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

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

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

27 and other pesticides (*see* Gerber et al. 2016; Volschenk et al. 2019, Mbongwe et al. 2003;

28 Govaerts et al. 2018 Viljoen et al. 2016; Wolmarans et al. 2018; Wolmarans et al. 2021). The

29 presence and environmental impacts of other World Health Organisation (WHO)

30 recommended pesticides for MVC (*see* WHO 2006) have not yet been examined in depth in

31 this region (Quinn et al. 2011). Simultaneous DDT and pyrethroid presence has, however,

32 been previously measured in human breastmilk from MVC regions in South Africa, with aquatic

33 ecosystems (water and fish) being identified as a possible vector for these pesticides into

34 humans (Bouwman et al. 2006).

35 Two pesticides were selected as representatives for MVC pesticides.

36 Dichlorodiphenyltrichloroethane as representative of the organochlorine pesticide group, and

37 deltamethrin (DTM) as representative of the pyrethroid pesticide group. These two pesticides

38 are often used in conjunction with one another for IRS in South Africa (Brooke et al. 2013).

39 Both DDT and DTM have broadly similar primary modes of action where binding affects cation

40 channels at neural synapses (Davies et al. 2007; Haschek et al. 2013). Secondary binding

41 activity plays a large role in sub-lethal effects, such as the endocrine disruption activity of DDT

42 (Hascheck et al. 2013). Both primary and secondary binding activity for these pesticides are

43 not extensively studied in non-target organisms such as amphibians.

44 Dichlorodiphenyltrichloroethane is highly persistent and bioaccumulates through the food web

45 due to its lipophilic nature (Bouwman et al. 2011). Deltamethrin is not considered to be

46 persistent in the environment (ATSDR 2003). The wider agricultural use and consequent

47 multiple sources of DTM to the natural environment could, however, theoretically lead to higher

48 exposure peaks to non-target organisms in the wild.

49 Given the potential hazard presented by these pesticides in the environment and uncertainty

50 as to sub-lethal effects in non-target organisms, the need was identified to assess the sub-

51 lethal effects of these pesticides on non-target anurans. In pursuit of this aim, metabolomics

52 and behavioural analysis were incorporated as possible effect indicators. The use of

53 metabolomics in an ecotoxicology context has proven useful in determining the biochemical

54 pathways upon which environmental contaminants act (Labine and Simpson 2020).

55 Metabolomics has previously been used in anuran ecotoxicology to assess the changes in

56 leopard frog liver metabolome towards herbicides, fungicides and insecticides (Van Meter et

57 al. 2018). As the use of metabolomics in contaminant exposure studies provides a link

58 between the environment and effects on the individual, behavioural analysis can be used as

59 a link between effects on the individual and cascading effects on the population, community,

60 or ecosystem. In many cases it has proven to be a more sensitive "early warning" of toxicity

61 than conventional mortality (LC50) testing based predictions (Hellou 2011). Hellou (2011) also

62 stipulated the importance of fate of chemicals and metabolic abilities of the subject organism

63 in understanding behavioural ecotoxicology in broader terms. Peterson et al. (2017)

64 suggested that the use of behaviour in ecotoxicology should focus on a framework that

65 incorporates causation, ontogeny, adaptation, and phylogeny (Tinbergen's four postulates).

66 According to Peterson et al. (2017), it is only through an integration of our understanding of

67 the mechanisms and causation by which contaminants affect behaviours, along with the

68 effects of these behaviours on fitness, that population level effects of contaminants can be

69 fully understood.

70 There is a distinct lack of sub-lethal toxicity literature related to anurans and MVC pesticides,

71 particularly in African species (Wolmarans et al. 2020). In this study, with the above mentioned

72 integration framework in mind, we combined chemical accumulation, metabolomics, and

73 behaviour as toxicological endpoints to explore the integrated effects of these pesticides on

74 the fully aquatic anuran *Xenopus laevis* in a laboratory setting. The African clawed frog (*X.*

75 *laevis*) was selected as test species for being an established model aquatic anuran as well as

76 having a natural distribution that coincides with areas where IRS is applied in Southern Africa

77 (*for distribution range see* De Villiers and Measy 2017; Du Preez and Carruthers 2017). Based

78 on the biological organisation hierarchy the aims were to link exposure, as measured through

79 pesticide bioaccumulation, to changes in the metabolome that in turn would result in

80 behavioural changes. We further determined whether mixtures of the pesticides would result

81 in additive effects.

Materials and Methods

Ethical research practise

84 All animal housing, handling, breeding and bioassays were completed under ethical approval

85 (NWU-0264-16-A5) by the AnimCare ethics committee of North-West University.

Animal husbandry and housing conditions prior to exposure

87 *Xenopus laevis* used in this study were bred and housed as in South et al. (2019; 2020) in

88 accordance with best practise as stipulated by Reed (2005). All frogs were 11 months old at

89 the time that experiments were carried out. Frogs were classified as sub-adult for the purpose

90 of this study as sexual maturity had been reached in some, but external sexual morphology

91 was not present in most animals yet.

Experimental setup and design

93 Pesticide treatments

94 The two pesticides tested, DDT and DTM, were selected in this study as representatives of

95 IRS pesticides. The scope of this study and ethical limitations on the use of vertebrate animals

96 did not allow for extensive LC50 testing on adult frogs, and no literature containing LC50 data

97 on adult *X. laevis* was found in a recent review (*see* Wolmarans et al. 2020). Therefore, the

98 $LC50_{FETAX}$ values (DDT= 35.7 mg/L and DTM = 0.19 mg/L, Channing 1998; Saka 2004) based

99 on toxicity to larval *X. laevis* were used as a starting point for lethality, even though sensitivity

100 between adult and developmental stages of animals can differ. After initial testing showed

101 mortality at higher concentrations, the exposure concentrations for this study were set at to

102 1% and 0.1% of the respective $LC50_{FETAX}$ for DDT and DTM where no mortality was observed

103 after 96 h. Fractions of the lethal concentrations were used in order to assess mixtures in

104 terms of equal toxic units. The different exposure treatments used in this study are outlined in

105   Table 1. For environmental relevance commercially available formulations of these pesticides

106   were used for exposure treatments.

107   Exposure chemicals

108   The technical grade DDT (AVIDDT 750, Avima (Pty) Ltd, RSA) consisted of 71.8% ∑DDT (of

109   which 80.5% = *p,p*-DDT and 19.5% = *o,p*-DDT) and concentrations were prepared in terms of

110   active ingredient (i.e. ∑DDT). For DTM a commercial formulation (Decatix 3, Cooper

111   Veterinary Products (Pty) Ltd, RSA) was also used consisting of 2.5% m/v DTM and exposure

112   concentrations were also prepared in terms of active ingredient. Stock solutions and dilutions

113   were prepared in fresh housing media.

114   Exposure setup

115   The frogs were allowed to acclimate in the experimental tanks for 2 h prior to exposure (3 L

116   clear polycarbonate tanks) with the temperature gradually being lowered from the housing

117   temperature (23°C) to experimental temperature (20°C) during this period. Static exposures

118   were performed and animals were not fed during the exposure period. Eight replicates of sub-

119   adult *X. laevis* were individually exposed per treatment. Four of these individuals per treatment

120   were selected prior to exposure and were used for behaviour recordings throughout the

121   exposure as per South et al. (2019, 2020).

122   Control animals underwent the exact same procedure and handling as exposed animals.

123   Behaviour was recorded for 2 h immediately at the onset of the exposure period followed by

124   another 2 h recording initiated at 94 h after exposure. At 96 h the frogs were euthanized

125   through means of mechanical stunning followed by double pithing. Chemical euthanasia

126   methods were not used to reduce the possible effect of euthanasia on the liver metabolome.

127   Liver samples were collected weighed and flash frozen at -80°C for metabolome analysis. The

128   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 H_2 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 $R^2 > 0.999$ for all compounds except DTM that had an $R^2 = 0.998$. Samples were analysed in batches of seven with quality control standards run between batches. Concentrations reported were adjusted according to recovery of the

156 internal standard added to each sample, which was 68 ± 11% (mean ± standard deviation) for

157 the analysed water samples and 80 ± 14% for the analysed tissue samples. Instrumental limit

158 of detection (LOD) and limit of quantitation (LOQ) was calculated individually for each

159 compound as 3 times and 10 times (respectively) the standard error of the gradient of the

160 calibration curve. All instrumental LODs were below 50 µg/L.

Metabolome analysis

162 Frog liver metabolome analyses were performed at the North-West University National

163 Metabolomics Platform (NMP). The method of Venter et al. (2016) was used for the extraction,

164 and oximation and silylation extracting 50 mg of liver sample (n=8 per treatment).

165 The GC/TOF-MS analysis was also performed according to (Venter et al. 2016) with slight

166 modifications. These modifications included the use of splitless injection (1 µL) and separation

167 through an Rxi-5Sil-MS (30 m, 0.25 mm, 0.25 µm; RESTEK, USA) column at a constant flow

168 of 1.5 mL/min. The primary oven temperature program was initiated at 70°C and held for 1

169 min followed by an initial ramp of 7°C/min to a final temperature of 300°C, which was held for

170 1 min (*see* Venter et al. 2016 for full extraction, analysis and peak data processing

171 information).

