Sub-lethal exposure to malaria vector control pesticides causes alterations in liver metabolomics and behaviour of the African clawed frog (**Xenopus laevis**)
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Abstract

In this study we explore the sub-lethal effects of two malaria vector control pesticides, deltamethrin and dichlorodiphenyltrichloroethane (DDT), on *Xenopus laevis* by incorporating different levels of biological organisation. Pesticide accumulation in frog tissue was measured alongside liver metabolomics and individual swimming behaviour to assess whether changes presented at these different levels, and if such changes could be linked between levels. Results showed evidence of concentration dependent accumulation of DDT and its metabolites, but no measurable accumulation of deltamethrin in adult *X. laevis* after 96h of exposure. Both DDT and deltamethrin were shown to cause alterations in the liver metabolome of *X. laevis*. We also showed that some of these changes can be enhanced in exposure to a mixture of these two pesticides. Initial behavioural responses recorded directly after exposure were seen in the form of decreased activity, less alterations between mobility states, and less time spent at the water surface. This response persisted after 96h of exposure to a mixture of the two pesticides. This study shows that sub-lethal exposure to pesticides can alter the biochemical homeostasis of frogs with the potential to cascade onto behavioural and ecological levels in mixture exposure scenarios.

Keywords

Amphibian ecotoxicology, DDT, Deltamethrin, bioaccumulation, pesticide mixture, synergistic effects

Introduction

There has been a recent resurgence of research confirming the presence of dichlorodiphenyltrichloroethane (DDT) in tropical aquatic ecosystems in Africa where malaria vector control (MVC), through Indoor residual spraying (IRS), is actively carried out using DDT
and other pesticides (see Gerber et al. 2016; Volschenk et al. 2019, Mbongwe et al. 2003; Govaerts et al. 2018 Viljoen et al. 2016; Wolmarans et al. 2018; Wolmarans et al. 2021). The presence and environmental impacts of other World Health Organisation (WHO) recommended pesticides for MVC (see WHO 2006) have not yet been examined in depth in this region (Quinn et al. 2011). Simultaneous DDT and pyrethroid presence has, however, been previously measured in human breastmilk from MVC regions in South Africa, with aquatic ecosystems (water and fish) being identified as a possible vector for these pesticides into humans (Bouwman et al. 2006).

Two pesticides were selected as representatives for MVC pesticides. Dichlorodiphenyltrichloroethane as representative of the organochlorine pesticide group, and deltamethrin (DTM) as representative of the pyrethroid pesticide group. These two pesticides are often used in conjunction with one another for IRS in South Africa (Brooke et al. 2013).

Both DDT and DTM have broadly similar primary modes of action where binding affects cation channels at neural synapses (Davies et al. 2007; Haschek et al. 2013). Secondary binding activity plays a large role in sub-lethal effects, such as the endocrine disruption activity of DDT (Hascheck et al. 2013). Both primary and secondary binding activity for these pesticides are not extensively studied in non-target organisms such as amphibians. Dichlorodiphenyltrichloroethane is highly persistent and bioaccumulates through the food web due to its lipophilic nature (Bouwman et al. 2011). Deltamethrin is not considered to be persistent in the environment (ATSDR 2003). The wider agricultural use and consequent multiple sources of DTM to the natural environment could, however, theoretically lead to higher exposure peaks to non-target organisms in the wild.

Given the potential hazard presented by these pesticides in the environment and uncertainty as to sub-lethal effects in non-target organisms, the need was identified to assess the sub-lethal effects of these pesticides on non-target anurans. In pursuit of this aim, metabolomics and behavioural analysis were incorporated as possible effect indicators. The use of metabolomics in an ecotoxicology context has proven useful in determining the biochemical
pathways upon which environmental contaminants act (Labine and Simpson 2020). Metabolomics has previously been used in anuran ecotoxicology to assess the changes in leopard frog liver metabolome towards herbicides, fungicides and insecticides (Van Meter et al. 2018). As the use of metabolomics in contaminant exposure studies provides a link between the environment and effects on the individual, behavioural analysis can be used as a link between effects on the individual and cascading effects on the population, community, or ecosystem. In many cases it has proven to be a more sensitive “early warning” of toxicity than conventional mortality (LC50) testing based predictions (Hellou 2011). Hellou (2011) also stipulated the importance of fate of chemicals and metabolic abilities of the subject organism in understanding behavioural ecotoxicology in broader terms. Peterson et al. (2017) suggested that the use of behaviour in ecotoxicology should focus on a framework that incorporates causation, ontogeny, adaptation, and phylogeny (Tinbergen’s four postulates). According to Peterson et al. (2017), it is only through an integration of our understanding of the mechanisms and causation by which contaminants affect behaviours, along with the effects of these behaviours on fitness, that population level effects of contaminants can be fully understood.

There is a distinct lack of sub-lethal toxicity literature related to anurans and MVC pesticides, particularly in African species (Wolmarans et al. 2020). In this study, with the above mentioned integration framework in mind, we combined chemical accumulation, metabolomics, and behaviour as toxicological endpoints to explore the integrated effects of these pesticides on the fully aquatic anuran *Xenopus laevis* in a laboratory setting. The African clawed frog (*X. laevis*) was selected as test species for being an established model aquatic anuran as well as having a natural distribution that coincides with areas where IRS is applied in Southern Africa (for distribution range see De Villiers and Measy 2017; Du Preez and Carruthers 2017). Based on the biological organisation hierarchy the aims were to link exposure, as measured through pesticide bioaccumulation, to changes in the metabolome that in turn would result in
behavioural changes. We further determined whether mixtures of the pesticides would result in additive effects.

**Materials and Methods**

**Ethical research practise**

All animal housing, handling, breeding and bioassays were completed under ethical approval (NWU-0264-16-A5) by the AnimCare ethics committee of North-West University.

**Animal husbandry and housing conditions prior to exposure**

*Xenopus laevis* used in this study were bred and housed as in South et al. (2019; 2020) in accordance with best practise as stipulated by Reed (2005). All frogs were 11 months old at the time that experiments were carried out. Frogs were classified as sub-adult for the purpose of this study as sexual maturity had been reached in some, but external sexual morphology was not present in most animals yet.

**Experimental setup and design**

Pesticide treatments

The two pesticides tested, DDT and DTM, were selected in this study as representatives of IRS pesticides. The scope of this study and ethical limitations on the use of vertebrate animals did not allow for extensive LC50 testing on adult frogs, and no literature containing LC50 data on adult *X. laevis* was found in a recent review (see Wolmarans et al. 2020). Therefore, the LC50\textsubscript{FETAX} values (DDT = 35.7 mg/L and DTM = 0.19 mg/L, Channing 1998; Saka 2004) based on toxicity to larval *X. laevis* were used as a starting point for lethality, even though sensitivity between adult and developmental stages of animals can differ. After initial testing showed mortality at higher concentrations, the exposure concentrations for this study were set at to 1% and 0.1% of the respective LC50\textsubscript{FETAX} for DDT and DTM where no mortality was observed after 96 h. Fractions of the lethal concentrations were used in order to assess mixtures in terms of equal toxic units. The different exposure treatments used in this study are outlined in
Table 1. For environmental relevance commercially available formulations of these pesticides were used for exposure treatments.

Exposure chemicals

The technical grade DDT (AVIDDT 750, Avima (Pty) Ltd, RSA) consisted of 71.8% $\sum$DDT (of which 80.5% = $p,p$-DDT and 19.5% = $o,p$-DDT) and concentrations were prepared in terms of active ingredient (i.e. $\sum$DDT). For DTM a commercial formulation (Decatix 3, Cooper Veterinary Products (Pty) Ltd, RSA) was also used consisting of 2.5% m/v DTM and exposure concentrations were also prepared in terms of active ingredient. Stock solutions and dilutions were prepared in fresh housing media.

Exposure setup

The frogs were allowed to acclimate in the experimental tanks for 2 h prior to exposure (3 L clear polycarbonate tanks) with the temperature gradually being lowered from the housing temperature (23°C) to experimental temperature (20°C) during this period. Static exposures were performed and animals were not fed during the exposure period. Eight replicates of sub-adult $X. laevis$ were individually exposed per treatment. Four of these individuals per treatment were selected prior to exposure and were used for behaviour recordings throughout the exposure as per South et al. (2019, 2020).

