Assessment of the effects of PPPs' mixtures on animal health

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1. Summary

The current report presents the results of the assessment of the effects of PPPs' mixtures on animal health (D4.4) related to Task 4.2 of the Horizon 2020 project Sustainable Plant Protection Transition; A global health approach (SPRINT). A tiered approach, has been used in the SPRINT project based on the EFSA guidelines for combined exposure to multiple chemicals using a component-based approach (EFSA, 2019). Tier 1 aimed to use available data and in silico modelling to assess the top 20 PPPs of concern and prioritize the PPPs of concern to be studied via in vitro models (Tier 2). The PPP selection was performed in three steps: A - Occurrence, B - hazard and C – synthesis. PPPs have been prioritized following quantitative ranking (by weighted hazard quotient, wHQ). The decision to select the PPPs to be tested for the in vitro studies was through occurrence via SPRINT CSS data on environmental and human samples, and by toxicity, based on updates of the EFSA guidance on Admittable Daily Intake (ADI) and Acceptable Operator Exposure Level (AOEL). In Tier 2, we used New Sprint Indicators (porcine ileum organoids) to test for potential negative effects of plant protection products (PPPs) and PPP mixtures on animal health in in vitro models. These new indicators are based on the great progress in organoid technologies over the past 5 years. Porcine ileum organoids were selected as the target tissue because small intestine is exposed to relatively high concentrations of PPPs via oral ingestion of food, dust, and swallowed mucus from the upper respiratory tract. The top 8 PPPs ranked in Tier 1 were selected along with 8 PPPs found frequently in food, faeces or urine on the SPRINT case study sites, including piperonyl butoxide, a compound used as a synergist for insecticides. Eleven mixtures were also selected. A relevant dose-range was defined as ranging from low to median concentrations found in faeces from human subjects up to 100 * ADI (Acceptable Daily Intake) level, which was used as an 'assessment factor' to cover uncertainties regarding interspecies and intraspecies extrapolations. Decreases in cell viability (quantified as ATP concentration in lysed cells)

occurred after 5 days of exposure to tebucanazole, imazalil, cyprodinil and fludioxonil and the pyrethoid synergist, piperonyl butoxide occurred at concentrations > 10 x ADI. The effects of individual PPPs and mixtures was also investigated using RNAseq. Notable findings from the transcriptomics study were the significant upregulation of genes in mitochondrial oxidative phosphorylation and the ubiquitin-proteosome pathway in piperonyl butoxide, Fludioxinil, Cyprodinil and Tebuconazole treated samples vs the DMSO control. These genes are also altered in neurodegenerative diseases such as Parkinson's and Alzheimer's where mitochondrial dysfunction plays a major role in the pathogenesis degeneration of neurons. For some compounds, pathways involved in barrier integrity through adherens and tight junction (TJ) functioning were downregulated. The permeability of the intestine is important for mucosal and systemic health and the potential effect of PPPs on barrier integrity warrants further study. In Tier 3, we investigated the potential effects of diet glyphosate to parent hens on egg hatchability and chicken health. Parent hens received diets with no amendment of (Control) or planned amendment of 1.5 ($Gly_{1.5}$), 4.5 ($Gly_{4.5}$) and 13.5 ($Gly_{13.5}$) mg glyphosate per kg diet. Even though we observed no effects of the applied diet glyphosate levels to parent hens on hatchability of fertilised eggs (proportions were 0.95, 0.93, 0.91 and 0.90 for Con, $Gly_{1.5}$, $Gly_{4.5}$ and $Gly_{13.5}$, respectively) or on one day-old chicken quality, a post hoc analysis indicated that among several quality traits, a navel score was best for chickens from the Con group compared to chickens from the combined Gly groups. Chicken growth until 8 days of age was not affected (ADG were 7.06, 7.03, 7.49 and 7.31 g/d, respectively). Regarding immune competence and leukocyte subset counts in whole blood from parent hens and one day-old chickens, the results indicated some immune imbalances due to diet glyphosate in both hens and chickens. The new models used (new SPRINT indicators), both in vitro (porcine organoids) and in vivo (Ross 308 chicken), proved to be sensitive and relevant for different toxicological endpoints of interest for risk assessment. Our tiered approach was successful and allowed to optimize the resources on

the pesticides and mixtures of greatest concern, since all the pesticides that have been tested in Tier 2 and 3 showed at least one positive signal.

2. Introduction

The current report pertains the assessment of the effects of PPPs' mixtures on animal health (D4.4) related to Task 4.2 of the Horizon 2020 project Sustainable Plant Protection Transition; A global health approach (SPRINT). A tiered approach, from simple assessments at low-tier to more complex assessments at high-tier, has been implemented in the SPRINT project based on the EFSA guidelines for combined exposure to multiple chemicals using a component-based approach (EFSA, 2019). Furthermore, the SPRINT project aimed to use alternatives to animal studies wherever reasonable, in particular in WP4, where the (eco)toxicological assessment was undertaken. Specifically, Task 4.2 (assessment of the effects of PPPs' mixtures on animal health) and Task 4.3 (assessment of the effects of PPPs' mixtures on human health) aimed to include the following tiers as described in Grant Agreement (GA) no 862568: using available data and in silico modelling to assess the top 20 PPPs of concern (Tier 1), then studying in vitro models for the top 10 PPPs of concern (Tier 2) and then studying in vivo only the Top 3 substance of concern (Tier 3) (Figure 2.1).

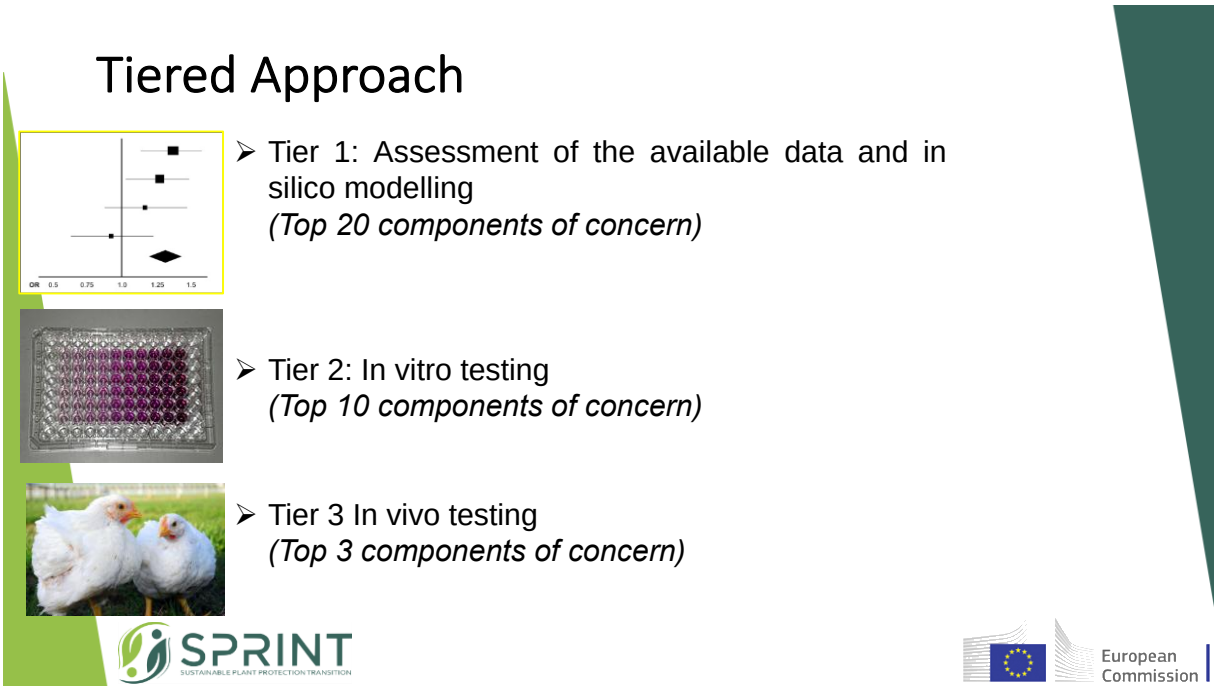

Figure 2.1 *SPRINT Tiered Approach: Task 4.2 (assessment of the effects of PPPs' mixtures on animal health) and Task 4.3 (assessment of the effects of PPPs' mixtures on human health) aimed to include the following tiers as described in Grant Agreement no. 862568*

Furthermore, the *in vitro* (organoids) and *in vivo* (farm animals) testing are based on New Sprint Indicators (Table 2.1), which are new models that could be of interest for risk assessment, but that are currently not yet embedded in standard guidelines. Any change or amendment from the original GA is reported and explained in the specific Tier subsection. Notably, other SPRINT Tasks will be relevant and informative for animal toxicology, in particular Task 4.3 (assessment of the effects of PPPs' mixtures on human health) which includes other *in vivo* assays performed in laboratory animals that are used as models for human health (rats and mice) and Task 4.1 (assessment of the effects of PPPs' mixtures on ecosystem health) which includes toxicological assays performed with fish. However, D 4.4 specifically focuses on the toxicological effects on *in vitro* and *in vivo* models with farm animals.

Table 2.1 *New SPRINT indicators for Task 4.2 (assessment of the effects of PPPs' mixtures on animal health) described in Table 1.7 of the Grant Agreement no. 862568*

Test organisms	Indicators (references)	Duration (days)	Source for single a.S./Me data	Exp. units
Animal: new SPRINT indicators				
In vitro: Bovine intestinal organoids.	Transcriptomics and differential gene expression analyses, growth, viability and cell lineage differentiation.	70	Literature.	105 (*4 repl. = 420)
In vivo: Ross 308 chicken	Health status, growth rate.	90	Literature	17 (*2*10=340)

3. Tier 1: Assessment of the available data

1. BACKGROUND and AIMS

Tier 1 aimed to use available data and in silico modelling to assess the top 20 PPPs of concern and prioritize the Top 10 PPPs of concern to be studied via in vitro models (Tier 2). A systematic procedure to prioritise PPPs for toxicology testing has been developed in WP3 and WP4 (Scheepers et al., 2022). The PPP selection was performed in three steps: A - Occurrence, B - hazard and C - synthesis and started with the SPRINT list of 209 PPPs (long-list) included in the scope of the SPRINT field study in 11 case study sites (CSS) (Silva et al., 2021). After the initial screening of the 209 substances, the methodology developed in WP3 and WP4 (Scheepers et al., 2022), served also as a base for the assessment of the 20 substances described here for Tier 1. The decision to select the Top 10 PPPs to be tested for the in vitro studies was through occurrence via SPRINT CSS data on environmental and human samples, and by toxicity, based on updates of the EFSA guidance on Admittable Daily Intake (ADI) and Acceptable Operator Exposure Level (AOEL).

2. METHODS

2.1 Conversion of PPP residue concentrations to units of exposure assessment

For the use of exposure and risk data in a risk matrix, the PPP residue concentrations in human samples needed to be converted to standard units of exposure assessment using a framework of limits based on the toxic properties of each substance. Human samples from the CSS (farmers, neighbours and consumers) were considered a good proxy of farm animal exposure. This conversion is explained below in more detail.

Conversions of PPP residue concentration were made to ADI (C) and AOEL (farmers/applicators). If no ADI or AOEL was available (e.g. for chlorpyrifos) a published OEL was used. Conversion of an OEL in mg/m3 to mg/kg/d is done assuming:

Average inhalation volume over an 8 hour period is 10 m3 (light exercise). A work shift of 8 hours is converted to 24 hours by multiplying with a factor of 3. We used 70 kg as an average person's body weight for adult (M/F). This leads to the following conversion:

OEL (mg/m3) x 10 (m3) x 3 / 70 kg bw => mg/kg bw/day

2.2 Calculation of hazard quotient

Below is a brief description of the calculation used for the hazard quotient (HQ).

The hazard quotient is commonly used to assess how exposures relate to reference values. It is calculated as follows:

HQ = Exposure level / Reference value

The reference value and exposure level should have the same unit resulting in the HQ as a dimensionless parameter. For interpretation, it may be assumed that no elevated health risk is anticipated when HQ < 1. For farmers:

HQ = PPPL / AOEL

With AOEL = acute occupational exposure level (in mg/kg bw/d) and PPPL = PPP level are expressed in mg/kg bw/d. For consumers:

HQ = PPPL/ADI

With ADI = acceptable daily intake (in mg/kg bw/d) and PPPL = PPP level are expressed in mg/kg bw/d

Given the small numbers anticipated, we will express the HQ as a %.

2.3 Weight factor

The detection rate is used as a weight factor for each PPP and each matrix considered.

3. RESULTS AND DISCUSSION

The 20 highest ranking PPPs from the SPRINT list of 209 PPPs were initially identified as described in Figure 3.1 derived from Scheepers et al., 2022.

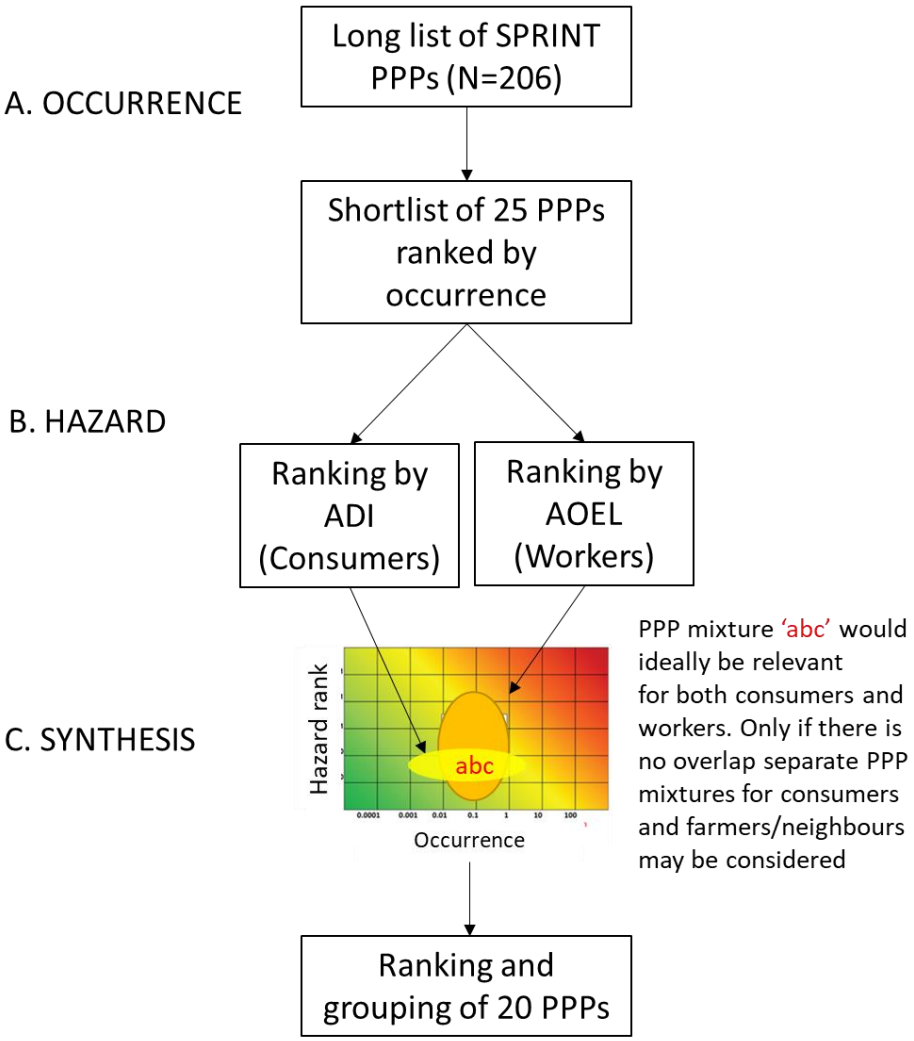

Figure 3.1 *Flow diagram describing the three-step process followed to select and prioritize PPPs and PPP mixtures by Scheepers et al 2022 out of 209 PPPs.*

PPPs have been prioritized following quantitative ranking (by weighted hazard quotient, wHQ). This resulted in a list of 14 PPPs for which quantitative data on occurrence and toxicity data were available and resulting in the wHQ (Table 3.1). However, Folpet (originally ranking as 4th PPP) was removed because of limitations of the occurrence data (metabolite is not unique for folpet) and was replaced by tebuconazole. This fungicide was already ranked as a priority PPP based on occurrence (5th most important). To compensate for the urine data from the SPRINT CSS (that were not yet available in 2022 when Tier 1 was performed), data from the Dutch OBO study (Oerlemans et al, 2019) were used to calculate a wHQ for neighbours and

for consumers (see Appendix 1). This resulted in a ranking of 6 for residents living close to agricultural fields and 5 for controls living far from agricultural fields.

Table 3.1 *Integrated proposed list and separate lists for F+N and C. Revised prioritisation based on rank by HQs (weighted by detection rate) have been indicated with each PPP name.*

Rank	Integrated PPP selection	For F+N (based on OEL)	For C (based on ADI)
1	**lambda-Cyhalothrin (N=66)**	**lambda-Cyhalothrin (N=66)**	**lambda-Cyhalothrin (N=34)**
2	**Cypermethrin (N=100)**	**Cypermethrin (N=100)**	**Deltamethrin (N=39)**
3	**Deltamethrin (N=55)**	MCPA (N=3)a	**Cypermethrin (N=49)**
4	**Tebuconazole (N=99)**	**Tebuconazole**	**Cyprodinil (N=9)**
5	**Glyphosate (N=74)**	**Deltamethrin (N=55)**	**Glyphosate (N=42)**
6	**Acetamiprid (M) (N=22)**	**Glyphosate (N=74)**	**Imazalil (N=9)**
7	**Cyprodinil metabolite (N=19)**	**Acetamiprid (N=22)**	**Pirimiphos-methyl (N=3)**
8	**Piperonyl butoxide (N=37)**	**Cyprodinil metabolite (N=19)**	Tebuconazole
9	**Fluopyram (N=26)**	**Piperonyl butoxide (N=37)**	Boscalid (N=3)a
10	**Imazalil (N=10)**	**Fluopyram (N=26)**	**Piperonyl butoxide (N=22)**
11	**Pendimethalin (N=10)**	**Imazalil (N=10)**	Flonicamid (N=2)a
12	**Trifloxystrobin (N=5)**	**Pendimethalin (N=10)**	**Pendimethalin (N=6)**
13	**Flonicamid (N=2)**	**Trifloxystrobin (N=5)**	Metalaxyl-M (N=3)a
14	**Fludioxonil (N=9)**	Dimethomorph (N=3)a	**Acetamiprid (N=4)**
15	[to be decidedb]	Pyrimethanil (N=1)a	S-metolachlor (N=2)a
16	[to be decidedb]	S-Metolachlor (N=1)a	Pyrimethanil (N=3)a
17	[to be decidedb]	Metalaxyl-M (N=2)a	Fludioxonil (N=6)a
18	[to be decidedb]	Flonicamid (N=2)a	**Trifloxystrobin (M) (N=3)**
19	[to be decidedb]	Fludioxonil (N=9)a	Fluopyram (N=1)a
20	[to be decidedb]	-	Azoxystrobin (N=2)a

The non-bold printed PPPs represent low-abundancy findings of PPPs that may still be selected as relevant for F+N or C subpopulations; Bold: matching rank; Underlined: detects in blood; [a] Too small number of observations to be considered for ranking.

For the selection of the PPPs to be studied then in vitro, a component-based approach was used (single components and mixture according to EFSA, 2019), where a Standard SPRINT Mixture

of the top 8 highest ranking PPPs by wHQ was identified (Table 3.2). The Standard SPRINT Mixture was considered relevant to be studied in all in vitro studies both for animal health and human health. Additional to the Standard SPRINT Mixture, at least 3 other substances and their mixtures should be tested. Therefore the selection of the PPPs should include a total of at least 11 components and their mixture that should be tested via in vitro models. Apart from the Standard SPRINT Mixture, the other at least 3 substances and their mixture should be selected among the top highest ranking 20 PPPs from the SPRINT (Table 3.1) and selection criteria should be based on prior knowledge from (predicted) interaction mixture components in certain specific internal organs (e.g. intestine) or organ systems (immune system, central nervous system or reproductive/developmental system) that are relevant for the in vitro model used in Tier 2. The justification to test at least 11 components, instead of the originally planned 10, was based on the fact that one of the component included in the Standard SPRINT Mixture is a co-formulant (piperonyl butoxide). Further considerations for in vitro modelling are reported in Appendix 1.

