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Rachid Jellali, Perrine Zeller, Françoise Gilard, Audrey Legendre, Marie José Fleury, et al.. Effects of DDT and permethrin on rat hepatocytes cultivated in microfluidic biochips: Metabolomics and gene expression study. *Environmental Toxicology and Pharmacology*, 2018, 59, pp.1-12. <10.1016/j.etap.2018.02.004>. <hal-02357463>

HAL Id: hal-02357463

<https://hal.science/hal-02357463v1>

Submitted on 4 Feb 2024

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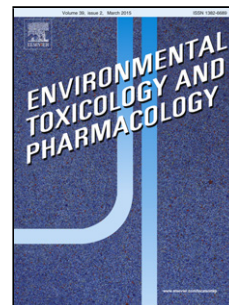


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Accepted Manuscript

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PII: S1382-6689(18)30023-1
DOI: <https://doi.org/10.1016/j.etap.2018.02.004>
Reference: ENVTOX 2961

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 21-10-2017
Revised date: 3-2-2018
Accepted date: 9-2-2018

Please cite this article as: Jellali R, Zeller P, Gilard F, Legendre A, Fleury MJ, Jacques S, Tcherkez G, Leclerc E, Effects of DDT and permethrin on rat hepatocytes cultivated in microfluidic biochips: metabolomics and gene expression study, *Environmental Toxicology and Pharmacology* (2018), <https://doi.org/10.1016/j.etap.2018.02.004>

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Effects of DDT and permethrin on rat hepatocytes cultivated in microfluidic biochips: metabolomics and gene expression study

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Highlights

- Rat hepatocytes cultivated in microfluidic biochip were exposed to DDT and permethrin
- Hepatocytes response was investigated by metabolomics coupled with RT-qPCR analysis
- Low doses of DDT and PMT were detoxified after 24 h, without major changes in the metabolome
- DDT at 150 μM causes cell death and induces cytochrome P450
- Sugar / lipid homeostasis was affected by DDT and PMT exposure at high doses

Abstract:

Dichlorodiphenyl-trichloroethane (DDT) and permethrin (PMT) are amongst most prevalent pesticides in the environment. Although their toxicity has been extensively studied, molecular mechanisms and metabolic effects remain unclear, including in liver where their detoxification occurs. Here, we used metabolomics, coupled to RT-qPCR analysis, to examine effects of DDT and PMT on hepatocytes cultivated in biochips. At 150 μM , DDT caused cell death, cytochrome P450 induction and modulation of estrogen metabolism. Metabolomics analysis showed an increase in some lipids and sugars after 6 h, and a decrease in fatty acids (tetradecanoate, octanoate and linoleate) after 24 h exposure. We also found a change in expression associated with genes involved in hepatic estrogen, lipid, and sugar metabolism. PMT at 150 μM perturbed lipid/sugar homeostasis and estrogen signaling pathway, between 2 and 6 h. After 24 h, lipids and sugars were found to decrease, suggesting continuous energy demand to

detoxify PMT. Finally, at 15 μM , DDT and PMT appeared to have a small effect on metabolism and were detoxified after 24 h. Our results show a time-dependent perturbation of sugar/lipid homeostasis by DDT and PMT at 150 μM . Furthermore, DDT at high dose led to cell death, inflammatory response and oxidative stress.

Keywords: microfluidic biochips, hepatocytes, DDT, permethrin, metabolomics, RT-qPCR

1. Introduction

For many decades, pesticides have been used in agriculture to enhance food production by eradicating undesirable insects and controlling disease vectors. Exposure to pesticides is widespread and on the long-term, poses a potential health risk. Population exposure occurs primarily through the ingestion of food that contains low levels of pesticide residues or through inhalation and/or dermal exposure at home and in the environment. Long-term pesticides exposure, even at low doses, endangers human life and can disturb the function of different organs in the body (Mostafalou et al., 2013). Furthermore, the World Health Organization (WHO) estimates that approximately 220,000 deaths are caused by acute pesticides poisoning each year (Sarwar, 2015).

Dichlorodiphenyl-trichloroethane (DDT) is a persistent organochlorine pesticide that has been used for several decades to control insects affecting crops or carrying various diseases such as malaria and typhus (Rogan and Chen, 2005). The use of DDT has been banned in many

countries since 1970s because of its persistence and bioaccumulation (Rogan and Chen, 2005). However, DDT is still used for the control of insect-transmitted diseases and for agriculture in some developing countries (Gaspar et al., 2015; He et al., 2017). Toxic effects of DDT and its metabolites have been extensively studied in humans and laboratory animals. DDT is reported to have a competitive effect in binding the androgen receptor (Lemaire et al., 2004) and to be an estrogen receptor agonist (Lemaire et al., 2006). Estrogen properties of DDT have been observed *in vivo* in rat, mouse and human (Diel et al., 2000; Das et al., 1998; Saxena et al., 1987). Furthermore, Sierra-Santoyo and *al.*, reported that DDT exposure increases testosterone metabolism in rat (Sierra-Santoya et al., 2005). In addition to hormonal effects, toxicity of DDT in humans has been related to several types of cancer, including breast cancer (Wolff et al., 1993; Rogan and Chen, 2005). Exposure to DDT impacts on liver development and metabolism, including hepatic-cell hypertrophy, necrosis, increased liver weight and hepatic oxidative stress and up-regulation of liver cytochrome P450, *CYP2B* and *CYP3A* (Kostka et al., 2004; Rogan and Chen, 2005; Harada et al., 2016). The effects of DDT on the metabolome have been examined in earthworms (McKelvie et al., 2009) and mussels (Song et al., 2016) and suggested alteration in amino acid and energy metabolism. Zuluaga *et al.* (2016) have also reported the perturbation of sugars and amino acid metabolism in HepG2/C3A culture exposed to DDT. In humans, GC-MS metabolomics carried out on serum samples from patients that have been exposed to organochlorine pesticides of the same family as DDT have shown that metabolic alterations are mostly associated with lipid metabolism (Salihovic et al., 2016). However, to our knowledge, there is no specific data on metabolomics effects of DDT in Vertebrates, in particular in liver, which contributes to DDT detoxification along with kidney.

Since toxicity and persistence in the ecosystem of organochlorine and organophosphates are regarded as serious issues (Toft, 2014; Clarkson, 1995), the use of pyrethroid such as permethrin (PMT) and its derivatives has considerably increased worldwide. These broad-spectrum products are widely used in agriculture and for indoor pest control in the public health sector and housing (Bjorling-Poulsen et al., 2008). Pyrethroids show high selectivity toward their intended target species and are effective against insects at extremely low doses. Furthermore, pyrethroids that reach systemic circulation are rapidly metabolized to less toxic metabolites (Nakamura et al., 2007; Tomalik-Scharte et al., 2005). However, recent biomonitoring studies, using measurements of urinary metabolites, have shown significant human exposure to PMT or similar compounds, such as 3-phenoxybenzoic acid (3-PBA) and cis or trans (3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane) carboxylic acid (DCCA) (Fréry et al., 2013). PMT is suspected to induce neuronal dysfunction such as paresthesia or headaches (Abdel-Rahman et al., 2004; Bradberry et al., 2005; Wolansky and Harill, 2008) and was also associated with modifications of semen quality (Meeker et al., 2008; Imai et al., 2014; Manikkam et al., 2012). Endocrine disruptor agonist properties have been reported *in vitro* and *in vivo* suggesting that PMT have potential estrogen-like effects on rodents (Kim et al., 2005; Issam et al., 2011, Jin et al., 2011). In liver, PMT can lead to severe alterations and cell death by apoptosis, increased hepatic oxidative stress and liver weights (Kostka et al., 2000; Das et al., 2008). However, there is a gap of knowledge about toxic effects of PMT on cellular metabolism. Some metabolomics analyses of the impact of PMT or its derivatives on primary carbon metabolism have been conducted in *Drosophila* (PMT) (Brinzer et al., 2015), golden fish organs (cyhalothrin) (Li et al., 2014) and rat plasma (PMT combined with propoxur) (Liang et al., 2012). They all suggested a general perturbation of amino acid metabolism, in addition of species-specific effects like oxidative stress. A general biomarker survey in rat plasma showed little effect of PMT on

proteins (except for interleukin-11 and stem cell factor) and an increase in plasmatic glutamate (while deltamethrin causes a decrease in plasmatic alanine) (Moser et al., 2012). However, the specific effect of PMT on primary metabolism of liver cells, which are responsible for PMT metabolism and detoxification, is not well documented.