Control animals underwent the exact same procedure and handling as exposed animals. Behaviour was recorded for 2 h immediately at the onset of the exposure period followed by another 2 h recording initiated at 94 h after exposure. At 96 h the frogs were euthanized through means of mechanical stunning followed by double pithing. Chemical euthanasia methods were not used to reduce the possible effect of euthanasia on the liver metabolome. Liver samples were collected weighed and flash frozen at -80°C for metabolome analysis. The remainder of each carcass was frozen at -20°C for bioaccumulation analysis.
**Chemical analysis**

For confirmation of dosing concentrations, 100 mL water samples (n=4 per treatment) were spiked with 100 μL of 100 μg/L chlorinated biphenyl (CB #143, LGC Standards Ltd, UK) as internal standard. Liquid-Liquid extraction was performed with hexane 100 mL of sample extracted with 200 mL hexane and filtered through sodium sulphate. The extract was evaporated down to near-dryness under a gentle nitrogen stream at 34°C. The extract was reconstituted to 100 μL n-Decane (Sigma-Aldrich, USA) containing 100 µg/L Dr Erhenstorfer (GmbH, DEU) Tetrachloro-m-Xylene (LGC Standards Ltd, UK) for final volume calculation before analysis.

Tissue sample extraction method performed as in Wolmarans et al. (2018, 2021).

Quantification of pesticide bioaccumulation and confirmation of nominal dosing concentrations was performed on a HP 6890 Gas Chromatograph coupled with a micro-Electron Capture Detector (GC-μECD). Compound separation was achieved using an HT8-MS (30 m, 0.25 mm, 0.25 μm Trajan Scientific Australia Pty Ltd, AUS) column with H₂ as carrier gas. Splitless injection (1 μL) was used with the inlet temperature set at 225°C. The oven program followed initiation at 100°C which was held for 1 min and ramped up at 20°C/min to 200°C where the ramp was changed to 6°C/min until at 260°C and held for 20 min. Target compounds were identified based on retention time (RT in min: 11.862 = o,p-DDE; 12.752 = p,p-DDE; 13.131 = o,p-DDD; 13.788 = CB#143(IS); 13.908 = o,p-DDT; 14.321 = p,p-DDD; 15.163 = p,p-DDT; 32.225 = DTM). Method validation for both extraction and analysis was performed using Dr Erhenstorfer (GmbH, DEU) pesticide mix 1037 (LGC Standards) containing 22 organochlorine pesticide isomers including all six major DDT isomers and metabolites, as well as Dr Erhenstorfer (GmbH, DEU) DTM standard (LGC Standards Ltd., UK). Instrumental calibration for all DDT isomers and DTM were performed on 7-point calibration curves (averaged over 5 runs) ranging between 5 and 1000 μg/L with R² > 0.999 for all compounds except DTM that had an R² = 0.998. Samples were analysed in batches of seven with quality control standards run between batches. Concentrations reported were adjusted according to recovery of the
internal standard added to each sample, which was 68 ± 11% (mean ± standard deviation) for
the analysed water samples and 80 ± 14% for the analysed tissue samples. Instrumental limit
of detection (LOD) and limit of quantitation (LOQ) was calculated individually for each
compound as 3 times and 10 times (respectively) the standard error of the gradient of the
calibration curve. All instrumental LODs were below 50 μg/L.

Metabolome analysis

Frog liver metabolome analyses were performed at the North-West University National
Metabolomics Platform (NMP). The method of Venter et al. (2016) was used for the extraction,
and oximation and silylation extracting 50 mg of liver sample (n=8 per treatment).

The GC/TOF-MS analysis was also performed according to (Venter et al. 2016) with slight
modifications. These modifications included the use of splitless injection (1 μL) and separation
through an Rxi-5Sil-MS (30 m, 0.25 mm, 0.25 μm; RESTEK, USA) column at a constant flow
of 1.5 mL/min. The primary oven temperature program was initiated at 70°C and held for 1
min followed by an initial ramp of 7°C/min to a final temperature of 300°C, which was held for
1 min (see Venter et al. 2016 for full extraction, analysis and peak data processing
information).

Behavioural analysis

For the first 2 h post-exposure, experimental treatments (n=4 per treatment) were recorded
and movement behaviour metrics were recorded as per the methods of South et al. (2019,
2020). Detailed metrics are given in the Statistical analysis – Behavioural analyses section
and Table 5

Statistical analysis

Chemical accumulation analyses
Initially a zero-filter was used on the data disregarding compounds (per treatment group) with
detection rates (values above LOQ) less than 50% for each group. For all further statistical
analyses remaining values below LOD were replaced with 0.5 LOD values. Comparison groups were set to compare each exposure treatment to the control treatment. Multiple t-tests were performed on each comparison group coupled with Dunn’s post hoc test with p-value adjustment for multiple comparison using the Holm-Sidak method with significance set at $\alpha \leq 0.05$.

Metabolomics analyses

Non-parametric analyses for metabolome data consisted of a fold change (FC) analysis performed on untransformed MS peak intensity data. This was performed for each exposure treatment compared to the control treatment for each detected spectral peak. This analysis provides a sense of practical significance to changes in the screening data with significance set at $|\text{FC}| \geq 2$.

Prior to parametric analyses a zero-filter was used excluding all compounds with detection rates below 50% in samples from both groups within a comparison. Log transformed ($\log_{10}(X+1)$) metabolome data were used to achieve normalisation for parametric testing. The parametric analysis consisted of independent samples multiple t-tests with adjustment for multiple testing (Benjamini Hochberg adjustment for the control of false discovery rates; FDR). Significance was set at adjusted $p < 0.1$ because of the number of variables measured. As a parametric measure of practical significance in the changes observed, effect sizes (Cohen’s --d value) were calculated with significance set at $|d| \geq 0.8$ (Lakens 2013).

Both effect size and FC were used as effect size takes into account the standard deviation between samples whereas the FC is based only on the mean per treatment, but more sensitive to changes in this mean.

A partial least squares-discriminant analysis (PLS-DA) was performed on the metabolome data for each of three pesticide groupings (DDT, DTM, and Mix) in order to assess separation variables between treatments in terms of the severity of exposure. Data included in this analysis were filtered based on effect sizes and FC. The PLS-DA models were used to identify
predictor variables based on their variable importance in projection (VIP) scores for separation between the control, 0.1%, and 1% treatments for each analysis (and overall separation in final PLS-DA). For predictive ability only VIP scores >1 were considered viable. All metabolomics statistical analyses were performed using Metaboanalyst 4.0 (Chong et al. 2019).

Pathways analysis was performed using the Metaboanalyst pathway analysis package (Xia and Wishart 2010). As *X. laevis* is not a species option within this package the zebrafish, *Danio rerio*, was used as the analysis organism in the model as done by Onjiko et al. (2016), and Portero and Nemes (2019). Pathway analysis algorithms consisted of pathway enrichment analysis by global test and pathway topology analysis by relative-between-ness centrality. All pathways referred to in this study were confirmed through the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database (KEGG 2020; also see Kanehisa et al. 2010; Tanabe and Kanehisa 2012).

Behavioural analyses

Various metrics were calculated from the two hour recordings of the frogs based on centre point tracking data as described in Table 5. Total distance moved, mean velocity, cumulative duration spent moving, the mean acceleration, and meander were the movement metrics measured. Meander is a factor of turn angle and total distance moved and is thus dependent on the direction of movement in the tank with right handed turns resulting in a positive turn angle and left handed turns resulting in a negative turn angle. The frequency of visits to, and cumulative duration spent at, the top zone of the tank were also measured. Lastly the frequency of being in, and cumulative duration spent in each of three mobility states were measured. An immobile state was set a 0-20% of the maximum velocity. A mobile state was set as 20-60% of the maximum velocity, and a highly mobile state being above 60% of the maximum velocity.
The behavioural metric data in this study were analysed individually using Kruskal-Wallis analyses for comparison between groups coupled with Dunn’s post-hoc test for mean rank comparison. Significance was set at $p \leq 0.05$. Three sets of comparison groups were analysed for the behaviour data. A time factor was assessed consisting of each treatment at 0 h vs that same treatment at 96 h. Two treatment factors were assessed consisting of the control at 0 h vs every exposure treatment at 0 h, as well as control at 96 h vs every exposure treatment at 96 h. For a measure of practical significance effect sizes (Cohen’s $d$ value) were also calculated with significance was set at $|d| \geq 0.8$.