Behavioural analysis

173 For the first 2 h post-exposure, experimental treatments (n=4 per treatment) were recorded

174 and movement behaviour metrics were recorded as per the methods of South et al. (2019,

175 2020). Detailed metrics are given in the Statistical analysis – Behavioural analyses section

176 and Table 5

Statistical analysis

178 Chemical accumulation analyses

179 Initially a zero-filter was used on the data disregarding compounds (per treatment group) with

180 detection rates (values above LOQ) less than 50% for each group. For all further statistical

181 analyses remaining values below LOD were replaced with 0.5 LOD values. Comparison

182 groups were set to compare each exposure treatment to the control treatment. Multiple t-tests

183 were performed on each comparison group coupled with Dunn's post hoc test with p-value

184 adjustment for multiple comparison using the Holm-Sidak method with significance set at $\alpha \leq$

185 0.05.

186 Metabolomics analyses

187 Non-parametric analyses for metabolome data consisted of a fold change (FC) analysis

188 performed on untransformed MS peak intensity data. This was performed for each exposure

189 treatment compared to the control treatment for each detected spectral peak. This analysis

190 provides a sense of practical significance to changes in the screening data with significance

191 set at $|FC| \geq 2$.

192 Prior to parametric analyses a zero-filter was used excluding all compounds with detection

193 rates below 50% in samples from both groups within a comparison. Log transformed

194 ($Log_{10}X+1$) metabolome data were used to achieve normalisation for parametric testing. The

195 parametric analysis consisted of independent samples multiple t-tests with adjustment for

196 multiple testing (Benjamini Hochberg adjustment for the control of false discovery rates; FDR).

197 Significance was set at adjusted $p < 0.1$ because of the number of variables measured. As a

198 parametric measure of practical significance in the changes observed, effect sizes (Cohen's

199 –d value) were calculated with significance set at $|d| \geq 0.8$ (Lakens 2013).

200 Both effect size and FC were used as effect size takes into account the standard deviation

201 between samples whereas the FC is based only on the mean per treatment, but more sensitive

202 to changes in this mean.

203 A partial least squares-discriminant analysis (PLS-DA) was performed on the metabolome

204 data for each of three pesticide groupings (DDT, DTM, and Mix) in order to assess separation

205 variables between treatments in terms of the severity of exposure. Data included in this

206 analysis were filtered based on effect sizes and FC. The PLS-DA models were used to identify

207   predictor variables based on their variable importance in projection (VIP) scores for separation

208   between the control, 0.1%, and 1% treatments for each analysis (and overall separation in

209   final PLS-DA). For predictive ability only VIP scores >1 were considered viable. All

210   metabolomics statistical analyses were performed using Metaboanalyst 4.0 (Chong et al.

211   2019).

212   Pathways analysis was performed using the Metaboanalyst pathway analysis package (Xia

213   and Wishart 2010). As *X. laevis* is not a species option within this package the zebrafish, *Danio*

214   *rerio*, was used as the analysis organism in the model as done by Onjiko et al. (2016), and

215   Portero and Nemes (2019). Pathway analysis algorithms consisted of pathway enrichment

216   analysis by global test and pathway topology analysis by relative-between-ness centrality. All

217   pathways referred to in this study were confirmed through the Kyoto Encyclopedia of Genes

218   and Genomes (KEGG) pathways database (KEGG 2020; *also see* Kanehisa et al. 2010;

219   Tanabe and Kanehisa 2012).

220   Behavioural analyses

221   Various metrics were calculated from the two hour recordings of the frogs based on centre

222   point tracking data as described in Table 5. Total distance moved, mean velocity, cumulative

223   duration spent moving, the mean acceleration, and meander were the movement metrics

224   measured. Meander is a factor of turn angle and total distance moved and is thus dependent

225   on the direction of movement in the tank with right handed turns resulting in a positive turn

226   angle and left handed turns resulting in a negative turn angle. The frequency of visits to, and

227   cumulative duration spent at, the top zone of the tank were also measured. Lastly the

228   frequency of being in, and cumulative duration spent in each of three mobility states were

229   measured. An immobile state was set a 0-20% of the maximum velocity. A mobile state was

230   set as 20-60% of the maximum velocity, and a highly mobile state being above 60% of the

231   maximum velocity.

232 The behavioural metric data in this study were analysed individually using Kruskal-Wallis

233 analyses for comparison between groups coupled with Dunn's post-hoc test for mean rank

234 comparison Significance was set at $p \leq 0.05$. Three sets of comparison groups were analysed

235 for the behaviour data. A time factor was assessed consisting of each treatment at 0 h vs that

236 same treatment at 96 h. Two treatment factors were assessed consisting of the control at 0 h

237 vs every exposure treatment at 0 h, as well as control at 96 h vs every exposure treatment at

238 96 h. For a measure of practical significance effect sizes (Cohen's –d value) were also

239 calculated with significance was set at $|d| \geq 0.8$.

240 A principal component analysis was performed on log transformed ($\text{Log}_{10}X+1$) behavioural

241 data to determine group separation based on behavioural metrics and identify possible driving

242 metrics behind group separations.

243 To assess whether links of association could be established between the datasets measured

244 at different levels of biological organisation, redundancy analyses (RDAs) were performed for

245 the various combinations of variable sets (exposure treatment, chemical accumulation, 96h

246 behaviour, and metabolomics) using CANOCO v.5 software. In dealing with the large number

247 of variables within these datasets interactive forward selection of variables was applied to

248 each RDA in order to identify the most important variables for explaining variation in the data

249 in each analysis. P-value cut-off was set at 0.1. Significant variables selected from these RDAs

250 were re-analysed and priority variables were identified based on their influence in explaining

251 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.

277   The composition of DDT isomers (Table 3) measured in each sample was inconsistent

278   between treatments with large variation within the treatments. The prominent compound in the

279   DDT 1% and Mix 0.1% treatments was *p,p*-DDT making up more than 63% of the ∑DDT. The

280   *p,p*-DDD was the major contributor in the DDT 0.1% treatment (58.8%) while *o,p*-DDT made

281   up only 32.8% of the ∑DDT in the Mix 1% treatment.

Metabolomics

283   The whole metabolome screening analysis detected 562 spectral compound peaks in the frog

284   livers. Based on FC and effects sizes a total of 259 spectral peaks with notable up- or down

285   regulation (i.e. practical significance as defined under statistical analysis) between groups

286   were identified. Of these spectral peaks, 41 showed statistically significant differences to

287   control based on multiple t-tests (supplementary Table S2). The DDT 0.1% and DTM 0.1%

288   treatments had the most spectral peaks (18 and 17 in total, respectively) with statistical

289   significance. The least significant changes, only three peaks, were found in the Mix 0.1%

290   treatment. The DDT 1% and DTM 1% treatments showed significant up- or down-regulation

291   in 13 and 10 metabolites respectively. The Mix 1% treatment also had 10 statistically

292   significant changes, but had only two spectral peaks in common with the individual 1%

293   treatments in terms of statistical significance. In addition to the 42 peaks with statistical

294   significance, 13 peaks were also considered important for being either completely absent or

295   only present in the analysed treatments compared to control (supplementary Table S2).

296   Difficulty in compound identification through the screening databases used, resulted in a large

297   number of unidentified analytes among those that showed significant differences.

298   The PLS-DA analysis across five components for all DDT treatments showed separation

299   between the control and DDT 1% treatment along the first two components (Figure 1A)

300   accounting for 27.7% (20.2% and 7.5% for component one and two respectively) of the

301   variation. The DDT 0.1% treatment did not separate from either the control or DDT 1%

302   treatment, but its distribution was rather uniformly split between the two other treatments

303   resulting in a clear concentration based shift from the control in the bottom left to the DDT 1%

304   in the top right. Cross validation of the model showed the highest Q_2 value (predictive capacity

305   measure) at only one component (0.60) with the highest accuracy (0.83) at three components.

306   The VIP scores were used to identify the top metabolic variables in predicting concentration

307   groups of DDT exposure across the first two components of which 60 had scores >1. The

308   variable with the highest VIP scores was phosphoric acid, with scores >1.76 for both

309   components.   The PLS-DA analysis performed for all DTM treatments showed clear

310   separation between all three treatments (Figure 1B) across the first two components

311   accounting for 24.8% (15.3% and 9.5% for component one and two respectively) of the

312   variation. The first and third components (not shown) accounted for more variation (component

313   three explained 13% of the variation), but in this combination the DTM 0.1% treatment showed

314   overlap with the control. Cross validation of the model also showed the highest Q_2 value at

315   five components (0.72) with the highest accuracy (0.91) at two components. The VIP scores

316   were again used to identify the top variables in predicting groups across the first two

317   components, of which 77 had scores >1. L-Sorbitol had the highest VIP scores (>1.9 across

318   the first two components). For analysis of both Mix groups the PLS-DA analysis showed less

319   separation between control and mixture exposure treatments than the individual pesticide

320   treatments. There was slight overlap between the Control and the Mix 1% with the Mix 0.1%

321   once again falling in between the two along the first two components (Figure 1C). The group

322   separation was skewed resulting in greater separation between groups toward the top left and

323   convergence toward the bottom right. This suggests the two components having an inverse

324   relationship toward each other.   Component one accounted for 22.4% of the total variation

325   and component two for 10.4% (32.8% in total). The variable with the highest VIP score (>1.77

326   on the first two components) was an unknown peak (Analyte 458 UM: 159, RT: 1376.42). The

327   top 62 variables showed VIP scores >1. Cross validation of this model indicated two

328   components as the optimal in terms of predictive value (Q_2 = 0.48) and accuracy (0.74).