Here, to obtain a better understanding of potential PMT and DDT toxicity on metabolism, we examined their effect on liver cells. An approach combining toxico-metabolomics and organ-on-chip experiment was conducted, based on our methodology published previously (Shintu et al., 2012; Choucha Snouber et al., 2013, Legendre et al., 2013, 2014). Metabolomics were used to monitor changes in metabolic pathways and may thus provide information about hepatocyte dysfunction or cellular stress occurring after exposure to xenobiotics (Song et al., 2016). In other words, metabolomics can be used to identify potential biomarkers of toxicity. In practice, rat hepatocytes were cultivated in a microscale biochip system used as a 3D dynamic environment mimicking *in vivo* conditions. Fluid samples were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) to obtain metabolomics profiles. mRNA levels of selected genes were also determined in order to identify mechanisms of action of pesticides.

2. Material and methods

2.1. Hepatocytes extraction

Primary hepatocytes were isolated from 5-week-old male Sprague–Dawley rats with a body mass of about 250 - 300 g, using the two-step method of Seglen (1973). Rats were housed

in ventilated, humidity and temperature-controlled rooms with a 12/12-h light/dark cycle, with food and water ad libitum. All procedures were performed with the approval of the Veterinary Authorities of France in accordance with the European Communities Council Directive of 22 September 2010:63/UE. This study was approved by the ethics committee of the Université de Technologie de Compiègne. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Centravet, Gondreville, France). After perfusion with HEPES/EGTA and digestion with collagenase, the liver was extracted, dissected in Dulbecco's Modified Eagle Medium (DMEM, Gibco – Life Technologies) and filtered through 100 μm filters (cell strainer 100 μm nylon, Falcon®, USA). The obtained cell suspension was centrifuged and washed three times with DMEM. Percoll isogradient centrifugation was performed to separate and discard living cells from dead cells and non-parenchymal cells which were in the top layer. Hepatic cells were then suspended in the culture medium as described below. Cell viability was assessed by trypan blue dye exclusion and hepatocytes with viability greater than 90% were used. Hepatocytes were prepared from three independent rats in this study.

2.2. Biochips and bioreactors

The entire set up is abbreviated IDCCM (for Integrated Dynamical Cell Culture in Microsystems) and comprised several assembled microfluidic biochips. Biochips were made of polydimethylsiloxane (PDMS) conventional moulding. Details on the structure of the microchannels and microchambers, and the principles of IDCCM are provided in our previous papers (Baudoïn et al., 2011, 2012, 2013). PDMS biochips were interconnected at the bottom of IDCCM set by plugs. This allowed an “easy plug and display” of biochips like the use for microscopic observations. The frame of the IDCCM was a manufactured polycarbonate box with

a conventional of 24-well plate format. Each microfluidic biochip was connected to two adjacent wells. Wells were used as inlet and outlet reservoirs connected to biochips. A specific lid was designed to allow hermetic closure of the polycarbonate frame, continuous flow perfusion and medium sampling.

2.3. Hepatic dynamic cell culture

Freshly isolated hepatocytes were cultured for the first 24 hours in seeding medium. Hepatocytes seeding medium is composed of William's E Glutamax medium (Fisher Scientific) supplemented with 6.25 $\mu\text{g}/\text{mL}$ Insulin-Transferrin-Selenium (ITS) (Becton Dickinson, Biosciences), 100 units/mL penicillin, 100 mg/mL streptomycin (Fisher Scientific) and fetal bovine serum (10%). To enhance cell adhesion, the inner surface of the biochips was coated with rat tail type 1 collagen (0.3 mg/mL; Becton Dickinson Biosciences, Le Pont de Claix, France) prepared in a phosphate-buffer solution (PBS, Gibco) for 1 h at 37°C in a humidified atmosphere supplied with 5% CO₂. After washing with seeding medium, the cells were seeded at a density of 0.5×10^6 cells per biochip (0.25×10^5 cells cm⁻²) using a pipette. Each well was completed by 2 mL of seeding medium. After 24 h of static conditions to promote cell adhesion, the seeding medium was replaced by basal medium. The basal medium was based on DMEM-F12 with sodium bicarbonate (1.2 g/L) supplemented with ITS (6.25 $\mu\text{g}/\text{mL}$), Hepes (15 mM), 100 units/mL penicillin, and 100 mg/mL streptomycin. The IDCCM was connected to the peristaltic pump by PTFE tubes and the perfusion was launched for 24h at 37°C. During cell adhesion and cell culture, primary hepatocytes were incubated at 37°C under a humidified atmosphere at 5% CO₂. After 24h of perfusion, hepatocytes were exposed to pesticides for 24 h. At the end of the perfusion, biochips were separated to assess cell viability by calcein AM-IP

staining and collect cells for trypan blue analysis. Thus, each individual experiment took 72 h (Fig.1).

2.4. Exposure to pesticides

The p-pDDT and PMT were supplied at 15 or 150 μM . These concentrations were selected based on literature data of LD50 or IC50 (Willemin et al., 2015; Leibold and Schartz 1993; Zucchini Pascal et al., 2012; Ramos-Chavez et al., 2015) and rat PBPK mathematical models extracting blood and liver concentrations from *in vivo* assays in which rat were feed with PMT corn oil (Willemin et al., 2014). In these studies, we found values ranging between 2.5 and 125 μM . Stock solutions were 50 mM in DMSO, leading to working solution with a DMSO highest concentration of 0.3%. This DMSO concentration was used because in the literature, no significant toxic effect has been reported at this dose (Zuluaga et al., 2016; El-Shenawy, 2010). Tuschl and Mueller (2006) reported that DMSO addition (0.5%) did not cause substantial gene expression alterations in primary rat hepatocytes culture. Furthermore, previous study carried out on HepG2/C3A cells showed that cell metabolism is not significantly influenced at DMSO concentrations up to 0.5% (Niklas et al., 2009)

Three distinct biochips were used as replicates and each experiment was repeated 3 times (corresponding to 9 biochips). Collected samples were stored at $-80\text{ }^{\circ}\text{C}$ before analysis.

2.5. Biological assays

Viability was analyzed using the 'live dead' assay. Hepatocytes were exposed for 20 min to the live dead mix (2 μ M calcein AM, 50 nM propidium iodide (IP) and Hoechst 10 μ g/ml for counterstaining in PBS). Images were acquired with a Leica epifluorescent microscope at 488 nm (calcein), 565 nm (IP) and 345 nm (Hoechst). At least 5 representative fields were acquired per biochip. In addition, a trypan blue staining was performed. To do so, cells were detached by trypsin perfusion on a dedicated set of biochips and counted with Malassez cell.