Table 3.2 *SPRINT standard mixture (in yellow) and the top ten ranking PPPs for in vitro studies (with the number referring to the rank by occurrence, wHQ and N as the number of samples in the current SPRINT CSS database (data from Table 12 in Scheepers et al., 2022).*

Rank	PPP active ingredients[a]	Group	F+N wHQ	C wHQ	Water solubility (mg/L at 25°C)	Log Po/w[b]
1	lambda-Cyhalothrin (N=66)	Insecticide	2.5E-03	6.1E-04	None	6.8
2	Cypermethrin (N=100) [c]	Insecticide	8.6E-04	2.7E-04	0.01	6.6
3	Deltamethrin (N=55) [c]	Insecticide	3.3E-04	2.6E-04	<0.002	6.2
4	Tebuconazole (N=99)	Fungicide	2.4E-04[c]	1.1E-04[c]	36	3.1
5	Glyphosate (N=74)	Herbicide	1.3E-04	3.1E-05	12000	-3.4
6	Acetamiprid (M) (N=22)	Insecticide	9.8E-05	5.8E-06	4250	0.60
7	Cyprodinil (N=19)	Fungicide	3.2E-05	4.2E-05	13.9	3.59
8	Piperonyl butoxide (N=37)	Pesticide synergist	2,6E-05	1.8E-05	14.3	4.75
9	Fluopyram (N=26)	Fungicide	2.2E-05	1.3E-06	16	3.3
10	Imazalil (N=10)	Fungicide	1.09E-05	2.7E-05	14	4.6

F + N = farmers + neighbours; C = consumers; [a]Folpet was removed from rank 4 because the metabolite used for occurrence is not specific for this chemical; [b]British Crop Protection Council C, ed; e-Pesticide Manual. 15th ed., ver. 5.1, Alton, UK; [c]see Table A1 in Appendix 1.

4. Tier 2: In vitro testing

BACKGROUND and AIMS

Organoids are considered a new tool for *in vitro* studies on different organs and tissues of animal and human origin. The organoid stem cell models are self-renewing and more closely mimic host physiology than cancer or transformed cell lines (Sato et al. 2011; Lukovac and Roeselers 2015). In contrast, cancer or transformed cells display aneuploidy and undergo significant genotypic alterations within a few passages *in vitro* leading to altered pathway expression compared to primary cells (Liu et al. 2019). Organoids can be generated from pluripotent stem cells that can be expanded seemingly indefinitely in culture, maintain a normal karyotype and have the potential to generate any cell type in the body. Alternatively adult stem cells present in the tissue or organ of interest can also be used to generate many types of organoids. The advantage of using adult stem cells rather than induced pluripotent stem cells to generate intestinal organoids is that they maintain functions associated with the specific intestinal segment from which they were generated (Middendorp et al. 2014). We recently described the generation of porcine intestinal organoids which mimic the crypt-villus axis with a similar spatial organization of the heterotypic cell lineages found in the tissue of origin (van der Hee et al. 2020; van der Hee et al. 2018a).

The effect of pesticides and pesticide mixtures on the intestinal epithelium is important to study because of the crucial barrier function of the intestine, restricting entry of harmful substances and microorganisms into the body (Wells et al. 2011; van der Hee et al. 2018b). Injury of the intestinal epithelium can lead to 'leaky gut syndrome' increasing permeability to toxins and bacteria, which may enter the bloodstream and generate inflammatory responses. Therefore, integrity of the intestinal epithelium barrier is crucial in maintaining health. Ileum porcine organoids were selected as the target tissue for this study on animal health as the ileum

epithelium is exposed to relatively high concentrations of PPPs via oral ingestion of food, dust, and swallowed mucus from the upper respiratory tract. Pigs were chosen over bovine organoids because many antibodies developed against human intestinal markers can be used for immunofluorescent histology in pig tissue due to high conservation of protein sequences. Furthermore, the recent efforts of FAANG consortium and EU funded GeneSwitch project have advanced the functional annotation of the pig genome and developed new tools and resources for RNAseq. Furthermore, pigs have a similar intestinal physiology to humans, thus the results will contribute to our understanding of the potential risks of PPPs to the intestinal health of humans and other species and inform decision-making related to regulation of the use of PPPs.

The effects of PPPs on the epithelium of the porcine ileum were assessed via a stepwise approach. In step 1, single PPPs and mixtures were incubated with porcine ileum organoids at relevant dose ranges for 96 hours to identify synergistic, antagonist or combinatorial effects on cell viability and growth by measuring cell ATP production (Figure 3.2). In step 2, RNAseq was performed on organoids exposed for 5 days to individual PPPs and mixtures of PPPs selected based on results in Tier 1. The data was used to identify significantly differentially expressed genes as well as potential effects on differentiation into differ cell lineages (goblet cells, Paneth cells, etc) in treated vs control samples.

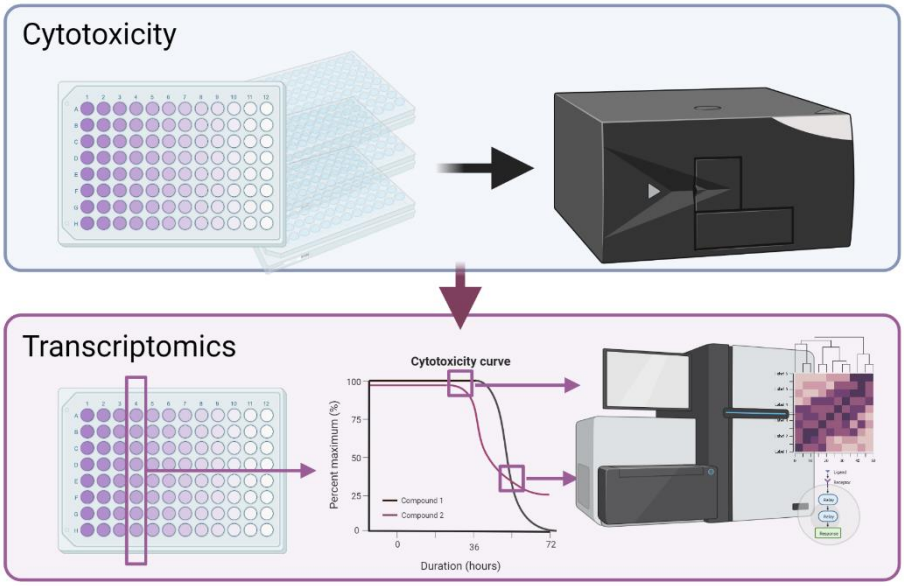

Figure 4.1 *Graphical overview of testing steps taken in Tier 2. Initial cytotoxicity assessment using a dose-response curve was measured and subsequently the compounds were added to organoids for transcriptomics analysis.*

Step 1 – Cytotoxicity testing (cellular ATP production)

Viability and growth of organoids were assessed for 11 mixtures and 15 individual compounds over a relevant dose-range.

Step 2 – Transcriptomics

Cell lineage differentiation and gene expression were assessed for 8 selected mixtures and 5 individual compounds at a single concentration.

1.1 Accordance with Grant Agreement:

In the formulation of the project plan we mentioned using bovine intestinal organoids for the animal health study reported in deliverable 4.4 but we decided that porcine was the best for the objectives.

However, in the last rounds of editing the proposal, not all the requested changes were incorporated. Both pigs and bovines are relevant animals in the sense that they are exposed to environmental and feed sources of pesticides. However, we find bovine organoids less easy to characterise in terms of the different epithelial cell types because of the lack of commercially available antibodies for staining markers in the bovine intestine whereas antibodies binding to human proteins often react with the homologous proteins in pigs due to high level of sequence homology. Moreover, the bovine organoid line we established was less stable in long term culture than porcine organoids and they could not generate monolayers of polarised organoid epithelial cells for the apical exposure to pesticides. In pigs, this was possible by modifications of a protocol published for human organoids. We also considered that pigs are monogastric and have a similar intestinal physiology to humans, thus translation of the results to humans is expected to be more reliable than for bovine and will benefit other research on human organoids being done in SPRINT. For these reasons, we chose pigs as a species to study the effects of pesticides on the intestine.

In the grant agreement it was proposed to select one mixture for each CSS (i.e., a maximum of 11 mixtures, containing a maximum of 5 PPPs). Selecting a separate mixture for each CSS was not feasible as the compounds that were measured most frequently in faeces, urine and blood did not differ sufficiently between CSS to select CSS specific mixtures. Therefore, the selected mixtures do not directly reflect the risk to subjects at each CSS.

METHODS

Organoid development

Ileum tissue was obtained from 4-week-old control piglets used for another study approved by the ethical committee of Wageningen University. Colonic organoids were derived from the crypts using as previously described methods with minor modifications (van der Hee et al. 2020; van der Hee et al. 2018a) as follows: a 1 cm section of ileum epithelium was opened longitudinally and washed three times in D-PBS (Gibco) containing 1% penicillin/streptomycin (v/v) to remove residual fecal matter (Figure 4.2). The tissue was then placed epithelial side up and gently scrapped with the blunt side of a scalpel to remove the upper mucosal layer. The epithelium was then gently lifted and transversely cut to remove the muscle layer. The remaining ileal epithelium was then divided into smaller 5 mm sections and transferred into ice-cold D-PBS (Gibco) containing 30 mM EDTA and incubated at room temperature for 15 minutes on a rotating platform. The PBS-EDTA was then removed and replaced by pre-warmed D-PBS+EDTA (30 mM) and then incubated for a further 10 min with gently shaking at 37 °C. The crypt mixture was then washed with ice-cold DMEM-F12 (Gibco) supplemented with 5% P/S (v/v) and vortexed to disperse the crypts.

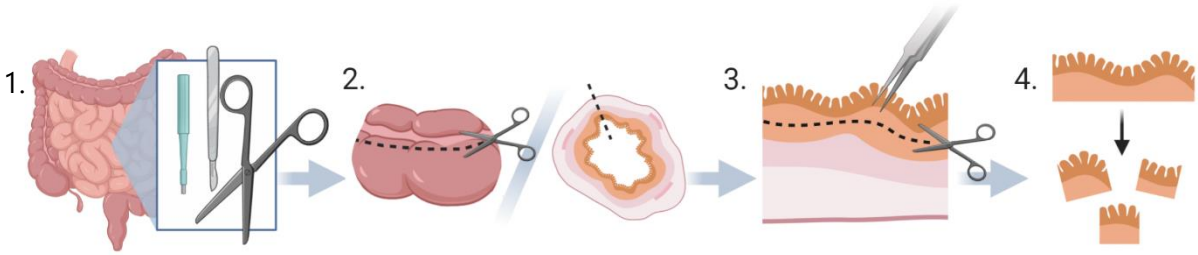

***Figure 4.2**. Graphical overview of tissue handling for organoid generation. Tissue was resected from the distal section of the ileum (1) and opened longitudinally (2). This resulted in opening the luminal compartment and was subsequently removed from the serosal layer by gently cutting in between the epithelium and muscle layer (3). The resulting tissue was then further dissociated and processed for crypt extraction (4).*

The crypt mixture was then strained over a 100 µm filter into ice-cold DMEM-F12 (Gibco) supplemented with 5% fetal bovine serum (FBS, v/v) and 1% P/S (v/v). The crypts were then pelleted at 300 x g for 5 min and suspended in Matrigel (Basement Membrane, Growth factor reduced, REF 356231, Corning, Bedford, MA, United States). The Matrigel/crypt mixture was then added as small 5 µl droplets to a pre-warmed 24-well plate (6-9 droplets per well) and inverted to polymerize at 37 °C for 10 min. Gelation was observed and 600 µl/well basal culture medium (BCM) was added. W-ENR consisted of DMEM-F12 (Gibco) supplemented with 1% P/S (10378016), 100 µg Primocin (Invivogen, ant-pm-1), 10 mM HEPES buffer (HyClone), B-27 supplement (Gibco, 17504044), 1.25 mM N-acetylcysteine (Sigma), 50 ng/ml human epidermal growth factor (hEGF; R&D Systems), 7.5 nM gastrin, 10 mM nicotinamide, 10 µM p38 MAPK inhibitor (Sigma), 600 nm transforming growth factor-beta (TGF-b) receptor inhibitor A83-01, and conditioned media for Noggin (15% v/v), R-Spondin (15% v/v), and WNT3A (30% v/v) provided by Dr. Kuo and Hubrecht Institute (Utrecht, The Netherlands). Culture medium was exchanged every other day and the organoids were sub-cultured every 5 days and passaged as spherical structures by mechanical dissociation in pre-warmed TrypLE and plating as previously described in Matrigel GFR BME. The organoid differentiation was initiated by reducing the total concentration of conditioned media components WNT3A and R-

Spondin to 5% to decrease stem cell numbers and initiate increased differentiated cell populations (Diff-Med).

Two-dimensional methodsPolarised organoid monolayers

The differentiated ileum organoids containing all epithelial cell lineages present in *in vivo* were extracted from the wells by repeated pipetting with ice-cold DMEM-F12 (Gibco) containing 1% P/S (v/v) and transferred to a 15 ml conical tube. The organoids were pelleted at 500 x g for 5 min and the supernatant was removed. The pellet was subsequently suspended in 750 µl pre-warmed TrypLE and transferred to a 37 °C water bath for 5 min to dissociate the cells. After 5 min incubation, the dissociated organoid cell mixture was agitated with a P200 pipette by vigorous pipetting and placed in the water bath for an additional 5 min at 37 °C (see previous methods REF). The enzymatic reaction was stopped by addition of 5 ml ice-cold DMEM-F12 and the tube was centrifuged at 750 x g for 5 min. The dissociated cells were resuspended in 100 µl pre-warmed Diff-Med and an additional 5 ml Diff-Med was added. The cells were counted using a Bürker chamber and added to tissue culture-treated imaging 96- (viability assays) or 24-well (RNA-sequencing assay) plates (Corning) at a density of 2.5 x 104 cells/well. The Diff-Med was replaced daily to remove cellular debris with pre-warmed media.

Step 1 - cytotoxicity testing

Compound selection for viability testing in intestinal organoids

As described in Tier 1, existing databases were explored for hazard documentation and a ranked list created for compound prioritization. The top 8 PPPs were automatically chosen for viability testing. Additionally, 4 compounds from rank 9-14 were selected based on their detection in

human feces, food, blood and urine. Five additional compounds were selected that were often found in food, feces and/or urine. For example, Boscalid was often found in food and urine. Boscalid is ranked 9 for consumers (Table 3.1). Spirotetramat was often found in food and in feces. This PPP was not included in an earlier ranking as insufficient data was available. Pirimiphos-methyl was often found in food and is ranked 8 for consumers (Table 3.1) and was observed in relatively high frequency in animal blood. Axoxystrobin was often detected and food and ranked 20 for consumers. Metalaxyl-M was often found in food and was ranked 17 for farmers and neighbours and 13 for consumers. The selected compounds were lambda-Cyhalothrin, Cypermethrin, Deltamethrin, Tebuconazole, Glyphosate, Acetamiprid, Cyprodinil, Piperonyl butoxide (Top 8), Fluopyram, Imazalil, Trifloxystrobin, Fludioxonil (ranked 9-14), and additionally Boscalid, Spirotetramat, Azoxystrobin, Metalaxyl-M and Pirimiphos-methyl which were not included in the mixtures tested.

Concentrations and exposure for viability testing

A relevant dose-range was defined as ranging from low to median concentrations found in feces from human subjects up to 100 * ADI (Acceptable Daily Intake) level, which used as an 'assessment factor' to cover uncertainties regarding interspecies and intraspecies extrapolations. ADI is often defined as the (No Observed Adverse Effect Level)/100. So, 100*ADI level corresponds to the NOAEL level. Therefore, any indications of toxicity in this dose range would be of scientific relevance. The ADI was translated to a concentration using the following equation, based on an approach used by Truzzi & Mandrioli et al (2021; (Truzzi et al. 2021)).

ADI (mg/kg/day) * average_bodyweight (kg)/ average_water_intake (L/day) = concentration (mg/L)

using the assumptions of an average bodyweight of 70 kg and average water intake of 2 L.

The polarized organoid monolayers were grown to confluence and exposed to 5 concentrations of each compound for 4 days by replacing the medium containing the PPP every 24 hours. Despite having no problems dissolving the compounds in dimethyl sulfoxide (DMSO), Compounds lambda-cyhalothrin, cypermethrin, tebuconazole, cyprodinil, and fludioxonilspreciptated immediately precipitation when added to the culture medium at a concentration corresponding to 100x the ADI-level. However, diluting the compound to give 50 x ADI exposure remedied the precipitation issues. No solubility problems were observed at 10 x ADI or lower.

PPP mixtures selected for viability testing

Eleven mixtures were selected containing 2 or more of the individual compounds that were selected from the top 14 in Table 3.1. Imazalil was not included in the mixtures as it showed low co-occurrence with other compounds in human feces, blood and urine.

The following mixtures were selected based on considerations above:

1. Glyphosate, Lambda-Cyhalothrin, Deltamethrin, Cypermethrin, Tebuconazole, Acetamiprid, Cyprodinil, Piperonyl butoxide
2. Glyphosate, Lambda-Cyhalothrin, Deltamethrin, Cypermethrin, Tebuconazole, Acetamiprid, Cyprodinil, Piperonyl butoxide, Fluopyram, Fludioxonil, Trifloxystrobin
3. Glyphosate, Piperonyl butoxide, Tebuconazole, Fludioxonil, Trifloxystrobin
4. Glyphosate, Tebuconazole, Piperonyl butoxide, Deltamethrin, Cypermethrin
5. Lambda-cyhalothrin, Cypermethrin, Deltamethrin, Piperonyl butoxide
6. Lambda-cyhalothrin, Cypermethrin, Deltamethrin
7. Cyprodinil, Fludioxonil
8. Fluopyram, Piperonyl butoxide
9. Cyprodinil, Fluopyram

10. Tebuconazole, Trifloxystrobin
11. Acetamiprid, Lambda-Cyhalothrin, Cypermethrin, Piperonyl butoxide

- * Piperonyl butoxide is primarily used as synergist co-formulant in pyrethroid-based insecticides and for that reason was included in some mixtures

Rationale for the formulation of the mixtures:

- Combination of top-ranking compounds as described in Tier 1
- Occurrence in food samples (mixture 3)
- Presence in human and animal feces, urine, and blood (relevant for mixtures 4 to 11)
- Co-occurrence in human and animal feces, urine, and blood (mixture 4, 5, 6) *Glyphosate and Tebuconazole were present in almost all samples and therefore co-occur with all other compounds. Multiple pyrethroids and their synergist Piperonyl butoxide were often found together (from the samples that contained glyphosate, >50% also contained tebuconazole.*
- Compounds from the same PPP subclass (i.e. with a similar mode of action) were grouped together (mixture 5 and 6)
- Insecticides were grouped with other insecticides and fungicides were grouped with other fungicides.

  This was not done for herbicides since Glyphosate was the only selected herbicide. (mixtures 4, 5, 7, 9, 10, 11)
- Piperonyl butoxide is a synergist that enhances the plant protective effects of multiple PPPs including pyrethroids and Fluopyram. Two mixtures containing Piperonyl butoxide were designed to assess its possible synergistic or combinatorial effects with pyrethroids and fluopyram in the animal gut. (Mixtures 5 and 8)
- Compounds that are also combined in commercial mixtures Mixtures 7, 9, and 10)

Cytotoxicity testing of organoids exposed to PPPs

The 3D organoid cells were grown to confluence in 2-dimensional monolayers in white-walled 96-well plates as previously described. On the day of the experiment, the medium was replaced of all wells with Diff-med. The top row was subsequently stimulated with 100x or 50x ADI of the compounds or mixtures and serially diluted ten-fold downwards to 0.001x ADI-level, which, for most compounds, corresponds well with concentrations found in feces. The chemical solvent (DMSO) and medium control (MC) were added to the final two rows to control for potential effects. The following 4 days consisted of similar stimulations, of refreshing culture media and ten-fold dilutions of the stimulations into the wells. On the fifth day, the cells were lysed for ATP concentrations following manufacturer's instructions (Cell-titer glo 2.0, Promega). Briefly, the cells and compound were left to acclimatize to room temperature for 30 minutes. The cells were then lysed by addition of 1 volume *v/v* of cell titer glo solution and mixed by vortexing on a plate shaker at 500 rpm for 2 minutes. The plates were then incubated in a dark environment for 10 minutes and measured on a luminescence plate reader (Molecular devices) with top read at 500ms. The resulting relative fluorescence units (RFU) were then normalized using the DMSO-treated wells as relative control.