A principal component analysis was performed on log transformed ($\log_{10}X+1$) behavioural data to determine group separation based on behavioural metrics and identify possible driving metrics behind group separations.

To assess whether links of association could be established between the datasets measured at different levels of biological organisation, redundancy analyses (RDAs) were performed for the various combinations of variable sets (exposure treatment, chemical accumulation, 96h behaviour, and metabolomics) using CANOCO v.5 software. In dealing with the large number of variables within these datasets interactive forward selection of variables was applied to each RDA in order to identify the most important variables for explaining variation in the data in each analysis. P-value cut-off was set at 0.1. Significant variables selected from these RDAs were re-analysed and priority variables were identified based on their influence in explaining the variation in the data of each group with a p-value cut-off at 0.1.
Results

Pesticide concentrations in the exposure medium

Analysis of sample water from the exposure tanks taken directly after exposure (from those not used for behaviour analysis) indicate that both DDT and DTM were present in the water column at detectable levels (Table 1).

Bioaccumulation

Deltamethrin was not detected in any of the analysed tissue samples. In some of the DTM 1% and Mix 1% tissue samples small GC-ECD peaks were observed on the chromatograms at the appropriate retention time (RT = 32.225), but these were all below the instrumental LOD and thus excluded from analysis.

All control, DTM 0.1% and DTM 1% samples did not have any DDT isomers present. For this reason, DTM treatments are absent from accumulation analyses. For statistical analyses purposes control sample values and values below LOD within the DDT and Mix treatments were substituted with 0.5 of the LOD values for each respective compound in all analyses.

The DDT isomers were successfully detected at measurable concentrations in samples from the DDT 0.1%, DDT 1%, Mix 0.1% and Mix 1% exposure groups (supplementary Table S1). Multiple t-tests showed significant differences between all DDT exposed treatments (DDT and Mix groups) compared to control (Table 2). The o,p-DDE isomer was not detected in any of the samples. The p,p-DDE isomer was detected in 100% of the DDT 1% and Mix 1% treatment samples. In the 0.1% treatments for DDT and Mix, p,p-DDE was also only detected in one sample, respectively. The o,p-DDD detection rate was above 87.5% for all treatments that received DDT except the Mix 0.1% treatment where it was only detected in one sample. For o,p-DDT the detection rate was above 50% in the DDT 0.1%, Mix 0.1%, and Mix 1% treatments, but was not detected in the DDT 1% treatment. The detection rates of both p,p-DDD and p,p-DDT were above 50% for all treatments.
The composition of DDT isomers (Table 3) measured in each sample was inconsistent between treatments with large variation within the treatments. The prominent compound in the DDT 1% and Mix 0.1% treatments was \( p,p\)-DDT making up more than 63% of the \( \Sigma\)DDT. The \( p,p\)-DDD was the major contributor in the DDT 0.1% treatment (58.8%) while \( o,p\)-DDT made up only 32.8% of the \( \Sigma\)DDT in the Mix 1% treatment.

**Metabolomics**

The whole metabolome screening analysis detected 562 spectral compound peaks in the frog livers. Based on FC and effects sizes a total of 259 spectral peaks with notable up- or down-regulation (i.e. practical significance as defined under statistical analysis) between groups were identified. Of these spectral peaks, 41 showed statistically significant differences to control based on multiple t-tests (supplementary Table S2). The DDT 0.1% and DTM 0.1% treatments had the most spectral peaks (18 and 17 in total, respectively) with statistical significance. The least significant changes, only three peaks, were found in the Mix 0.1% treatment. The DDT 1% and DTM 1% treatments showed significant up- or down-regulation in 13 and 10 metabolites respectively. The Mix 1% treatment also had 10 statistically significant changes, but had only two spectral peaks in common with the individual 1% treatments in terms of statistical significance. In addition to the 42 peaks with statistical significance, 13 peaks were also considered important for being either completely absent or only present in the analysed treatments compared to control (supplementary Table S2). Difficulty in compound identification through the screening databases used, resulted in a large number of unidentified analytes among those that showed significant differences.

The PLS-DA analysis across five components for all DDT treatments showed separation between the control and DDT 1% treatment along the first two components (Figure 1A) accounting for 27.7% (20.2% and 7.5% for component one and two respectively) of the variation. The DDT 0.1% treatment did not separate from either the control or DDT 1% treatment, but its distribution was rather uniformly split between the two other treatments resulting in a clear concentration based shift from the control in the bottom left to the DDT 1%
in the top right. Cross validation of the model showed the highest $Q^2$ value (predictive capacity measure) at only one component (0.60) with the highest accuracy (0.83) at three components. The VIP scores were used to identify the top metabolic variables in predicting concentration groups of DDT exposure across the first two components of which 60 had scores >1. The variable with the highest VIP scores was phosphoric acid, with scores >1.76 for both components. The PLS-DA analysis performed for all DTM treatments showed clear separation between all three treatments (Figure 1B) across the first two components accounting for 24.8% (15.3% and 9.5% for component one and two respectively) of the variation. The first and third components (not shown) accounted for more variation (component three explained 13% of the variation), but in this combination the DTM 0.1% treatment showed overlap with the control. Cross validation of the model also showed the highest $Q^2$ value at five components (0.72) with the highest accuracy (0.91) at two components. The VIP scores were again used to identify the top variables in predicting groups across the first two components, of which 77 had scores >1. L-Sorbitol had the highest VIP scores (>1.9 across the first two components). For analysis of both Mix groups the PLS-DA analysis showed less separation between control and mixture exposure treatments than the individual pesticide treatments. There was slight overlap between the Control and the Mix 1% with the Mix 0.1% once again falling in between the two along the first two components (Figure 1C). The group separation was skewed resulting in greater separation between groups toward the top left and convergence toward the bottom right. This suggests the two components having an inverse relationship toward each other. Component one accounted for 22.4% of the total variation and component two for 10.4% (32.8% in total). The variable with the highest VIP score (>1.77 on the first two components) was an unknown peak (Analyte 458 UM: 159, RT: 1376.42). The top 62 variables showed VIP scores >1. Cross validation of this model indicated two components as the optimal in terms of predictive value ($Q^2 = 0.48$) and accuracy (0.74).

When all treatments were analysed together (Figure 1D) clear separation from the control could be established for DTM 0.1%, DDT 1%, and DTM 1%. The Mix 0.1% and Mix 1%
treatments only showed minor overlap with the control whilst the DDT 0.1% grouped closer to control than the other exposure treatments. All exposure treatments overlapped with each other across the first two components. Only 18.3% of the variation was accounted for. This model however showed poor predictive capacity ($Q_2 = 0.24$) and accuracy (0.38). In total 81 variables showed VIP scores > 1 with L-sorbitol showing the greatest predictive capacity (VIP > 2.5 across the first two components).
The metabolic pathway analysis results (Table 4) indicated four pathways were significantly influenced in the DDT 0.1% treatment frogs. The inositol phosphate metabolism and galactose metabolism pathways showed pathway impact (PI) > 0 (see Xia and Wishart 2010 for description of pathway impact). In the DDT 1% treatment the fructose mannose metabolism pathway and galactose metabolism pathways showed PI > 0. The DTM 0.1% influenced two pathways significantly, but the net impact was zero for both. Five different pathways were significantly affected in the DTM 1% treatment. Three of which (pentose phosphate pathway, pyruvate metabolism and the citrate cycle) showed PI > 0. Four pathways were significantly affected in the Mix 0.1% treatment. The glycine, serine and threonine metabolism pathway as well as the valine, leucine and isoleucine biosynthesis pathway both had PI > 0. The Mix 1% treatments showed only one pathway affected, but with zero PI. Using a less strict cut-off (false discovery rate adjusted p < 0.1) affected pathways were placed into their respective categories. All exposure treatments resulted in carbohydrate-, lipid- and amino acid metabolism changes along several pathways. The aminoacyl-tRNA biosynthesis pathway (under translation pathways) was uniquely affected by the DDT 1% treatment and both Mix treatments with 11 amino acids affected in each treatment and a 12th, L-proline, also affected in Mix treatments.
Behaviour