Glucose consumption and urea production were measured using a biochemical analyzer, the Konelab 20 (Thermo Fisher Electron Corporation, Courtaboeuf, France). The protocols have been described in detail in our previous works (Jellali et al., 2016a, 2016b). Briefly, glucose was measured using the glucose oxidase method and a modified Trinder color reaction catalyzed by the enzyme peroxidase GOD-POD Kit (Thermo Electron Corporation). For urea measurements, a QuantiChrom™ Urea Assay Kit (DIUR-500) was used. This method utilizes a chromogenic reagent that forms a colored complex specifically with urea. Measured values were normalized to cell number.

2.6. Metabolomic analysis

At each time point, metabolomics analysis was carried out on at least 4 to 5 samples (culture medium collected after 2, 6 and 24 h exposure to pesticides) for both control and treated conditions. GC-MS analyses were done as described in Tcherkez *et al.*, (2009). Briefly, gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) was performed on a LECO Pegasus III with an Agilent (Massy, France) 6890N GC system and an Agilent 7683 automatic liquid sampler. The column was an RTX-5 w/integra-Guard (30 m \times 0.25 mm internal

diameter + 10 m integrated guard column) (Restek, Evry, France). Culture mediums (150 μL) were completed with 300 μL methanol 80% (v:v), in which ribitol (100 mmol L^{-1}) was added as an internal standard. After centrifugation and spin-drying, extracts of the culture medium (for exometabolome analysis) were derivatised with methoxyamine (in pyridine) and N-methyl-N(trimethyl-silyl)trifluoroacetamide (MSTFA). Before loading into the GC autosampler a mix of a series of eight alkanes (chain lengths: C_{10} – C_{36}) was included. Analyses were performed by injecting 1 mL in splitless mode at 230°C (injector temperature). The chromatographic separation was performed in helium as a gas-carrier at 1 mL min^{-1} in the constant flow mode and using a temperature ramp ranging from 80 to 330°C between minute 2 and 18, followed by 6 min at 330°C. Ionization was made by electron impact at 70 eV and the MS acquisition rate was 20 spectra s^{-1} over a m/z range 80–500. Peak identity was established by comparison of the fragmentation pattern with available MS databases (NIST), using a match cut-off criterion of 700/1000 and retention time using the alkane series as retention standards. The integration of peaks was performed using the LECO Pegasus (Garges-lès-Gonesse, France) software. Because automated peak integration was occasionally erroneous, integration was verified manually for each compound in all analyses. Statistical significance between control and treated conditions was examined using Student t-tests.

2.7. RTqPCR analysis

Total RNA was extracted using the Nucleospin® RNA XS isolation kit (Macherey-Nagel EURL, Hoerdt, France). The quantity of RNA was measured with a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). The quality of extracted RNA was assessed

using the Agilent 2100 Bioanalyzer (using the Agilent RNA 6000 Nano and Pico kits). All RNA samples had an RNA Integrity Number of more than 6.1.

cDNA samples were synthesized from 7.5 ng of total RNA with Maxima first strand cDNA synthesis kit (ThermoScientific), according to the manufacturer's protocol. Quantitative RT-PCR was performed using the LightCycler480 Syber Green I Master and the LightCycler480 Probes Master (Roche), for the housekeeping genes and the targets genes respectively, and the Real Time PCR system LC480 (Roche). Pairs of primers for each targets genes were designed using the Roche Real Time ready (UPL probes) configurator tool. Expression stability of reference genes was assayed using GeNorm software application, which allows selecting the two most stable housekeeping genes, *GAPDH* and *PGK1*, in our model. For each gene, quantitative RT-PCR was performed in triplicate. Data from target genes were normalized with two selected reference genes. Data were analyzed using the ΔC_t method (Pfaffl, 2001) after validation of each triplicate (i.e. elimination of outliers from triplicate with a standard deviation above 0.3).

We examined the impact of DDT and PMT on the expression of selected genes: genes of the estrogen pathway (*ESR1*, *CYP1A1*, *CYP2A2*, *CYP2A1*, *HSD17B2*), lipid, sugar metabolisms (*PCK*, *CPT1A1*, *HMGSC2*, *ACSS2*, *HSPA5*), hepatic metabolism (*PXR*, *CAR*, *CYP2B1*, *CYP3A1*, *CYP2B3*, *CYP2D1*, *ESD*, *CES3*), inflammatory response (*IL1A*, *IL1B*, *IL6*, *TNF*, *TGFB3*), and oxidative stress (*NFE2L2*, *GCLC*, *GCLM*, *GPX2*, *GSS*, *GSTA2*, *SOD1*, *SOD2*). The full names of studied genes are summarized in Table S1. To allow comparisons between conditions, statistics were performed using the Mann and Whitney test.

2.8. Statistical analyses and Biomarker identification

The multivariate data analysis was performed with the SIMCA-P 13.0 software (Umetrics AB, Umea, Sweden). The supervised OPLS-DA was carried out to discover best discriminators between the groups. Metabolites that best discriminate treated group from control samples were identified using loadings along axis 1 (discriminating) and the variable importance for the projection (VIP). All other statistical tests (Mann and Witney test, Student t-tests, hierarchical clustering and two-way ANOVA) were performed using MeV 4.9 open source software (Saeed et al., 2003).

The metabolites with significant changes in the groups ($P < 0.05$) were selected as biomarkers. The pathways analysis of potential biomarkers was performed with MetaboAnalyst 3.0 (Xia et al., 2012).

3. Results

3.1. Morphology and cell viability

Hepatocytes were cultivated for 72 h in the biochips including 24 h of adhesion followed by 48h of perfusion. The first 24 h of perfusion were used to establish the hepatocyte response to dynamic culture conditions. The last 24 h were dedicated to pesticides exposure. Fig.2 presents the cell morphology, and DAPI, calcein AM and propidium iodide staining in control and biochips exposed to pesticides after 72 of culture. At the end of the perfusion period, live dead assay and trypan blue staining showed that 98% of cells were alive under control conditions. By contrast, DDT addition led to a higher proportion of dead cells, as shown by intense red staining,

with $10\pm 5\%$ and $20\pm 10\%$ trypan blue positive cells at $15\ \mu\text{M}$ and $150\ \mu\text{M}$ DDT, respectively. It should be noted morphological change of culture at $150\ \mu\text{M}$ DDT, because hepatocytes clustered at the center of the micro-chambers and the cell layer presented cell-free areas (white arrows in Fig.2). PMT addition was not associated with an increase in cell death as compared to control. Nevertheless, at $150\ \mu\text{M}$ of PMT, the cells were spherical and tend to aggregate on the walls of microchannels (white arrow in Fig.2). We also found a decrease of fluorescence intensity after staining with calcein.

3.2. Basal metabolism

Glucose consumption and urea production were measured at the end of experiments. Glucose consumption was slightly higher while urea production was not modified by pesticides (Fig.3A and 3B). For comparative purpose, we represented the glucose and urea amount extracted at the same time by the metabolomics. We found lower glucose concentration in treated cells (consistent with a higher consumption) and any modulation of the urea signals (Fig.3C and 3D).