Step 2 – Transcriptomics analysis

Compound and mixture selection for transcriptomics

Three of the above mixtures were selected for transcriptomics;

1. Top 8 (later referred to as 'Mix-1'); This mixture was selected as it contains the top 8 compounds expected to be most hazardous for human and animal health and is used to explore possible combinatorial effects between these 8 compounds.

5. Pyrethroids + synergist (later referred to as ‘Mix-2’); Pyrethroids have a similar mode of action and might have similar effects in the body; they might have synergistic/additive/combinatorial effects. At high concentration, piperonyl butoxide affected organoid viability.

7. Cyprodinil + Fludioxonil (later referred to as ‘Mix-3’); Both compounds affected organoid viability at high concentrations. The combination of Cyprodinil and Fludioxonil is sold as a commercial mixture.

Five individual compounds were selected for transcriptomics. The selected compounds had to be present in at least one of the 3 selected mixtures.

1. Cyprodinil (strongly affected organoid viability at 50x ADI)
2. Fludioxonil (affected organoid viability at 10x ADI)
3. Piperonyl butoxide (slightly affected organoid viability at 100x ADI)
4. Glyphosate (present in >75% feces samples)
5. Tebuconazole (present in >75% urine samples)

RNA-sequencing and analysis

For transcriptomic analysis, cell monolayers were grown to confluency within 24h in 24-well plates, as previously described and treated with control (DMSO), and compounds (listed above), for 5 days with daily exposure recurrence. After treatment, the cells were then washed with prewarmed D-PBS, lysed using RLT buffer and placed on ice for extraction using the RNeasy mini kit (ID: 74104, Qiagen) following manufacturer’s instructions. The extracted RNA was then eluted in 30 µl RNAse- and DNAse-free water (Promega) and measured for concentration with Qubit (RNA HS assay kit, Thermo-Fisher). The RNA was then normalized to 250 ng/µl and stored at -80 ˚C.

RNA library preparation was performed using the TruSeq RNA sample kit at Novogene, as previously described (van der Hee et al. 2020). The samples were depleted for ribosomal RNA using the RiboZero kit and enriched for mRNA with oligo(dT) beads. The RNA was subsequently fragmented and synthesized into cDNA using mRNA template and hexamer primers. Second strand synthesis initiation was performed with the addition of second strand synthesis buffer (Illumina), dNTP's, RNAse H, and DNA polymerase I and following a series of terminal repair the cDNA library construction was completed by size selection and PCR enrichment. The libraries were then paired-end sequenced on a Illumina Hi-Seq 4000 (at Novogene, Hong Kong) at 9 GB raw data (±55million reads) per sample with 150 bp length. The raw data was then checked for quality using FastQC (Andrews 2017) and trimmed for quality, polyA, and adapter using trim-galore (Krueger 2017). The trimmed reads were then mapped to sus scrofa (Ensembl, version 11.1.91, (Zerbino et al. 2018; Kersey et al. 2018)) using TopHat (Trapnell et al. 2012) and analyzed in R using the Cufflinks package (Trapnell et al. 2012). Differential gene expression was analyzed with false discovery rate (FDR) p-value < 0.05 (P_{adj}). Genes expressed with >1 RPKM were considered expressed. Figures display z-score normalized values for range optimization or RPKM values. Biological functions were analyzed by gene set enrichment analysis using Gene Ontology (GO, (Tuggle et al. 2016), Reactome, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapper and differentially tested from control (expressed as p value and gene set)

Calculations

The mixtures consisted of equal parts of each of the component solutions at 100x ADI and its 10-fold dilutions. The highest concentration of each of the individual components in the mixture therefore corresponds to 100xADI/n, where n is the number of components present in the mixture.

It was not possible to create mixtures that contained each of the individual components at a 100xADI concentration due to low solubility of some of the compounds.

RESULTS

Viability of cells exposed to different PPPs was measured using a luminescence-based ATP assay because only viable cells can synthesize ATP and this method is less prone to artefacts than other viability assays. Most compounds did not affect the cellular ATP concentrations, but we did observe a sharp decrease in available ATP in cells exposed to Cyprodinil (50x ADI) and Imazalil (100x ADI). Furthermore, a decline in available ATP was noticeable for Fludioxinil starting at 10x ADI (approx. 15 % reduction) to 100 x ADI (25 % reduction) and piperonyl butoxide at 100 x ADI (20 % reduction). In contrast, the mixtures did not seem to significantly affect cell viability (Figure 4.3). This could be attributed to the maximal volume of DMSO and solubility limit for PPP mixtures. For example, the concentration of each of the 8 compounds in mixture 1 was only 12.5x ADI.

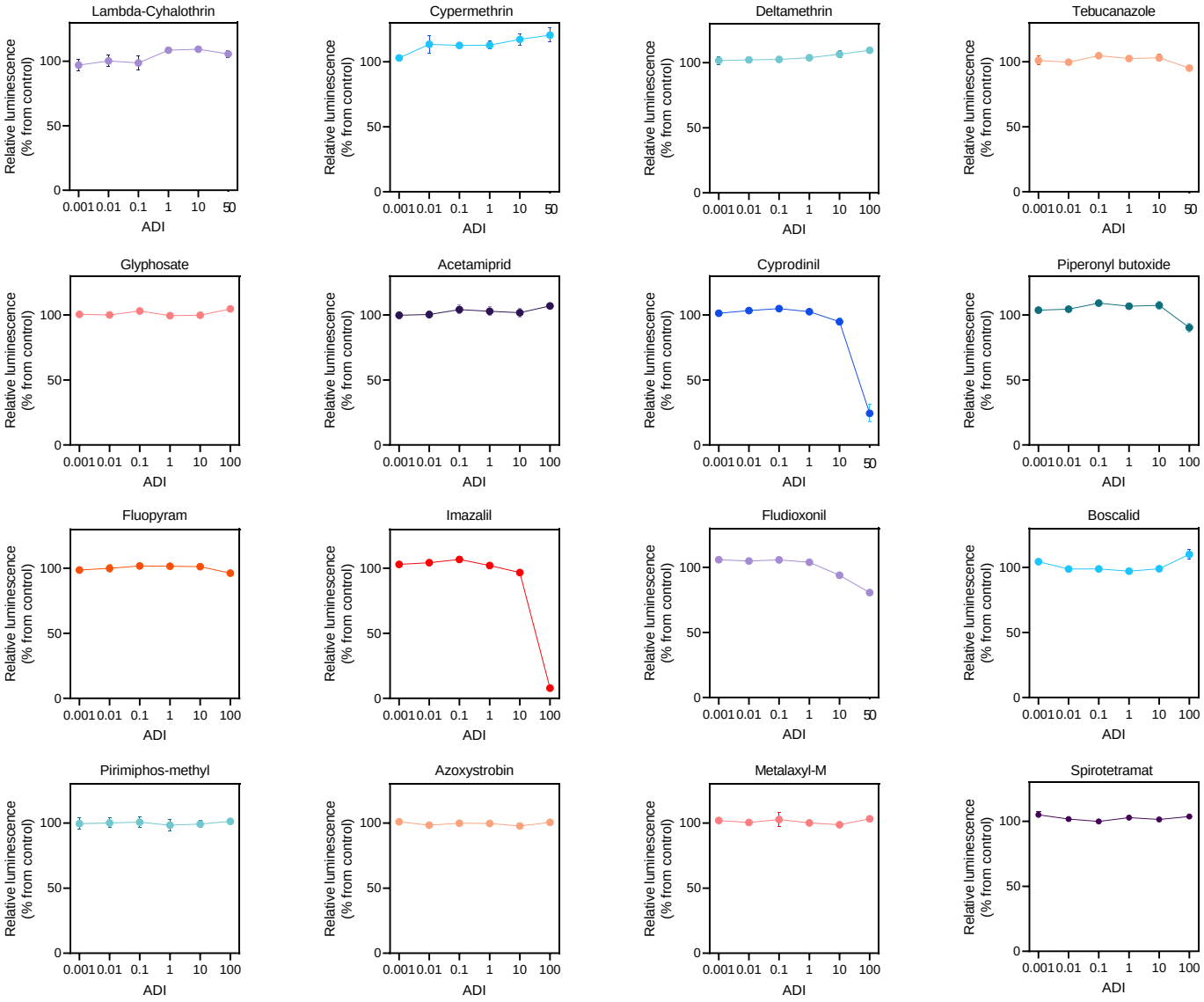

Figure 4.3 *Viability response curves of ileum organoids exposed to individual PPPs. n = 4 per PPP per experiment, n = 2 experiments per treatment. Data is shown as mean ± SEM.*

In contrast, the mixtures did not seem to significantly affect cell viability. This could be attributed to the maximal solubility limit for PPP mixtures as described above.

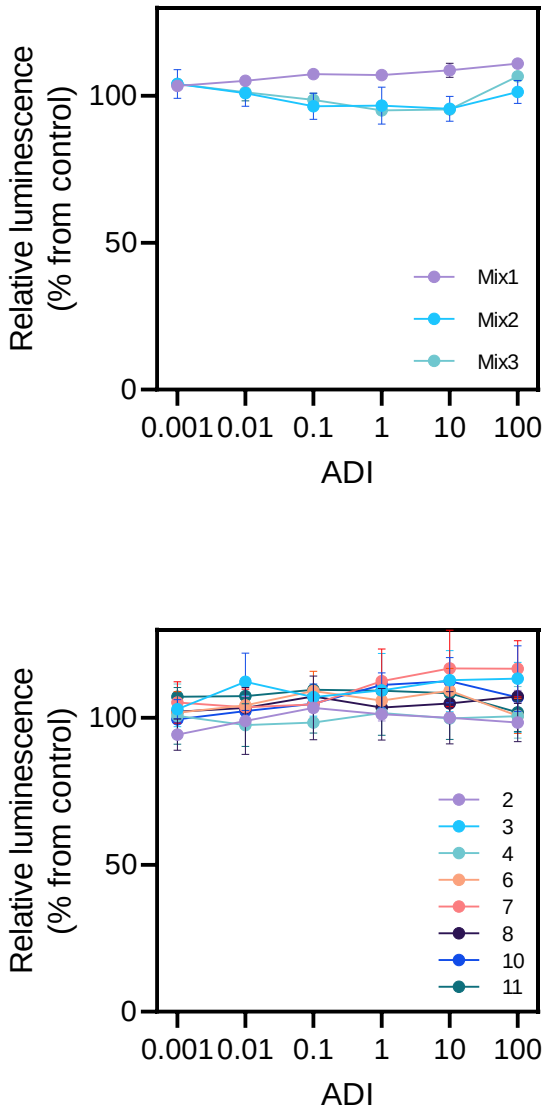

Figure 4.4 *Viability response curves of ileum organoids treated with PPP mixtures. Top graph showing relative viability of cells treated with Mix1-3 as specified in the methods section, which also have been tested for RNA-sequencing. Bottom figure shows the relative viability of ileum organoid cells treated with the remaining mixtures as specified in the methods section. n = 4 per treatment per experiment, n = 2 experiments per treatment. Data is shown as mean ± SEM. The x-axis refers to the concentration of the individual compound in comparison to their individual ADI times the number of components present in the mixture For example; in a*

mixture with 4 components, at '100 ADI' each of the individual components is present at 100/4 = 25x their ADI level.

Effects of PPP exposure on gene expression in ileum organoids

The transcriptomes of each sample were mapped for similarity ordination using principal component analysis (Figure A2.3). Each sample replicate clusters together, showing low intra-treatment variability, except for one sample treated with cyprodinil showing larger variation and clustering away from its replicates in the first dimension. It is also apparent that glyphosate and tebuconazole cluster closer to the control samples when compared to the mixtures, Cyprodinil, Fludioxonil and piperonyl butoxide, indicating their effects on the whole transcriptome to be relatively small compared to other treatments. The piperonyl butoxide samples clustered further away from the control than the two mixtures containing this compound. This could be due to the limitation in maximal solubility of the compounds and the need to dilute piperonyl butoxide 1:8 in mixture 1 and 1:4 mixture 2 compared to samples to which only contained piperonly butoxide. Organoids treated with both cyprodinil and fludioxinil cluster furthest away from the controls, and mixture 3, consisting of both compounds, clusters in between them. This indicates that, although both compounds might largely affect cell transcriptomics, fludioxinil has the largest effect on the transcriptome of these cells. In the cytotoxicity assays cyprodinil had a larger effect on ATP production than fludioxinil at 100 x ADI.

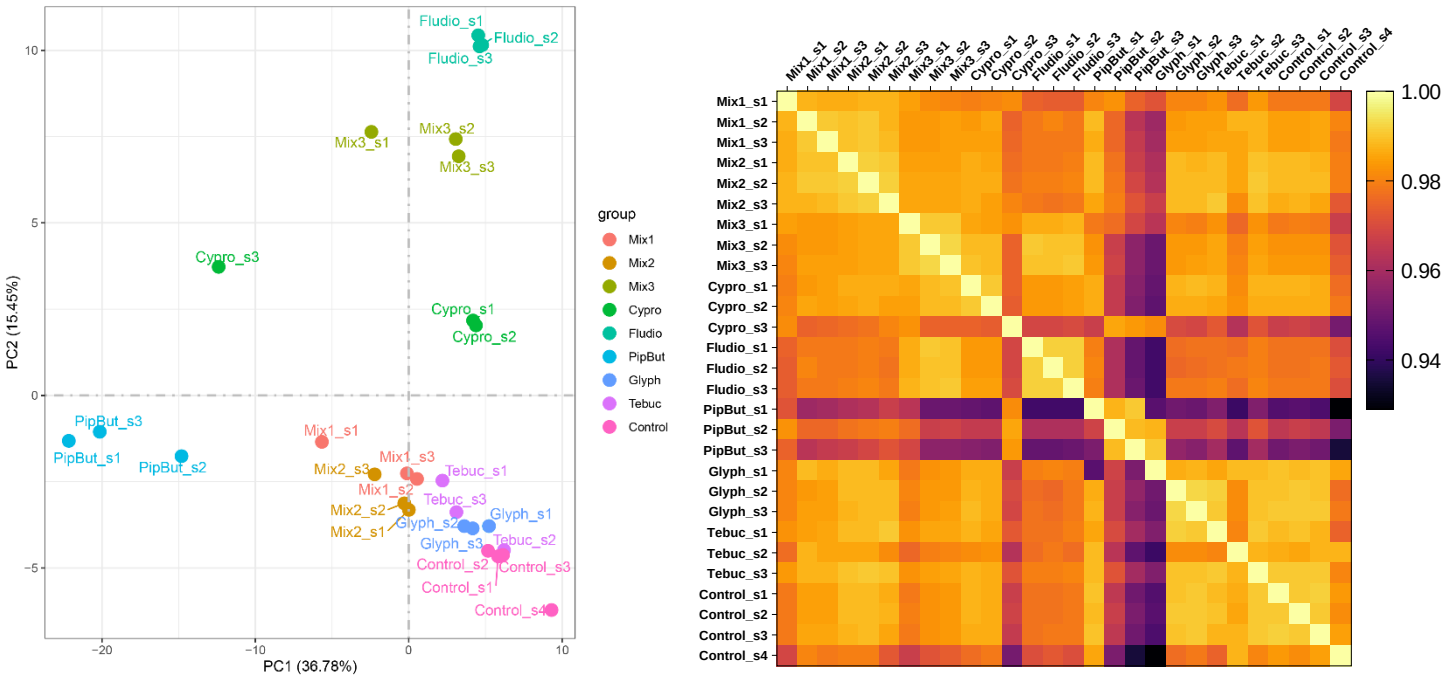

Figure 4.5 *Effects of different PPP's and mixtures on gene expression in ileum organoids. (A) Principal component analysis plot visualizing the sample distribution between different treatments (Mix1, Mix2, Mix3, PipBut; fluido , Tebuc; Tebucanazole, Cypro; Cyprodinil, Fludio; Flludioxonil, Glyph; Glyphosate, Control; medium and solvent control). (B) Correlation matrix comparing transcriptomes of ileum organoid after exposure to PPP's or control. Replicate samples are indicated S1, S2 and S3 for each compound or mixture.*

The quantification of gene expression in RNA-seq experiments is directly inferred from the abundance of transcripts. This abundance, which is determined by the count of sequenced transcripts that successfully map to the genome or exon regions, serves as a reliable indicator of gene expression levels. It is important to note that the read count is influenced by several factors, including gene expression level, gene length, and sequencing depth. Among the various methods used to estimate normalized gene expression levels, the most common is FPKM, which stands for "expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced." This accounts for variations in sequencing depth and gene length, ensuring a more accurate estimation of gene expression levels in RNA-seq analyses.

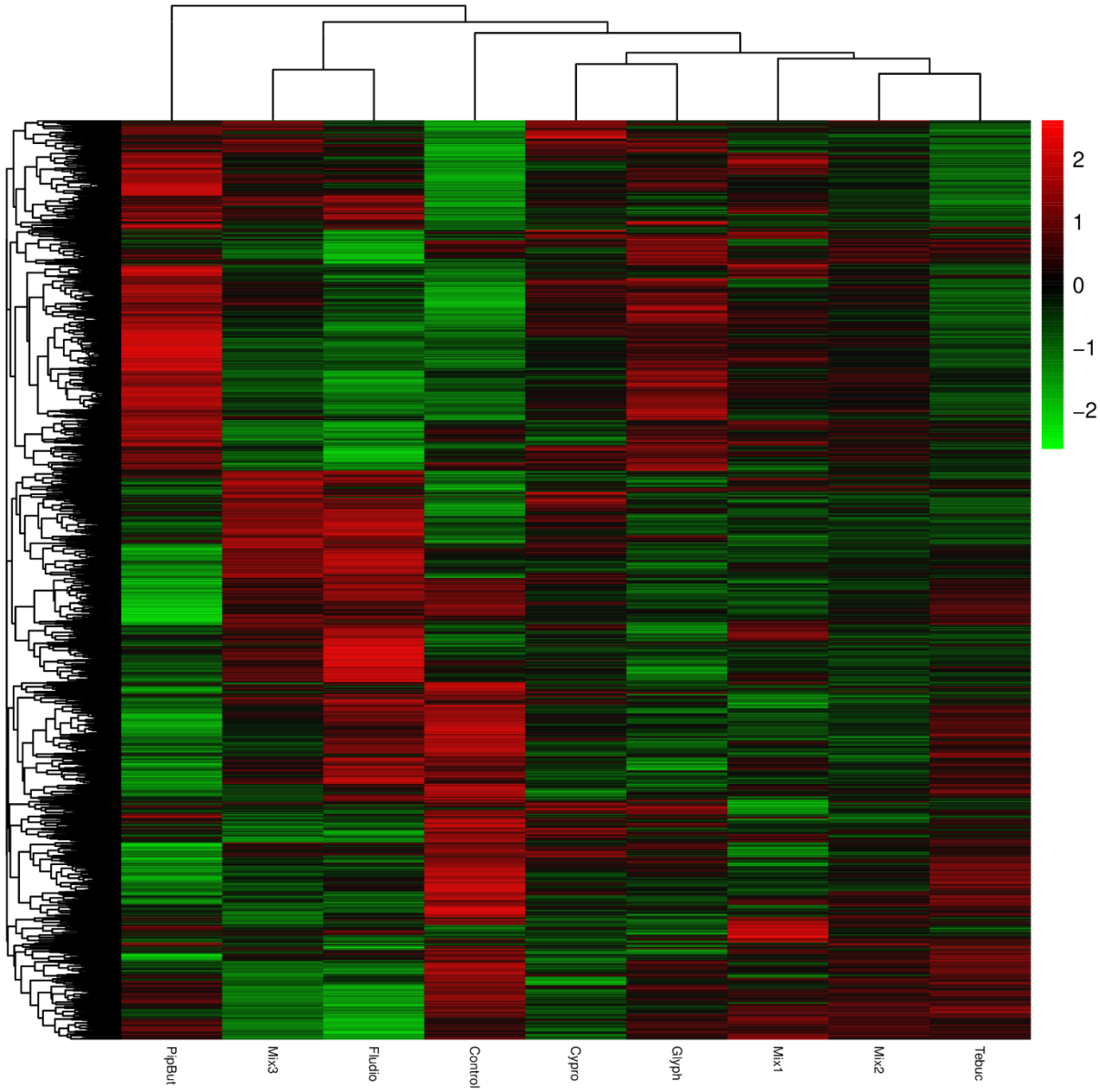

Figure 4.6 *Hierarchical clustering plot of transcriptome expression patterns for all individual treatment groups. Left clustering tree indicates individual genes (gene names too small to include in figure), top clustering for treatment groups.*

On average, 11544 genes were expressed at > 1 FPKM in our dataset and 24440 genes were not expressed < 1 FPKM. Thus 32.08% of the porcine genes were expressed, which is in line with previous expression studies in porcine organoids (supplementary Figure A2.1).