Analysis of behavioural data showed no statistical differences between immediate (0 h) and 96 h post-exposure recordings per treatment groups. Effect sizes (Cohen’s-d value) did, however, show large changes for most treatments (Table 5; Visual representations of all behavioural data are provided in supplementary Figures S1 and S2). Control frogs showed a temporal decrease in the frequency of being in either a highly mobile, mobile or immobile state, with the cumulative duration spent in a highly mobile and mobile state, and the cumulative duration in the top zone also decreased at 96 h compared to 0h. For the DDT 0.1% treatment no changes were observed. For the DDT 1% only a decrease in the frequency of being in an immobile state along with an increase in the cumulative duration spent in an immobile state was observed. The DTM 0.1% treatment showed a decrease in the distance moved, mean velocity, and frequency of visits to the top zone. It also showed an increase in the frequency of being in a highly mobile state. The DTM 1% treatment showed a decrease in the total distance moved, mean velocity, and cumulative duration spent moving, with an increase in the cumulative duration in an immobile state. The Mix 0.1% treatment showed a decrease in the frequency of being in a mobile state, and a decrease in the cumulative duration of being both in a mobile state and in an immobile state. The Mix 1% treatment showed a decrease in the frequency of being in both a highly mobile, mobile, and immobile state as well as the cumulative duration of being in highly mobile and mobile states.

For treatment factor comparisons (Table 6) statistical significance was shown in some group comparisons at 0 h. The DDT 0.1% treatment had significant decreases from the control for the frequency of visits to- and the cumulative duration spent in the top zone. There was also a significant decrease (p<0.05) in the frequency of being in a mobile state and immobile state as well as in the percentage of the total duration spent in a mobile state. The Mix 1% treatment showed significant decrease from the control in the cumulative duration spent in the top zone. There were multiple changes based on large effect sizes (Table 6). Markedly the cumulative duration spent at the top, the frequency in a highly mobile state, and the cumulative duration
in a highly mobile state were lower for all exposure treatments at 0 h compared to the control
treatment. The frequency and duration in a mobile state were also lower for all exposure
treatments except the Mix 1% treatment. The frequency of visits to the top zone was lower for
the DTM 1% and both of the Mix treatments. The frequency of being in an immobile state was
lower for the DTM 1% and Mix 0.1% treatments while the cumulative duration in an immobile
state was lower for the DDT 1%, DTM 0.1% and DTM 1% treatments. The only metrics that
increased at 0 h were the cumulative duration in an immobile state for the Mix 1% and the
mean meander for the Mix 0.1% treatment.

After 96 h no exposure treatment groups showed significant differences to the control group.
Effect sizes did however once again show multiple notable changes, but in less treatments
than at those measured 0 h. The mean meander showed a notable increase for the DDT 0.1%,
DDT 1%, Mix 0.1% and Mix1% treatment groups. The cumulative duration in an immobile
state and the mean acceleration both showed a decrease for the Mix 0.1% treatment, but and
increase for the Mix 1% treatment. Along with the prior changes mentioned, the Mix 1%
treatment also showed a decrease in the frequency of visits to the top zone, cumulative
duration spent in the top zone, frequency of being in a mobile state, frequency of being in an
immobile state and cumulative duration spent in a mobile state. Furthermore, the DTM 1%
treatment showed a decrease in total distance moved, mean velocity, cumulative duration
spent moving, frequency of visits to the top zone, frequency of being in a mobile state,
frequency of being in an immobile state and the cumulative duration spent in a mobile state.

A principle component analysis (PCA) of the behavioural metrics (not included) did not show
any clear group separation along the first two axes (Eigen values 0.50 and 0.17 respectively)
of the total variation in the data. Meander was shown to have a negative correlation to all other
metrics along the first axis.
An RDA performed on the bioaccumulation data with the metabolome data as selection variables. Forward selection of variables (based on raw p-values) indicated 20 variables of importance in explaining the variation in accumulation data. Forward selection with FDR adjusted p-values only showed conditional significance (adjusted p < 0.1) of 16 variables under the inclusion of all 20 (Figure 2). In total 99% of the variation in chemical accumulation was explained by the selected variables (56.3% was explained by significant variables) with 81.3% explained across the first two axes (eigenvalues of 0.63 and 0.18 for axis one and two respectively). The DDT 0.1% and 1% treatments as well as the DTM 1% treatment separated from the control based on 95% confidence intervals.

A second RDA performed on the 96 h behaviour metrics with the individual compound bioaccumulation data as selection variables (Figure 3) did not indicate pesticide bioaccumulation as having a significant influence on the separation of behaviour data. There was a quadrant based separation of the Mix 1% treatment samples from all the other treatment samples on both the first and second axis. The Mix 1% treatment showed strong association with increased \(o,p\)-DDT, \(o,p\)-DDD, and \(p,p\)-DDD which correlated positively with mean acceleration and the cumulative duration spent immobile. This RDA however only explained 10.5% of the total variation in the data cumulatively between the first two axes.

A third RDA was performed on the behaviour data using the metabolome data as selection variables. In order to reduce the number of variables an initial analysis was performed where after metabolome data were filtered based on raw p-values < 0.1 from that analysis. This resulted in 23 remaining variables. From a second round analysis using only these variables and applying forward selection, nine spectral peaks were identified as significant based on
FDR p-values < 0.1 (Figure 4). The nine selected variables accounted for 75.5% of the total variation in behaviour data with 58.5% explained across the first two axes (eigenvalues of 0.44 and 0.14 for axis one and two respectively). No group separation was visible due to very large spatial variation between individuals within each treatment (Figure 4). Of the nine variables selected six were unknown analytes. Alpha-D-glucopyranose, mannobiose, and L-arabiniose were the only known metabolites of significance.

Discussion

In assessing the effects of DTM and DDT on *X. laevis*, effect metrics need to be assessed individually and in conjunction with one another to gain an understanding of the mechanisms at play. Linking bioaccumulation to both exposure and effects can provide a better understanding of the hazard posed by these pesticides in natural environments.

The bioaccumulation of DTM in frog tissue could not be confirmed through the methods used in this study. The validation results of the extraction and analysis methods for DTM used in this study indicated that the method was effective for the extraction and analysis of DTM. No published literature is available on the accumulation of DTM in *Xenopus* tissue (see Wolmarans et al. 2020). Studies on fish such as Szegletes et al. (1995) also failed to measure DTM residues in tissue samples. Kim et al. (2008) did successfully measure DTM residues in various tissues of rats when orally exposed to 2 and 10 mg/Kg DTM. The authors calculated the maximum accumulation time to be ≤ 6 h for all tissue types except skin (12 h) and the terminal elimination half-life was only > 96 h for fat, muscle and skin. These values are not comparable to a constant immersion exposure scenario with aquatic organisms where skin permeability plays a major role in uptake, but do speak to the tendency of DTM to eliminate from tissue rapidly.