3.3. Metabolomic analysis

In culture mediums collected after 2, 6 and 24 h of culture, we were able to identify 87 compounds in GC/TOF-MS analyses (supplementary Table S2). To investigate metabolic differences amongst the DDT, PMT and control groups, supervised multivariate analyses were

performed by orthogonal projection on latent structures-discriminant analysis (OPLS-DA). Two OPLS-DA analyses (one for each pesticide) were performed with all samples (with three groups: control vs. 15 μ M vs. 150 μ M). However, these analyses could not sufficiently separate the groups (Fig.S1) simply because there was an overlap (mixing of classes) between control and 15 μ M (DDT or PMT) samples. In other words, the treatment at 15 μ M generated samples that were intermediate between controls and 150 μ M treated samples and that could not be statistically differentiated. That said, overlapping was less important between the 15 μ M and 150 μ M samples, thereby suggesting that hepatocytes exposed to 150 μ M of pesticides (either DDT or PMT) had a clearly different metabolome compared to controls.

OPLS-DA was then performed using the data of control and samples treated with pesticides at 150 μ M (that is, control vs. DDT 150 μ M and control vs. PMT 150 μ M) only. In the OPLS-DA score plot of DDT 150 μ M experiments (Fig.4A), DDT-treated samples were clearly separated from the control group. Similar results were obtained for PMT (Fig.5A). For both pesticides, the OPLS-DA model fitted well the data ($R^2 = 0.854$ [DDT] and 0.718 [PMT]) and was significant ($P = 0.020$ [DDT] and 0.018 [PMT]). In the OPLS-DA analysis, metabolites that were most altered by pesticides were found using the volcano plot, which represents the variable importance for the projection (by multivariate regression) against the weight in axis-1 composition (loading) (Fig.4B and 5B). DDT exposure led to an increase in stearate, palmitate, glycerate, levoglucosan, fructose, gluconate, and a decrease in arginine, oxalate and glycerol (Fig.4B). PMT exposure was associated with an increase in sugars (fructose, levoglucosan, tagatose, glucuronate) and fatty acids (stearate, palmitate, hexanoate), and a decrease in glycine, glycerol, malate, succinate, ethanolamine and oxalate (Fig.5B). These results were confirmed by

univariate analysis carried out with a two-way ANOVA (Table S3, DDT and Table S4, PMT), in which the same metabolites appeared to be significant.

3.3.1. Specific effects of DDT

Metabolites significantly changed by DDT treatments (15 and 150 μM) are summarized in Table 1 (comparison with control, student t-tests, $P < 0.05$). At 15 μM , there was almost no effect after 2 h of exposure to DDT. Then, we found a modulation of few amino acids, lipids and sugars after 6h (up-regulation of aspartate, cysteine, glycine, palmitate, fructose and myo-inositol) and 24h (down-regulation of glycine, laurate, octanoate, glucose and fructose). For DDT at 150 μM , important changes were observed with a total of 25, 20 and 9 metabolites significantly modified after 2, 6 and 24 h, respectively. After 2 h, exposure to DDT 150 μM mostly caused a decrease in the concentration of many amino acids (such as asparagine, glycine, isoleucine, leucine, lysine, methionine, ornithine, proline, serine and valine). The metabolomic analysis at 6 h revealed an increase in lipids (palmitate, laurate, tetradecanoate, stearate, benzoate and hexanoate) and sugars (glucarate, gluconate, and fructose). Finally, after 24 h with DDT 150 μM , we found a decrease in 2-oxoglutarate, asparagine, cysteine, glycine and fatty acids (linoleate, octanoate and tetradecanoate).

Metabolites affected by DDT were mapped with MetaboAnalyst 3.0 to identify disturbed metabolic pathways. After the treatment with DDT 15 μM , only galactose metabolism and glycine, serine and threonine pathways appeared to be significantly altered after 6 h and 24 h (Table S5). A slight oxidative stress response is also possible, with one molecule (glycine) of glutathione metabolism altered at both 6 h and 24 h. At 150 μM DDT, more pathways were

significantly altered with a high proportion of altered metabolites (11 and 36%; Table S5). As a result, five, two and one pathways were altered after 2, 6 and 24 h, respectively. Most striking changes were observed after 2 h in protein biosynthesis (36%), ammonia recycling (22%) and catecholamine biosynthesis (40%). After 6h, significantly affected pathways were exclusively related to lipids/fatty acids metabolism (glycerolipid metabolism and β -oxidation of long chain fatty acids).

3.3.2. Specific effects of PMT

Table 2 summarizes the metabolites significantly modulated by PMT treatment (15 and 150 μ M) and their direction of change compared to control (Student t-tests, $P < 0.05$). Both PMT concentrations (15 and 150 μ M), caused an early hepatic response after 2 h of exposure with 39 and 34 metabolites significantly changed. We found a decrease in many metabolites including sugars/polyols (myo-inositol, galactose, ribose, erythritol, glycerol-3-phosphate), organic acids (ϵ -aminocaproate, fumarate, succinate, oxalate, glycolate, glucuronate) and amino acids (valine, isoleucine, leucine, proline, threonine, histidine, serine, glycine). After 6 h, the number of significantly affected metabolites declined, as compared to the situation after 2 h exposure. In total, only 14 and 9 metabolites were modulated for PMT 15 and 150 μ M, respectively. In particular, the changes consist of an increase in sugars (fructose, gluconate and myo-inositol) and fatty acids (octanoate, palmitate and stearate) levels. Finally, after 24 h, PMT at 15 μ M induces minor changes in the metabolome (only ascorbate, aspartate, glucose and S-methyl-L-cysteine were altered). By contrast, PMT 150 μ M caused important changes in culture medium composition (18 metabolites significantly modulated). Most of these metabolites appeared to be

decreased compared to control after 24 h (asparagine, aspartate, cysteine, fructose, glucose, glycine, laurate, linoleate, myo-inositol, octanoate, 2-oxoglutarate, succinate and tetradecanoate).

After 2 h exposure to PMT at 15 μM , changes in metabolites suggest a clear early effect on 6 pathways (Table S5). Three pathways were altered, with a high proportion of altered metabolites: protein biosynthesis (31%), ammonia recycling (22%) and galactose metabolism (40%). Then, only galactose metabolism appeared to be affected after 6 h (Table S5). At PMT 150 μM , protein biosynthesis, ammonia recycling and mitochondrial electron transport were affected after 2 h ($P < 0.05$). The number of metabolites altered in protein metabolism (8 metabolites) was relatively high, suggesting that protein turn-over was particularly affected at. After 6 h, we found an alteration in three pathways ($P < 0.05$) with a low level of altered metabolites (maximum two modulated metabolites in each pathway, Table S5). Finally, five pathways appeared to be affected after 24 h at 150 μM (2 to 5 metabolites affected per pathway).

3.4. mRNA levels after pesticides exposure

3.4.1. mRNA expression in response to DDT treatment

RTqPCR analysis was performed to investigate gene expression after 24 h exposure to DDT and PMT. mRNA levels were normalized to control levels and expressed as a mRNA ratio (mRNA levels in treated samples / mRNA levels in control; Fig.6). The estrogenic effect of DDT was already visible at 15 μM with the significant increase in *ESR1* mRNA expression (estrogen receptor α , 2-fold higher compared with control). The levels of steroid metabolism-related CYPs450 genes such as *CYP1A1* and *CYP2A1/2* (involved in estradiol and testosterone

metabolism, respectively) were also increased 2 to 3.5-fold by both DDT concentrations. No effect was observed on *HSD17B2* (steroidogenesis). mRNA expression of nuclear receptors (*PXR* and *CAR*) and *CYP2D1* increased at the same level in both DDT conditions ($P < 0.05$). The *PXR* related CYPs450 gene (*CYP2B3*) was up-regulated only after exposure at 15 μM ($P < 0.05$, fold change of 4). mRNA expression of efflux pump involved in xenobiotic transport, such as P-glycoprotein coding gene (*ABCB1*), increased at 15 μM , as for *ESD* (esterase D - metabolism of DDT) and *CYP1A2* (metabolism of xenobiotic and estradiol). *CES3* gene (which encodes a carboesterase involved in DDT metabolism) was more induced at 15 μM than at 150 μM , being 5- and 16-fold higher compared with control. No induction of *CYP2C6* activity was observed after the DDT treatment.