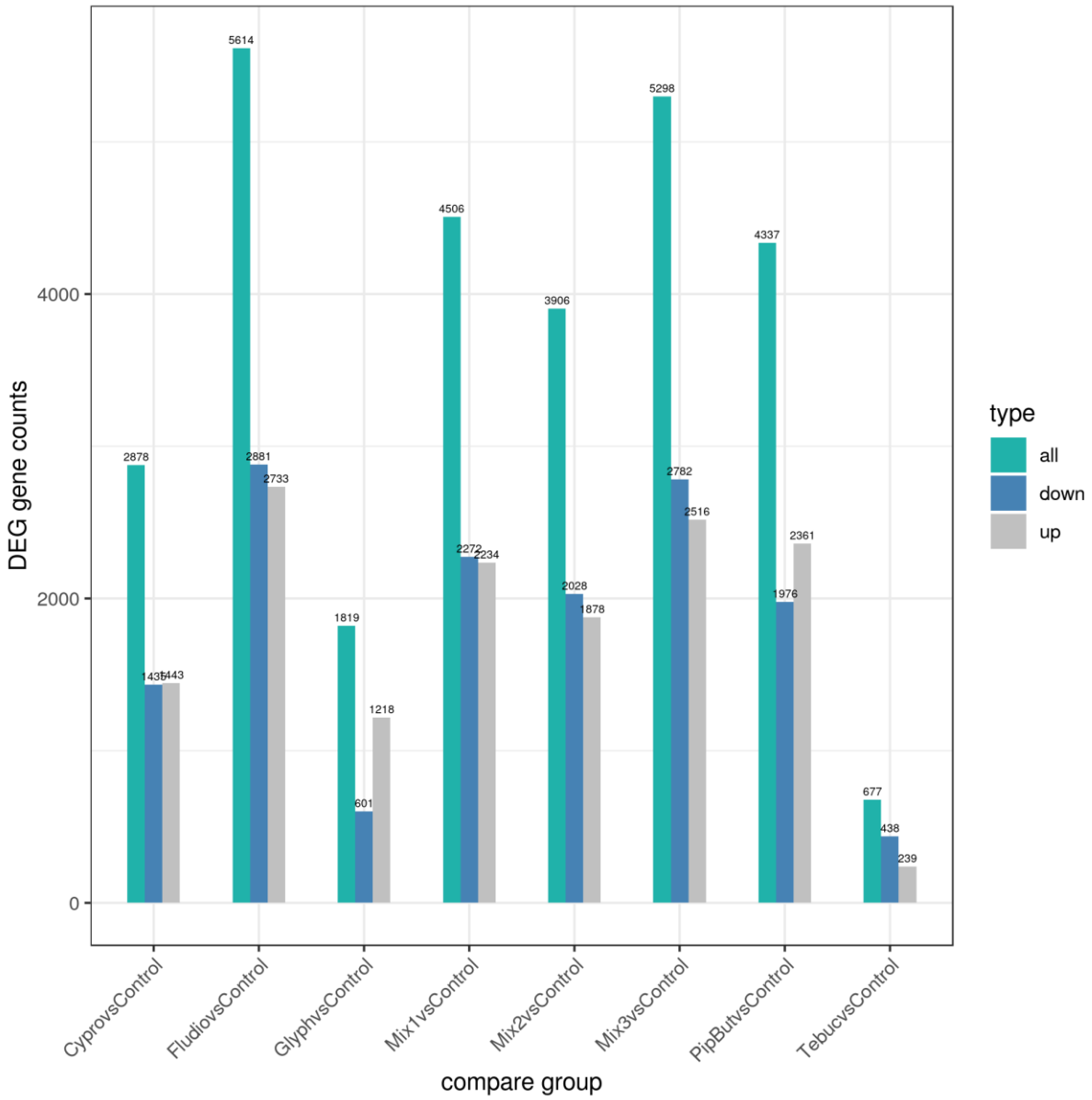

Figure 4.7 *Differential gene expression statistics between ileum organoid transcriptomes treated with PPPs compared to control. The ileum organoid cells were grown to confluency and treated with individual and mixed PPPs for 5 days. The transcriptomics data resulting from RNA-sequencing was tested for differential expression compared to control using DeSeq2 with a cut-off of* $P_{adj} < 0.05$ *and FDR* $q < 0.05$.

In line with the PCA analysis, the lowest number of differentially expressed genes compared to control were found in glyphosate- and tebuconazole-treated ileum organoid monolayers (Figure 4.7). A total of 1819 genes were differentially regulated in glyphosate-treated cells with 1218 up- and 601 down-regulated genes. For tebuconazole a total of 677 genes were differentially regulated with 239 up- and 438 down-regulated genes. The largest effects were found in cells

treated with fludioxinil (5614 genes) and piperonyl butoxide (4337 genes), with a smaller difference with cyprodinil (2878 genes).

Increasing P-value stringency (P_{adj}) for organoids treated with piperonyl butoxide yields a total of 1472 genes significantly differentially expressed genes (DEGs). The number of significantly DEGs (P_{adj}) shared between datasets obtained for piperonyl butoxide and the two mixtures was 720 genes. For only mix 1 and piperonyl butoxide it was 285 shared DEGs and for only mix 2 and piperonyl butoxide it was 117 DEGs. A total of 350 DEGs were specific to piperonyl butoxide treatment and not shared with either mixture. Mapping of these 350 genes for pathway analysis point towards the involvement of genes encoding enzymes related to the biological oxidation of eicosanoids and cytochrome P450 (discussed in more in-depth below). Eicosanoids do not play a direct role in metabolism of pesticides and are a group of signaling molecules that play important roles in inflammation and other physiological processes (Gupta et al. 2009). They are derived from arachidonic acid (AA) and can be converted into biologically active compounds via metabolism by cyclooxygenases (COX). Eicosanoids are involved in a variety of biological processes, including insect biology and psychiatric disorders (Yui et al. 2015). Other studies have suggested that exposure to pesticides can affect eicosanoid metabolism and signaling pathways. For example, one study found that exposure to the pesticide chlorpyrifos led to changes in eicosanoid metabolism in rats. Another study found that exposure to the pesticide dichlorodiphenyltrichloroethane (DDT) led to changes in eicosanoid signaling in human breast cancer cells (Gupta et al. 2009).

When mapping the differentially expressed genes in sample groups treated with PPPs, we observed upregulation of pathways associated with drug metabolism, neurodegenerative diseases and oxidative phosphorylation for all PPPs (all $P < 0.001$; Tables A2.1-5). The mapping of these pathway DEGs using the KEGG pathway database indicates upregulation of drug metabolism pathways, mitochondrial oxidative phosphorylation, the ubiquitin pathways

and mitophagy. For instance, the number of up regulated genes in Parkinson's disease pathways were highest in cells treated with piperonyl butoxide (n = 126 genes) and fludioxonil (n = 90 genes), with a smaller gene set for glyphosate (n = 61 genes), Cyprodinil (n = 62 genes), and Tebuconazole (n = 17 genes). The genes upregulated within the mitochondrial pathway are mainly cytochrome c oxidase (COS) genes and these were mainly upregulated in cells treated with piperonyl butoxide. They are vital for the mitochondrial respiration chain and could signify malfunction of the mitochondria and oxidative stress. The COS genes play a crucial factor in etiology, progression, and prevalence of numerous human neurodegenerative diseases (Valente et. al, 2004).

Cyprodinil, piperonyl butoxide, and fludioxonil downregulated genes associated with adherens or tight junctions when compared with control samples. The tight junctions (TJs) and adherens junctions (AJs) in the intestine play important roles in maintaining the integrity of cell-cell junctions and maintaining the gut barrier. From the gene lists of the affected pathways, it is evident that expression of cell tubulin which is important for delivery of junctional proteins to cell-cell contact sites is affected by the treatments. Additionally, expression of vinculin (*VNC*), which plays a role in adherens junction formation, is affected by exposure to PPPs. Together these findings support the notion that barrier function might be negatively affected by certain PPPs and warrants further studies in the follow up *in vitro* studies using human organoids.

We observed altered expression of pathways involving xenobiotic metabolism and cellular stress for all PPP exposures. Pathways for metabolism of xenobiotics by cytochrome P450 were predicted to be upregulated by all tested PPPs. The cytochrome P450 family of enzymes metabolize a wide variety of xenobiotic drugs and toxins, as well as endogenous compounds like steroids and fatty acids (Stavropoulou, Pircalabioru, and Bezirtzoglou 2018). Moreover, cytochromes are essential for cellular respiration and are part of the electron transport chain in mitochondria, where they help transfer electrons to oxygen, ultimately producing ATP. This

explains the efficacy of some insecticides that target the energy supplies for the wings to stop insects from flying (Hollingworth et. al, 1994).

All results from the RNA-sequencing will be deposited in the GEO database and all enrichment (pathway) information uploaded to a shared folder on the sharepoint (as example, see KEGG pathway in supplementary figure A.2.4). This will contain all regulated pathways, their individual genes, and direction of expression, with relevant visualization of expressed location within the pathway. This will contribute to the development of new SPRINT indicators.

DISCUSSION

The effect of PPPs on gut health

Glyphosate exposure has been shown to have negative effects on animal gut health by altering the gut microbiota and metabolites. Studies have shown that glyphosate exposure can lead to alterations in the gut microbiota of farm animals given feed containing glyphosate (Mao et al. 2018). Glyphosate exposure has also been associated with alterations in the gut microbiota of humans (Puigbo et al. 2022). Recent studies have suggested that glyphosate exposure may lead to changes in the urine metabolome and may be associated with intestinal diseases and neurological as well as endocrine problems in animals and humans (Van Bruggen et al. 2018; Puigbo et al. 2022). Glyphosate exposure has also been shown to affect the gut microbiota of bumblebees, leading to decreased gut microbiota diversity (Helander et al. 2023). Glyphosate exposure has been linked to changes in the development of the nervous system, neurotransmission systems, and oxidative stress in animals (Costas-Ferreira, Duran, and Faro 2022).

Tebuconazole exposure has also been shown to alter the gut microbiota and has been shown to induce colitis in mice via regulation of the gut microbiota. Specifically, exposure to tebuconazole can cause structural damage and inflammatory cell infiltration in colon tissue, activate the expression of inflammation-related genes, disrupt the expression of barrier function-related genes, and induce colonic inflammation in mice (Meng et al. 2022). Similarly, exposure to tebuconazole can also exacerbate DSS-induced colitis in mice. The effects of pyrethroids, piperonyl butoxide, cyprodinil and fludioxonil on animal gut health has not been extensively studied previously although some studies suggest that pyrethroids may have negative impacts on the animal gut microbiota (Giambo et al. 2021; Wu et al. 2022).

Our results show that piperonyl butoxide, cyprodinil and fludioxonil all decreased cell viability resulting in less ATP production. Decreases in viability of the organoids were only visible after 5 days of exposure to specific PPS at high concentrations (>10 x ADI). Using the 2D monolayer approach described here it is not possible to assess if lower concentrations would have harmful effects after longer exposure periods. Effects on other organs and immune system remain to be tested in other models. Biotransformation of exogenous compounds can occur in intestinal cells but the liver is the major site of endogenous and exogenous compounds. Biotransformation might activate exogenous compounds to a more toxic metabolite. *In vivo*, the microbiota may also play a role in biotransformation of PPPs, something which is not included in this study. In this experiment the organoids were only exposed to the original compound and not to metabolized components.

In the grant agreement it was proposed to select one mixture for each CSS (i.e., a maximum of 11 mixtures, containing a maximum of 5 PPPs). Selecting a separate mixture for each CSS was not feasible as the compounds that were measured most frequently in feces, urine and blood did not differ sufficiently between CSS to select CSS specific mixtures. Therefore, the selected mixtures do not directly reflect the risk to subjects at each CSS. A technical challenge with combining mixtures of compounds concerns their low solubility and limited volume of the solvent that can be added. This meant that some mixtures could not be combined at the highest desired concentrations.

For these reasons the PPP mixtures were designed according to the criteria described in the methods section. This allowed us to test 11 different mixtures, which lead to increased information on additive and combinatorial effects and gave more information about the roles of each individual compound in these mixtures at the cellular level. Our study does not provide an exhaustive list of all potentially relevant PPP mixtures but includes relevant and scientifically interesting mixtures. Two of the 11 mixtures contained more than 5 components.

These mixtures were included to reflect the physiological situation in which subjects are often exposed to more than 5 components. In addition, these mixtures help to identify possible synergistic interactions between their components to be further investigated in future studies.

Transcriptome effects of PPP's on the ileum organoids

Further evaluation of the genes associated with the Parkinson's and Alzheimer's disease pathway shows an important contribution of the ubiquitin and the mitochondrial pathway, which in these diseases is associated with the formation of Lewy bodies, alpha synuclein aggregation, mitochondrial dysfunction cell toxicity, and cell apoptosis. Studies have shown a link between exposure to certain pesticides that are now prohibited in many countries and an elevated risk of developing Parkinson's disease (PD). For example, rotenone, an isoflavone broad-spectrum insecticide, has been linked to PD (Tanner et al. 2011). The compound inhibits the electron transport chain by blocking transfer of electrons in mitochondrial complex I to ubiquinone (Hay 1992). Our results also signify an important contribution of genes associated with oxidative phosphorylation in mitochondria affecting genes for cytochrome oxidases, complex I, NADH dehydrogenase and F-type ATPase. This warrants future investigation especially the contribution of piperonyl butoxide. This could be investigated using assays for oxygen consumption rates or ATP homeostasis. These findings would also explain why the decline in ATP production in the cytotoxicity assays.

Further evaluation of the genes downregulated by PPPs suggest a negative effect on barrier integrity through adherens and tight junction (TJ) functioning. Specifically, the TJs regulate the paracellular movement of various substances, including ions, solutes, and water across the intestinal epithelium, and provide a physical barrier to luminal inflammatory molecules. The AJs, on the other hand, establish the epithelial cell barrier and regulate the paracellular passage of ions, water, and macromolecules. Together, the TJs and AJs help to prevent the intrusion of extracellular substances such as microorganisms, antigens, and xenobiotics into the body.

Dysfunction of the TJs and AJs can lead to various diseases, including metabolic and inflammatory diseases and further research is needed to fully understand the effects of PPPs on intestinal TJ and AJ function. Loss of barrier functioning could increase luminal outflow of pesticides and other xenobiotic compounds into host circulation. This could then induce systemic effects in several parts of the body, for example low-grade pesticide exposure could decrease cell bioenergetics of the substantia nigra, a key component of the ventral midbrain and its dopaminergic neurons. It could therefore be interesting to further study the direct effects of pesticide compounds on the passage of the blood-brain barrier (BBB) and human ventral midbrain organoids *in vitro* (Sozzi et al. 2022).

Organoids as model for toxicity testing: strengths and limitations

The use of laboratory animals in toxicity testing can be further reduced by employing other strategies such as organoid models. Organoids models more closely mimic the physiology of its derived tissue than cancer or transformed cell lines. Intestinal organoids are suitable to assess the effects of toxins on cell viability and cell growth, gut permeability, and differential gene expression in the gut. The method is easy to upscale for larger screening studies with multiple compounds in multiple concentrations. The method is non-invasive and poses less ethical challenges compared to *in vivo* animal studies. However, long-term exposure is not feasible in the organoid model used here, as the 2D monolayers do not remain stable for more than 7 days. 3D organoid models remain stable for at least multiple weeks to months, depending on the tissue, but are not yet suitable for long-term (>1 year) exposure assays. Therefore, organoid models are less suited to assess carcinogenicity compared to *in vivo* models. The ongoing efforts to develop (multi-)-organ-on-a-chip models may ultimately allow the interactions between intestinal epithelium and microbes or other organs to be studied, for instance models that could mimic the gut-brain axis. This could be important to assess the (in)direct effects of PPPs on the intestinal epithelium, gut hormone signaling, and nerve signaling. Moreover, intestinal models

which include immune cells and other organoid types have already been described in the literature (Skardal, Shupe, and Atala 2016). This could be useful to study effect of PPPs on allergic or inflammatory reactions. Further development of such models is anticipated to open up opportunities for future toxicology studies and further reduction of animal studies.

5. Tier 3: In vivo testing

1. Introduction

The issue of using glyphosate-based pesticides is repeatedly discussed, not the least in periods when glyphosate is undergoing periodic re-evaluation of registration in the EU. Glyphosate is the active substance in commercial pesticides e.g. Roundup. Among its applications, glyphosate-based herbicides are used on genetically modified glyphosate-resistant (GR) crops like Roundup Ready soya beans for weed control, for general weed control especially in conservation agriculture, and for pre-harvest desiccation of conventional crops like soybeans and cereals. This application leaves residues in feed for livestock, and we therefore investigated the transfer of diet-related glyphosate to blood and eggs of laying hens (Carlsvig et al., 2022). When the diet contained 0.03 mg/kg glyphosate, the concentration was 0.29 µg/L in blood serum, 0.29 ng/g in egg yolk and 0.01 ng/g in egg white, and when the diets were amended with glyphosate to levels of 18.5 and 192 mg/kg, the concentration in blood serum was 25.0 and 129 µg/L, in egg yolk it was 21.4 and 223 ng/g and in egg white it was 0.25 and 3.09 ng/g (Carlsvig et al., 2022).

It has been shown that glyphosate incorporated in the egg may affect the embryo. Ruuskanen et al. (2020) indicated an adverse effect of the glyphosate-based herbicide Roundup on embryo development in birds, and Estienne et al. (2022) found that a hen layer diet containing 1250 mg/kg of glyphosate caused hatchability of fertilized eggs to be reduced to 9% after three weeks of exposure as compared to 98% for the control diet containing 0.21 mg/kg glyphosate. In a survey based on data from a commercial hatchery (DanHatch Denmark A/S), we found a negative association between residues of glyphosate in the diet for broiler breeders and egg hatchability (Foldager et al., 2021).

A dietary glyphosate level of 1250 mg/kg as used by Estienne et al. (2022) is substantially exceeding the levels of glyphosate residues in feed crop found in Danish surveys, e.g. soybean meal (up to 5.1 mg/kg; Fødevarestyrelsen, 2017) and wheat grain (up to 4.1 mg/kg; Miljøstyrelsen, 2014) as well as the EU-defined maximum residue levels (MRL) for common feed crops such as wheat grain has a MRL of 10 mg/kg and soyabeans that has a MRL of 20 mg/kg (EFSA, 2019). The glyphosate levels used by Carlsvig et al. (2022) are also exceeding or at the high end of MRL.

With this background, we studied whether hen diet levels of glyphosate, covering the range that may occur under real-life circumstances, can affect egg hatchability and chicken health.

