM paraoxon-ethyl were used for incubating planarians²⁵, while concentrations ranging from 13 μM up to 57 μM were reported for diazinon, fenitrothion, parathion, chlorpyrifos and malathion^{26,27}. The viability of *D. japonica* for concentrations up to 500 μM was also described and the authors showed that dichlorvos was 100 times more toxic than chlorpyrifos suggesting that toxicity is strongly dependent on the insecticide²². This difference was supposed to be related with the oxon form of dichlorvos which is probably more toxic than thiono insecticides. The toxicity of oxon insecticides was further highlighted in *Djap*-AChE and diazinon oxon was shown to have the highest effect²⁴. In this study we found that paraoxon-ethyl was highly toxic in *S. mediterranea*, causing complete mortality at concentrations down to 50 μM . Thiono insecticides were found to be less toxic, causing complete mortality at concentrations down to 200 μM for diazinon and 400 μM for both parathion-ethyl and fenitrothion. After enzymatic degradation of insecticides, the survival of planarians was drastically enhanced. Diazinon and parathion-ethyl have two distinct leaving groups but generate the same phosphorothionate diester. The differences observed in the toxicity of these compounds after decontamination may thus be strongly ascribable to their variable leaving group. Moreover, because only low impacts of the degradation products on *Smed*-AChE activity were observed, their neurotoxicity appears lower as compared to insecticides.

The effect of insecticides on planarian mobility was also evaluated. At sub-lethal concentrations, paraoxon-ethyl and diazinon induced mobility disruption down to 10 μM , while the observable effects of parathion-ethyl and fenitrothion occurred only at 200 μM . At low but lethal concentrations, the mobility of planarians was strongly altered in the early days of exposure and was followed by death. Enzymatic decontamination of insecticides before exposure of planarians enhanced mobility. For most assayed concentrations, a non-exposed type behavior was recovered and enzyme degradation enhanced the survival rate. However, for the highest concentrations of paraoxon-ethyl and fenitrothion, death still occurred but was delayed in comparison to insecticide-treated samples. These results highlight that the enzyme improved planarian mobility, although for highest assayed concentrations, degradation products may also cause disruption, while only a low impact on *Smed*-AChE activity was observed. Although degradation products induced deleterious effects on mobility from 200 μM for 4-nitrophenol and 6-methyl-2-propan-2-yl-1H-pyrimidin-4-one or 400 μM for 3-methyl-4-nitrophenol their impact on planarian behavior was reduced as compared to insecticides.

To further investigate the toxicity of insecticides and their degradation products, their impact on planarian regeneration was observed. Planarians have the capacity to regenerate an entire flatworm from a tissue fragment thanks to abundant stem cells. Alteration of this capacity by low-level OP exposure was followed by a 15 day long full regeneration cycle. The worms were cut into two, allowing both head and tail regeneration from tail and head fragments respectively. Surprisingly, both fragments appeared to be more sensitive to OP exposure than full worms and were characterized by lower NOEC values, as previously discussed. The lowest concentrations did not induce any delay in regeneration, but increasing insecticide concentration either delayed regeneration or caused mortality. In a few cases, planarians died after one week, although the regeneration process had started. Impressively, for all the conditions that were assayed, enzymatic decontamination improved survival and/or regeneration of planarian fragments. No disruption was observed after paraoxon-ethyl, parathion-ethyl and diazinon degradation and only a slight delay was observed for fenitrothion degradation products. Taken together, these results suggest that insecticides may have a stronger impact on planarian development than their degradation products. However, the degradation products were only found to slightly alter *Smed*-AChE activity in contrast to insecticides, underlining a lower neurotoxicity⁴⁰.

Enzymatic remediation of insecticides appears to be a promising approach to decreasing the deleterious effects induced by OP exposure. *SsoPox*, which is active towards a broad range of OPs and which is highly stable may thus be further considered for decontamination purposes. Previous reports have shown that the enzyme can be produced at a large scale and retain its catalytic activity after immobilization into alginate beads or polyurethanes which may be considered in particular for incorporation into filtration devices^{16,17}. Although degradation products may not be completely innocuous, these compounds were found to be much less toxic than insecticides and reduced poisoning effects by increasing NOEC values by up to eight-fold. Enzymatic decontamination also drastically limited the detrimental consequences to planarian mobility and enabled recovery of a non-exposed like regeneration process.