329   When all treatments were analysed together (Figure 1D) clear separation from the control

330   could be established for DTM 0.1%, DDT 1%, and DTM 1%. The Mix 0.1% and Mix 1%

331 treatments only showed minor overlap with the control whilst the DDT 0.1% grouped closer to

332 control than the other exposure treatments. All exposure treatments overlapped with each

333 other across the first two components. Only 18.3% of the variation was accounted for. This

334 model however showed poor predictive capacity (Q_2 = 0. 24) and accuracy (0.38). In total 81

335 variables showed VIP scores > 1 with L-sorbitol showing the greatest predictive capacity (VIP

336 > 2.5 across the first two components).

337

338   The metabolic pathway analysis results (Table 4) indicated four pathways were significantly

339   influenced in the DDT 0.1% treatment frogs. The inositol phosphate metabolism and galactose

340   metabolism pathways showed pathway impact (PI) > 0 (*see* Xia and Wishart 2010 for

341   description of pathway impact). In the DDT 1% treatment the fructose mannose metabolism

342   pathway and galactose metabolism pathways showed PI > 0. The DTM 0.1% influenced two

343   pathways significantly, but the net impact was zero for both. Five different pathways were

344   significantly affected in the DTM 1% treatment. Three of which (pentose phosphate pathway,

345   pyruvate metabolism and the citrate cycle) showed PI > 0. Four pathways were significantly

346   affected in the Mix 0.1% treatment. The glycine, serine and threonine metabolism pathway as

347   well as the valine, leucine and isoleucine biosynthesis pathway both had PI > 0. The Mix 1%

348   treatments showed only one pathway affected, but with zero PI. Using a less strict cut-off (false

349   discovery rate adjusted $p < 0.1$) affected pathways were placed into their respective

350   categories. All exposure treatments resulted in carbohydrate-, lipid- and amino acid

351   metabolism changes along several pathways. The aminoacyl-tRNA biosynthesis pathway

352   (under translation pathways) was uniquely affected by the DDT 1% treatment and both Mix

353   treatments with 11 amino acids affected in each treatment and a 12th, L-proline, also affected

354   in Mix treatments.

355

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

383   in a highly mobile state were lower for all exposure treatments at 0 h compared to the control

384   treatment. The frequency and duration in a mobile state were also lower for all exposure

385   treatments except the Mix 1% treatment. The frequency of visits to the top zone was lower for

386   the DTM 1% and both of the Mix treatments. The frequency of being in an immobile state was

387   lower for the DTM 1% and Mix 0.1% treatments while the cumulative duration in an immobile

388   state was lower for the DDT 1%, DTM 0.1% and DTM 1% treatments. The only metrics that

389   increased at 0 h were the cumulative duration in an immobile state for the Mix 1% and the

390   mean meander for the Mix 0.1% treatment.

391   After 96 h no exposure treatment groups showed significant differences to the control group.

392   Effect sizes did however once again show multiple notable changes, but in less treatments

393   than at those measured 0 h. The mean meander showed a notable increase for the DDT 0.1%,

394   DDT 1%, Mix 0.1% and Mix1% treatment groups. The cumulative duration in an immobile

395   state and the mean acceleration both showed a decrease for the Mix 0.1% treatment, but and

396   increase for the Mix 1% treatment. Along with the prior changes mentioned, the Mix 1%

397   treatment also showed a decrease in the frequency of visits to the top zone, cumulative

398   duration spent in the top zone, frequency of being in a mobile state, frequency of being in an

399   immobile state and cumulative duration spent in a mobile state. Furthermore, the DTM 1%

400   treatment showed a decrease in total distance moved, mean velocity, cumulative duration

401   spent moving, frequency of visits to the top zone, frequency of being in a mobile state,

402   frequency of being in an immobile state and the cumulative duration spent in a mobile state.

403   A principle component analysis (PCA) of the behavioural metrics (not included) did not show

404   any clear group separation along the first two axes (Eigen values 0.50 and 0.17 respectively)

405   of the total variation in the data. Meander was shown to have a negative correlation to all other

406   metrics along the first axis.

407 Association between MVC Pesticide accumulation and metabolome

408 An RDA performed on the bioaccumulation data with the metabolome data as selection

409 variables. Forward selection of variables (based on raw p-values) indicated 20 variables of

410 importance in explaining the variation in accumulation data. Forward selection with FDR

411 adjusted p-values only showed conditional significance (adjusted p < 0.1) of 16 variables

412 under the inclusion of all 20 (Figure 2). In total 99% of the variation in chemical accumulation

413 was explained by the selected variables (56.3% was explained by significant variables) with

414 81.3% explained across the first two axes (eigenvalues of 0.63 and 0.18 for axis one and two

415 respectively). The DDT 0.1% and 1% treatments as well as the DTM 1% treatment separated

416 from the control based on 95% confidence intervals.

417

418 Association between MVC Pesticide accumulation and behaviour

419 A second RDA performed on the 96 h behaviour metrics with the individual compound

420 bioaccumulation data as selection variables (Figure 3) did not indicate pesticide

421 bioaccumulation as having a significant influence on the separation of behaviour data. There

422 was a quadrant based separation of the Mix 1% treatment samples from all the other treatment

423 samples on both the first and second axis. The Mix 1% treatment showed strong association

424 with increased o,p-DDT, o,p-DDD, and p,p-DDD which correlated positively with mean

425 acceleration and the cumulative duration spent immobile. This RDA however only explained

426 10.5% of the total variation in the data cumulatively between the first two axes.

427 Association between metabolome and behaviour

428 A third RDA was performed on the behaviour data using the metabolome data as selection

429 variables. In order to reduce the number of variables an initial analysis was performed where

430 after metabolome data were filtered based on raw p-values < 0.1 from that analysis. This

431 resulted in 23 remaining variables. From a second round analysis using only these variables

432 and applying forward selection, nine spectral peaks were identified as significant based on

433 FDR p-values < 0.1 (Figure 4). The nine selected variables accounted for 75.5% of the total

434 variation in behaviour data with 58.5% explained across the first two axes (eigenvalues of 0.44

435 and 0.14 for axis one and two respectively). No group separation was visible due to very large

436 spatial variation between individuals within each treatment (Figure 4). Of the nine variables

437 selected six were unknown analytes. Alpha-D-glucopyranose, mannobiose, and L-arabiniose

438 were the only known metabolites of significance.

439 **Discussion**

440 In assessing the effects of DTM and DDT on *X. laevis*, effect metrics need to be assessed

441 individually and in conjunction with one another to gain an understanding of the mechanisms

442 at play. Linking bioaccumulation to both exposure and effects can provide a better

443 understanding of the hazard posed by these pesticides in natural environments.

444 The bioaccumulation of DTM in frog tissue could not be confirmed through the methods used

445 in this study. The validation results of the extraction and analysis methods for DTM used in

446 this study indicated that the method was effective for the extraction and analysis of DTM. No

447 published literature is available on the accumulation of DTM in *Xenopus* tissue (*see*

448 Wolmarans et al. 2020). Studies on fish such as Szegletes et al. (1995) also failed to measure

449 DTM residues in tissue samples. Kim et al. (2008) did successfully measure DTM residues in

450 various tissues of rats when orally exposed to 2 and 10 mg/Kg DTM. The authors calculated

451 the maximum accumulation time to be ≤ 6 h for all tissue types except skin (12 h) and the

452 terminal elimination half-life was only > 96 h for fat, muscle and skin. These values are not

453 comparable to a constant immersion exposure scenario with aquatic organisms where skin

454 permeability plays a major role in uptake, but do speak to the tendency of DTM to eliminate

455 from tissue rapidly.

456 Accumulation of DDT was successfully measured in all individuals exposed to DDT, both

457 individually and in a mixture. The accumulation data showed large variation in terms of total

458 accumulated concentration, as well as isomeric and breakdown product contribution. Large

459 variation in DDT accumulation data is consistent with field results in *Xenopus sp.* from areas

460 of South Africa where DDT is applied for MVC (Viljoen et al. 2016; Wolmarans et al. 2018).

461 The mean ∑DDT tissue concentration in the DDT 0.1% (21.7 ng/g ww) and Mix 0.1% (23.13

462 ng/g ww) correspond to the highest survey mean for *X. muelleri* from Wolmarans et al. (2018)

463 of 17.79 ng/g ww. Frogs from Viljoen et al. (2016) had higher levels with male *Xenopus* sp.

464 having a mean ∑DDT concentration of 206 ng/g ww making it comparable to the DDT 1%

465 treatment from this study (244 ng/g ww), but these concentrations from Viljoen et al. (2016)

466 were measured in lipid bodies only and not whole organism tissues. It should also be noted

467 that there is limited value in a direct comparison of accumulation between laboratory exposure

468 data and long term field exposure data where the latter is subjected to long-term diffuse

469 exposure. Interestingly the fraction of the total accumulated DDT consisting of *p,p*-DDT itself

470 was lower than expected for such a short exposure period relative to the general half-life of

471 *p,p*-DDT, which is 28 days in river water and 56 days in lake water (USEPA 1989). The

472 accumulation of ∑DDT was very similar between the DDT 0.1% treatment and Mix 0.1%

473 indicating that the addition of DTM did not affect the accumulation of DDT at the lower

474 concentration. At higher concentration though the Mix 1% treatment only accumulated

475 approximately half of the ∑DDT accumulated in the DDT 1% treatment. This suggests some

476 interaction in either the uptake or storage of these two chemicals at the higher exposure

477 concentrations tested in this study. Although literature in this regard is scarce, interactions

478 between chemicals in a mixture have been shown to affect its biological uptake in plants in

479 some cases. Measured in aquatic macrophytes (*Juncus effuses L.*), the variability in

480 chlorpyrifos uptake was shown to be affected in mixtures whereas for atrazine it was not (Lytle

481 and Lytle 2002). In the current study, uptake could only have occurred through dermal

482 absorption. While *X. leavis* skin is known to be highly permeable, there is very little published

483 literature on how chemical interactions might affect the selectivity in permeability. The skin of

484 *X. laevis* contains peptides, such as magainin, that can form ioniphores in bilipid layers as part

485 of the neurosecretory system (Duclohier et al. 1989). Atrazine has been shown to reduce the

486 protein content of *X. laevis* skin peptides, but with no effect on the antimicrobial properties

487   these peptides have (Gibble and Baer 2011). Both permethrin and DTM have been shown to

488   interact with ion transport pathways in *Rana esculenta* skin causing absorption of Na^+ and

489   excretion of Cl^- ions, but the mechanism behind this activation could not be confirmed

490   (Cassano et al. 2009). The possibility that DTM acts on frog skin and subsequently alters

491   dermal DDT uptake in *X. laevis*, therefore cannot be excluded at this time.