1.1 Accordance with Grant Agreement:

The reported experiment is adjusted relative to the experiment described in the Grant Agreement following discussion among the AU Principal Investigator, the WP4 Leader and other WP4 members, the SPRINT management, including the Overall Project Coordinator and the Scientific Coordinator. Originally, the top 3 pesticides were planned to be included, each at one diet concentration. Instead, it was decided to focus on glyphosate (top 1) at three diet concentrations covering the concentrations that reflect real life circumstances. Furthermore, instead of a chicken growth-health experiment, it was decided to extend the experiment to a poultry reproduction-health experiment, i.e. effects of glyphosate diet to parent hens on egg hatchability and chicken health. Instead of the originally planned 340 chickens, the adjusted experiment included 128 parent hens and 680 chickens. The adjusted experiment was conducted within the original budged despite the considerably increased number of experimental animals.

2. Material and methods

2.1. Animals and housing

Laying hens. A total 128 Ross 308 laying hens of age 25 weeks at start of the dietary glyphosate exposure were used. The hens were housed in a room divided into pens. Each pen (100 × 165 cm) had sawdust bedding and was equipped with a nest and a perch. Flocks of four hens were allocated to pens that were assigned to one of four treatments (see below). Room temperature was 21 °C and duration of lights-on was 13 h per day.

Chickens. Room, pens and bedding were as described for the laying hens. Room temperature decreased gradually from 33.5 °C at day one to 31.5 °C at day seven after hatching. Lights-on duration decreased from 24 h at day one to 18 h per day at day seven.

2.2. Diets and treatments

Each pen was assigned to one of four dietary treatments, i.e. a control (Con) diet that was intended to be free of glyphosate residues by using organic ingredients and three diets where the control diet was supplemented with glyphosate (glyphosate isopropylamine, IPA) salt, CAS no. 38641-94-0, catalogue no. QV-4224 Combi-Blocks Inc.) at 1.5 ($Gly_{1.5}$), 4.5 ($Gly_{4.5}$) or 13.5 mg/kg ($Gly_{13.5}$). A content of 13.5 mg glyphosate per kg is in between the above-mentioned EU-defined maximum residue limit MRL of different common feed crops. The composition of the control diet is shown in Table 5.1. The laying hens commenced the dietary treatments at 25 weeks of age and were fed restrictedly with 130 g per hen per day and gradually increasing to 162 g at 32 weeks of age at termination of egg collection for incubation. In addition, approximately 10 g of corn silage were provided per hen per day to reduce the risk of feather picking and cannibalism. The chickens were fed ad libitum a commercial conventional starter diet (Ruge Start 1, DLG Group) based on soybean meal, wheat, oat, sunflower meal, soya oil,

minerals and vitamins (percent composition was not given). All animals had free access to drinking water. Diet samples were collected and stored at -20 °C until analyses for glyphosate.

Table 5.1 *Basal diet used for the hens during the experiment*

Ingredient	**%**	**Nutrient**	
Wheat, organic	43.83	Energy, MJ/kg	10,800
Oat, organic	21.98	Energy incl. enzyme effects, MJ/kg	11,235
Soya beans, organic, toasted	18.21	Crude ash, %	9.48
Calcium carbonate	6.97	Crude fat, %	6.54
Sunflower cake, organic	5.00	Crude protein, %	15.75
Fish meal	2.07	Crude fibre, %	6.08
Monocalcium phosphate	0.95	Lysine, g/kg	7.66
Vitamin mix	0.40	Methionine, g/kg	5.03
DL-methionine	0.25	Cysteine, g/kg	2.87
Sodium chloride	0.18	Threonine, g/kg	5.57
Sodium bicarbonate	0.10	Tryptophan, g/kg	2.02
Choline chloride, 50%	0.05	Calcium, g/kg	31.00
Enzyme mix	0.01	Phosphorus, g/kg	5.99

2.3. Protocol

Originally one round of data collection was planned, however, due to lower than anticipated egg fertilization rate, two rounds were carried out.

Laying hens and eggs for incubation. In Round 1, hens were exposed to the experimental diets for 11 to 20 days when collecting eggs for incubation and in Round 2, hens were exposed to the experimental diets for 8 to 16 days when collecting eggs for incubation. There was a period of 7 days between Round 1 and 2 where the Control-diet was used for all hens in order to have enough of the other glyphosate-amended diets for Round 2. In both Rounds, hens were inseminated with a mix of newly harvested semen from approximately 20 White Leghorn roosters two times, i.e. three days before collecting the first eggs for incubation and again five days later. Inseminations were completed approximately 45 minutes after harvesting the semen. In Round 1, all regular eggs of minimum 46 g were used and in Round 2, all regular eggs of minimum 50 g were used for incubation. These minimum egg weight cut-offs according to hen age are similar to those applied by the DanHatch Denmark Ltd. hatchery. Blood (2 ml) was collected from the jugular vein into Vacuette® tubes coated with 18 IU/ml Sodium Heparin (Greiner Bio-One) of one hen per pen in Round 1 after 23 days of feeding the treatment diets. Fresh whole blood was used for flow cytometry where after plasma was harvested and stored at -20 °C until analyses. Incubated eggs were candled at day 7 and 14 of incubation to monitor fertilization, and unfertilized eggs were discharged. On day 22 after start of incubation, hatched eggs were monitored.

Chickens. One-day old chickens (day 22 after start of incubation) were weighed individually and scored for quality traits according to Tona et al. (2003) except that we used 0, 1 and 2 for scores and reduced the number of scoring levels from three to two for three quality traits: down, (clean and dry=1 vs wet and/or dirty=0), remaining membrane (yes=1 vs no=0) and remaining yolk (no=1 vs yes=0); for further scoring details, see Tona et al. (2003). There were two scoring

teams. Blood was collected from the jugular vein into tubes containing 20 IU/ml Heparin "LEO" (LEO Pharma) of two chickens per pen from either Round 1 or 2 and these animals were then sacrificed. Fresh whole blood was used for flow cytometry where after plasma was harvested and stored at -20 °C until analyses. In Round 1 after collection of eggs for incubation, eggs were collected for another two days to hatch chickens for the blood sampling. In Round 2, blood was sampled in order to attain a sample from two chickens per pen. Chickens not used for blood sampling were allocated to a room with pens as described in section 2.1 and had ad libitum access to a commercial conventional starter diet and drinking water. The chickens were weighed by pen at 8 days of age and then sacrificed.

2.4. Sample analyses

Glyphosate and AMPA analyses

Glyphosate and glyphosate degradation product aminomethylphosphonic acid (AMPA) in plasma and feed were analysed according to the microLC-MS/MS method described and validated by Nørskov et al. (2019). The microLC-MS/MS method for egg yolk, is described in Carlsvig et al. (2022); LoQ were 0.1 ng/g for glyphosate and 1.0 ng/g for AMPA.

Flow cytometric analyses

All flow cytometric analyses were conducted on a BD FACSCelesta™ flow cytometer with a flow rate of 0.5-1 µL per second and analyses of acquired samples were performed in the FACS Diva software.

a) Whole blood phagocytosis

Assessment of phagocytic activity in whole blood was performed using a no-lyse-no-wash flow cytometry-based method (Naghizadeh et al., 2019) with a few modifications. Briefly, heparinized blood were diluted 100-fold in RPMI-1640 (containing 25 mM HEPES and 300 mg/L l-Glutamine; Lonza) and 50 µL were added per well in round bottom 96-well cell culture plates (Thermo Fisher Scientific) and mixed with CD45-PerCP-Cy5.5 antibody (clone UM16-6, BioRad). Conjugation of anti-CD45 with PerCPCy5.5 was beforehand performed using Lightning-Link® (Innova Bioscience) according to the manufacturer's instructions. Subsequently, samples were incubated for 60 min at 41 °C with 2.5 µL of FITC-Salmonella serotype Infantis (S. 123445). The bacteria were kindly donated by John Elmerdahl Olsen, Copenhagen University, Denmark. After incubation, the samples were placed on ice and fixed with paraformaldehyde (Thermo Fisher Scientific) at a final concentration of 1%. Eighty microliters of each sample were acquired in the flow cytometer.

b) Whole blood leucocyte counts

Enumeration of different cell subsets in peripheral blood was determined using a no-lyse-no-wash flow cytometric method as previously described (Kjærup et al., 2016; Seliger et al., 2012) with a few modifications. Fifty microliters of heparin-stabilized blood were diluted 100 times with RPMI-1640 (containing 25 mM HEPES and 300 mg/L l-Glutamine; Lonza). Fifty microliters of this solution were then mixed with 50 µL of antibody master mix in 96-well U-bottom plates. The antibodies used were: CD41/61-FITC (clone 11C3, BioRad); TCR1-PE (clone TCR-1, Southern Biotech); Kul-01-A647 (clone KUL01, Southern Biotech); CD45-PerCPCy5.5 (clone UM-16, BioRad); CD3-A700 (clone CT3, Southern Biotech). Optimal antibody concentrations were determined beforehand and FMO controls included. Staining was done for 20 minutes in darkness at 4 °C and immediately before acquisition 123count eBeads™

Counting Beads (Thermofisher), diluted 1:10 in FACS buffer and 4 mM EDTA were added. Forty microliters of each sample were acquired in the flow cytometer. Absolute number of cells was calculated according to 123count eBeads™ manufactures instructions.

2.5. Statistical analyses

Statistical analyses were carried out using the statistical software R 4.3.0 (R Core Team, 2023) and with a significance level of 0.05. Generally, the initial models contained diet treatment as a fixed effect.

Estimated marginal means (aka least-squares means) with standard errors of means (SE) were calculated by use of the *emmeans* package v. 1.8.5 with back-transformation to the original scale. Overall differences among diet treatment groups as well as the need for a precision model in beta regressions were tested by likelihood ratio tests using the *lrtest* function from the *lmtest* R package v. 0.9-40 (Zeileis and Hothorn, 2002). Pairwise comparisons between Controls (Con) and each of the GLY diet treatment groups were done with the approximation to the Dunnett correction for multiple testing implemented in the *emmeans* R package. The P-values in the 'P-value' column of tables 5.2-6 are for the overall test of differences among treatment groups. The P-values indicated in footnote number 3 of tables 5.5-6 are for the pairwise comparisons between Con and each of the GLY diet treatment groups. Pairwise comparisons were only performed when the overall test was significant ($P<0.05$).

2.6 Ethics approval

All experimental procedures involving animals were approved by the Danish Animal Experimentation Council [approval no. 2017-15-0201-01229].

3. Results

3.1. Egg hatchability, and chicken viability and growth

Results concerning egg hatchability, and chicken viability and growth are shown in Tables 5.2-4. In total, 740 eggs were fertilized (35.7% of 2074 eggs) and from these 683 chicken hatched (92.3%); there were no significant effects of diet glyphosate on fertilization or hatchability (Table 5.2).

Table 5.2 *Fertility. Proportion of live chicken out of the fertilized eggs. Proportion of fertilized eggs out of hatch eggs (i.e. eggs put into the incubator). Hens were fed basal diets amended glyphosate as indicated (treatment). Results are shown as EM-means (proportions) with SE in subscripted parentheses. Results were back-transformed by the inverse link function and by the Delta-method from mixed effects logistic regressions diet treatment as the effect of interest and adjusted for Round (1 and 2), and with pen within batch as random effect.*

	Glyphosate treatment[1]				
	Con	$Gly_{1.5}$	$Gly_{4.5}$	$Gly_{13.5}$	P-value
Proportion live chicken out of fertilized eggs	0.95 (0.018)	0.93 (0.018)	0.91 (0.021)	0.90 (0.025)	0.42
Proportion fertilized eggs out of hatch eggs	0.26 (0.043)	0.39 (0.051)	0.36 (0.050)	0.34 (0.049)	0.30

[1] Glyphosate amended per kg diet feed; Con: 0 mg, $Gly_{1.5}$: 1.5 mg, $Gly_{4.5}$: 4.5 mg, $Gly_{13.5}$: 13.5 mg.

Among the fertilized eggs, 27 were dead at day 7 of incubation (7 Con, 6 $Gly_{1.5}$, 4 $Gly_{4.5}$ and 10 $Gly_{13.5}$), 27 did not hatch (0 Con, 8 $Gly_{1.5}$, 13 $Gly_{4.5}$ and 6 $Gly_{13.5}$) and 3 only hatched partly (1 Con and 2 $Gly_{13.5}$). Four chickens died from day 1 to 8, one from each of the four diet treatment groups.

One chicken died ($Gly_{1.5}$) before scoring and two were weak and killed right after scoring (Con and $Gly_{4.5}$). The first of these three was excluded from all analyses except those concerning mortality and fertility. Most of the quality traits being scored were too rare to be analysed statistically and are therefore described by counts. None of the chickens were assigned an eyes score of 0, two had only 1 clear eye (both from $Gly_{1.5}$) and the remaining 680 chickens had 2 clear and open eyes. Six chickens were found to have non-absorbed yolk (big, hard belly); none from the $Gly_{1.5}$ group and two from each of the other three groups. Three chickens had remaining membrane; none from the Con group and one from each of the other diet treatment groups. All chickens had two good legs. Remaining yolk was observed in only one chicken. When placed on their back, most chickens turned within 2 s (activity score) and only 35 were inactive for more than 2 s. These chickens were quite evenly distributed among diet treatment groups with 4%, 5%, 7% and 4% in Con, $Gly_{1.5}$, $Gly_{4.5}$ and $Gly_{13.5}$, respectively. A mixed effects logistic regression including treatment as fixed effect and scoring team within round as random effect revealed no differences among the diet treatment groups ($p = 0.49$).

Results from two traits with sufficient frequencies to allow statistical analyses are shown in Table 5.3 but the treatment means within trait were not significantly affected by diet with glyphosate.

Table 5.3 *Chicken quality scoring. The chickens were scored on day 1 after hatching. Hens were fed basal diets amended glyphosate as indicated (treatment). Results are shown as EM-means (estimated proportions) with SE in subscripted parentheses as back-transformed by the inverse link function from mixed effects logistic regressions diet treatment as the effect of interest and adjusted for Round (1 and 2), and with pen within round as random effect.*

	Glyphosate treatment[1]				
	Con	$Gly_{1.5}$	$Gly_{4.5}$	$Gly_{13.5}$	P-value
Navel score (0 or 1) vs 2	0.09 $_{(0.028)}$	0.17 $_{(0.039)}$	0.18 $_{(0.041)}$	0.19 $_{(0.043)}$	0.16
Wet/dirty down	0.05 $_{(0.027)}$	0.02 $_{(0.011)}$	0.03 $_{(0.017)}$	0.03 $_{(0.015)}$	0.38

[1] Glyphosate amended per kg diet feed; Con: 0 mg, $Gly_{1.5}$: 1.5 mg, $Gly_{4.5}$: 4.5 mg, $Gly_{13.5}$: 13.5 mg.

To enable parametric analysis, the two lowest navel scores (0=not closed and discolored and 1=not completely closed and not discolored) were combined and analysed by a mixed effects logistic regression with treatment and round as fixed effects, and scoring team within round and pen within team and batch as random effects. The Con group appeared to have more chickens with score 2 (=completely closed and clean) than the three GLY groups (see Table 5.3). The three GLY groups were not significantly different ($p = 0.95$) and after post hoc combining these, the odds of having a score of 0 or 1 was then significantly higher in the combined GLY group than in the Con group ($p = 0.024$), as indicated by an odds ratio of 2.2 (95% confidence interval: 1.1 - 4.5). In total, 50 chickens were found to have wet/dirty down but with a larger proportion (11%) in the Con group than in the three GLY groups (5%, 8% and 6%, respectively). A mixed effects logistic regression as described for the navel score was run but no significant differences among diet treatment groups were found (see Table 5.3).

Results concerning body weight on day 1, day 8 and the average daily gain between these days (ADG) are shown in Table 5.4. In Round 2, 31 chickens were used for blood sampling after scoring and thus not followed for viability and growth from day 1 to 8. Moreover, the weight at day 8 was omitted for one chicken that stopped growing when its only companion chicken died. Thus, 644 were available for weighing on day 8 and thus for analysis of ADG, calculated for each pen as the difference between the average weights on day 8 and day 1 divided by 7.

Table 5.4 *Weight and growth. Average chicken bodyweight day 1, day 8 and average daily gain from day 1 to 8. Hens were fed basal diets amended glyphosate as indicated (treatment) and bodyweights day 1 were averaged within hen-pen before analysis. Bodyweight day 8 were average weight per chicken within pen. Results are shown as EM-means (SE) from linear normal models with diet treatment as the effect of interest and adjusted for round (1 and 2). The model of bodyweight on day 1 furthermore included heterogeneous treatment-dependent variances.*

	Glyphosate treatment[1]				
	Con	$Gly_{1.5}$	$Gly_{4.5}$	$Gly_{13.5}$	P-value
Bodyweight day 1, g	38.3 (0.63)	39.1 (0.37)	38.7 (0.47)	38.0 (0.29)	0.10
Bodyweight day 8, g	87.7 (1.63)	88.4 (1.63)	90.9 (1.63)	89.1 (1.58)	0.31
ADG day 1 to 8, g/d	7.06 (0.204)	7.03 (0.204)	7.49 (0.204)	7.31 (0.198)	0.33

[1] Glyphosate amended per kg diet feed; Con: 0 mg, $Gly_{1.5}$: 1.5 mg, $Gly_{4.5}$: 4.5 mg, $Gly_{13.5}$: 13.5 mg.

3.2. Blood leucocytes

For the analyses of hens, no random effects will be relevant as there is only one hen measured from each pen and they were all taken after Round 1 was finished. Thus, the only available

explanatory variable was diet treatment – either as a categorical factor or by a continuous regression against the dose.

Whole blood phagocytosis percentages were changed to proportions (divided by 100%) and analysed by a beta regression using the *betareg* function from the *betareg* R package v. 3.1-4 (Cribari-Neto and Zeileis, 2010). Capacity for bacteria uptake was highly elevated for one of the $Gly_{1.5}$ hens, especially in the monocyte subset (almost 73% larger than the second largest from the diet group) but also in the lymphocyte subset (48% larger than second largest). It is not unlikely that the biologically variation is this large and that the distributions would be fine in a larger sample, but we excluded this hen for the analyses of monocyte and lymphocyte to keep a reasonable distributional fit. The variance was allowed to vary with diet treatment via the precision model for heterophils and lymphocytes, whereas for monocyte phagocytosis this was not needed. Results are shown in Table 5.5. Whole blood leucocyte counts per µL and heterophil- lymphocyte ratio was analysed by Gamma regressions, again with diet treatment as the sole explanatory variable. Results are shown in Table 5.5.

Table 5.5 *Hen whole blood phagocytosis proportions and leucocyte counts per µL. Hens were fed basal diets amended glyphosate as indicated (treatment[1]) and the blood samples were taken from one hen per pen (in total 8 hens per treatment[2]). Results are shown as back-transformed EM-means (SE) from beta (phagocytosis proportion) and Gamma (leucocyte count per µL) regressions with diet treatment as the only explanatory variable. The models for heterophil and lymphocyte phagocytosis proportion included a precession model, allowing the variance to vary among treatment groups. Pairwise comparisons between Con and each of the GLY diet treatment groups were done with (an approximation to) the Dunnett correction for multiple testing[3].*

	Glyphosate treatment[1]				
	Con	$Gly_{1.5}$	$Gly_{4.5}$	$Gly_{13.5}$	P-value
Heterophil, phagocytosis %	18.0 (0.53)	21.7^{*} (1.34)	20.2^{**} (0.55)	$20.7^{\#}$ (1.17)	0.017
Monocyte[2], phagocytosis %	22.4 (1.30)	19.3 (1.31)	23.4 (1.32)	22.7 (1.30)	0.18
Lymphocyte[2], phagocytosis %	15.5 (0.50)	16.5 (0.29)	16.7 (0.73)	16.7 (0.29)	0.30
Thrombocyte, count per µL	17430 (2635)	24800 (3748)	18520 (2799)	19480 (2945)	0.33
γδT-cell, count per µL	1910 (187)	2170 (213)	2140 (210)	2000 (196)	0.74
B-cell, count per µL	750 (105)	870^{ns} (121)	1090^{ns} (152)	1540^{**} (214)	0.005
Heterophil, count per µL	4780 (394)	4720 (389)	5340 (440)	5640 (465)	0.31
Monocyte, count per µL	1570 (183)	1640 (190)	2060 (240)	2260 (262)	0.075
T-cell, count per µL	3810 (454)	3760 (447)	4210 (501)	4050 (482)	0.88
Heterophil-lymphocyte ratio	0.76 (0.084)	0.73 (0.080)	0.73 (0.080)	0.81 (0.089)	0.86

[1] Glyphosate amended per kg diet feed; Con: 0 mg, $Gly_{1.5}$: 1.5 mg, $Gly_{4.5}$: 4.5 mg, $Gly_{13.5}$: 13.5 mg.