Accumulation of DDT was successfully measured in all individuals exposed to DDT, both individually and in a mixture. The accumulation data showed large variation in terms of total accumulated concentration, as well as isomeric and breakdown product contribution. Large
variation in DDT accumulation data is consistent with field results in *Xenopus* sp. from areas of South Africa where DDT is applied for MVC (Viljoen et al. 2016; Wolmarans et al. 2018). The mean $\Sigma$DDT tissue concentration in the DDT 0.1% (21.7 ng/g ww) and Mix 0.1% (23.13 ng/g ww) correspond to the highest survey mean for *X. muelleri* from Wolmarans et al. (2018) of 17.79 ng/g ww. Frogs from Viljoen et al. (2016) had higher levels with male *Xenopus* sp. having a mean $\Sigma$DDT concentration of 206 ng/g ww making it comparable to the DDT 1% treatment from this study (244 ng/g ww), but these concentrations from Viljoen et al. (2016) were measured in lipid bodies only and not whole organism tissues. It should also be noted that there is limited value in a direct comparison of accumulation between laboratory exposure data and long term field exposure data where the latter is subjected to long-term diffuse exposure. Interestingly the fraction of the total accumulated DDT consisting of $p,p'$-DDT itself was lower than expected for such a short exposure period relative to the general half-life of $p,p'$-DDT, which is 28 days in river water and 56 days in lake water (USEPA 1989). The accumulation of $\Sigma$DDT was very similar between the DDT 0.1% treatment and Mix 0.1% indicating that the addition of DTM did not affect the accumulation of DDT at the lower concentration. At higher concentration though the Mix 1% treatment only accumulated approximately half of the $\Sigma$DDT accumulated in the DDT 1% treatment. This suggests some interaction in either the uptake or storage of these two chemicals at the higher exposure concentrations tested in this study. Although literature in this regard is scarce, interactions between chemicals in a mixture have been shown to affect its biological uptake in plants in some cases. Measured in aquatic macrophytes (*Juncus effuses* L.), the variability in chlorpyrifos uptake was shown to be affected in mixtures whereas for atrazine it was not (Lytle and Lytle 2002). In the current study, uptake could only have occurred through dermal absorption. While *X. leavis* skin is known to be highly permeable, there is very little published literature on how chemical interactions might affect the selectivity in permeability. The skin of *X. laevis* contains peptides, such as magainin, that can form ionophores in bilipid layers as part of the neurosecretory system (Duclohier et al. 1989). Atrazine has been shown to reduce the protein content of *X. laevis* skin peptides, but with no effect on the antimicrobial properties
these peptides have (Gibble and Baer 2011). Both permethrin and DTM have been shown to interact with ion transport pathways in *Rana esculenta* skin causing absorption of Na\(^+\) and excretion of Cl\(^-\) ions, but the mechanism behind this activation could not be confirmed (Cassano et al. 2009). The possibility that DTM acts on frog skin and subsequently alters dermal DDT uptake in *X. laevis*, therefore cannot be excluded at this time.

In the current study, the PLS-DA results showed that the metabolic profiles of *X. laevis* liver can be used to identify exposure to pesticides under laboratory conditions, however, only at the higher concentration range assessed in this study due to separation from the control observed for both the DDT-, DTM-, and Mix 1% treatments, but not at their respective 0.1% treatments. Distinguishing between frogs exposed to different pesticides based on their metabolic profiles was not possible with the metabolome screening methods used due to large overlap in metabolite changes observed.

While some unique responses could be identified through univariate comparisons, a lot of overlap in metabolic profiles emerge during multivariate analysis indicating some general response to exposure independent of the pesticide. This becomes clear from the pathway analyses where carbohydrate, amino acid, and lipid metabolism are all affected across the board. This is similar to the results of Van Meter et al. (2018) that found carbohydrate and amino acid metabolism affected in leopard toads exposed to individual and mixtures of herbicides, fungicides, and insecticides. Van Meter et al. (2018) also attributed this to a general exposure response, which is supported by the current study results, even though different pesticide classes were used on a different species. Alanine appears to be a suitable biomarker for insecticide exposure. Increased alanine was found in frogs exposed to malathion and malathion containing mixtures in the study by Van Meter et al. (2018), but not in exposures only containing herbicides and fungicides. The increased alanine to glycine ratio measured by McKelvie et al. (2009) in earthworms for both DDT and endosulfan, and the alanine increase observed in mussels exposed to lindane (Tuffnail et al. 2009), indicate that this increase upon exposure occurs across different classes of organisms. Li et al. (2014) found an increase in
alanine in brain tissue of goldfish exposed to \( \lambda \)-cyhalothrin, but a decrease in kidney tissue.

Tissue specific differences in metabolomic response or variability is common (Simmons et al. 2015). In the current study, several derivatives of alanine were spectrally detected with the major peak (RT: 310.19) indicating an increase (based on effect size) in the DTM 0.1% treatment and both mixture treatments. The DTM 1% treatment, however, showed a decrease. The ratio between alanine and glycine could not be calculated from the untargeted method used, but based on the results from McKelvie et al. (2009) we suspect this ratio may hold greater promise as a biomarker of insecticide exposure across different species than alanine changes alone.

The alanine changes discussed, along with similarities in sugar and amino acid metabolism, between the current study and Van Meter et al. (2018) suggest a secondary effect of exposure stress regardless of the pesticide, which could affect a wide range of organisms. A possible explanation for changes observed in carbohydrate and lipid metabolism could be a part of a stress response not unlike general adaptation syndrome (GAS), initiated through activation of the hypothalamus-pituitary-interrenal (HPI) axis, increasing cortisterone levels (Selye 1950). Direct evidence of cortisol or corticosterone changes in the liver itself could not be confirmed in the current study, but the down-regulation of cholesterol observed in DTM and Mix treatments could be the result of liver response toward HPI axis activation, as it serves a precursor for cortisone and corticosteroid synthesis through the steroid hormone biosynthesis pathway (KEGG 2020). DDT exposure did not cause cholesterol changes, but squalene was down-regulated in all exposures except the Mix 0.1% treatment. Squalene serves as a precursor to cholesterol formation through the steroid biosynthesis pathway (KEGG 2020). It should be noted that cholesterol hydroxylase, involved in the breakdown of cholesterol into primary bile acids (KEGG 2020), can be induced by pregnane X receptor (PXR) activity, which is known to be activated by DDE (You 2004).

Based on the current evidence the aminoacyl-t-RNA biosynthesis pathway is uniquely affected by DDT at the higher exposure concentration, with the responses exacerbated in mixture
exposure. The majority of the amino acids involved in the aminoacyl-t-RNA biosynthesis pathway were down-regulated. The decrease in amino acids could indicate increased protein synthesis depleting amino acid resources, but aminoacyl-t-RNA has also been linked to other biochemical processes including aminoacylation of phospholipids in the cell membrane, and antibiotic biosynthesis (Raina and Ibba 2014). Evidence of nucleotide metabolism changes was seen in all individual exposures and the higher concentration mixture exposure through changes in the purine metabolism and pyrimidine metabolism (only in DTM 1%) pathways. This suggests that both pesticides have the potential to cause some form of DNA or RNA alteration, and that this potential persists in mixture exposures. DNA damage correlating to DDT exposure in humans is well documented (ATSDR 2002; Yáñez et al. 2004), and Petrovici et al. (2020) indicated the DNA damaging potential of DTM on Zebrafish gonads. Damage to DNA (both genome and epigenome) links back to oxidative stress and oxidative damage (Sies et al. 2017), which increases due to increased mitochondrial activity (Barzilai and Yamamoto 2004). The redox system of the body can also be triggered by any disruption to the metabolic state of equilibruium, as redox signalling occurs through changes in a multitude of metabolic pathways interconnected (Sies et al. 2017). Based on the evidence we hypothesise that, similar to GAS, a general stress response reaction occurs upon exposure to low concentrations of pesticides leading to exhaustion of key components (e.g. L-Sorbitol was down-regulated to the point of depletion in all but one exposure treatment). General stress can also lead to redox signalling changes causing oxidative damage and subsequent DNA damage (Sies et al. 2017).