492   In the current study, the PLS-DA results showed that the metabolic profiles of *X. laevis* liver

493   can be used to identify exposure to pesticides under laboratory conditions, however, only at

494   the higher concentration range assessed in this study due to separation from the control

495   observed for both the DDT-, DTM-, and Mix 1% treatments, but not at their respective 0.1%

496   treatments. Distinguishing between frogs exposed to different pesticides based on their

497   metabolic profiles was not possible with the metabolome screening methods used due to large

498   overlap in metabolite changes observed.

499   While some unique responses could be identified through univariate comparisons, a lot of

500   overlap in metabolic profiles emerge during multivariate analysis indicating some general

501   response to exposure independent of the pesticide. This becomes clear from the pathway

502   analyses where carbohydrate, amino acid, and lipid metabolism are all affected across the

503   board. This is similar to the results of Van Meter et al. (2018) that found carbohydrate and

504   amino acid metabolism affected in leopard toads exposed to individual and mixtures of

505   herbicides, fungicides, and insecticides. Van Meter et al. (2018) also attributed this to a

506   general exposure response, which is supported by the current study results, even though

507   different pesticide classes were used on a different species. Alanine appears to be a suitable

508   biomarker for insecticide exposure. Increased alanine was found in frogs exposed to malathion

509   and malathion containing mixtures in the study by Van Meter et al. (2018), but not in exposures

510   only containing herbicides and fungicides. The increased alanine to glycine ratio measured by

511   McKelvie et al. (2009) in earthworms for both DDT and endosulfan, and the alanine increase

512   observed in mussels exposed to lindane (Tuffnail et al. 2009), indicate that this increase upon

513   exposure occurs across different classes of organisms. Li et al. (2014) found an increase in

514   alanine in brain tissue of goldfish exposed to λ-cyhalothrin, but a decrease in kidney tissue.

515   Tissue specific differences in metabolomic response or variability is common (Simmons et al.

516   2015). In the current study, several derivatives of alanine were spectrally detected with the

517   major peak (RT: 310.19) indicating an increase (based on effect size) in the DTM 0.1%

518   treatment and both mixture treatments. The DTM 1% treatment, however, showed a decrease.

519   The ratio between alanine and glycine could not be calculated from the untargeted method

520   used, but based on the results from McKelvie et al. (2009) we suspect this ratio may hold

521   greater promise as a biomarker of insecticide exposure across different species than alanine

522   changes alone.

523   The alanine changes discussed, along with similarities in sugar and amino acid metabolism,

524   between the current study and Van Meter et al. (2018) suggest a secondary effect of exposure

525   stress regardless of the pesticide, which could affect a wide range of organisms. A possible

526   explanation for changes observed in carbohydrate and lipid metabolism could be a part of a

527   stress response not unlike general adaptation syndrome (GAS), initiated through activation of

528   the hypothalamus-pituitary-interrenal (HPI) axis, increasing cortisterone levels (Selye 1950).

529   Direct evidence of cortisol or corticosterone changes in the liver itself could not be confirmed

530   in the current study, but the down-regulation of cholesterol observed in DTM and Mix

531   treatments could be the result of liver response toward HPI axis activation, as it serves a

532   precursor for cortisone and corticosteroid synthesis through the steroid hormone biosynthesis

533   pathway (KEGG 2020). DDT exposure did not cause cholesterol changes, but squalene was

534   down-regulated in all exposures except the Mix 0.1% treatment. Squalene serves as a

535   precursor to cholesterol formation through the steroid biosynthesis pathway (KEGG 2020). It

536   should be noted that cholesterol hydroxylase, involved in the breakdown of cholesterol into

537   primary bile acids (KEGG 2020), can be induced by pregnane X receptor (PXR) activity, which

538   is known to be activated by DDE (You 2004).

539   Based on the current evidence the aminoacyl-t-RNA biosynthesis pathway is uniquely affected

540   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 equilibruim, 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).

567   When considering the behavioural responses, the changes over time have to be considered

568   in order to distinguish persistence of effects. The differences to control observed at 0 h are

569   drastically reduced at 96 h. For the mobility state metrics, the reduction over time seen in the

570   control appears to be the reason for this shift as the Mix 1% treatment also shows some time

571   differences for mobility state metrics and thus also shows differences to control at 96 h. The

572   only persistent differences were in the distance moved, velocity, cumulative duration spent

573   moving and frequency of visits to the top from the Mix 1% treatment, and the increase in

574   meander seen in DDT exposures at both concentrations. There thus seems to be an initial

575   response at the onset of all the exposure treatments. A general decrease in activity is

576   observed. Organophosphate pesticides are known to elicit a concentration dependent

577   decrease in swimming behaviour and heart rate on *X. laevis* and *D. rerio* larvae (Watson et al.

578   2014). The effects of chronic DTM exposure between 0.25 and 2 µg/L on *D. rerio* behaviour

579   showed increase in aggression after 6 days at all concentrations indicating that time

580   dependent responses can occur and that longer term exposure may be required for conclusive

581   analysis of behavioural effects of pesticides (Strungaru et al. 2019). If the reduction in activity

582   observed in the current study was an anti-threat response based on human activity when

583   initiating the exposures, then this should be reflected in the control organisms as well, but it is

584   not. It thus seems logical that the initial behavioural responses are due to the chemical change

585   in the environment and may be a direct or indirect result of toxicological interaction of the

586   pesticides. From an ecological perspective this behaviour is unlikely to lead to increased

587   predation. Because the acceleration, velocity and distance moved are unaffected (except in

588   DDT 0.1% for the latter two factors) the ability to escape predation would likely remain

589   unaffected. While the ability to recognise and detect predators may be impaired under DDT

590   exposure, the reduction in mobility and duration spent at the top zone would likely increase

591   camouflage and reduce the chance of being spotted by a predator (South et al. 2019).

592   *Xenopus laevis*' dorsal eye position means threats are mainly perceived from above (Reed

593   2005), making the bottom zone the safest position for the frog. *X. laevis* tadpoles also show

594   reduced activity in the presence of predators (Kruger et al. 2019). That being said, literature

595   indicates that pesticides generally reduce escape responses of frogs (Sievers et al. 2019).

596   The fact that the exposed animal activity is more similar to the control after 96 h suggests that

597   while there was an initial behavioural reaction, some adaptation to the initial chemical stressor

598   occurred over the 96 h period. The time dependent variability in behaviour of DTM exposed

599   *D. rerio* observed by Strungaru et al. (2019) could also explain the temporal variation observed

600   in the present study. The persistence of these effects at the Mix 1% treatment indicates an

601   additive or synergistic effect between these pesticides at the higher concentration tested. The

602   increase in meander with no change in velocity, seen in the DDT exposures, could be an

603   indication of disorientation effects by the DDT exposure. Pesticide induced disorientation may

604   originate from neurotoxic effects. In a study on the swimming pattern differences between

605   aquatic and terrestrial frogs Jizhuang et al. (2017) noted that not only foot shape, but also

606   movement patterns such as ankle rotation and lateral leg rotation play a role in the efficiency

607   of swimming behaviour. Changes to nerve firing due to pesticide activity has the potential to

608   disrupt these movement patterns. From a meta-analysis by Sievers et al. (2019) insecticides

609   predominantly increase abnormal swimming behaviour and decrease general activity in frogs.

610   Granted that the majority of studies analysed used tadpoles, these data correspond to what

611   was observed in the current study. Data on surface activity caused by insecticides was widely

612   varied (Sievers et al. 2019). South et al. (2019) showed that the immediate behavioural

613   changes seen at the DDT 0.1% treatment of the current study can alter predator-prey

614   interactions between *X. laevis* and *Culex* sp. larvae. These interaction effects described by

615   South et al. (2019) may be expressed similarly at the higher DDT exposure concentrations

616   and could also be evident in both DTM exposures based on the similarity in behavioural

617   changes (from control) observed between these treatments. More importantly we predict that,

618   as the mixture exposure treatments showed persistent changes in behaviour after 96 h,

619   ecological interaction effects as those shown by South et al. (2019) may also be persistent

620   after 96 h in mixture exposure scenarios.