[2] One $Gly_{1.5}$ hen was left out in analyses of monocyte and lymphocyte phagocytosis proportion.

[3] * $p_{adj} < 0.05$; ** $p_{adj} < 0.01$; *** $p_{adj} < 0.001$; $^{\#}$ $p_{adj} < 0.10$; ^{ns} $p_{adj} \geq 0.10$; p_{adj}: Dunnett correction.

For the analyses of chickens, random effect of pen could in principle be included for some of the analyses while for other analyses this lead to convergence problems or random effects close to zero. From each pen, blood was sampled from zero (1 pen), one (1 pen), two (28 pens) or three chickens (2 pens), but for 10 pens with one or two from each of Round 1 and 2. Moreover, the hatching eggs were from 4 hens per pen inseminated with a mix semen from 20 roosters. Thus, all-in-all, correlations among 2-3 chickens from the same pen may be modest and it was therefore decided to use the same models as those used for the hens. In addition to the categorical treatment diet variable, Round was included as a categorical variable to adjust for (in some cases quite large) differences between the two rounds. The effect of Round was generally significant (results not shown) but also kept in the models even when not statistically significant. Interaction between Round and treatment group was tested but were not significant with exception of the model for B-cell (results not shown).

For the analysis of phagocytosis percentages in chickens, also a few values had to be excluded to keep a reasonable fit for the beta distributions. Heterophil phagocytic capacity was relatively large for two of the $Gly_{4.5}$ chickens (from the same room but different rounds), being 118% and 56% larger than the second largest from the same diet group and round. The latter of these $Gly_{4.5}$ chickens were also extreme for monocyte (74% larger) and lymphocyte (71% larger). One $Gly_{1.5}$ chicken had relatively large values for all three phagocytosis measures compared to the second largest measure from same round and diet treatment group: 42% larger for heterophil, 144% for monocyte and 65% larger for lymphocyte. Moreover, not really disturbing the distribution though, one Con chicken had large heterophil value (47% larger). In addition, for monocyte, one Con and one $Gly_{4.5}$ had zero value, which is outside the support for the implemented beta model. When changing these two zeroes to a low value, say 1.5%, they appeared being outliers disturbing the distributions as much as one $Gly_{4.5}$ having a value of only 1.6%. The lowest monocyte phagocytosis percentages were otherwise between 5% and 7%, and

it was therefore decided to also exclude these three monocyte values being below 2%. These exclusions did not change any of the conclusions that would have been made, had they been included, but means and standard errors would of course be slightly different. The variance was allowed to vary with diet treatment via the precision model of the *betareg* function for heterophil and lymphocyte phagocytosis proportion. Results are shown in Table 5.6.

Whole blood leucocyte counts per µL and heterophil- lymphocyte ratio in chickens were analysed by Gamma regressions, again with diet treatment as the effect of interest and with adjustment for Round. No exclusions were made for these analyses. Results are shown in Table 5.6.

Table 5.6 *Chicken whole blood phagocytosis proportions and leucocyte counts per µL. Hens were fed basal diets amended glyphosate as indicated (treatment[1]) and the blood samples were taken from a total of 15, 16, 15 and 17 chickens[2] (34 and 29 in Round 1 and 2). Results are shown as back-transformed EM-means (SE) from beta (phagocytosis percentages) and Gamma (leucocyte counts per µL) regressions with diet treatment as the effect of interest and adjusted for Round (1 and 2). The models for heterophil and lymphocyte phagocytosis proportion included a precession model, allowing the variance to vary among treatment groups. When overall test of differences among treatments (P-value) was significant, pairwise comparisons between Con and each of the GLY groups were done with (an approximation to) the Dunnett correction for multiple testing[3].*

	Glyphosate treatment[1]				
	Con	$Gly_{1.5}$	$Gly_{4.5}$	$Gly_{13.5}$	P-value
Heterophil[2], phagocytosis %	16.2 (1.23)	15.8 (0.64)	16.2 (0.81)	16.8 (0.91)	0.88
Monocyte[2], phagocytosis %	13.3 (1.10)	13.7 (1.09)	11.3 (1.08)	15.2 (1.02)	0.091
Lymphocyte[2], phagocytosis %	14.8 (0.73)	13.0 (0.35)	14.0 (0.56)	14.0 (0.32)	0.079

Thrombocyte, count per µL	14340 (2130)	21950 (3139)	11900 (1744)	19860 (2746)	0.054
γδT-cell, count per µL	251 (22.5)	353* (30.4)	266^{ns} (23.5)	328$^{\#}$ (27.4)	0.014
B-cell, count per µL	127 (16.4)	168^{ns} (20.9)	100^{ns} (12.8)	169^{ns} (20.4)	0.009
Heterophil, count per µL	2470 (275)	3790* (405)	2610^{ns} (286)	3750* (388)	0.005
Monocyte, count per µL	286 (28.6)	383$^{\#}$ (36.9)	230^{ns} (22.7)	341^{ns} (31.7)	0.002
T-cell, count per µL	728 (81.0)	697 (74.7)	819 (90.0)	781 (80.9)	0.70
Heterophil-lymphocyte ratio	2.47 (0.310)	3.27 (0.396)	2.46 (0.305)	3.06 (0.358)	0.21

[1] Glyphosate amended per kg diet feed; Con: 0 mg, $Gly_{1.5}$: 1.5 mg, $Gly_{4.5}$: 4.5 mg, $Gly_{13.5}$: 13.5 mg.
[2] Exclusions: 1 Con, 1 $Gly_{1.5}$ and 2 $Gly_{4.5}$ for Heterophil; 1 Con, 1 $Gly_{1.5}$ and 3 $Gly_{4.5}$ for monocyte; 1 $Gly_{1.5}$ and 1 $Gly_{4.5}$ for lymphocyte phagocytosis proportion
[3] * $p_{adj} < 0.05$; ** $p_{adj} < 0.01$; *** $p_{adj} < 0.001$; $^{\#}$ $p_{adj} < 0.10$; ^{ns} $p_{adj} \geq 0.10$; p_{adj}: Dunnett correction.

4. Discussion

Glyphosate is available in its pure form and in commercially available glyphosate-based herbicides (GBH) (e.g. Roundup). We decided not to use a GBH as the source of glyphosate in order to exclude potential confounding effects of any surfactants that may be present in a GBH. Glyphosate exists as an acid or with different salts. We decided to use a salt since salts are commonly used in GBHs. Moreover, we found that generally the glyphosate IPA-salt was more toxic to in vitro cultures of various bacteria than the glyphosate acid (Katholm, 2016).

Diet glyphosate is deposited in eggs and was shown to negatively affect bird embryos or egg hatchability (Foldager et al. 2021, Ruuskanen et al. 2020, Estienne et al. 2022). We found however no effects of diet glyphosate on the hatchability of fertilized eggs. Nevertheless, these results may not be contradictory. The results of Foldager et al. (2021) were from a survey and the range of diet glyphosate residues was narrow (0.004 to 0.19 mg/kg), which calls for cautious interpretation of the results and in line with this, the authors conclude that an experiment with

diet glyphosate over a range covering the conditions in commercial settings is warranted. Ruuskanen et al. (2020) found that a dietary glyphosate concentration of 200 mg/kg gave tendencies ($p=0.09$) of lower percentage of normal development of embryos in a quail model, and Estienne et al. (2022) found that a diet glyphosate concentration of 1250 mg/kg increased embryo death during incubation and that hatchability of fertilized eggs was decimated; Foldager et al. and Estienne et al. as well as the present study used a Ross 308 animal model. Thus, overall it seems that tendencies of negative effects on bird embryos/egg hatchability appear at around 200 mg glyphosate/kg diet and that extensive effects are established at 1250 mg/kg diet, while effects of levels that may be found in diets composed of common feed ingredients could not be confirmed. The hens in the present study had been exposed to the dietary treatments for 8 to 22 days when collecting eggs for incubation, thus at or above the 8-day exposure time needed to reach maximum glyphosate deposition in eggs (Estienne et al., 2022). Scoring of one-day old chickens for vitality and other quality traits did not reveal indications that the hatched chickens were negatively affected by being exposed the egg content of glyphosate except that a post hoc analysis indicated that the navel score was best for chickens from the Control (Con) group compared to chickens from the Gly groups when the two lowest navel score levels were combined. Hens fed a diet containing even a rather low concentration of glyphosate (0.03 mg/kg) will lay eggs containing glyphosate (0.26 ng/g yolk)(Carlsvig et al., 2022), however diet concentration up to 13.5 mg/kg seems not to affect chicken early life since there were no short-term effects on day-one old chicken weight or on growth during an eight-day period following hatching.

The number of studies on the effect of glyphosate on immunological parameters in both mammals and non-mammals, mostly fish, are few and inconsistent, and studies with poultry are even more scarce. Phagocytosis may be reflective of the immune competence of the individual as a first line of host defence (Sun et al. 2008). In our study, a no-lyse-no-wash,

whole blood assay was used to assess the phagocytic activity of different leukocyte populations *in vitro*. In the parent hens, glyphosate was observed to have an immunostimulatory effect on phagocytic ability of the heterophils, while no effect on phagocytic ability was observed for the monocyte and lymphocyte populations. The increase in phagocytotic activity is in contrast to findings in fish where a reduction in phagocytic activity was observed (Kreutz et al, 2011; Terech-Majewska et al, 2004). No effect was observed for phagocytosis in any of the leukocyte populations in the chickens. The lower phagocytic activity for heterophils in the chickens, in contrast to the hens, may be due to phagocytosis capacity being underdeveloped in chickens until the age of 7 days after hatching (Wells et al, 1998), while the samples in our study was taken on one-day old chickens.

The effect of glyphosate on the immunological profile in blood was furthermore determined by leukocyte subset counts. In the hens, the B-cell count was higher for hens in the $Gly_{13.5}$ group compared to Con, with a significant dose-dependency. For monocytes in hens, there was a similar dose-dependency. In chickens, there were differences (or tendency in one case) between Con and two of the Gly groups ($Gly_{1.5}$ and $Gly_{13.5}$) for count of heterophils and γδT-cells, but no tendency of dose dependency; the $Gly_{4.5}$ group seems to deviate from the other two Gly groups for which we have no explanation. Both decreased and increased numbers of immune cells have been shown in other studies (reviewed by Maddalon et al; 2021; Marino et al, 2021; Peillex & Pelletier, 2020). The most numerous granulocytes in chicken blood are the heterophils, and they are considered to be the avian counterpart of neutrophils in mammals. The increased number of heterophils in chickens from the $Gly_{1.5}$ and $Gly_{13.5}$ groups compared to Con, may be a sign of an inflammatory response, since heterophils will increase in number and proliferate in response to infections and irregularities related to diet and stress (Gross & Siegel, 1983; Maxwell & Robertson, 1998). γδT-cells are a subpopulation of T-cells with high frequencies. Their role and function in an immune response in poultry has not yet been fully

elucidated, but they are able to perform a wide range of functions and considered important for the outcome of several infectious diseases (Berndt et al, 2006). The existing literature indicates that glyphosate exposure may cause an imbalance of the immune system (Maddalon et al; 2021; Marino et al, 2021; Peillex & Pelletier, 2020). The present study also indicate some immune imbalances due to diet glyphosate, including leucocyte subset counts in hens, however the results are not clear-cut.

5. Ross 308 chicken as model for toxicity testing: strengths and limitations

A literature search in Web of Science using "Ross 308" AND "chicken" (All fields) and "toxicity" OR "toxic" (All fields) as keywords gave 82 references. The intention of many of these references were to describe optimal conditions for rearing chickens, i.e. establishing at which concentration favourable feed substances (essential nutrients like minerals and amino acids) give optimum performance and health or at which concentration unfavourable grain feed substances (e.g. mycotoxins in grains) negatively affects performance and health. Unfavourable substances may also be present in bi-products from the industry (e.g. the biofuel industry) that have potential as feed items. Nevertheless, the broad range of substances that have been tested indicates that the chicken model may be applied more broadly than merely to optimize chicken performance in the poultry industry. In addition to the feed route, test substances can also be injected in ova.

Among the 82 references, there was none on carcinogenicity or other slowly developing illnesses. This is likely because farm animals seldom have a lifespan long enough to develop such illnesses.

The results of the current experiment suggest that this a sensitive and relevant farm animal model for different developmental and immunological outcomes. Further strengths of the Ross

308 chicken model included two routes of application: feed amendment and in ova injection. Limitation of the model mainly include the lack of data on studies available regarding chronic long-term illnesses (for example carcinogenicity). A conceivable limitation may be that chickens are not mammals and thus further apart from human in the phylogenetic tree than e.g. rodents.

6. Conclusion

Even though we observed no effects of diet glyphosate to parent hens in the range of up to 13.5 mg/kg on hatchability of fertilised eggs or on one day-old chicken quality, a post hoc analysis indicated that the navel score was best for chickens from the Con group compared to chickens from the combined Gly groups. Chicken growth until 8 days of age was not affected. Regarding immune competence and leukocyte subset counts in whole blood from parent hens and one-day-old chickens, the results indicated some immune imbalances due to diet glyphosate in both hens and chickens, although further studies are needed to clarify if the observed immune imbalances impact e.g. disease outcome in an experimental challenge model.

6. Synthesis and conclusions

The current report presents the results of the assessment of the effects of PPPs' mixtures on animal health (D4.4) related to Task 4.2 of the Horizon 2020 project Sustainable Plant Protection Transition; A global health approach (SPRINT). A tiered approach, has been used in the SPRINT project based on the EFSA guidelines for combined exposure to multiple chemicals using a component-based approach (EFSA, 2019). In Tier 1, based on the available data from the SPRINT CSS on over 200 PPPs and in silico modelling, the top 20 PPPs of concern were assessed and prioritized for in vitro testing based on occurrence and wHQ. In Tier 2, in vitro organoid models were used to test more than 10 PPPs and mixtures of concern, showing altered expression of pathways involving xenobiotic metabolism and cellular stress for all PPP exposures, along with alteration of tight junction and cell viability. Finally, in vivo testing in chicken models of the component of greatest concern (glyphosate) at real-life exposure condition indicated that the navel score was altered in glyphosate-exposed groups and that immune imbalances were observed in both hens and chickens exposed to glyphosate. The new models used (new SPRINT indicators), both in vitro (porcine organoids) and in vivo (Ross 308 chicken), proved to be sensitive and relevant for different toxicological endpoints of interest for risk assessment. Our tiered approach was successful and allowed to optimize the resources on the pesticides of greatest concern, since all the pesticides that have been tested in Tier 2 and 3 showed at least one positive signal. PPP mixtures also proved to be often more detrimental, however technical limits (such as solubility of the mixtures and number of compounds that can be tested at once) represent still important challenges for adequate modelling and dose-response.

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8. Appendix

APPENDIX 1

Considerations for modelling

Reference

It is preferable to not use vehicles, but if a vehicle should be used for testing, this will be an organic solvent (e.g. DMSO) used to prepare a clear solution as a stock. Therefore, if needed, as a first step, the substance would have to be dissolved in DMSO. The maximum allowable final concentration of this solvent in the test solution will depend on the assay characteristics but should be the same in all solutions tested reference and doses.

Controls

A negative control group should always be used (if a vehicle is used, the vehicle alone is the negative control). If appropriate, for a check on the sensitivity of the toxicity assay it is useful to include testing a substance with a known positive response in the essay. This can be the active ingredient of a PPP but the solubility of some of the substances may also be a limiting factor in the preparation of solutions, especially at high doses.

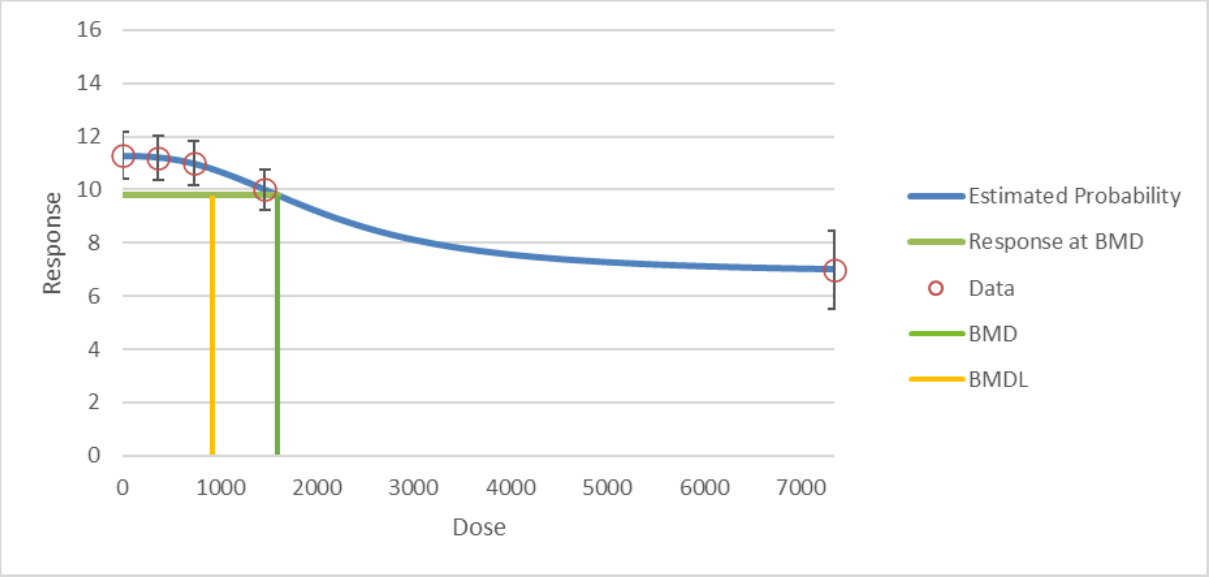


Figure A1.1 *Dose-response modelling for nitrous oxide (Menon et al., 2021) with an appropriate number and distribution of doses in the low range for determination of BMD and BMDL.*

Dose response modelling

For *in vitro* tests, it is useful to aim for a dose-response curve as the main outcome. This means that the dose-range will include one or more high doses of single PPPs that may not have relevance to real life exposure. For dose-response *in vitro* testing the minimum number of 5 doses in addition to a reference condition (negative control) should result in five valid observations with 3 replicates for each dose (mean ± sd) as promised in the grant agreement. The low-dose range should be well covered to allow estimation of the point of departure (cf. BMDS and PROAST web-based software). See an example above of good practice (Figure A1.1). The maximal difference between adjacent doses should be 10x, while the minimal difference should be 3x. Note that the dilutions tested should all contain the same concentration of organic solvent to aid the poor water solubility. The maximum vol% of this solvent is determined by the assay characteristics.

Calculation of HQ and wHQ for tebuconazole

For tebuconazole the data from the OBO study (Oerlemans et al., 2019) in The Netherlands was used. In this study, morning urine samples were collected during the use and non-use period. Samples were available from residents (flower bulb farmers and their neighbours) and from controls (consumers). For the calculation of HQ and wHQ, the excretion of 5-OH-tebuconazole was back-calculated to uptake of tebuconazole using a conversion factor determined in a human volunteer study by Oerlemans et al., (2019). The wHQ for residents resulted in a rank of 6 and for controls in a rank of 5 (both times ranking higher than glyphosate).