A limitation in assessing the metabolomics data from this study was the large number of unidentified analytes, which form the majority of important variables identified. This is a common issue with untargeted metabolomics methods, but is a compromise against the cost of chemical standards and specificity (requires marker metabolites) in using targeted methods (Cajka and Fiehn 2016).
When considering the behavioural responses, the changes over time have to be considered in order to distinguish persistence of effects. The differences to control observed at 0 h are drastically reduced at 96 h. For the mobility state metrics, the reduction over time seen in the control appears to be the reason for this shift as the Mix 1% treatment also shows some time differences for mobility state metrics and thus also shows differences to control at 96 h. The only persistent differences were in the distance moved, velocity, cumulative duration spent moving and frequency of visits to the top from the Mix 1% treatment, and the increase in meander seen in DDT exposures at both concentrations. There thus seems to be an initial response at the onset of all the exposure treatments. A general decrease in activity is observed. Organophosphate pesticides are known to elicit a concentration dependent decrease in swimming behaviour and heart rate on *X. laevis* and *D. rerio* larvae (Watson et al. 2014). The effects of chronic DTM exposure between 0.25 and 2 μg/L on *D. rerio* behaviour showed increase in aggression after 6 days at all concentrations indicating that time dependent responses can occur and that longer term exposure may be required for conclusive analysis of behavioural effects of pesticides (Strungaru et al. 2019). If the reduction in activity observed in the current study was an anti-threat response based on human activity when initiating the exposures, then this should be reflected in the control organisms as well, but it is not. It thus seems logical that the initial behavioural responses are due to the chemical change in the environment and may be a direct or indirect result of toxicological interaction of the pesticides. From an ecological perspective this behaviour is unlikely to lead to increased predation. Because the acceleration, velocity and distance moved are unaffected (except in DDT 0.1% for the latter two factors) the ability to escape predation would likely remain unaffected. While the ability to recognise and detect predators may be impaired under DDT exposure, the reduction in mobility and duration spent at the top zone would likely increase camouflage and reduce the chance of being spotted by a predator (South et al. 2019). *Xenopus laevis*’ dorsal eye position means threats are mainly perceived from above (Reed 2005), making the bottom zone the safest position for the frog. *X. laevis* tadpoles also show reduced activity in the presence of predators (Kruger et al. 2019). That being said, literature
indicates that pesticides generally reduce escape responses of frogs (Sievers et al. 2019). The fact that the exposed animal activity is more similar to the control after 96 h suggests that while there was an initial behavioural reaction, some adaptation to the initial chemical stressor occurred over the 96 h period. The time dependent variability in behaviour of DTM exposed *D. rerio* observed by Strungaru et al. (2019) could also explain the temporal variation observed in the present study. The persistence of these effects at the Mix 1% treatment indicates an additive or synergistic effect between these pesticides at the higher concentration tested. The increase in meander with no change in velocity, seen in the DDT exposures, could be an indication of disorientation effects by the DDT exposure. Pesticide induced disorientation may originate from neurotoxic effects. In a study on the swimming pattern differences between aquatic and terrestrial frogs Jizhuang et al. (2017) noted that not only foot shape, but also movement patterns such as ankle rotation and lateral leg rotation play a role in the efficiency of swimming behaviour. Changes to nerve firing due to pesticide activity has the potential to disrupt these movement patterns. From a meta-analysis by Sievers et al. (2019) insecticides predominantly increase abnormal swimming behaviour and decrease general activity in frogs. Granted that the majority of studies analysed used tadpoles, these data correspond to what was observed in the current study. Data on surface activity caused by insecticides was widely varied (Sievers et al. 2019). South et al. (2019) showed that the immediate behavioural changes seen at the DDT 0.1% treatment of the current study can alter predator-prey interactions between *X. laevis* and *Culex* sp. larvae. These interaction effects described by South et al. (2019) may be expressed similarly at the higher DDT exposure concentrations and could also be evident in both DTM exposures based on the similarity in behavioural changes (from control) observed between these treatments. More importantly we predict that, as the mixture exposure treatments showed persistent changes in behaviour after 96 h, ecological interaction effects as those shown by South et al. (2019) may also be persistent after 96 h in mixture exposure scenarios.
When integrating the data from all analyses, the metabolomics data were separated into responses unique to a pesticide (i.e. occurring in either the DDT or DTM treatments, but not both) and shared responses. This distinction identified adenine as metabolite of interest in DTM exposure both in individual and mixture exposure. Up-regulation of adenine played an important role in exposure identification (treatment group separation) and pathway analysis (purine metabolism), and may prove a useful biomarker in identifying DTM (or similar pyrethroid) exposure in frogs. In the DDT exposures L-arabinopyranose proved useful in identifying DDT exposure (separation from control) in *X. laevis*. However, the effects seem concentration dependent and are not markedly visible in mixture exposures. L-Arabinopyranose also showed a notable statistical relationship to the variation in behavioural data. L-Arabinopyranose plays an important role in the interconversion of sugars and is linked to the ascorbate and aldarate metabolism, pentose and glucoronate interconversions, and amino sugar and nucleotide sugar metabolism pathways (KEGG 2020). DDT is known to affect carbohydrate metabolism, such as the inhibition of glycolytic pyruvate production in insects (Agosin et al. 1961).

Shared responses between pesticides indicate metabolites that may be used as general stress response markers. In this regard further identification of unknown metabolites will also illuminate the possible mechanisms behind the general response observed. L-sorbitol and myo-inositol was shown to be important in both pathway analysis and general exposure identification (i.e. separation of exposed from control regardless of pesticide or concentration). Myo-inositol plays a role in mitigating the oxidative damage in plants where it inhibits lesion formation, and cell death due to increases in H$_2$O$_2$ and salicylic acid (Chaouch and Noctor 2010). When the fish cells were treated with myo-inositol after copper exposure it reversed oxidative damage in the form of cell injury, lipid peroxidation, and protein oxidation (Jiang et al. 2013). We suggest that myo-inositol production may be activated in *X. laevis* as a means to combat oxidative damage due to pesticide exposure. Sorbitol degradation has been shown to be down-regulated in (*Carassius auratus*) by exposure to a mixture of fungicides and
herbicides (Gandar et al. 2017). Such impairment of the degradation pathway could result in elevated sorbitol levels, as observed in this study, and subsequently force interconversion into fructose, glucose or glycogen (Gandar et al. 2017). Oleic acid, cadaverine were shared responses that showed importance in explaining variation in accumulation. The increase in fatty acids due to exposure can lead to disruptions in redox signalling and increase oxidative stress (Mels et al. 2011) and may stem from endocrine disruption (Strong et al. 2015). Cadaverine levels were down-regulated. Theoretically this could be the result of increased GSH metabolism into glutathionylaminopropylcadaverine (Wagner et al. 2012), but GSH oxygenation through GST seems more likely under increased oxidative stress. Cadaverine is produced though lysine decarboxylation (KEGG 2020), but no lysine changes were observed.

While the evidence presented in the present study cannot outright prove causality for any of these responses, the controlled conditions under which the study was performed increases the probability of a causal relationship between exposure and measured effect (Adams 2003). In-depth targeted analysis of the metabolites of interest identified in this study, as well as further correlation analyses between metabolites and exposure in real-world or simulated real-world settings, may be necessary to provide robust biomarkers of exposure for these pesticides in frogs. The variation explained by the selected variables in the RDA analyses was higher (< 50%) for the two consecutive levels of organisation (i.e. accumulation vs. metabolome, and metabolome vs. behaviour) compared to the accumulation vs behaviour analysis where only 10.5% of the variation in data was explained by the selected variables. This indicates the importance of multi-level approaches in assessing the effects of pesticides as it shows the variation increase when levels of organisation are skipped, reducing the efficacy of such analysis. It is for this reason that many unknowns still exist regarding the mechanisms involved in pesticide toxicology, and that adverse outcome pathways (AOPs) and computational modelling are increasing in popularity in ecotoxicological studies (Wu et al. 2020).
Conclusion

This study successfully showed that acute exposure to DDT and DTM can result in sub-lethal effects on *X. laevis* in terms of changes to the liver metabolome, with some of the effects enhanced in exposure to a mixture of these pesticides. The behaviour of *X. laevis* exposed to DDT and DTM at sub-lethal concentrations showed initial responses in the form of decreased activity, less alterations between mobility states, and less time spent at the top with persistence of this response in mixture exposure after 96 h. This shows that sub-lethal exposure to pesticides can alter the biochemical homeostasis of frogs with the potential to cascade onto behavioural changes in mixture exposure scenarios. Further targeted analysis, and perhaps a multi-analysis approach to enhance identification of metabolites, is required in order to fully link the metabolomic effects to accumulation and uptake. The potential shown for behavioural changes with links to known ecological interactions to persist, validates further study into pesticide mixtures and ecological interactions in both laboratory and field settings. Potential unique marker metabolites including adenine (for DTM), L-arabinopyranose (for DDT), and glycerol-3-phosphate (for DDT) were identified. General pesticide exposure markers possibly linked to a general stress response were also identified. These included L-sorbitol, myo-inositol, oleic acid, and cadaverine. These metabolites of interest may serve as a useful starting point for future targeted metabolomics research. This study provides necessary toxicological data on frogs, as non-target victims of MVC pesticide exposure, which can be used improve *in silico* models for future research into AOPs associated with these pesticides.