Materials and Methods

Production-purification of *SsoPox*- αsD6 . *SsoPox*- αsD6 was cloned in a pET32b- Δtrx plasmid and produced using an *E. coli* BL21(DE₃)-pGro7/GroEL (TaKaRa) chaperone expressing strain as previously described^{11,12}. In short, auto-inducible ZYP medium (supplemented with 100 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin and 34 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol) was used for growth. When OD_{600nm} reached a value of 0.8–1, CoCl₂ and L-arabinose was added (final concentrations respectively 0.2 mM and 2 $\text{g}\cdot\text{l}^{-1}$) to induce chaperones GroEL/ES expression, the temperature was decreased to 23 °C for 16–20 hours. Cells were harvested by centrifugation (4,400 g, 4 °C, 20 minutes) and the pellet was resuspended in *lysis buffer* (50 mM HEPES pH 8.0, 150 mM NaCl, CoCl₂ 0.2 mM, lysozyme 0.25 $\text{mg}\cdot\text{ml}^{-1}$, PMSF 0.1 mM and DNaseI 10 $\mu\text{g}\cdot\text{ml}^{-1}$) and stored at –80 °C. The lysate was thawed and lysed by three 30-second steps of sonication (Qsonica, Q700; Amplitude 45). Cell debris were removed by centrifugation (14,000 g, 4 °C, 15 minutes). As *SsoPox*- αsD6 is hyperthermostable a pre-purification step was conducted by heating the lysate for 30 minutes at 70 °C. Precipitated *E. coli* proteins were removed by centrifugation (14,000 g, 4 °C, 15 minutes). *SsoPox*- αsD6 was concentrated by ammonium sulfate precipitation (75%) and resuspended in 8 ml of *activity buffer* (50 mM HEPES pH 8.0, 150 mM NaCl, CoCl₂ 0.2 mM). The remaining ammonium sulfate was eliminated by desalting column (HiPrep 26/10 desalting, GE Healthcare; ÄKTA Avant), concentrated to 2 ml and injected on size exclusion chromatography (HiLoad 16/600 Superdex™ 75 pg, GE Healthcare; ÄKTA Avant). Final purity was verified by 10% SDS-page and protein concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Planarian. Freshwater planarians belonging to the *Schmidtea mediterranea* species (asexual clonal line CIW4) were used for all experiments. The planarians were maintained in autoclaved water at 19 °C in the dark and fed twice per week with calf liver. The animals were starved for at least one week prior to the experiments. The water was changed every two days and did not contain antibiotics.

Acetylcholinesterase activity assays. Acetylcholinesterase activity was measured using Ellman's assay³⁶. Worms were manually selected to fall within a certain range of sizes, around 0.8–1 cm in length. Thirty animals were processed in a Fastprep 24 5 g (three cycles of 10 seconds, 6,0 m/s) in 600 µL of activity buffer (20 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol, 100 mM NaCl, pH 8.0). Experiments were conducted in the same buffer complemented with 4 mM 5,5'-Dithiobis (2-nitrobenzoic acid). 10 µL of the planarian supernatant was added to 140 µL of activity buffer in a 96-well plate. 50 µL of 0.01 M acetylthiocholine iodide in the activity buffer was added to each well to start the reaction and the OD_{412nm} was followed with a microplate reader (Synergy HT, BioTek, USA) for 10 minutes. This activity was normalized to 100%. As negative controls, experiments without planarian supernatant and without acetylthiocholine iodide were carried out. The reproducibility of the crude homogenate preparation was demonstrated with three independent replicates (Supplementary Figure 4).

Experiments with pesticides (paraoxon-ethyl, parathion-ethyl, diazinon and fenitrothion) were conducted as previously described after incubation of the supernatant from the planarian with the pesticide for 45 minutes to ensure reaching inhibition plateau (Supplementary Figure 5). An additional control was carried out using only pesticide in the activity buffer.

Experiments with degradation products of pesticides were carried out as previously described after a degradation by *SsoPox-αSD6* variant at a final concentration of 0.25 µM. The reaction was monitored by measuring the absorbance of the product released until complete degradation. The planarians were then incubated for 45 minutes in the decontaminated solution (Supplementary Figure 1). Supplementary controls were conducted using only the degradation products in the activity buffer.

The activity results after incubation with pesticides or degradation products were normalized as compared to the activity value without inhibition set to 100%.

Toxicity assays and perturbation of behavior. Worms were manually selected to fall within a certain range of sizes, around 0.8–1 cm in length. The animals were starved for at least one week prior to the experiments. Observations were carried out using a Leica M165 FC lens connected to the IC Capture 2.4 software. Four pesticides (Sigma Aldrich) were used for these experiments at different concentrations (paraoxon-ethyl, parathion-ethyl, diazinon and fenitrothion).

4 mL of solution was prepared for each organophosphorus compound at different concentrations (paraoxon-ethyl: 10 µM–400 µM, parathion-ethyl: 10 µM–1000 µM, diazinon: 10 µM–800 µM and fenitrothion: 10 µM–800 µM) without and with degradation by *SsoPox-αSD6* variant at a final concentration of 0.25 µM into a 12-well plate. Five planarians were used in each well and experiments were conducted in duplicate. In the same well, mortality and mobility were analyzed over 12 days. As a negative control, the same experiments were carried out without pesticides, with enzyme, and with the pesticide solvent alone (ethanol) at the higher concentration of pesticides.