Table A1.2 *OBO data and calculations of HQ and wHQ for tebuconazole.*

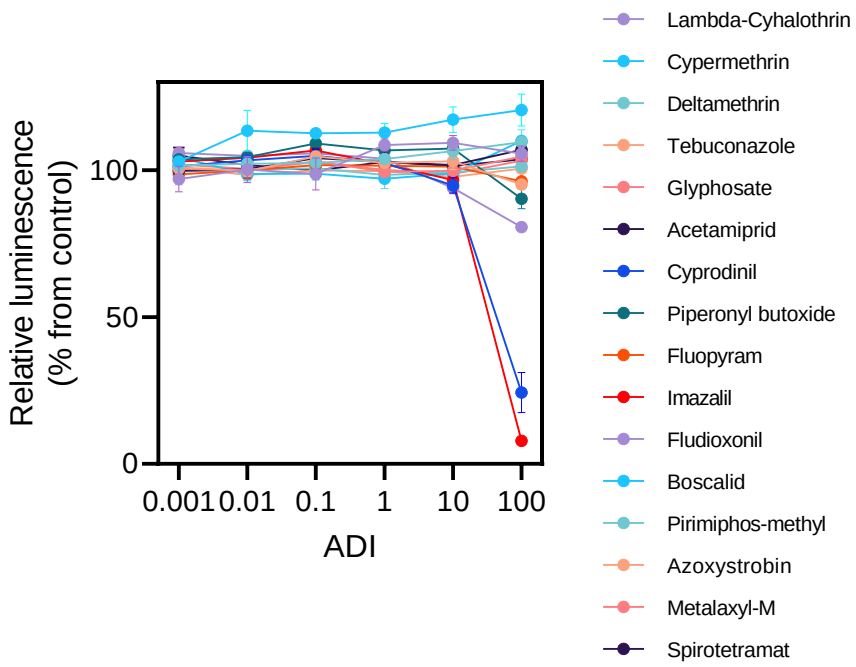
Parameters and input data from OBO	**Residents**	**Controls**
Number of participants in the use period (growth season)	99	64
Median in urine (ug/L)	0.16	0.10
Detects (>LOD)	61	43
Conversion factor for metabolism (Oerlemans et al., 2019)	0.38	0.38
Assumed urine production per day (L)	2	2
Estimated uptake per day (mg/kg/day)	1.2×10^{-5}	7.5×10^{-6}
ADI (mg/kg/day)[a]	n/a[b]	0.03
AOEL (mg/kg/day)[a]	0.03	n/a[b]
HQ	4.0×10^{-4}	2.5×10^{-4}
wHQ	2.4×10^{-4}	1.1×10^{-4}

[a] EU pesticides database available at https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances/?event=search.as (accessed October 30th 2022)

[b] not available

APPENDIX 2

Viability assays



Sample sequencing information

sample	library	raw_reads	raw_bases	clean_reads	clean_bases	error_rate	Q20	Q30	GC_pct
Control_s1	ERAS230036513-1r	51376914	7.71G	50708030	7.61G	0.03	97.85	94.26	52.71
Control_s2	ERAS230036514-1r	48554126	7.28G	47914220	7.19G	0.02	98.04	94.69	53.01
Control_s3	ERAS230036515-1r	52254486	7.84G	51598422	7.74G	0.02	97.94	94.42	52.53
Control_s4	ERAS230036516-1r	51956682	7.79G	51120942	7.67G	0.02	98.04	94.6	51.24
Cypro_s1	ERAS230036498-1r	59892566	8.98G	59273136	8.89G	0.02	97.9	94.39	52.95
Cypro_s2	ERAS230036499-1r	52251966	7.84G	51568872	7.74G	0.02	97.99	94.58	52.62
Cypro_s3	ERAS230036500-1r	53719606	8.06G	53117594	7.97G	0.03	97.64	93.85	52.86
Fludio_s1	ERAS230036501-1r	47487582	7.12G	46970070	7.05G	0.02	98.03	94.6	52.09
Fludio_s2	ERAS230036502-1r	50860596	7.63G	50329538	7.55G	0.02	98.06	94.72	51.85
Fludio_s3	ERAS230036503-1r	47718654	7.16G	47224378	7.08G	0.03	97.88	94.32	52.49
Glyph_s1	ERAS230036507-1r	59270520	8.89G	58681580	8.8G	0.02	97.98	94.58	52.25
Glyph_s2	ERAS230036508-1r	57193364	8.58G	56298698	8.44G	0.03	97.85	94.24	53.52
Glyph_s3	ERAS230036509-1r	51643918	7.75G	50406020	7.56G	0.03	97.79	94.11	53.22
Mix1_s1	ERAS230036489-1r	51651878	7.75G	49989860	7.5G	0.02	98.03	94.77	52.78
Mix1_s2	ERAS230036490-1r	49993204	7.5G	49150096	7.37G	0.02	97.97	94.52	52.04
Mix1_s3	ERAS230036491-1r	46298194	6.94G	45759332	6.86G	0.02	97.99	94.51	52.58
Mix2_s1	ERAS230036492-1r	50755972	7.61G	50082006	7.51G	0.02	97.96	94.52	52.83
Mix2_s2	ERAS230036493-1r	64907660	9.74G	64083108	9.61G	0.02	97.98	94.52	52.84
Mix2_s3	ERAS230036494-1r	61924760	9.29G	61215384	9.18G	0.03	97.69	93.84	53.12
Mix3_s1	ERAS230036495-1r	52645970	7.9G	51874364	7.78G	0.02	97.95	94.5	52.9
Mix3_s2	ERAS230036496-1r	60725318	9.11G	59847728	8.98G	0.02	98	94.52	52.88
Mix3_s3	ERAS230036497-1r	58388408	8.76G	57732292	8.66G	0.02	97.92	94.35	52.82
PipBut_s1	ERAS230036504-1r	59645260	8.95G	59020242	8.85G	0.02	97.95	94.4	52.07
PipBut_s2	ERAS230036505-1r	57722192	8.66G	57182516	8.58G	0.02	97.99	94.58	52.19
PipBut_s3	ERAS230036506-1r	49958480	7.49G	49481248	7.42G	0.03	97.84	94.19	52.64
Tebuc_s1	ERAS230036510-1r	59371808	8.91G	58607504	8.79G	0.02	97.93	94.45	53.44
Tebuc_s2	ERAS230036511-1r	55827172	8.37G	55061628	8.26G	0.02	98	94.56	51.64
Tebuc_s3	ERAS230036512-1r	49307182	7.4G	48695062	7.3G	0.02	98.01	94.61	52.91

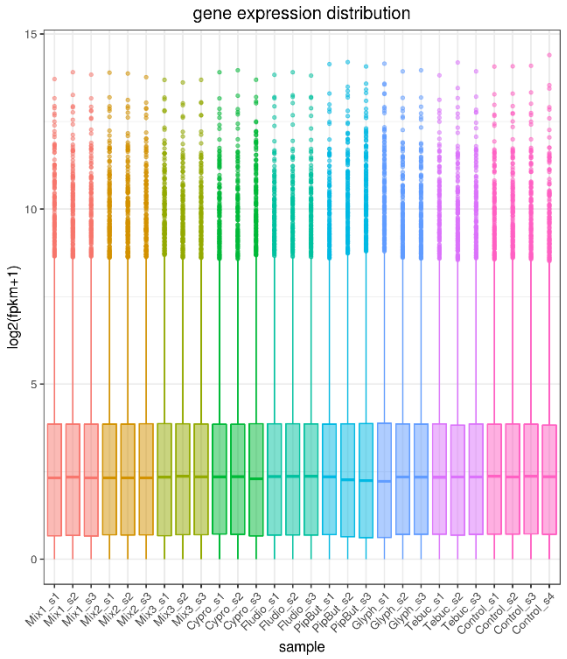
Disclaimer: This report is part of a project that has received funding by the European Union's Horizon 2020 research and innovation program under grant agreement number 862568.

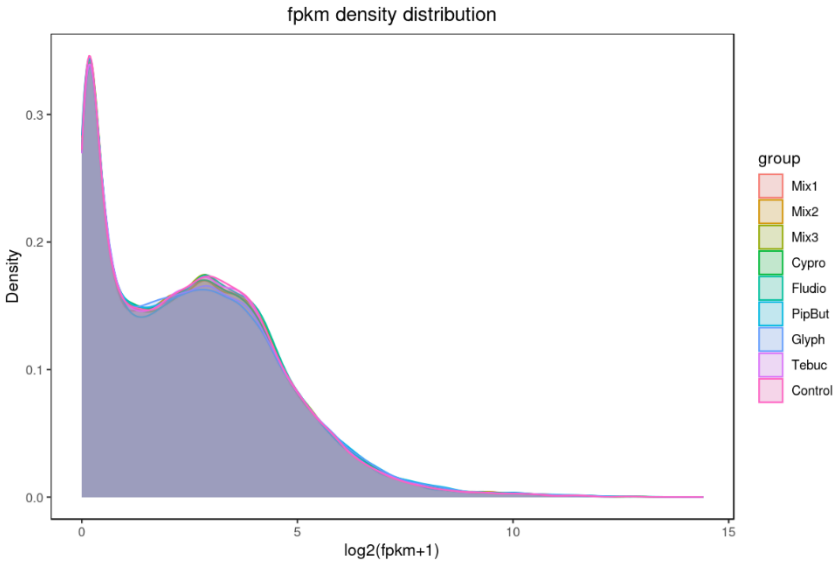
Sample mapping information

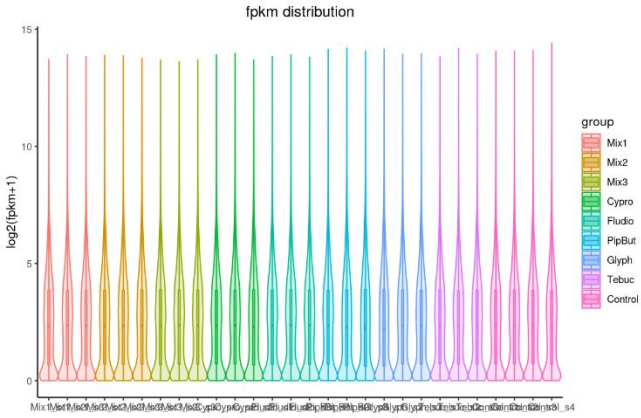
sample	total_reads	total_map	unique_map	multi_map	read1_map	read2_map	positive_map	negative_map	splice_map	unsplice_map	proper_map
Control_s1	50708030	48245112 (95.14%)	46289593 (91.29%)	1955519 (3.86%)	23205892 (45.76%)	23083701 (45.52%)	23084732 (45.52%)	23204861 (45.76%)	21488619 (42.38%)	24800974 (48.91%)	43997240 (86.77%)
Control_s2	47914220	45674840 (95.33%)	43855327 (91.53%)	1819513 (3.8%)	21960277 (45.83%)	21895050 (45.7%)	21868455 (45.64%)	21986872 (45.89%)	20688722 (43.18%)	23166605 (48.35%)	41674790 (86.98%)
Control_s3	51598422	49183707 (95.32%)	47338008 (91.74%)	1845699 (3.58%)	23724180 (45.98%)	23613828 (45.76%)	23608506 (45.75%)	23729502 (45.99%)	22159725 (42.95%)	25178283 (48.8%)	45006150 (87.22%)
Control_s4	51120942	48711536 (95.29%)	46872518 (91.69%)	1839018 (3.6%)	23473849 (45.92%)	23398669 (45.77%)	23366545 (45.71%)	23505973 (45.98%)	21621219 (42.29%)	25251299 (49.4%)	44355528 (86.77%)
Cypro_s1	59273136	56410004 (95.17%)	54131346 (91.33%)	2278658 (3.84%)	27119336 (45.75%)	27012010 (45.57%)	27000845 (45.55%)	27130501 (45.77%)	25338898 (42.75%)	28792448 (48.58%)	51704150 (87.23%)
Cypro_s2	51568872	49175122 (95.36%)	47309694 (91.74%)	1865428 (3.62%)	23697886 (45.95%)	23611808 (45.79%)	23601364 (45.77%)	23708330 (45.97%)	22403241 (43.44%)	24906453 (48.3%)	45388938 (88.02%)
Cypro_s3	53117594	50251966 (94.61%)	47777045 (89.95%)	2474921 (4.66%)	23893543 (44.98%)	23883502 (44.96%)	23823015 (44.85%)	23954030 (45.1%)	22644531 (42.63%)	25132514 (47.31%)	45569220 (85.79%)
Fludio_s1	46970070	44832963 (95.45%)	43600049 (92.83%)	1232914 (2.62%)	21836007 (46.49%)	21764042 (46.34%)	21768677 (46.35%)	21831372 (46.48%)	20616743 (43.89%)	22983306 (48.93%)	42044502 (89.51%)
Fludio_s2	50329538	48032267 (95.44%)	46657779 (92.7%)	1374488 (2.73%)	23368530 (46.43%)	23289249 (46.27%)	23291699 (46.28%)	23366080 (46.43%)	21952726 (43.62%)	24705053 (49.09%)	44888744 (89.19%)
Fludio_s3	47224378	44942618 (95.17%)	43376003 (91.85%)	1566615 (3.32%)	21750012 (46.06%)	21625991 (45.79%)	21645917 (45.84%)	21730086 (46.01%)	20702519 (43.84%)	22673484 (48.01%)	41771176 (88.45%)
Glyph_s1	58681580	56115680 (95.63%)	53423269 (91.04%)	2692411 (4.59%)	26745793 (45.58%)	26677476 (45.46%)	26650386 (45.42%)	26772883 (45.62%)	24287090 (41.39%)	29136179 (49.65%)	51389012 (87.57%)
Glyph_s2	56298698	53605774 (95.22%)	51560276 (91.58%)	2045498 (3.63%)	25843105 (45.9%)	25717171 (45.68%)	25719646 (45.68%)	25840630 (45.9%)	24411185 (43.36%)	27149091 (48.22%)	49108676 (87.23%)
Glyph_s3	50406020	47945844 (95.12%)	46110888 (91.48%)	1834956 (3.64%)	23125114 (45.88%)	22985774 (45.6%)	23001510 (45.63%)	23109378 (45.85%)	21528631 (42.71%)	24582257 (48.77%)	43833330 (86.96%)
Mix1_s1	49989860	47638096 (95.3%)	45457164 (90.93%)	2180932 (4.36%)	22737664 (45.48%)	22719500 (45.45%)	22665834 (45.34%)	22791330 (45.59%)	21867479 (43.74%)	23589685 (47.19%)	43974916 (87.97%)
Mix1_s2	49150096	46853082 (95.33%)	45019024 (91.59%)	1834058 (3.73%)	22551711 (45.88%)	22467313 (45.71%)	22452737 (45.68%)	22566287 (45.91%)	21596334 (43.94%)	23422690 (47.66%)	43156064 (87.8%)

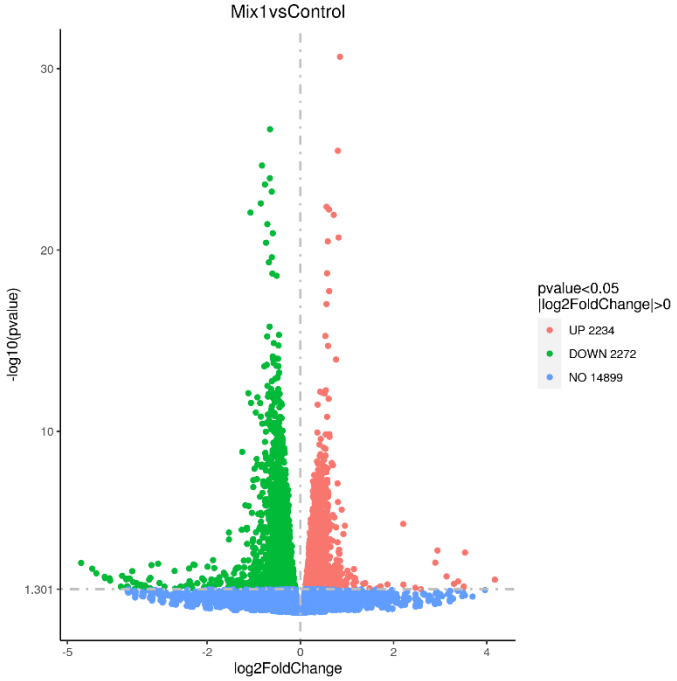
Mix1_s3	45759332	43678509 (95.45%)	41824953 (91.4%)	1853556 (4.05%)	20945867 (45.77%)	20879086 (45.63%)	20848960 (45.56%)	20975993 (45.84%)	20143048 (44.02%)	21681905 (47.38%)	40096764 (87.63%)
Mix2_s1	50082006	47711801 (95.27%)	45698444 (91.25%)	2013357 (4.02%)	22892634 (45.71%)	22805810 (45.54%)	22788008 (45.5%)	22910436 (45.75%)	22116947 (44.16%)	23581497 (47.09%)	43598572 (87.05%)
Mix2_s2	64083108	61069079 (95.3%)	58417641 (91.16%)	2651438 (4.14%)	29263617 (45.67%)	29154024 (45.49%)	29127662 (45.45%)	29289979 (45.71%)	28198878 (44.0%)	30218763 (47.16%)	55700760 (86.92%)
Mix2_s3	61215384	58114961 (94.94%)	55912116 (91.34%)	2202845 (3.6%)	27967538 (45.69%)	27944578 (45.65%)	27891871 (45.56%)	28020245 (45.77%)	26967920 (44.05%)	28944196 (47.28%)	53261206 (87.01%)
Mix3_s1	51874364	49278586 (95.0%)	47235684 (91.06%)	2042902 (3.94%)	23661414 (45.61%)	23574270 (45.44%)	23560644 (45.42%)	23675040 (45.64%)	22622299 (43.61%)	24613385 (47.45%)	45234054 (87.2%)
Mix3_s2	59847728	57032041 (95.3%)	55305877 (92.41%)	1726164 (2.88%)	27713966 (46.31%)	27591911 (46.1%)	27607271 (46.13%)	27698606 (46.28%)	26772739 (44.73%)	28533138 (47.68%)	53195934 (88.89%)
Mix3_s3	57732292	54850839 (95.01%)	52711162 (91.3%)	2139677 (3.71%)	26417598 (45.76%)	26293564 (45.54%)	26297233 (45.55%)	26413929 (45.75%)	25271108 (43.77%)	27440054 (47.53%)	50418760 (87.33%)
PipBut_s1	59020242	56391435 (95.55%)	54186883 (91.81%)	2204552 (3.74%)	27157350 (46.01%)	27029533 (45.8%)	27022959 (45.79%)	27163924 (46.02%)	25744137 (43.62%)	28442746 (48.19%)	52009204 (88.12%)
PipBut_s2	57182516	54611038 (95.5%)	52163950 (91.22%)	2447088 (4.28%)	26128803 (45.69%)	26035147 (45.53%)	26017558 (45.5%)	26146392 (45.72%)	24617738 (43.05%)	27546212 (48.17%)	50109792 (87.63%)
PipBut_s3	49481248	47157517 (95.3%)	44842889 (90.63%)	2314628 (4.68%)	22483785 (45.44%)	22359104 (45.19%)	22365475 (45.2%)	22477414 (45.43%)	21006081 (42.45%)	23836888 (48.17%)	42923430 (86.75%)
Tebuc_s1	58607504	55758515 (95.14%)	53832712 (91.85%)	1925803 (3.29%)	26978789 (46.03%)	26853923 (45.82%)	26857181 (45.83%)	26975531 (46.03%)	25916509 (44.22%)	27916203 (47.63%)	51242724 (87.43%)
Tebuc_s2	55061628	52470037 (95.29%)	50520042 (91.75%)	1949995 (3.54%)	25311689 (45.97%)	25208353 (45.78%)	25190645 (45.75%)	25329397 (46.0%)	24064819 (43.71%)	26455223 (48.05%)	47814424 (86.84%)
Tebuc_s3	48695062	46360563 (95.21%)	44440779 (91.26%)	1919784 (3.94%)	22258841 (45.71%)	22181938 (45.55%)	22161216 (45.51%)	22279563 (45.75%)	21278903 (43.7%)	23161876 (47.57%)	42169252 (86.6%)

Disclaimer: This report is part of a project that has received funding by the European Union's Horizon 2020 research and innovation program under grant agreement number 862568.