Changes in genes involved in energy metabolism were also observed. Fatty acid synthesis related genes were significantly induced. *CPT1A1* was up-regulated by DDT at 15 μM (3 times higher in comparison with control), and *ACSS2* mRNA increased in both 15 and 150 μM groups ($P < 0.05$, fold change of 3.7 and 3.2). However, no significant effect was observed for *PCK* and *HMGCS2* expression. Regarding inflammation, *TGFB3* gene was significantly induced at 15 μM , whereas the levels of *IL6* mRNA were down-regulated in both DDT groups (fold change of 0.22 and 0.7 for 15 and 150 μM , respectively). No significant effect was observed for *TNF*, *IL1A*, nor *IL1B*. Expression of genes associated with oxidative stress was unchanged at 150 μM . By contrast, several mRNA were significantly induced at 15 μM , such as *GCLC* and *GSTA2* that were increased of 3 and 40-fold, compared with control.

Other specific stress targets were analysed. The level of *HSPA5* (an actor of endoplasmic reticulum response; ER stress) was significantly increased 3.5 times, after the treatment at DDT

15 μM . Apoptosis-related genes were affected as well, such as *FASN* that was significantly down-regulated at 15 μM (3-fold), while *FASLG* was up-regulated at 150 μM ($P < 0.05$).

3.4.2. mRNA expression in response to PMT treatment

mRNAs were quantified in control samples and samples treated for 24 h with PMT (at 15 or 150 μM). mRNA ratios (mRNA levels in treated samples / mRNA levels in control) in all samples are presented in Fig.7. An estrogenic effect of PMT was suggested by the slight change in *ESR1* mRNA expression (Fig.7). However, this change was not significant. Steroid metabolism-related CYPs450 genes and *17 β -HSD2* were not significantly induced by PMT. Expression of genes involved in xenobiotic metabolism (*PXR*, *CAR*, *CYP*, *ABCB1*, *CES* and *ESD*) was unchanged. *CYP3A1* was significantly induced by PMT at 150 μM (2-fold higher compared with control). No change in gene expression related to energy metabolism was observed. Regarding inflammation and oxidative stress, *IL6* and *NFE2L2* were significantly down-regulated (nearly 2-fold) by PMT at 150 and 15 μM , respectively. Finally, mRNA abundance associated with *SOD2* was significantly increased 1.8 times by PMT at 150 μM ($P < 0.05$).

4. Discussion

In this study, we have investigated metabolic effects of two insecticides (DDT and PMT) on rat hepatocytes cultured in biochips. To do so, hepatocytes were exposed for up to 24 h to these insecticides, at two concentrations. Metabolomics by GC-MS profiling and gene expression

by RT-qPCR were performed so as to examine specific signatures of DDT and PMT hepatic intoxication.

4.1 Exposure to DDT at high concentration affects estrogen, lipid and sugar metabolism

The earliest metabolic changes were observed in samples treated with DDT at 150 μ M for 2h. The associated response led to a decrease in several amino acids (such as valine, isoleucine, proline, leucine, tyrosine, and threonine) (Fig.8A) and 2-oxoglutarate (α -ketoglutarate). However, there was no change in glucose or glucose derivatives such as glucarate and gluconate, nor lipids. Set enrichment analysis suggests that it can be related to a change in protein biosynthesis and ammonia recycling pathways, that is, an increase in proteins/amino acids turn-over and recycling of ammonium produced from amino acids degradation. Therefore, one hypothesis could be that amino acids were degraded and used to feed the Krebs cycle and thus produce energy required to detoxify the DDT (consistent with *ACSS2* induction). However, the analysis of total protein, lipid content and glycogen metabolism (to compute an energy balance) would be necessary to provide a definitive conclusion.

DDT has been reported to bind competitively the androgen receptor (Lemaire et al., 2004) and to be an estrogen receptor agonist (Lemaire et al., 2006). Estrogen properties of DDT have been also observed *in vivo* (Diel et al., 2000; Das et al., 1998; Saxena et al., 1987). Furthermore, DDT is reported to increase testosterone metabolism in rat (Sierra-Santoya et al., 2005). Since estrogen has been reported to reduce hepatic ketogenesis and to increase the incorporation of fatty acids into triglycerides, it is assumed that estrogen regulates hepatic fatty acid metabolism from oxidative to lipogenic pathway (O'Sullivan et al., 1995). This assumption appeared to be

consistent with our findings whereby DDT (after 6 h at 150 μM) led to an increase in organic and fatty acids (benzoate, octanoate, tetradecanoate, hexanoate, stearate, laurate and palmitate, Fig.8C). Lipogenesis is fed by acetyl-CoA (that is usually produced by pyruvate dehydrogenase from pyruvate, which is in turn the product of glycolysis) and redox power in the form of NADPH (ordinarily coming from the pentose phosphate pathway, PPP). After 6 h at 150 μM DDT, there was an increase in fructose, glucarate (from glucose oxidation), gluconate (from the PPP) probably reflecting the up-regulation of sugar catabolism (Fig.8C). Also, the increase in histidine likely reflected the activity of the PPP. This interpretation is supported by genomic data showing the modulation of genes involved in hepatic estrogen, lipid, and sugar metabolism. In fact, we found an increase in mRNA level of several genes: *ESR1*, *CYP1A1*, *CYP2A1/2*, *CPT1A1*, *ACSS2*, *PXR*, *CAR*, *CYP2D1* and *CYP2B3*. However, further investigations would be required to explain why *FASN* (fatty acid synthase) was down-regulated.

Finally, after 24 h exposure at 15 μM , both the metabolomics profile and the viability analysis showed the recovery to healthy cellular state. By contrast, the situation at 150 μM is less clear. Actually, visibly degraded cells at 150 μM (morphology, high percentage of dead cells) were not associated with a clear metabolomics hepatic necrotic signature (such as a typical increase in 2-oxoglutarate, sebacic acid, taurocholate, or steroids) (Sun et al., 2013; Yang et al., 2012). In addition, it was associated with little change in mRNA levels as compared to controls, although necrosis has been reported to modify mRNA levels, protein processing and DNA degradation (Zong and Thompson, 2006; Shan et al., 2007). Nevertheless, after 24 h at 150 μM , we found a low concentration in 2-oxoglutarate (opposite to what has been found after 24 h at 15 μM or 6 h at 15 or 150 μM). 2-oxoglutarate is central to hepatic metabolism and can be formed by glutamate deamination by transaminases (alanine and aspartate transaminases) in healthy liver

and thus its decrease could have reflected a drop in general enzyme activity and thus necrosis and hepatotoxicity.