Perturbation of development. To study regenerating animals, specimens were cut into two, the head and the tail, using a scalpel just above the pharynx. 4 mL of solution was prepared in a 12-well plate for each organophosphorus compound at different sub-lethal concentrations determined from toxicity assays (paraoxon-ethyl: 2 µM–100 µM, parathion-ethyl: 10 µM–300 µM, diazinon: 10 µM–100 µM and fenitrothion: 10 µM–200 µM) without and with degradation by *SsoPox-αSD6* variant at a final concentration of 0.25 µM. After amputation of five planarians, the heads and tails were regrouped respectively in a well and regeneration was monitored over 14 days. As a negative control, the same experiments were conducted without pesticides, with enzyme, and with the pesticide solvent alone (ethanol).

GC/MS analysis of degradation products. In order to identify the degradation products, 100 µL of the pesticide solution degraded by *SsoPox-αSD6* variant was first extracted with 100 µL of chloroform. Organic extracts were analyzed using a Clarus 500 gas chromatograph equipped with a SQ8S MS detector (Perkin Elmer, Courtaboeuf, France). 1 µL of organic extract was volatilized at 220 °C (split 15 mL.min⁻¹) in a deactivated FocusLiner with quartz wool (SGE, Ringwood, Australia) and compounds were separated on an Elite-5MS column (30 m, 0.25 mm i.d., 0.25 mm film thickness) for 12 minutes using a temperature gradient (80–280 °C at 30 °C.min⁻¹, five minutes' hold). Helium flowing at 2 mL.min⁻¹ was used as carrier gas. The MS inlet line was set at 280 °C and electron ionization source at 280 °C and 70 eV. Full scan monitoring was performed from 40 to 400 *m/z* to identify chemicals by spectral database search using MS Search 2.0 operated with the Standard Reference Database 1 A (National Institute of Standards and Technology, Gaithersburg, MD, USA). Identification was confirmed with reverse and forward scores above 800. Product identities were then validated with the Kovats Retention Index from the injection of an alkane standard (Sigma Aldrich, France). In the case of weak degradation product signals (4-nitrophenol *m/z* 139, 6-methyl-2-propan-2-yl-1H-pyrimidin-4-one *m/z* 137, 3-methyl-4-nitrophenol *m/z* 136), extracted ion chromatograms were generated with base peak ions to confirm the absence of chemicals (Paraoxon-ethyl and parathion-ethyl *m/z* 109; Fenitrothion *m/z* 125; Diazinon *m/z* 179). All data were processed using Turbomass 6.1 (Perkin Elmer).

Phylogeny. To identify a planarian homolog of *Homo sapiens* AChE (*Hs*-AChE) isoform E4-E5 precursor (NP_056646.1), we examined, via TBLASTN, the planarian transcriptome database PlanMine (<http://planmine.mpi-cbg.de/planmine/begin.do>)³³ particularly the following flatworm species: *S. mediterranea*, *P. torva*,

P. tenuis, *D. lactum* and *S. polychroa*. We identified sequences producing a significant alignment with the genes of interest. The top BLAST hit was used to predict *Smed*-AChE, *Pto*-AChE, *Pte*-AChE, *DI*-AChE and *Sp*-AChE via FGENESH+ (<http://www.softberry.com/>). Homology at the protein level between predicted *Smed*-AChE and *Hs*-AChE was analyzed using BLAST.

The previous predicted sequences were used as template for BLAST in UNIPROT (<http://www.uniprot.org/blast/>) and 250 sequences were collected. Sequence alignment, including planarian predicted sequences and Phylogeny, were performed with MEGA^{34,35}. A maximum likelihood tree was constructed and tested using the bootstrap method with a 100 replications.

Statistical analysis. For *Smed*-AChE activity (Fig. 3) the values represent the mean \pm SD (standard deviation) of the percentage of three technical replicates. Black stars (*) indicate a significant difference between insecticide and degradation product for a given concentration. According to Student's t-test, one star indicates $p < 0.05$, two stars indicate $p < 0.005$ and three stars indicate $p < 0.0005$.

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Author Contributions

L.P., L.B., P.J., C.L., N.A., D.D., performed the experiments; L.P., L.B., P.J., C.L., N.A., C.T., D.D., E.C., E.G. analyzed the data; D.D., E.C. and E.G. conceived and designed the experiments; and L.P., P.J., D.D., E.C., E.G. wrote the manuscript.

Additional Information

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Competing Interests: E.C. has a patent WO2014167140 A1 licensed to Gene&GreenTK. D.D. and E.C. report personal fees from Gene&GreenTK during the course of the study.

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