621 When integrating the data from all analyses, the metabolomics data were separated into
622 responses unique to a pesticide (i.e. occurring in either the DDT or DTM treatments, but not
623 both) and shared responses. This distinction identified adenine as metabolite of interest in
624 DTM exposure both in individual and mixture exposure. Up-regulation of adenine played an
625 important role in exposure identification (treatment group separation) and pathway analysis
626 (purine metabolism), and may prove a useful biomarker in identifying DTM (or similar
627 pyrethroid) exposure in frogs. In the DDT exposures l-arabinopyranose proved useful in
628 identifying DDT exposure (separation from control) in *X. laevis*. However, the effects seem
629 concentration dependent and are not markedly visible in mixture exposures. L-
630 Arabinopyranose also showed a notable statistical relationship to the variation in behavioural
631 data. L-Arabinopyranose plays an important role in the interconversion of sugars and is linked
632 to the ascorbate and aldarate metabolism, pentose and glucoronate interconversions, and
633 amino sugar and nucleotide sugar metabolism pathways (KEGG 2020). DDT is known to affect
634 carbohydrate metabolism, such as the inhibition of glycolytic pyruvate production in insects
635 (Agosin et al. 1961).

636 Shared responses between pesticides indicate metabolites that may be used as general stress
637 response markers. In this regard further identification of unknown metabolites will also
638 illuminate the possible mechanisms behind the general response observed. L-sorbitol and
639 myo-inositol was shown to be important in both pathway analysis and general exposure
640 identification (i.e. separation of exposed from control regardless of pesticide or concentration).
641 Myo-inositol plays a role in mitigating the oxidative damage in plants where it inhibits lesion
642 formation, and cell death due to increases in H_2O_2 and salicylic acid (Chaouch and Noctor
643 2010). When the fish cells were treated with myo-inositol after copper exposure it reversed
644 oxidative damage in the form of cell injury, lipid peroxidation, and protein oxidation (Jiang et
645 al. 2013). We suggest that myo-inositol production may be activated in *X. laevis* as a means
646 to combat oxidative damage due to pesticide exposure. Sorbitol degradation has been shown
647 to be down-regulated in (*Carassuis auratus*) by exposure to a mixture of fungicides and

648 herbicides (Gandar et al. 2017). Such impairment of the degradation pathway could result in

649 elevated sorbitol levels, as observed in this study, and subsequently force interconversion into

650 fructose, glucose or glycogen (Gandar et al. 2017). Oleic acid, cadaverine were shared

651 responses that showed importance in explaining variation in accumulation. The increase in

652 fatty acids due to exposure can lead to disruptions in redox signalling and increase oxidative

653 stress (Mels et al. 2011) and may stem from endocrine disruption (Strong et al. 2015).

654 Cadaverine levels were down-regulated. Theoretically this could be the result of increased

655 GSH metabolism into glutathionylaminopropylcadaverine (Wagner et al. 2012), but GSH

656 oxygenation through GST seems more likely under increased oxidative stress. Cadaverine is

657 produced though lysine decarboxylation (KEGG 2020), but no lysine changes were observed.

658 While the evidence presented in the present study cannot outright prove causality for any of

659 these responses, the controlled conditions under which the study was performed increases

660 the probability of a causal relationship between exposure and measured effect (Adams 2003).

661 In-depth targeted analysis of the metabolites of interest identified in this study, as well as

662 further correlation analyses between metabolites and exposure in real-world or simulated real-

663 world settings, may be necessary to provide robust biomarkers of exposure for these

664 pesticides in frogs. The variation explained by the selected variables in the RDA analyses was

665 higher (< 50%) for the two consecutive levels of organisation (i.e. accumulation vs.

666 metabolome, and metabolome vs. behaviour) compared to the accumulation vs behaviour

667 analysis where only 10.5% of the variation in data was explained by the selected variables.

668 This indicates the importance of multi-level approaches in assessing the effects of pesticides

669 as it shows the variation increase when levels of organisation are skipped, reducing the

670 efficacy of such analysis. It is for this reason that many unknowns still exist regarding the

671 mechanisms involved in pesticide toxicology, and that adverse outcome pathways (AOPs) and

672 computational modelling are increasing in popularity in ecotoxicological studies (Wu et al.

673 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

Conflict of Interest

706 The authors declare they have no conflict of interest

Author Contributions

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

References

715 Adams MS (2003) Establishing Causality between Environmental Stressors and Effects on
716 Aquatic Ecosystems. Hum Ecol Risk Assess 9:17–35

717 Agosin M, Scaramelli N, Neghme A (1961) Intermediary carbohydrate metabolism of Triatoma
718 infestans (insecta; hemiptera)—I. Glycolytic and pentose phosphate pathway enzymes and
719 the effect of DDT. Comp Biochem Phys 2:143–159

720 ATSDR (2002) Toxicological profile for DDT, DDE, and DDD. Atlanta, GA: Agency for Toxic
721 Substances and Disease Registry, U.S. Department of Health and Human Services, Public
722 Health Service

723 ATSDR (2003) Toxicological profile for pyrethrins and pyrethroids. Atlanta, GA: Agency for
724 Toxic Substances and Disease Registry, U.S. Department of Health and Human Services,
725 Public Health Service

726 Barzilai A, Yamamoto K (2004) DNA damage responses to oxidative stress. DNA Repair
727 3:1109–1115

728 Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification, Can J
729 Biochem Physiol 37:911–917

730   Bouwman H, Sereda B, Meinhardt HM (2006) Simultaneous presence of DDT and pyrethroid
731   residues in human breast milk from a malaria endemic area in South Africa. Environ Pollut
732   144:902–917

733   Bouwman H, Van den Berg H, Kylin H (2011) DDT and Malaria Prevention: Addressing the
734   Paradox. Environ Health Perspect 119:744–747

735   Bratton MR, Frigo DE, Segar HC, Nephew KP, Mclachlan JA, Wiese TE, Burrow ME (2012)
736   The Organochlorine *o,p'*-DDT Plays a Role in Coactivator-Mediated MAPK Crosstalk in MCF-
737   7 Breast Cancer Cells. Environ Health Perspect 120:1291–1296

738   Brooke B, Koekemoer L, Kruger P, Urbach J, Misiani E, Coetzee (2013) Malaria vector control
739   in South Africa. S Afr Med J 103:784–788

740   Cajka T, Fiehn O (2016) Toward Merging Untargeted and Targeted Methods in Mass
741   Spectrometry-Based Metabolomics and Lipidomics Anal Chem 88:524–545

742   Cassano G, Bellantuono V, Ardizzone C, Lippe C (2009) Pyrethroid stimulation of ion transport
743   across frog skin. Environ Toxicol Chem 22:1330–1334

744   Chaouch S, Noctor G (2010) Myo-inositol abolishes salicylic acid-dependent cell death and
745   pathogen defence responses triggered by peroxisomal hydrogen peroxide. New Phytol
746   188:711–718

747   Chong J, Wishart DS, Xia J (2019) Using MetaboAnalyst 4.0 for Comprehensive and
748   Integrative Metabolomics Data Analysis. Curr Protoc Bioinformatics 68:e86

749   Channing A (1998) Tadpoles as bio-indicators of stream quality: a baseline study. Water
750   Research Commission of South Africa (WRC) Technical Report No. 718/1/98

751   Davies TGE, Field LM, Usherwood PNR, Williamson MS (2007) DDT, Pyrethrins, Pyrethroids
752   and Insect Sodium Channels. Life 59:151–162

753   De Villiers FA, Measey J (2017) Overland movement in African clawed frogs (*Xenopus laevis*):
754   empirical dispersal data from within their native range. PeerJ 5:e4039

755   Duclohier H, Molle G Spach G (1989) Antimicrobial peptide magainin I from *Xenopus* skin
756   forms anion-permeable channels in planar lipid bilayers. Biophys J 56:1017–1021

757   Du Preez LH, Carruthers V (2017) Frogs of Southern Africa: a complete guide. Struik Nature,
758   Random House Struik, Cape Town

759   Gandar A, Laffaile P, Marty-Gasset N, Viala D, Molette C, Jean S (2017) Proteome response
760   of fish under multiple stress exposure: Effects of pesticide mixtures and temperature increase.
761   Aquat Toxicol 184:61–77

762   Gerber R, Smit NJ, Van Vuren JHJ, Nakayama SMM, Yohannes YB, Ikenaka Y, Ishizuka M,
763   Wepener V (2016) Bioaccumulation and human health risk assessment of DDT and other
764   organochlorine pesticides in an apex aquatic predator from a premier conservation area. Sci
765   Total Environ 550:522–533

766   Gibble RE, Baer KN (2011) Effects of atrazine, agricultural runoff, and selected effluents on
767   antimicrobial activity of skin peptides in *Xenopus laevis*. Ecotox Environ Saf 74:593–599

768   Govaerts A, Verhaert V, Covaci A, Jaspers VLB, Berg OK, Addo-Bediako A, Jooste A,
769   Bervoets L (2018) Distribution and bioaccumulation of POPs and mercury in the Ga-Selati
770   River (South Africa) and the rivers Gudbrandsdalslågen and Rena (Norway). Environ Int
771   121:1319–1330

772   Haschek WM, Rousseaux CG, Wallig MA (2013) Haschek & Rousseaux's Handbook of
773   Toxicologic Pathology, 3rd Edition. Academic Press, Elsevier. London

774   Hellou J (2011) Behavioural Ecotoxicology, an "Early Warning" Signal to Assess
775   Environmental Quality. Environ Sci Pollut Res Int 18:1–11

776   Jiang WD, Lui Y, Jiang J, Hu K, Li SH, Feng L, Zhou XQ (2013) In vitro interceptive and
777   reparative effects of myo-inositol against copper-induced oxidative damage and antioxidant
778   system disturbance in primary cultured fish enterocytes. Aquat Toxicol 132–133:100–110