4.2 Exposure to permethrin impacts weakly on hepatocyte metabolism after 24 hours

PMT led to an early hepatic response within the two first hours of culture. The metabolomics signature at 2 h at either 15 or 150 μM was characterized by a decrease in amino acids such as valine, proline, isoleucine, tyrosine and a lower content in fatty acids as compared to controls (Fig.8A and B, respectively). As mentioned above, amino acids can be degraded and used in Krebs cycle to produce energy. In addition, we found a decrease in glycerol-3-phosphate, which could reflect a lower phosphorylation of glycerol, a higher production of dihydroxyacetone phosphate to feed glycolysis, or simply an increase in cleavage and recycling of lipids as a short-term response. Also, the content in galactose, glucose (both analytical derivatives glucose-1 and glucose-2) and ribose was lower suggesting higher sugar consumption (Fig.8B) after 2 h exposure. Thus, the metabolomics pattern obtained after 2 h with PMT likely reflected an increased energy demand.

After 6 h exposure, we found increased levels of three free fatty acids (palmitate, octanoate and stearate; Fig.8C) and sugars (fructose and gluconate; Fig.8C), suggesting a re-orchestration of lipid and sugar metabolism. This metabolic signature may reflect a weak modulation of estrogen dependent pathways (when compared to DDT) in agreement with the literature, in which few studies report estrogenic effects of PMT (Kim et al., 2005; Brander et al., 2012; Jin et al., 2008). The mRNA levels indicate a weak induction of estrogen-related genes and of lipid and sugars metabolism.

Finally, metabolomics profile (only 4 metabolites modulated), set enrichment analysis (no affected pathway) and the viability analysis revealed no apparent toxicity after 24 h with 15 μ M PMT. In addition, some early oxidative stress-dependent genes (*NFE2L2*, *GCLC*, *GSS*) were not significantly down- or up-regulated as compared to controls. These results suggest an efficient and continuous detoxification at 15 μ M, thereby preventing cellular damage from occurring. Concerning PMT 150 μ M after 24 h, the metabolome still exhibited alterations, with a decrease in sugars, fatty acids and sorbitol (a product of glucose metabolism (Levine et al., 1978) as compared to controls. In addition, higher *NFE2L2* mRNA levels were found, and a lower level for *CYP2B3*. Again, this probably reflects detoxification of PMT on the medium-to-long term. However, the live/dead assay and trypan blue staining did not reveal over-toxicity in hepatocytes. Thus, the metabolomics pattern after 24 h at 150 μ M simply reflects a moderate toxic hepatic response (when compared to a strong toxic response such as that obtained with DDT under similar conditions, 24 h at 150 μ M). Also, the RTqPCR measurements did not show significant variations (expected for *NFE2L2*, *CYP2B3*, *IL6*, *SOD2*, so 4 genes over 36 targets) because of the large dispersion of the data.

Conclusions and perspectives

In this study, metabolomics and RTqPCR analyses were used to investigate rat hepatocytes responses to the exposures of DDT and PMT. At low concentration (15 μ M), DDT and PMT were detoxified after 24 h and resulted only in little changes in the metabolomics pattern and genes expression. By contrast, at 150 μ M, important changes were observed. After 2 h exposure

to PMT, we found a high energy demand illustrated by the amino acids, sugars and lipids reduction in the culture media when compared to control. The impact of DDT after 2 h appeared to be modest. After 6 h, we observed an increase in lipids and sugar derivatives levels, and this situation was more pronounced with DDT compared to PMT. Concurrently, RTqPCR analysis revealed changes in mRNAs associated with genes of estrogen, lipid and sugar metabolisms. These results suggest that DDT and PMT had an estrogen-like modulation of metabolism after 6 h, as reported in the literature. On the long-term exposure to PMT (24 h), an increase of sugars and fatty acids consumption was found, illustrating continuous energy demand to detoxify PMT. In the case of DDT, important perturbations remained after 24 h, with visible cellular reorganization, cell death, inflammatory response and oxidative stress. Our innovative technique (combining liver cells on chip culture coupled to metabolomics profiling and genes expression analysis) allowed the analysis of DDT and PMT impacts on rat hepatocytes. Further studies with chronic exposure would be required to complement our results. It should also be noted that our technique also allows mid- to long-term hepatocyte culture and thus could be adapted to isotopic labelling to trace metabolic fluxes. For example, the use of ^{13}C -1-glucose as a substrate could be instrumental to quantify fluxes in glycolysis, PPP and the TCA cycle.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

The study was supported by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, project IMITOMICS-N°EST-2014/1/093). The molds to fabricate the PDMS devices used in this study were built by the LAAS in the frame of the RENATECH French network.

ACCEPTED MANUSCRIPT

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Figures captions

Fig.1. Experimental procedure of hepatocytes culture and exposition.

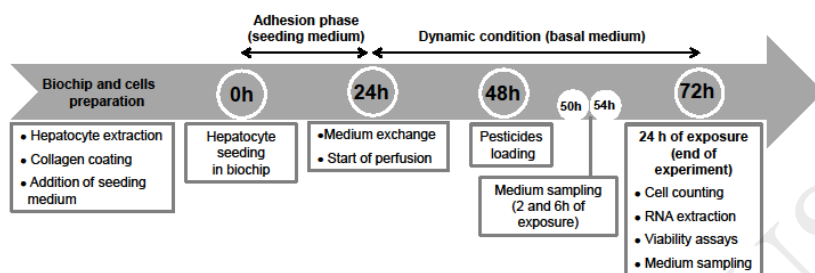


Fig.2. Cell morphology (white arrows indicate the cell-free areas); nucleus staining by DAPI; viability *via* calcein AM staining and propidium iodide staining at the end of the experiments (24h of perfusion culture) in control; DDT-15 μ M; DDT-150 μ M; PMT-15 μ M and PMT-150 μ M.

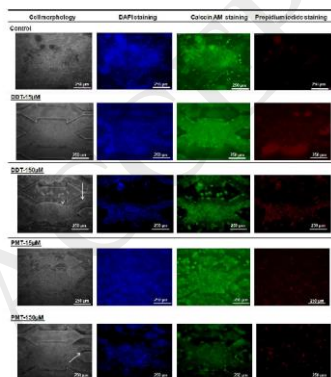


Fig. 3. (A) glucose consumption and (B) urea production in control, DDT and PMT treated samples after 24h of cultures measured by biochemical assay; (C, D) Glucose and urea levels in control, DDT and PMT treated samples after 24h of cultures extracted by metabolomics; (** P < 0.01).

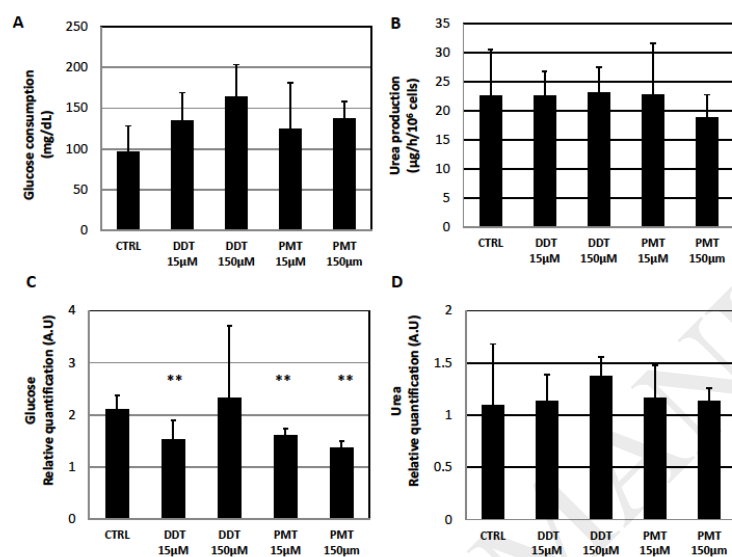


Fig.4. (A) OPLS-DA score plot based on GC-MS data (control vs. DDT-150 μ M), showing the separation between groups (each point represents one sample/replicate): CTRL_2_x and DDT_2_x = samples after 2 h of exposure; CTRL_6_x and DDT_6_x = samples after 6 h of exposure; CTRL_24_x and DDT_24_x = samples after 24 h of exposure. (B) Volcano plot (from OPLS-DA) showing discriminating metabolites using the loading along axis 1 and the variable importance for the projection (VIP) along axis 2. Variables with high VIP represent the best discriminating metabolites. Positive and negative values along axis 1 indicate metabolites that increased and decreased, respectively, after DDT exposure.