Acknowledgements

This study was partially funded by the Flemish Interuniversity Council (VLIR) to ECN (VLIR- OUS project—ZEIN21013PR396; PI’s Luc Brendonck and Victor Wepener), The South African National Research Foundation (NRF: Grant no. SFH150624120779; awarded to Nico Wolmarans), and the Chemical Industries Education & Training Authority (CHIETA; awarded to Nico Wolmarans). The Water Research Group NABF facilities, funded through the NRF
National Nanotechnology Equipment Program (Grant no. 99024), were used in this study. This is publication number 551 of the Water Research Group.

The Authors would like to acknowledge Dr. Tarryn-Lee Botha for providing assistance with behavioural analyses that contributed toward the completion of this study.

**Conflict of Interest**

The authors declare they have no conflict of interest

**Author Contributions**

Nico J Wolmarans: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft; Lieven Bervoets: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review and editing; Patrick Meire: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review and editing; Victor Wepener: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review and editing.

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Table 1: Experimental treatments with corresponding nominal concentrations of exposures and measured water concentrations taken from the four tanks, where behaviour was not measured, for each treatment. The percentage of the nominal exposure measured is also included.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nominal exposure concentration</th>
<th>Measured concentration (% of nominal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>DDT 0.1%</td>
<td>*DDT 20 μg/L ≈ 0.1% of LC50_{FETAX}</td>
<td>19.3 ± 2.7 μg/L (97%)</td>
</tr>
<tr>
<td>DDT 1%</td>
<td>DDT 357 μg/L = 1% of LC50_{FETAX}</td>
<td>301.6 ± 41.6 μg/L (84%)</td>
</tr>
<tr>
<td>DTM 0.1%</td>
<td>DTM 0.19 μg/L = 0.1% of LC50_{FETAX}</td>
<td>0.16 ± 0.07 μg/L (87%)</td>
</tr>
<tr>
<td>DTM 1%</td>
<td>DTM 1.9 μg/L = 1% of LC50_{FETAX}</td>
<td>1.66 ± 0.37 μg/L (87%)</td>
</tr>
<tr>
<td>Mix 0.1%</td>
<td>DDT 35.7 μg/L + DTM 0.19 μg/L</td>
<td>DDT 25 ± 2.8 μg/L (70%) + DTM 0.18 ± 0.4 μg/L (95%)</td>
</tr>
<tr>
<td>Mix 1%</td>
<td>DDT 357 μg/L + DTM 1.9 μg/L</td>
<td>DDT 265 ± 9.7 μg/L (74%) + DTM 1.69 ± 0.2 μg/L (89%)</td>
</tr>
</tbody>
</table>

*0.1% of DDT LC50_{FETAX} = 35.7 μg/L. Experimental animals from South et al. (2019) were used for this treatment for ethical compliance in reducing and re-using test organisms, therefore the concentration was chosen as required by South et al. (2019) and was used to represent the lower concentration of DDT.
Table 2: Concentrations of DDT measured in frog tissue (mean ± standard deviation; ng/g wet weight) with calculated metrics. Statistical significant differences (p≤0.05) from the control is indicated with * and in bold. Isomeric ratio significance is in comparison to the stock DDT used. Superscript lettering (*) and (b) indicate significant differences between exposure treatments for parent/daughter (P/D) ratios.