779   Jizhuang F, Wei Z, Bowen Y, Gangfeng L (2017) Propulsive efficiency of frog swimming with
780   different feet and swimming patterns. Biol Open 6:503–510

781   KEGG (2020) Kyoto Encyclopedia of Genes and Genomes. Kanehisa Laboratories.
782   www.kegg.jp (accessed: Jan 2020)

783   Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M (2010) KEGG for representation
784   and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res 38:D355-
785   D360

786   Keum YS, Kim JH, Li QX (2010) Metabolomics in Pesticide Toxicology. In: Krieger R (Ed)
787   Hayes' Handbook of Pesticide Toxicology 3rd ed. New York: Academic Press 627–643

788   Kim K, Anand SS, Kim HJ, White CA, Bruckner JV (2008) Toxicokinetics and Tissue
789   Distribution of Deltamethrin in Adult Sprague–Dawley Rats. Toxicol Sci 101:197–205

790 Kruger N, Measey J, Herrel A, Secondi J (2019) Anti-predator strategies of the invasive African
791 clawed frog, Xenopus laevis, to native and invasive predators in western France. Aquat
792 Invasions 14:433–443

793 Labine LM Simpson MJ (2020) The use of nuclear magnetic resonance (NMR) and mass
794 spectrometry (MS)–based metabolomics in environmental exposure assessment. Curr Opin
795 Environ Sci Health 15:7–15

796 Lakens D (2013) Calculating and reporting effect sizes to facilitate cumulative science: a
797 practical primer for t-tests and ANOVAs. Front Psychol 4:863

798 Li M, Wang J, Lu Z, Wei D, Yang M, Kong L (2014) NMR-based metabolomics approach to
799 study the toxicity of lambda-cyhalothrin to goldfish (*Carassius auratus*). Aquat Toxicol 146:82–
800 92

801 Lytle JS, Lytle TF (2002) Uptake and Loss of Chlorpyrifos and Atrazine by *Juncus effusus L.*
802 in a Mesocosm Study with a Mixture of Pesticides. Environ Toxicol Chem 21:1817–1825

803 Mbongwe B, Legrand M, Blais JM, Kimpe LE, Ridal JJ, Lean DR (2003)
804 Dichlorodiphenyltrichloroethane in the aquatic ecosystem of the Okavango Delta, Botswana,
805 South Africa. Environ Toxicol Chem 22:7–19

806 McKelvie JR, Yuk J, Xu Y, Simpson AJ, Simpson MJ (2009) ^{1}H NMR and GC/MS
807 metabolomics of earthworm responses to sub-lethal DDT and endosulfan exposure.
808 Metabolomics 5:84–94

809 Mels CM, Van der Westhuizen FH, Pretorius PJ, Erasmus E (2011) Unbalanced
810 biotransformation metabolism and oxidative stress status: implications for deficient fatty acid
811 oxidation. Health 3:43–48

812 Onjiko RM, Morris SE, Moody SA, Nemes P (2016) Single-cell Mass Spectrometry with Multi-
813 solvent Extraction Identifies Metabolic Differences between Left and Right Blastomeres in the
814 8-cell Frog (*Xenopus*) Embryo. Analyst 141:3648–3656

815 Petrovici A, Strungaru SA, Nicoara M, Rodea MA, Solcan C, Faggio C (2020) Toxicity of
816 Deltamethrin to Zebrafish Gonads Revealed by Cellular Biomarkers. J Mar Sci Eng 8:73–88

817 Peterson EK, Buchwalter DB, Kerby JL, LeFauve MK, Varian-Ramos CW, Swaddle JP (2017)
818 Integrative behavioral ecotoxicology: bringing together fields to establish new insight to
819 behavioral ecology, toxicology, and conservation. Curr Zool 63:185–194

820 Portero EP, Nemes P (2019) Dual cationic–anionic profiling of metabolites in a single identified
821 cell in a live *Xenopus laevis* embryo by microprobe CE-ESI-MS. Analyst 144:725–1018

822 Quinn LP, De Vos BJ, Fernandes-Whaley M, Roos C, Bouwman H, Kylin H, Pieters R, Van
823 den Berg J (2011) Pesticide Use in South Africa: One of the Largest Importers of Pesticides
824 in Africa. *in* Stoycheva M (Ed) Pesticides in the Modern World – Pesticide Use and
825 Management. InTech. Rijeka

826 Raina M, Ibba M (2014) tRNA as regulators of biological processes. Front Genet 5:171

827 Reed BT (2005) Guidance on the housing and care of the African clawed frog *Xenopus laevis*.
828 Royal Society for the Prevention of Cruelty to Animals, Horsham, United Kingdom. Report

829 Saka M (2004) Developmental toxicity of p,p'-dichlorodiphenyltrichloroethane, 2,4,6-
830 trinitrotoluene, their metabolites, and benzo[a]pyrene in Xenopus laevis embryos. Environ
831 Toxicol Chem 23:1065–1073

832 Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, Hollender J (2014) Identifying
833 Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence.
834 Environ Sci Technol 48:2097–2098

835 Selye H (1950) Stress and the General Adaptation Syndrome. Br Med J 1:1384–1392

836 Sies H, Berndt C, Jones DP (2017) Oxidative Stress. Annu Rev Biochem 86:715–48

837 Sievers M, Hale R, Parris KM, Melvin SD, Lanctôt CM, Swearer SE (2019) Contaminant-
838 induced behavioural changes in amphibians: A meta-analysis. Sci Total Environ 693:113570

839 South J, Botha TL, Wolmarans NJ, Wepener V, Weyl OLF (2019) Assessing predator-prey
840 interactions in a chemically altered aquatic environment: the effects of DDT on *Xenopus laevis*
841 and Culex sp. larvae interactions and behaviour. Ecotoxicology 28:771–780

842 Strong AL, Shi Z, Strong MJ, Miller DFB, Rusch DB, Buechlein AM, Flemmington EK,
843 Mclachlan JA, Nephew KP, Burow ME, Bunnel BA (2015) Effects of the Endocrine-Disrupting
844 Chemical DDT on Self-Renewal and Differentiation of Human Mesenchymal Stem Cells.
845 Environ Health Perspect 123:42–48

846 Strungaru SA, Plavan G, Ciobica A, Nicoara M, Robea MA, Solcan C, Petrovici A (2019)
847 Toxicity and chronic effects of deltamethrin exposure on zebrafish (*Danio rerio*) as a reference
848 model for freshwater fish community. Ecotox Environ Saf 171:854–862

849 Szegletes T, Bálint T, Szegletes ZS, Nemcsók J (1995) *In Vivo* Effects of Deltamethrin
850 Exposure on Activity and Distribution of Molecular Forms of Carp AChE. Ecotox Environ Saf
851 31:258–263

852 Tanabe M, Kanehisa M (2012) Using the KEGG Database Resource. Curr Protoc
853 Bioinformatics 38:1934–3396

USEPA (1989). Environmental Fate and Effects Division, Pesticide Environmental Fate One Line Summary: DDT (p, p'). United States Environmental Protection Agency, Washington, DC.

Tuffnail W, Mills GA, Cary P, Greenwood R (2009) An environmental 1H NMR metabolomic study of the exposure of the marine mussel Mytilus edulis to atrazine, lindane, hypoxia and starvation. Metabolomics 5:33–43

Venter L, Jansen van rensburg P, Loots DT, Vosloo A, Lindeque JZ (2016) Untargeted Metabolite Profiling of Abalone Using Gas Chromatography Mass Spectrometry. Food Anal Methods 9:1254–1261

Van Meter RJ, Glinski DA, Purucker ST, Henderon WM (2018) Influence of exposure to pesticide mixtures on the metabolomic profile in post-metamorphic green frogs (*Lithobates clamitans*). Sci Total Environ. 624:1348–1359

Viljoen IM, Bornman R, Bouwman H (2016) DDT exposure of frogs: A case study from Limpopo Province, South Africa. Chemosphere 159:335–341

Volschenk CM, Gerber R, Mkhonto MT, Ikenaka Y, Yohannes YB, Nakayama SMM, Ishizuka M, Van Vuren JHJ, Wepener V, Smit NJ (2019) Bioaccumulation of persistent organic pollutants and their trophic transfer through the food web: Human health risks to the rural communities reliant on fish from South Africa's largest floodplain. Sci Total Environ 685:1116–1126

Wagner AE, Ernst IMA, Birringer M, Sancak O, Barella L, Rimbach G (2012) A Combination of Lipoic Acid Plus Coenzyme Q10 Induces PGC1α, a Master Switch of Energy Metabolism, Improves Stress Response, and Increases Cellular Glutathione Levels in Cultured C2C12 Skeletal Muscle Cells. Oxid Med Cell Longev 2012:835970

Watson FL, Schmidt H, Turman ZK, Hole N, Garcia H, Gregg J, Tilghman J, Fradinger EA (2014) Organophosphate pesticides induce morphological abnormalities and decrease locomotor activity and heart rate in *Danio rerio* and *Xenopus laevis*. Environ Toxicol Chem 33:1337–1345

WHO (2006) Indoor residual spraying: Use of indoor spraying for scaling up global malaria control and elimination. World Health Organization, report http://whqlibdoc.who.int/hq/2006/WHO_HTM_MAL_2006.1112_eng.pdf Accessed 08/2018

Wolmarans NJ, Du Preez LH, Yohannes YB, Ikenaka Y, Ishizuka M, Smit NJ, Wepener V (2018) Linking organochlorine exposure to biomarker response patterns in Anurans: a case study of Müller's clawed frog (*Xenopus muelleri*) from a tropical malaria vector control region. Ecotoxicology 27:1203–1216