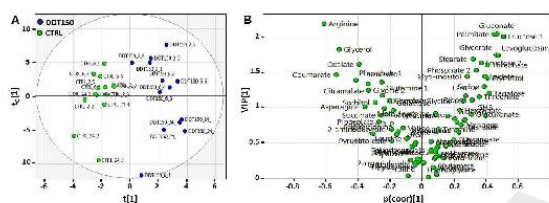


Fig.5. (A) OPLS-DA score plot based on GC-MS data (control vs. PMT-150 μ M), showing the separation between groups (each point represents one sample/replicate): CTRL_2_x and PMT_2_x = samples after 2 h of exposure; CTRL_6_x and PMT_6_x = samples after 6 h of exposure; CTRL_24_x and PMT_24_x = samples after 24 h of exposure. (B) Volcano plot (from OPLS-DA) showing discriminating metabolites using the loading along axis 1 and the variable importance for the projection (VIP) along axis 2. Variables with high VIP represent the best discriminating metabolites. Positive and negative values along axis 1 indicate metabolites that increased and decreased, respectively, after PMT exposure.

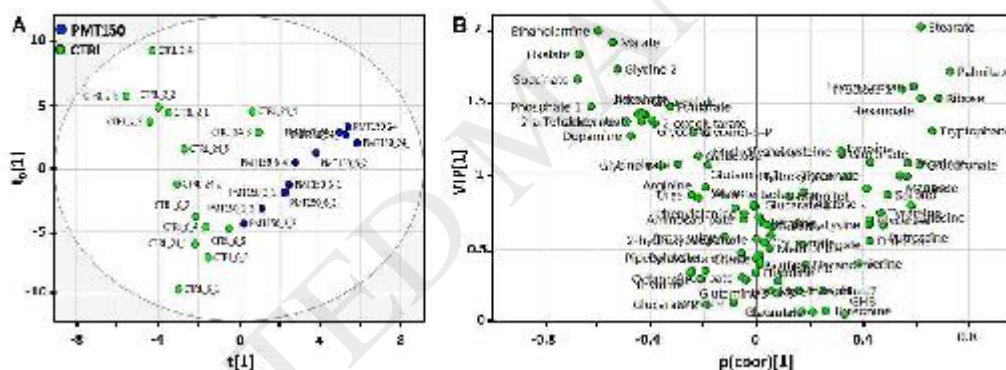


Fig.6. mRNA ratio (mRNA levels in DDT treated samples / mRNA levels in controls, n=3 for controls, n=3 for 15 μ M; n=3 for 150 μ M), +: denotes $P < 0.05$ for 150 μ M versus control, *: denotes $P < 0.05$ for 15 μ M versus control. mRNA ratio > 1 : gene upregulated and mRNA ratio < 1 : gene downregulated.

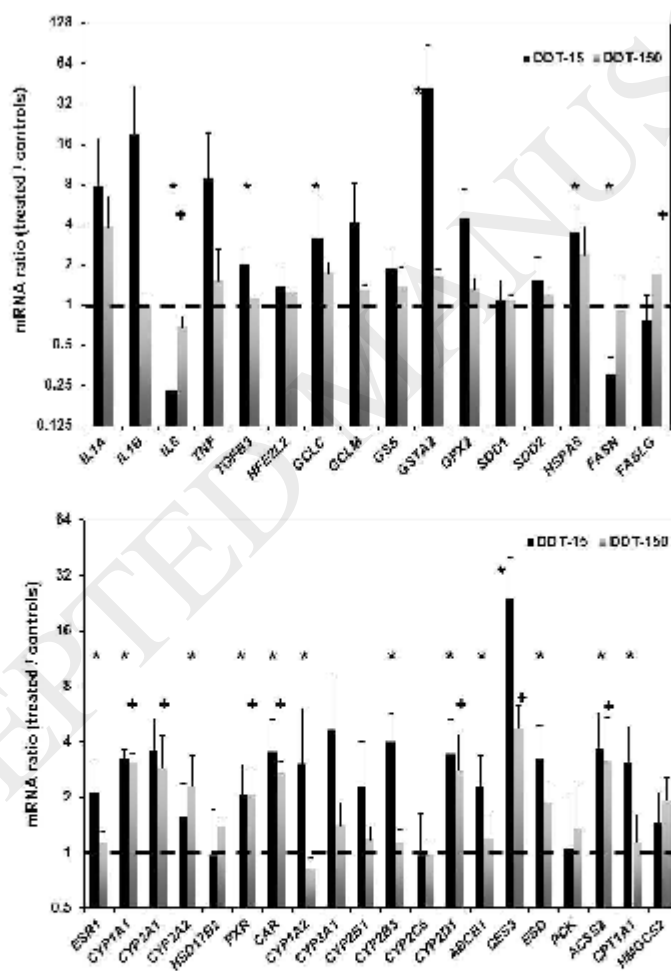


Fig.7. mRNA ratio (mRNA levels in PMT treated samples / mRNA levels in controls, n=3 for controls, n=3 for 15 μ M; n=3 for 150 μ M), +: denotes $P < 0.05$ for 150 μ M versus control, *: denotes $P < 0.05$ for 15 μ M versus control. mRNA ratio > 1 : gene upregulated and mRNA ratio < 1 : gene downregulated.

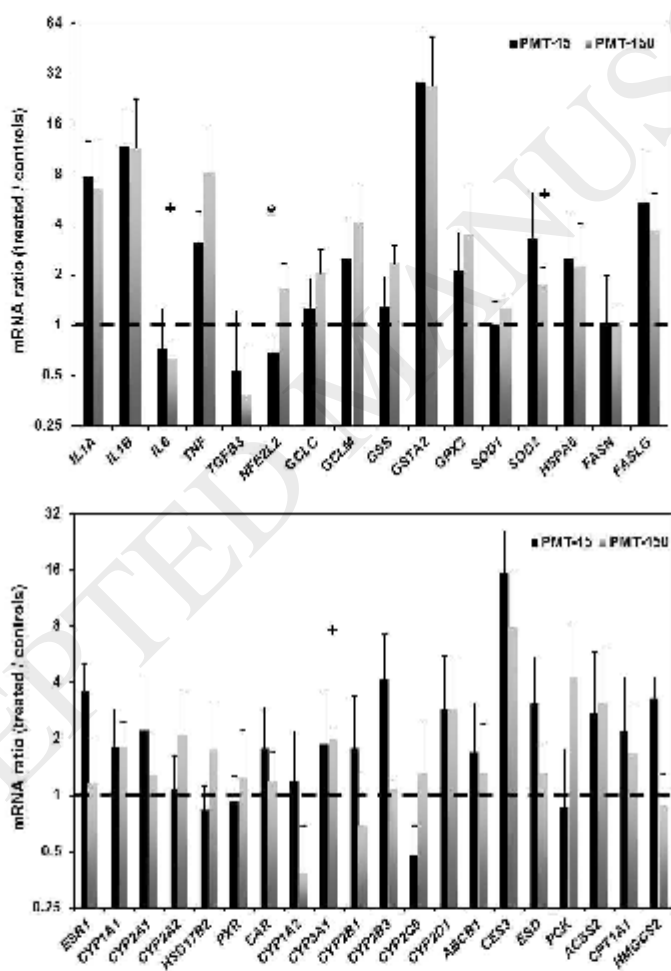


Fig.8. Heat maps of amino acids, lipids and sugars levels in control and samples exposed to DDT and PMT: (A) amino acids levels after 2h; (B) lipids and sugars after 2h and (c) lipids and sugars after 6h. The columns represent samples in different experimental conditions, and the rows represent different metabolites. Colors denote the concentrations of different samples (red = maximum; black = middle; and green = minimum concentrations of metabolites, respectively).