<table>
<thead>
<tr>
<th>treatment</th>
<th>Total DDT</th>
<th>P/D ratio (DDT/DDD+DDE)</th>
<th>Isomeric ratio (p,p-/o,p-)</th>
<th>stock = 4.13 ± 0.1</th>
<th>o,p-DDE</th>
<th>p,p-DDE</th>
<th>o,p-DDD</th>
<th>p,p-DDD</th>
<th>o,p-DDT</th>
<th>p,p-DDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;LOD</td>
<td>-</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>DDT 0.1%</td>
<td>21.7 ± 5.0</td>
<td>a0.69 ± 0.83</td>
<td>4.77 ± 2.07</td>
<td>&lt;LOD</td>
<td>0.35 ± 0.42</td>
<td>*2.1 ± 1.89</td>
<td>*11.62 ± 2.86</td>
<td>*1.91 ± 1.68</td>
<td>± 5.54 ± 6.44</td>
<td></td>
</tr>
<tr>
<td>DDT 1%</td>
<td>244 ± 121</td>
<td>a12.8 ± 6.40</td>
<td>&lt;LOD</td>
<td>*9.29 ± 2.69</td>
<td>*18.17 ± 4.74</td>
<td>*39.5 ± 19.5</td>
<td>&lt;LOD</td>
<td>*176 ± 102</td>
<td>± 15.55 ± 8.34</td>
<td></td>
</tr>
<tr>
<td>Mix 0.1%</td>
<td>23.13 ± 10.6</td>
<td>ab17.15 ± 16.8</td>
<td>3.08 ± 2.26</td>
<td>&lt;LOD</td>
<td>0.34 ± 0.46</td>
<td>0.24 ± 0.27</td>
<td>*1.06 ± 0.92</td>
<td>*5.77 ± 3.44</td>
<td>± 15.55 ± 8.34</td>
<td></td>
</tr>
<tr>
<td>Mix 1%</td>
<td>127.16 ± 49.06</td>
<td>b1.7 ± 0.69</td>
<td>*1.28 ± 0.20</td>
<td>&lt;LOD</td>
<td>*3.43 ± 1.57</td>
<td>*14.45 ± 8.61</td>
<td>*30.90 ± 13.24</td>
<td>*41.82 ± 17.65</td>
<td>± 36.39 ± 18.35</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Composition of DDT isomers as percentage of total DDT with the top contributing mean per treatment indicated in **bold**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>o,p-DDE</th>
<th>p,p-DDE</th>
<th>o,p-DDD</th>
<th>p,p-DDD</th>
<th>o,p-DDT</th>
<th>p,p-DDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT 0.1%</td>
<td>1.64 ± 3.26</td>
<td>± 1.4 ± 1.43</td>
<td>10.8 ± 8.3</td>
<td><strong>58.8 ± 22.6</strong></td>
<td>7.5 ± 6.28</td>
<td>19.9 ± 20.6</td>
</tr>
<tr>
<td>DDT 1%</td>
<td>0.13 ± 0.15</td>
<td>± 6.07 ± 7.37</td>
<td>± 15.1 ± 23.4</td>
<td>15.1 ± 6.67</td>
<td>0.29 ± 0.33</td>
<td><strong>63.28 ± 25.75</strong></td>
</tr>
<tr>
<td>Mix 0.1%</td>
<td>0.99 ± 0.73</td>
<td>± 1.5 ± 1.23</td>
<td>1.27 ± 1.28</td>
<td>5.08 ± 4.52</td>
<td>23.88 ± 10.82</td>
<td>± <strong>67.29 ± 11.12</strong></td>
</tr>
<tr>
<td>Mix 1%</td>
<td>0.15 ± 0.06</td>
<td>± 2.63 ± 0.25</td>
<td>± 11.07 ± 3.11</td>
<td>25.28 ± 8.06</td>
<td>± <strong>32.86 ± 5.1</strong></td>
<td>28.02 ± 5.36</td>
</tr>
</tbody>
</table>
Table 4: Significant results of metabolic pathway analysis for the different exposure treatments. Only Holm-corrected p-values <0.1 were included. Unknown analytes were excluded from analysis. PI = pathway impact.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pathway</th>
<th>Hits</th>
<th>p-value</th>
<th>PI</th>
<th>Compound hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT 0.1%</td>
<td><strong>Biosynthesis of unsaturated fatty acids</strong></td>
<td>4</td>
<td>0.012</td>
<td>0</td>
<td>Stearic acid; Oleic acid; Linoleic acid; Eicosapentaenoic acid</td>
</tr>
<tr>
<td></td>
<td><strong>Inositol phosphate metabolism</strong></td>
<td>1</td>
<td>0.016</td>
<td>0.09</td>
<td>Myo-inositol</td>
</tr>
<tr>
<td></td>
<td><strong>Ascorbate and aldarate metabolism</strong></td>
<td>1</td>
<td>0.016</td>
<td>0</td>
<td>Myo-inositol</td>
</tr>
<tr>
<td></td>
<td><strong>Galactose metabolism</strong></td>
<td>2</td>
<td>0.063</td>
<td>0.002</td>
<td>Alpha-D-Glucose; Myo-inositol</td>
</tr>
<tr>
<td>DDT 1%</td>
<td><strong>Fructose and mannose metabolism</strong></td>
<td>3</td>
<td>0.017</td>
<td>0.02</td>
<td>Sorbitol; Mannose 6-phosphate; alpha-D-Glucose</td>
</tr>
<tr>
<td></td>
<td><strong>Galactose metabolism</strong></td>
<td>4</td>
<td>0.035</td>
<td>0.04</td>
<td>alpha-D-Glucose; Glycerol; Sorbitol; Myo-inositol</td>
</tr>
<tr>
<td>DTM 0.1%</td>
<td><strong>Propanoate metabolism</strong></td>
<td>1</td>
<td>0.026</td>
<td>0</td>
<td>2-Hydroxybutyric acid</td>
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<tr>
<td></td>
<td><strong>Biosynthesis of unsaturated fatty acids</strong></td>
<td>2</td>
<td>0.032</td>
<td>0</td>
<td>Stearic acid; Eicosapentaenoic acid</td>
</tr>
<tr>
<td>DTM 1%</td>
<td><strong>Pentose phosphate pathway</strong></td>
<td>4</td>
<td>0.014</td>
<td>0.08</td>
<td>6-Phosphogluconic acid; Beta-D-Glucose-6-phosphate; Beta-D-Glucose; Gluconolactone</td>
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<tr>
<td></td>
<td><strong>Pyruvate metabolism</strong></td>
<td>2</td>
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<td>0.17</td>
<td>Pyruvic acid; L-Malic acid</td>
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<td></td>
<td><strong>Propanoate metabolism</strong></td>
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<td>Succinic acid; Hydroxypropionic acid; Beta-Alanine; 2-Hydroxybutyric acid</td>
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<tr>
<td></td>
<td><strong>Biosynthesis of unsaturated fatty acids</strong></td>
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<td>0.072</td>
<td>0</td>
<td>Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid</td>
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<tr>
<td></td>
<td><strong>Citrate cycle (TCA cycle)</strong></td>
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<td>0.088</td>
<td>0.17</td>
<td>Succinic acid; L-Malic acid; Pyruvic acid; Fumaric acid</td>
</tr>
<tr>
<td>Mix 0.1%</td>
<td><strong>Biosynthesis of unsaturated fatty acids</strong></td>
<td>3</td>
<td>0.023</td>
<td>0</td>
<td>Stearic acid; Oleic acid; Eicosapentaenoic acid</td>
</tr>
<tr>
<td></td>
<td><strong>Valine, leucine and isoleucine degradation</strong></td>
<td>3</td>
<td>0.087</td>
<td>0</td>
<td>L-Valine; L-Isoleucine; L-Leucine</td>
</tr>
<tr>
<td></td>
<td><strong>Glycine, serine and threonine metabolism</strong></td>
<td>3</td>
<td>0.093</td>
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<td>Glyceric acid; L-Serine; L-Threonine</td>
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<td></td>
<td><strong>Valine, leucine and isoleucine biosynthesis</strong></td>
<td>4</td>
<td>0.093</td>
<td>0.99</td>
<td>L-Threonine; L-Leucine; L-Valine; L-Isoleucine</td>
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<tr>
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<td>0.058</td>
<td>0</td>
<td>Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid</td>
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Table 5: Summary of time factor (differences between 0 h and 96 h) results for the analysed behavioural metrics. Increase (↑), and decrease (↓) from 0h to 96h indicated for each metric measured through an effect size (Cohen’s-d value) > 0.8. None of the data showed statistical significance only practical changes are included.

<table>
<thead>
<tr>
<th></th>
<th>Distance moved</th>
<th>Velocity</th>
<th>Cumulative duration moving</th>
<th>Frequency of visits to top zone</th>
<th>Cumulative duration in top zone</th>
<th>Frequency in highly mobile state</th>
<th>Cumulative duration in highly mobile state</th>
<th>Frequency in mobile state</th>
<th>Cumulative duration in mobile state</th>
<th>Frequency in immobile state</th>
<th>Cumulative duration in immobile state</th>
<th>Mean Acceleration</th>
<th>Mean Meander</th>
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Table 6: Summary of results of treatment factor (differences between exposure and control treatments) for the analysed behavioural metrics. Increase (↑), and decrease (↓) from control for each treatment is indicated for each metric measured through an effect size (Cohen’s-d value) > 0.8. Statistical significance from the Kruskal-Wallis analyses are indicated with an asterisk (*).

<table>
<thead>
<tr>
<th>Exposure treatment</th>
<th>Distance moved</th>
<th>Velocity</th>
<th>Cumulative duration</th>
<th>Frequency of visits to top zone</th>
<th>Cumulative duration in top zone</th>
<th>Frequency in immobile state</th>
<th>Frequency in mobile state</th>
<th>Frequency in highly mobile state</th>
<th>Mean Acceleration</th>
<th>Mean Meander</th>
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<tbody>
<tr>
<td><strong>0h; Control vs:</strong></td>
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<td>↓</td>
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</table>
Figure 1: PLSDA scores plots for each exposure group (DDT (A), DTM (B), Mix (C)) individually and all groups assessed together (D). Colours are indicated in each legend.
Figure 2: Redundancy analysis of tissue accumulation data with metabolome data as selection variables (left). Red arrows indicate significant explanatory variables (p < 0.1), with blue arrows indicating non-significant explanatory variables (p > 0.1). Sample distribution with 95% confidence interval ellipses displayed separately (right).
Figure 3: Redundancy analysis of behavioural data with tissue accumulation data as explanatory variables (left). Eigen values across first and second axis are 0.06 and 0.05 respectively. Blue arrows indicate non-significant explanatory variables (p > 0.1). Behavioural metrics short-labels are as follows: Distance = Total distance moved; Velocity = Velocity; Accel = Mean acceleration; CDMoving = Cumulative duration spent moving; TopF = Frequency of visits to top zone; TopCD = Cumulative duration in top zone; Meander = Mean meander; ImmobF = Frequency in immobile state; ImmobCD = Cumulative duration in immobile state; MobF = Frequency in mobile state; MobCD = Cumulative duration in mobile state; HMobF = Frequency in highly mobile state; HMobCD = Cumulative duration in highly mobile state. Sample distribution with 95% confidence interval ellipses are displayed separately (right).
Figure 4: Redundancy analysis of behavioural data with metabolome data as explanatory variables (left). Eigen values across first and second axis are 0.44 and 0.14 respectively. Red arrows indicate significant explanatory variables (p < 0.1). Behavioural metrics short-labels are as follows: Distance = Total distance moved; Velocity = Velocity; Accel = Mean acceleration; CDMoving = Cumulative duration spent moving; TopF = Frequency of visits to top zone; TopCD = Cumulative duration in top zone; Meander = Mean meander; ImmobF = Frequency in Immobile state; ImmobCD = Cumulative duration in immobile state; MobF = Frequency in mobile state; MobCD = Cumulative duration in mobile state; HMobF = Frequency in highly mobile state; HMobCD = Cumulative duration in highly mobile state. Sample distribution with 66% (due to high variation) confidence interval ellipses are displayed separately (right).