887 Wolmarans NJ, Bervoets L, Meire P, Wepener V (2020) Current Status and Future Prognosis
888 of Malaria Vector Control Pesticide Ecotoxicology and *Xenopus* sp. Rev Environ Contam
889 Toxicol 252:131–171

890 Wu Q, Achebouche R, Audouze K (2020) Computational Systems Biology as an Animal-free
891 Approach to Characterize Toxicological Effects of Persistent Organic Pollutants. Altern Anim
892 Ex 37:287–299

893 Xia J, Wishart DS (2010) MetPA: a web-based metabolomics tool for pathway analysis and
894 visualization. Bioinformatics 26:2342–2344

895 Yáñez L, Borja-Aburto V, Rojas E, De La Fuente H, González-Amaro R, Gómez H, Jongitud
896 AA, Díaz-Barriga F (2004) DDT induces DNA damage in blood cells. Studies in vitro and in
897 women chronically exposed to this insecticide. Environ Res 94:18–24

898 You L (2004) Steroid hormone biotransformation and xenobiotic induction of hepatic steroid
899 metabolizing enzymes Chem Biol 147:233–246

Tables and Figures

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

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

*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 in with * **and in bold**. Isomeric ratio significance is in comparison to the stock DDT used. Superscript lettering ([a] and [b]) indicate significant differences between exposure treatments for parent/daughter (P/D) ratios.

treatment	Total DDT	P/D ratio (DDT/DDD+DDE)	Isomeric ratio (p,p-/o,p-) stock = 4.13 ± 0.1	o,p-DDE	p,p-DDE	o,p-DDD	p,p-DDD	o,p-DDT	p,p-DDT
Control	<LOD	-	-	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
DDT 0.1%	21.7 ± 5.0	[a]**0.69 ± 0.83**	4.77 ± 2.07	<LOD	0.35 ± 0.42	***2.1 ± 1.89**	***11.62 ± 2.86**	***1.91 ± 1.68**	5.54 ± 6.44
DDT 1%	***244 ± 121**	2.40 ± 1.13	***12.8 ± 6.40**	<LOD	***9.29 ± 2.69**	***18.17 ± 4.74**	***39.5 ± 19.5**	<LOD	***176 ± 102**
Mix 0.1%	23.13 ± 10.6	[ab]**17.15 ± 16.8**	3.08 ± 2.26	<LOD	0.34 ± 0.46	0.24 ± 0.27	***1.06 ± 0.92**	***5.77 ± 3.44**	***15.55 ± 8.34**
Mix 1%	***127.16 ± 49.06**	[b]**1.7 ± 0.69**	***1.28 ± 0.20**	<LOD	***3.43 ± 1.57**	***14.45 ± 8.61**	***30.90 ± 13.24**	***41.82 ± 17.65**	***36.39 ± 18.35**

Table 3: Composition of DDT isomers as percentage of total DDT with the top contributing mean per treatment indicated in **bold**

Treatment	*o,p*-DDE	*p,p*-DDE	*o,p*-DDD	*p,p*-DDD	*o,p*-DDT	*p,p*-DDT
DDT 0.1%	1.64 ± 3.26	1.4 ± 1.43	10.8 ± 8.3	**58.8 ± 22.6**	7.5 ± 6.28	19.9 ± 20.6
DDT 1%	0.13 ± 0.15	6.07 ± 7.37	15.1 ± 23.4	15.1 ± 6.67	0.29 ± 0.33	**63.28 ± 25.75**
Mix 0.1%	0.99 ± 0.73	1.5 ± 1.23	1.27 ± 1.28	5.08 ± 4.52	23.88 ± 10.82	**67.29 ± 11.12**
Mix 1%	0.15 ± 0.06	2.63 ± 0.25	11.07 ± 3.11	25.28 ± 8.06	**32.86 ± 5.1**	28.02 ± 5.36

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.

Treatment	Pathway	Hits	p-value	PI	Compound hits
DDT 0.1%	Biosynthesis of unsaturated fatty acids	4	0.012	0	Stearic acid; Oleic acid; Linoleic acid; Eicosapentaenoic acid
	Inositol phosphate metabolism	1	0.016	0.09	Myoinositol
	Ascorbate and aldarate metabolism	1	0.016	0	Myoinositol
	Galactose metabolism	2	0.063	0.002	Alpha-D-Glucose; Myoinositol
DDT 1%	Fructose and mannose metabolism	3	0.017	0.02	Sorbitol; Mannose 6-phosphate; alpha-D-Glucose
	Galactose metabolism	4	0.035	0.04	alpha-D-Glucose; Glycerol; Sorbitol; Myoinositol
DTM 0.1%	Propanoate metabolism	1	0.026	0	2-Hydroxybutyric acid
	Biosynthesis of unsaturated fatty acids	2	0.032	0	Stearic acid; Eicosapentaenoic acid
DTM 1%	Pentose phosphate pathway	4	0.014	0.08	6-Phosphogluconic acid; Beta-D-Glucose-6-phosphate; Beta-D-Glucose; Gluconolactone
	Pyruvate metabolism	2	0.046	0.17	Pyruvic acid; L-Malic acid
	Propanoate metabolism	4	0.047	0	Succinic acid; Hydroxypropionic acid; Beta-Alanine; 2-Hydroxybutyric acid
	Biosynthesis of unsaturated fatty acids	4	0.072	0	Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid
	Citrate cycle (TCA cycle)	4	0.088	0.17	Succinic acid; L-Malic acid; Pyruvic acid; Fumaric acid
Mix 0.1%	Biosynthesis of unsaturated fatty acids	3	0.023	0	Stearic acid; Oleic acid; Eicosapentaenoic acid
	Valine, leucine and isoleucine degradation	3	0.087	0	L-Valine; L-Isoleucine; L-Leucine
	Glycine, serine and threonine metabolism	3	0.093	0.26	Glyceric acid; L-Serine; L-Threonine
	Valine, leucine and isoleucine biosynthesis	4	0.093	0.99	L-Threonine; L-Leucine; L-Valine; L-Isoleucine
Mix 1%	Biosynthesis of unsaturated fatty acids	4	0.058	0	Stearic acid; Oleic acid; Eicosapentaenoic acid; Alpha-Linolenic acid

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.

	Distance moved	Velocity	Cumulative duration moving	Frequency of visits to top zone	Cumulative duration in top zone	Frequency in highly mobile state	Cumulative duration in highly mobile state	Frequency in mobile state	Cumulative duration in mobile state	Frequency in immobile state	Cumulative duration in immobile state	Mean Acceleration	Mean Meander
Control					↓	↓	↓	↓	↓	↓			
DDT 0.1%													
DDT1%										↓	↑		
DTM 0.1%	↓	↓		↓		↑							
DTM 1%	↓	↓	↓								↑		
Mix 0.1%								↓	↓		↓		
Mix 1%						↓	↓	↓	↓	↓			

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 (*).