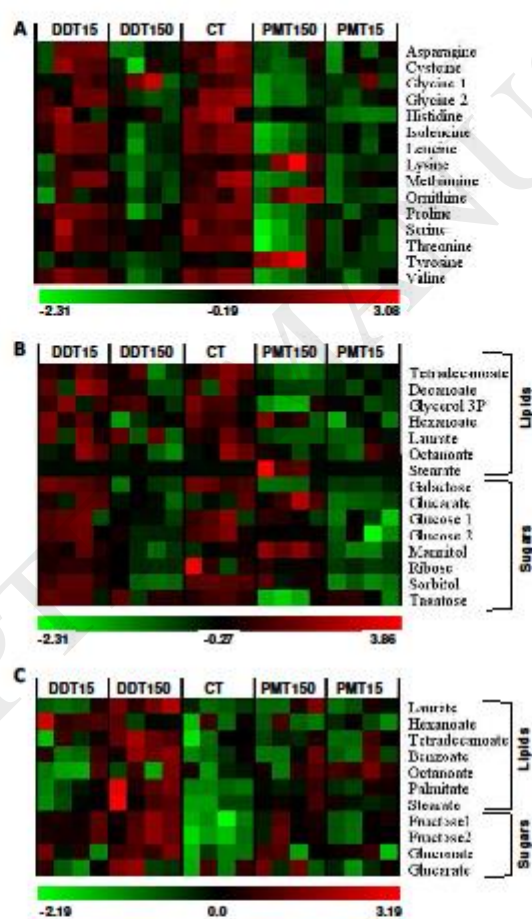


Table 1 Metabolites that change significantly ($P < 0.05$) after DDT treatments (compared with control, (↑) increase and (↓) decrease).

Samples	Metabolites
DDT 15μM 2h	Aaminocaproate (↓)
DDT 15μM 6h	Aspartate (↑), Cysteine (↑), Erythritol (↑), Fructose (↑), GHB (↑), Glycine (↑), Myo-inositol (↑), Phosphate (↑), Palmitate (↑), Threonine (↑)
DDT 15μM 24h	Ascorbate (↓), Fructose (↓), Glycine (↓), Glucose (↓), Laurate (↓), Octanoate (↓), S-methyl-L-cysteine (↓)
DDT 150μM 2h	Adenine (↓), Aminocaproate (↓), Ascorbate (↓), Asparagine (↓), Dopamine (↓), Galactose (↓), Glycine (↓), Glycolate (↓), Hydroxylysine (↓), Isoleucine (↓), Leucine (↓), Lysine (↓), Methionine (↓), Myo-inositol (↓), Ornithine (↓), Oxalate (↓), 2-oxoglutarate (↓), Phosphate (↓), Proline (↓), Serine (↓), Succinate (↓), Threonine (↓), Tyramine (↓), Tyrosine (↓), Valine (↓)
DDT 150μM 6h	Fructose (↑), Erythritol (↑), Aminocaproate (↑), Palmitate (↑), Gluconate (↑), Laurate (↑), Tetradecanoate (↑), Glucarate (↑), Myo-inositol (↑), Stearate (↑), Levoglucosan (↑), Glycine (↑), Glycerate (↑), Succinate (↑), Maleate (↑), Aspartate (↑), Benzoate (↑), Hexanoate (↑), Phosphate (↑), Histidine (↑)
DDT 150μM 24h	Ascorbate (↓), Asparagine (↓), Cysteine (↓), Glycine (↓), Linoleate (↓), Octanoate (↓), 2-oxoglutarate (↓), Tetradecanoate (↓) Levoglucosan (↑)

Table 2 Metabolites that change significantly ($P < 0.05$) after PMT treatments (compared with control, (↑) increase and (↓) decrease).

Samples	Metabolites
PMT 15μM 2h	Adenine (↓), Aminocaproate (↓), Coumarate (↓), Dopamine (↓), Erythritol (↓), Fumarate (↓), Galactose (↓), Glucarate (↓), Glucose (↓), Glycerol 3P (↓), Glycine (↓), Glycolate (↓), Glucuronate (↓), Hexanoate (↓), Histidine (↓), Hydroxylysine (↓), Isoleucine (↓), Itaconate (↓), Lactate(↓), Leucine (↓), Malate (↓), Mannitol (↓), Methylmaleate (↓), Myo-inositol (↓), Oxalate (↓), 2-oxoglutarate (↓), Phosphate (↓), Pipecolate (↓), Proline (↓), Ribose (↓), Serine (↓), S-methyl-L-cysteine (↓), Sorbitol (↓), Succinate (↓), Threonine (↓), Thymine (↓), Tyramine (↓), Tyrosine (↓), Valine (↓)
PMT 15μM 6h	Erythritol (↑), Fructose (↑), Gluconate (↑), Glycine (↑), Hydroxylysine (↑), Myo-inositol (↑), Octanoate (↑), Palmitate (↑), Phosphate (↑), S-methyl-L-cysteine (↑), Stearate (↑)
PMT 15μM 24h	Ascorbate (↓), Aspartate(↓), Glucose (↓), S-methyl-L-cysteine (↓)
PMT 150μM 2h	Aminocaproate (↓), Citrate (↓), Cysteine (↓), Erythritol (↓), Ethanolamine 3TMS (↓), Fumarate (↓), Galactose (↓), Glycerol 3P (↓), Glycine-1 (↓), Glycine (↓), Glycolate (↓), Glucuronate (↓), Histidine (↓), 2-hydroxybutyrate (↓), Isoleucine (↓), Laurate (↓), Leucine (↓), Methionine (↓), Malate (↓), Myo-inositol (↓), Oxalate (↓), Phosphate (↓), Proline (↓), Ribose (↓), Serine (↓), Succinate (↓), Tagatose (↓), Threonine (↓), Tetradecanoate (↓), Valine (↓) Glutamine (↑), Mannitol (↑), Stearate (↑), Tryptophan (↑), Tyrosine (↑)
PMT 150μM 6h	Fumarate (↓) Erythritol (↑), Fructose (↑), Glycerate (↑), Glycine (↑), Myo-inositol (↑), Palmitate (↑), Stearate (↑)

PMT 150 μ M Ascorbate (\downarrow), Asparagine (\downarrow), Aspartate (\downarrow), Cysteine (\downarrow), Fructose (\downarrow),
24h Glucose (\downarrow), Glycine (\downarrow), Laurate (\downarrow), Linoleate (\downarrow), Myo-inositol (\downarrow),
Octanoate (\downarrow), 2-oxoglutarate (\downarrow), Succinate (\downarrow), Tetradecanoate (\downarrow),
Levogluconan (\uparrow), Mannose (\uparrow), Sorbitol (\uparrow)

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