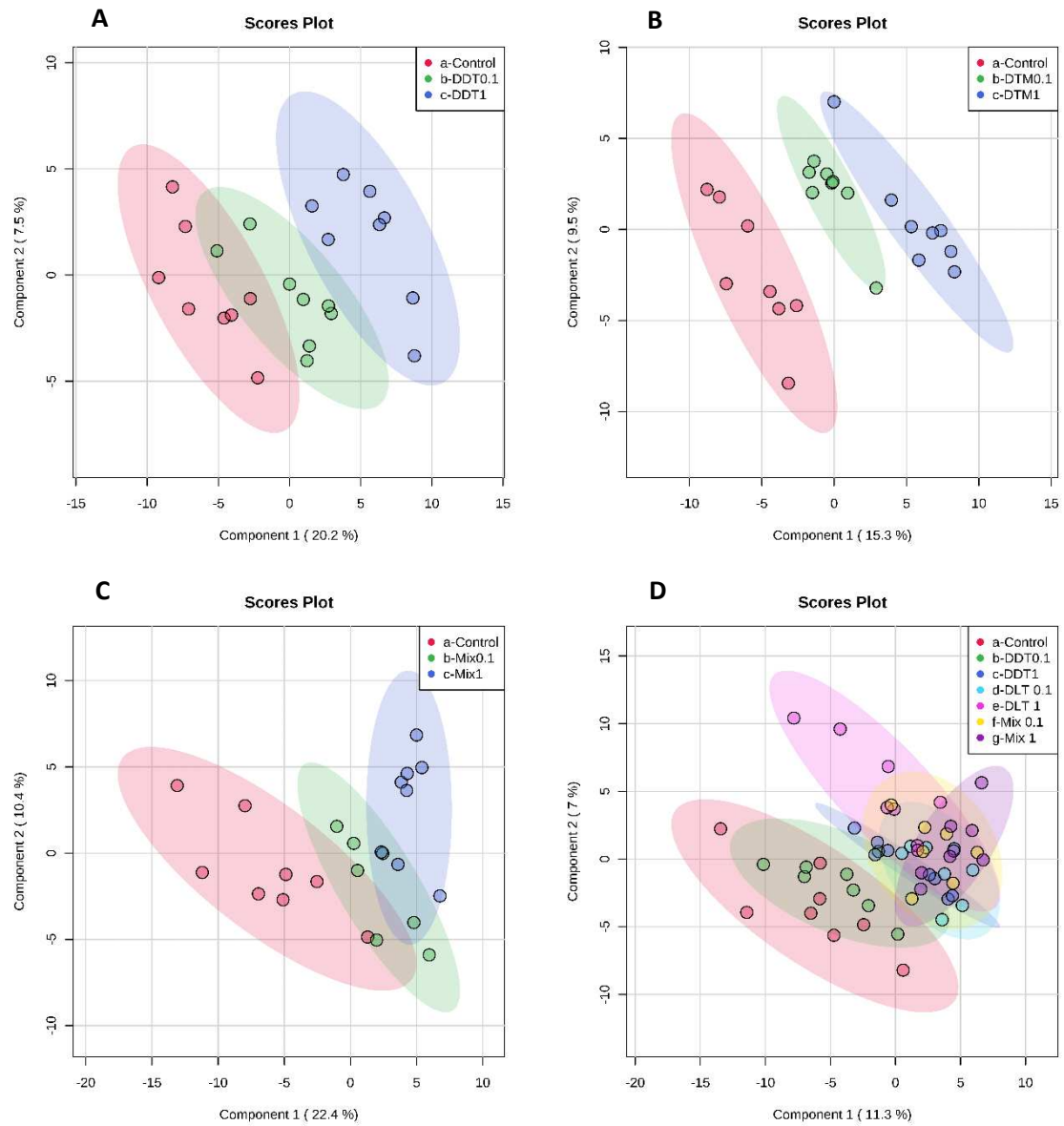| Exposure treatment		Distance moved	Velocity	Cumulative duration spent moving	Frequency of visits to top zone	Cumulative duration in top zone	Frequency in highly mobile state	Frequency in mobile state	Frequency in immobile state	Cumulative duration in highly mobile state	Cumulative duration in mobile state	Cumulative duration in immobile state	Mean Acceleration	Mean Meander
0h; Control vs:	DDT 0.1%	↓	↓	↓	↓*	↓*	↓	↓*	↓*	↓	↓*			
	DDT1%					↓	↓	↓		↓	↓	↓		
	DTM 0.1%					↓	↓	↓		↓	↓	↓		
	DTM 1%				↓	↓	↓	↓	↓	↓	↓	↓		
	Mix 0.1%				↓	↓	↓	↓	↓	↓	↓			↑
	Mix 1%				↓	↓*	↓			↓		↑		
96h; Control vs:	DDT 0.1%													↑
	DDT1%													↑
	DTM 0.1%													
	DTM 1%	↓	↓	↓	↓			↓	↓		↓			
	Mix 0.1%											↓	↓	↑
	Mix 1%				↓	↓		↓	↓		↓	↑	↑	↑

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
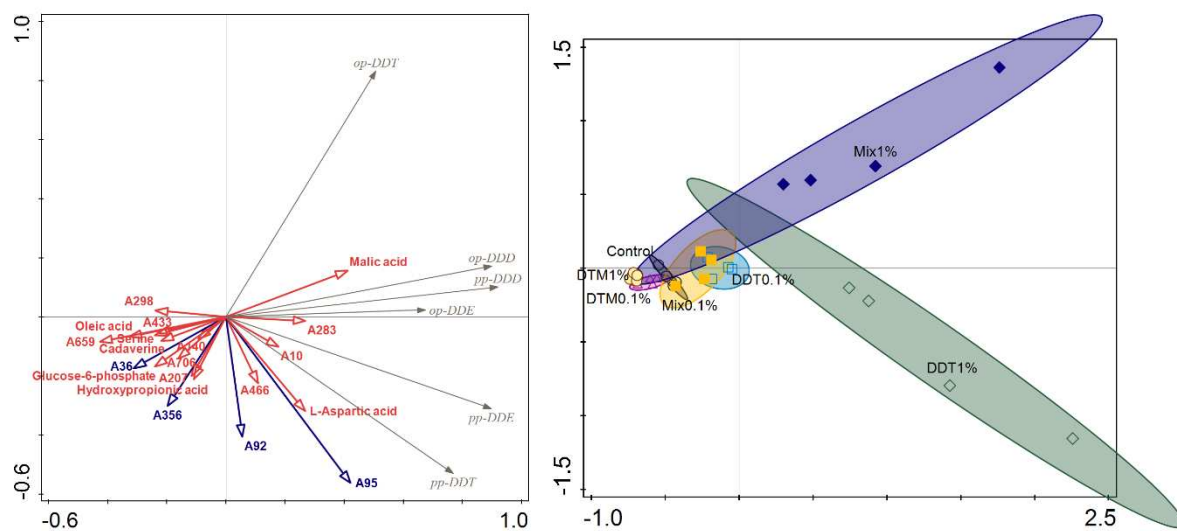
Figure 2: Redundancy analysis of tissue accumulation data with metabolome data as selection variables (left). Red arrows indicate significant explanatory variables (p < 0.1), with blue arrows indicating non-significant explanatory variables (p > 0.1) Sample distribution with 95% confidence interval ellipses displayed separately (right).
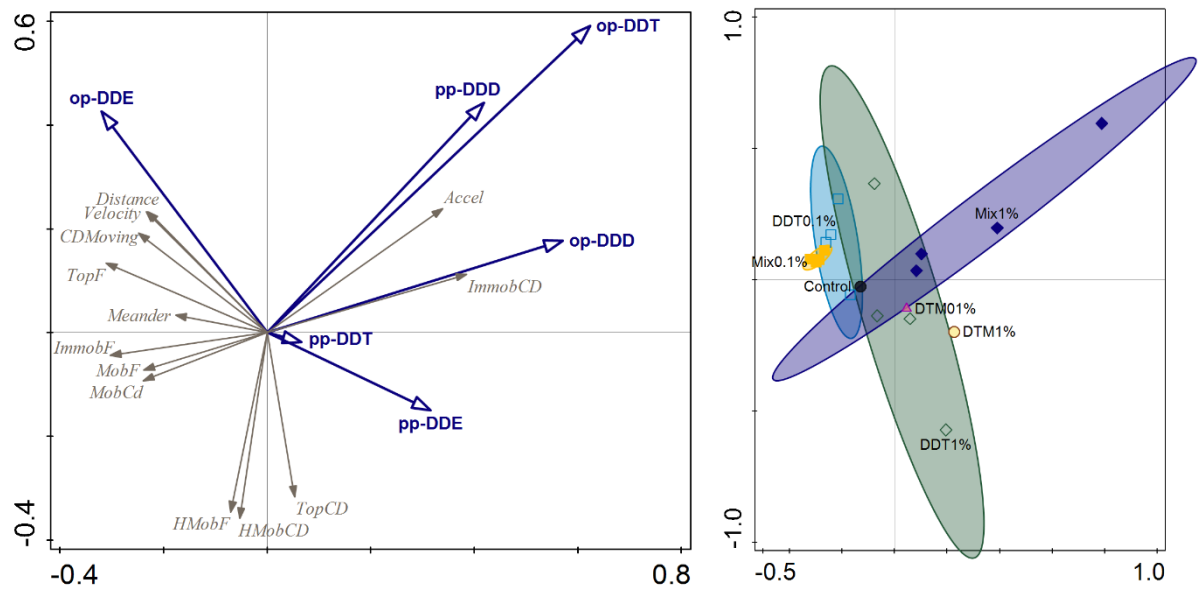
Figure 3: Redundancy analysis of behavioural data with tissue accumulation data as explanatory variables (left). Eigen values across first and second axis are 0.06 and 0.05 respectively. Blue arrows indicate non-significant explanatory variables (p > 0.1). Behavioural metrics short-labels are as follows: 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).
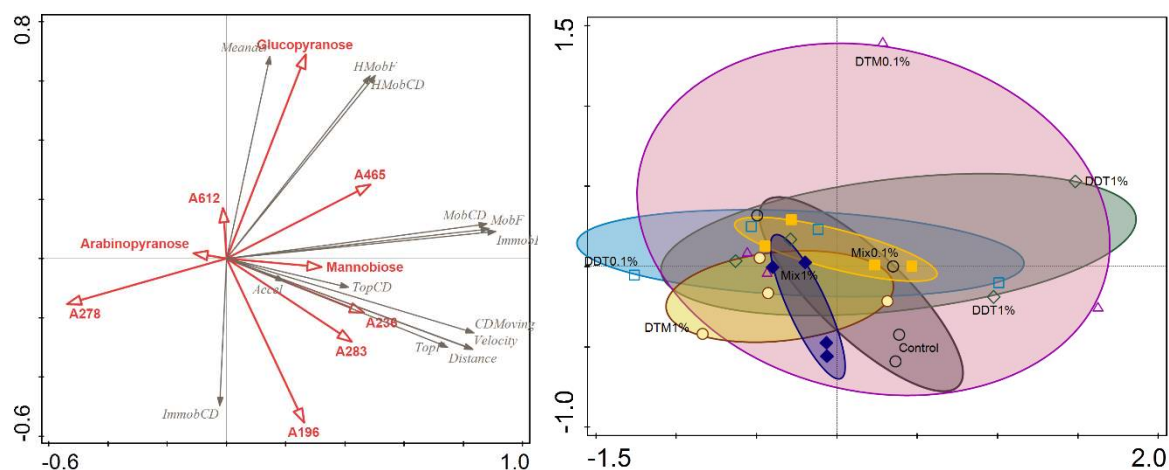
Figure 4: Redundancy analysis of behavioural data with metabolome data as explanatory variables (left). Eigen values across first and second axis are 0.44 and 0.14 respectively. Red arrows indicate significant explanatory variables (p < 0.1). Behavioural metrics short-labels are as follows: Distance = 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).