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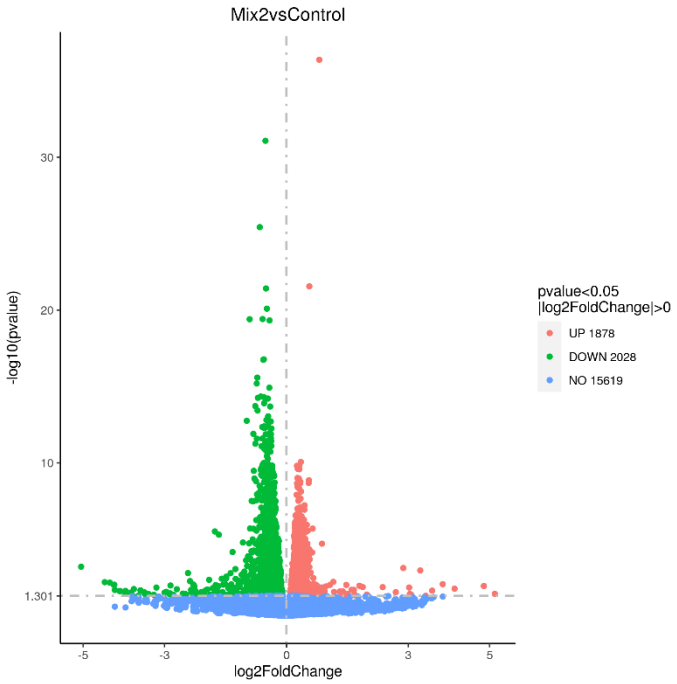
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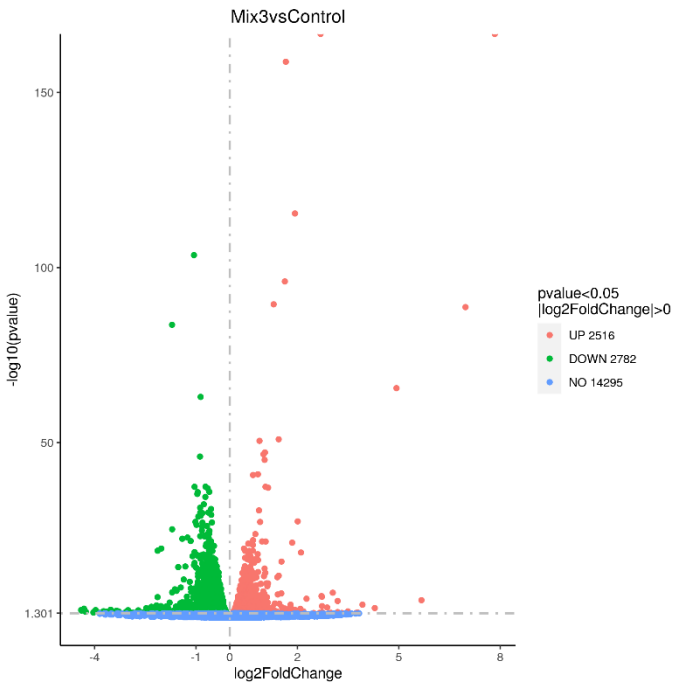
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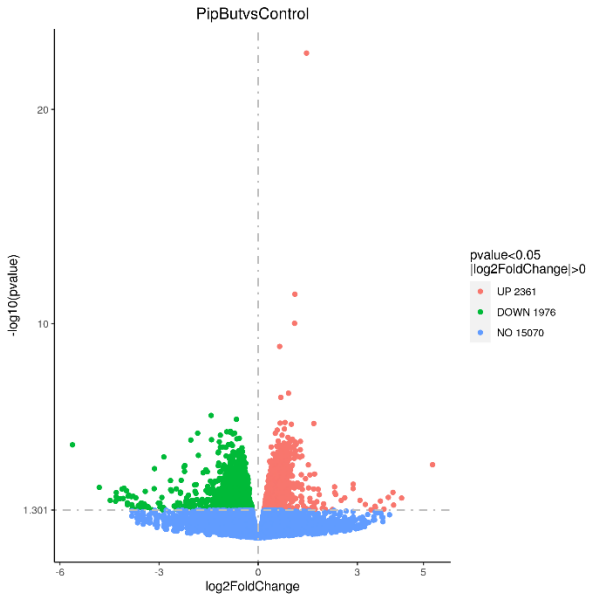

Figure A2.3 *Volcano plots representing the differences between control and PPP mixture-exposed ileum organoids.*

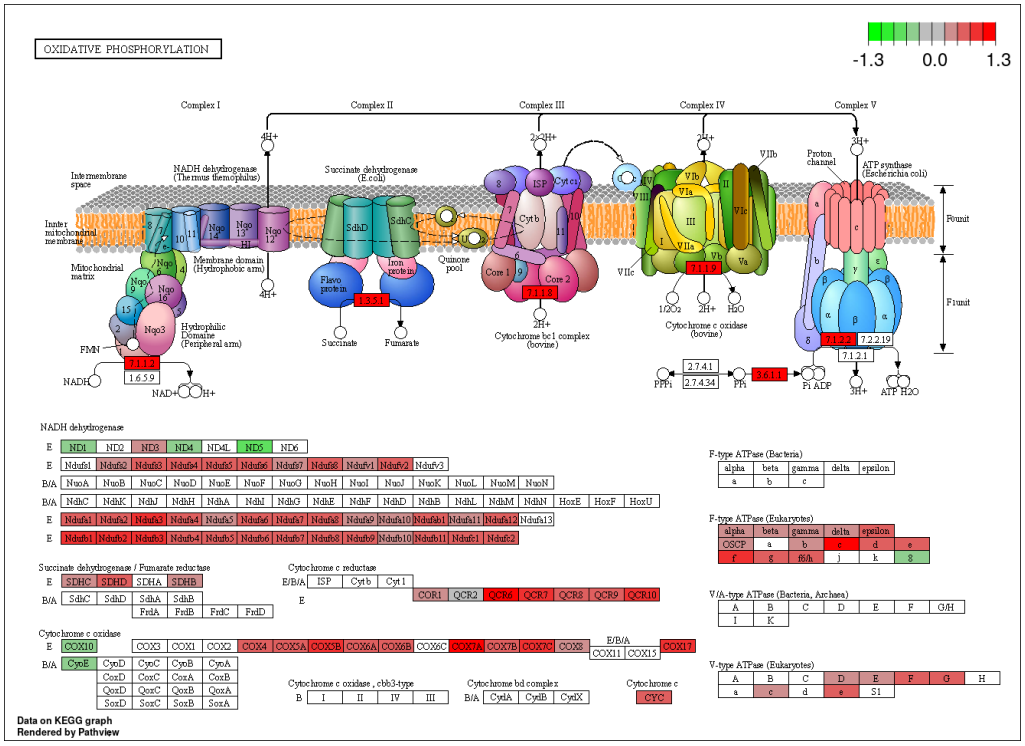

Figure A2.4 *KEGG pathway showing the up (red) and down (green) regulated genes by piperonyl butoxide. The expression levels indicate that the mitochondrial bioenergetics are in a stressed state by upregulating metabolism to compensate for energy loss (as observed with 100x ADI in the viability assay).*

Pathways differentially expressed by PPP's compared to control.

Table A2.1 *Effects of tebuconazole on ileum organoids (up-regulated denotes increased pathway expression and down-regulated denotes decreased pathway expression in PPP-treated ileum organoids).*

Up-regulated									
KEGGID	**Descriptio**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneNam**	**keggID**	**Count**
ssc05012	Parkinson	17/115	272/6738	3.00E-06	0.000426	ENSSSCG0	NDUFS2/A	ssc:100156	17
ssc05208	Chemical	15/115	223/6738	4.96E-06	0.000426	ENSSSCG0	NDUFS2/A	ssc:100156	15
ssc05016	Huntingto	17/115	303/6738	1.27E-05	0.000731	ENSSSCG0	NDUFS2/V	ssc:100156	17
ssc00190	Oxidative	11/115	144/6738	3.08E-05	0.001323	ENSSSCG0	NDUFS2/A	ssc:100156	11
ssc05020	Prion dise	15/115	268/6738	4.46E-05	0.001413	ENSSSCG0	NDUFS2/A	ssc:100156	15
ssc05010	Alzheime	18/115	371/6738	4.93E-05	0.001413	ENSSSCG0	NDUFS2/V	ssc:100156	18
ssc05022	Pathways	20/115	450/6738	6.25E-05	0.001535	ENSSSCG0	NDUFS2/V	ssc:100156	20
ssc04714	Thermoge	13/115	230/6738	0.000135	0.002907	ENSSSCG0	NDUFS2/A	ssc:100156	13
ssc05415	Diabetic c	11/115	199/6738	0.000549	0.010491	ENSSSCG0	NDUFS2/A	ssc:100156	11
ssc00790	Folate bio	4/115	25/6738	0.000772	0.013282	ENSSSCG0	ENSSSCG0	ssc:100522	4
ssc05014	Amyotrop	15/115	356/6738	0.000989	0.015469	ENSSSCG0	NDUFS2/V	ssc:100156	15
ssc05323	Rheumatc	6/115	80/6738	0.002334	0.033461	ENSSSCG0	AMCF-II/E	ssc:396900	6
ssc05134	Legionellc	5/115	58/6738	0.002959	0.03915	ENSSSCG0	ENSSSCG0	ssc:414904	5
Down-regulated									
KEGGID	**Descriptio**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneNam**	**keggID**	**Count**
ssc05205	Proteogly	16/181	192/6738	5.22E-05	0.012676	ENSSSCG0	FLNB/FN1	ssc:100514	16
ssc04510	Focal adhe	14/181	179/6738	0.000302	0.036739	ENSSSCG0	FLNB/FN1	ssc:100514	14

Table A2.2 *Effects of glyphosate on ileum organoids (up-regulated denotes increased pathway expression and down-regulated denotes decreased pathway expression in PPP-treated ileum organoids).*

Up-regulated									
KEGGID	**Descriptio**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneNam**	**keggID**	**Count**
ssc03010	Ribosome	65/534	185/6738	8.15E-27	2.42E-24	ENSSSCG0	ENSSSCG0	ssc:414392	65
ssc00190	Oxidative	48/534	145/6738	9.57E-19	1.42E-16	ENSSSCG0	ENSSSCG0	ssc:10052(	48
ssc05016	Huntingto	67/534	303/6738	2.10E-15	2.08E-13	ENSSSCG0	ENSSSCG0	ssc:10052(	67
ssc05171	Coronavir	60/534	258/6738	6.22E-15	4.62E-13	ENSSSCG0	ENSSSCG0	ssc:414392	60
ssc04714	Thermoge	56/534	231/6738	7.90E-15	4.69E-13	ENSSSCG0	ENSSSCG0	ssc:10052(	56
ssc05208	Chemical	55/534	225/6738	9.56E-15	4.73E-13	ENSSSCG0	HMOX1/EI	ssc:445512	55
ssc05012	Parkinson	61/534	274/6738	3.10E-14	1.31E-12	ENSSSCG0	ENSSSCG0	ssc:10052(	61
ssc05415	Diabetic c	49/534	200/6738	2.77E-13	1.03E-11	ENSSSCG0	ENSSSCG0	ssc:10052(	49
ssc05020	Prion dise	58/534	268/6738	5.12E-13	1.69E-11	ENSSSCG0	ENSSSCG0	ssc:10052(	58
ssc05014	Amyotrop	65/534	353/6738	4.18E-11	1.24E-09	ENSSSCG0	ENSSSCG0	ssc:10052(	65
ssc04932	Non-alcoh	38/534	154/6738	1.18E-10	3.19E-09	ENSSSCG0	ENSSSCG0	ssc:10052(	38
ssc05010	Alzheimer	65/534	371/6738	3.84E-10	9.50E-09	ENSSSCG0	ENSSSCG0	ssc:10052(	65
ssc05022	Pathways	73/534	451/6738	1.20E-09	2.74E-08	ENSSSCG0	ENSSSCG0	ssc:10052(	73
ssc00100	Steroid bi	9/534	19/6738	5.18E-06	0.00011	ENSSSCG0	SQLE/FDF	ssc:100113	9
ssc04723	Retrograd	24/534	123/6738	2.72E-05	0.000539	ENSSSCG0	ENSSSCG0	ssc:10052(	24
ssc00900	Terpenoic	7/534	22/6738	0.001123	0.020852	ENSSSCG0	HMGCS1/I	ssc:100524	7
Down-regulated									
KEGGID	**Descriptio**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneNam**	**keggID**	**Count**
ssc03013	Nucleocyt	13/216	108/6738	3.67E-05	0.009648	ENSSSCG0	NUP205/T	ssc:100523	13
ssc04110	Cell cycle	15/216	162/6738	0.0002	0.026242	ENSSSCG0	PDS5B/TIC	ssc:100154	15

Table A2.3 *Effects of Cyprodinil on ileum organoids (up-regulated denotes increased pathway expression in PPP-treated ileum organoids).*

Up-regulated									
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc00190	Oxidative phosphorylati	54/669	144/6727	3.10E-19	9.92E-17	ENSSSCG0	NDUFB4/N	ssc:100624	54
ssc05208	Chemical carcinogenesis	62/669	224/6727	1.57E-14	2.16E-12	ENSSSCG0	CYP1A1/C	ssc:403103	62
ssc04714	Thermogenesis	63/669	231/6727	2.02E-14	2.16E-12	ENSSSCG0	NDUFB4/A	ssc:100624	63
ssc05016	Huntington disease	70/669	304/6727	5.67E-12	4.54E-10	ENSSSCG0	NDUFB4/G	ssc:100624	70
ssc05020	Prion disease	63/669	266/6727	1.96E-11	1.26E-09	ENSSSCG0	CYBA/NDU	ssc:397507	63
ssc05415	Diabetic cardiomyopathy	51/669	201/6727	1.30E-10	6.75E-09	ENSSSCG0	CYBA/NDU	ssc:397507	51
ssc04932	Non-alcoholic fatty liver	43/669	154/6727	1.48E-10	6.75E-09	ENSSSCG0	SREBF1/N	ssc:397308	43
ssc05012	Parkinson disease	62/669	273/6727	1.87E-10	7.48E-09	ENSSSCG0	SLC39A11/	ssc:100518	62
ssc05014	Amyotrophic lateral scle	68/669	358/6727	6.61E-08	2.35E-06	ENSSSCG0	NDUFB4/G	ssc:100624	68
ssc03010	Ribosome	42/669	183/6727	1.33E-07	4.27E-06	ENSSSCG0	RPL35A/RI	ssc:100519	42
ssc01212	Fatty acid metabolism	19/669	54/6727	4.50E-07	1.31E-05	ENSSSCG0	FADS1/AC	ssc:444995	19
ssc05010	Alzheimer disease	66/669	370/6727	1.15E-06	2.87E-05	ENSSSCG0	SLC39A11/	ssc:100518	66
ssc05204	Chemical carcinogenesis	17/669	47/6727	1.17E-06	2.87E-05	ENSSSCG0	CYP1A1/C	ssc:403103	17
ssc00983	Drug metabolism - other	20/669	64/6727	1.95E-06	4.46E-05	ENSSSCG0	MGST1/EN	ssc:397567	20
ssc00980	Metabolism of xenobioti	17/669	49/6727	2.28E-06	4.87E-05	ENSSSCG0	CYP1A1/C	ssc:403103	17
ssc01240	Biosynthesis of cofactors	30/669	134/6727	1.43E-05	0.000286	ENSSSCG0	DHRS3/NC	ssc:100514	30
ssc05022	Pathways of neurodegen	72/669	452/6727	2.50E-05	0.000471	ENSSSCG0	NDUFB4/G	ssc:100624	72
ssc00100	Steroid biosynthesis	9/669	19/6727	3.33E-05	0.000592	ENSSSCG0	SQLE/FDF	ssc:100113	9
ssc04723	Retrograde endocannabi	27/669	122/6727	4.73E-05	0.000797	ENSSSCG0	NDUFB4/A	ssc:100624	27
ssc00280	Valine, leucine and isole	15/669	51/6727	8.25E-05	0.001321	ENSSSCG0	HMGCS1/A	ssc:100524	15
ssc00071	Fatty acid degradation	12/669	37/6727	0.000151	0.002281	ENSSSCG0	ACAT2/AC	ssc:100152	12
ssc00830	Retinol metabolism	14/669	48/6727	0.000157	0.002281	ENSSSCG0	CYP1A1/D	ssc:403103	14
ssc00480	Glutathione metabolism	15/669	55/6727	0.000211	0.002937	ENSSSCG0	GCLC/MGS	ssc:100522	15
ssc00520	Amino sugar and nucleot	14/669	51/6727	0.000317	0.004226	ENSSSCG0	GNPDA1/G	ssc:100512	14
ssc00140	Steroid hormone biosynt	14/669	52/6727	0.000395	0.005054	ENSSSCG0	CYP1A1/C	ssc:403103	14
ssc00982	Drug metabolism - cytocl	12/669	43/6727	0.000715	0.008805	ENSSSCG0	MGST1/EN	ssc:397567	12
ssc00760	Nicotinate and nicotinam	10/669	32/6727	0.000748	0.00887	ENSSSCG0	BST1/NAD	ssc:100625	10
ssc00900	Terpenoid backbone bio	8/669	22/6727	0.000826	0.009444	ENSSSCG0	FDPS/HM	ssc:100153	8
ssc01232	Nucleotide metabolism	17/669	77/6727	0.001204	0.013288	ENSSSCG0	CDA/TK2/	ssc:100515	17
ssc00240	Pyrimidine metabolism	13/669	52/6727	0.001348	0.014377	ENSSSCG0	CDA/TK2/	ssc:100515	13
ssc03320	PPAR signaling pathway	15/669	69/6727	0.002697	0.027835	ENSSSCG0	ANGPTL4/	ssc:397628	15
ssc00062	Fatty acid elongation	8/669	26/6727	0.00282	0.028201	ENSSSCG0	HSD17B12	ssc:100312	8
ssc00380	Tryptophan metabolism	10/669	38/6727	0.003169	0.029825	ENSSSCG0	CYP1A1/C	ssc:403103	10
ssc04975	Fat digestion and absorp	10/669	38/6727	0.003169	0.029825	ENSSSCG0	PLA2G12B	ssc:100152	10
ssc01040	Biosynthesis of unsatura	8/669	27/6727	0.003665	0.033508	ENSSSCG0	FADS1/FA	ssc:444995	8
Down-regulated									
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc04510	Focal adhesion	36/632	177/6727	5.57E-06	0.0017	ENSSSCG0	ITGA5/ME	ssc:100155	36
ssc05206	MicroRNAs in cancer	31/632	154/6727	3.09E-05	0.004708	ENSSSCG0	VIM/ITGA	ssc:100522	31
ssc05165	Human papillomavirus in	48/632	297/6727	0.000108	0.01084	ENSSSCG0	OASL/MX	ssc:595119	48
ssc04520	Adherens junction	20/632	88/6727	0.000142	0.01084	ENSSSCG0	MET/VCL/	ssc:654328	20
ssc04810	Regulation of actin cytos	36/632	208/6727	0.000202	0.012343	ENSSSCG0	FGFR4/C5/	ssc:100127	36
ssc04512	ECM-receptor interactior	17/632	73/6727	0.000326	0.014561	ENSSSCG0	ITGA5/NP	ssc:100155	17
ssc05203	Viral carcinogenesis	33/632	191/6727	0.000382	0.014561	ENSSSCG0	ENSSSCG0	ssc:110261	33
ssc05205	Proteoglycans in cancer	33/632	191/6727	0.000382	0.014561	ENSSSCG0	ITGA5/CD	ssc:100155	33
ssc04919	Thyroid hormone signali	22/632	116/6727	0.001005	0.03344	ENSSSCG0	BMP4/KA	ssc:100113	22
ssc04070	Phosphatidylinositol sigr	19/632	95/6727	0.001129	0.03344	ENSSSCG0	INPP5F/PI	ssc:100144	19
ssc05164	Influenza A	26/632	148/6727	0.001206	0.03344	ENSSSCG0	RSAD2/OA	ssc:396752	26

Table A2.4. *Effects of Piperonyl butoxide on ileum organoids (up-regulated denotes increased pathway expression and down-regulated denotes decreased pathway expression in PPP-treated*

Up-regulated								
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc03010	Ribosome	120/1142	181/6716	8.05E-51	2.61E-48	ENSSSCG0	ENSSSCG0	ssc:10062	120
ssc05012	Parkinson disease	126/1142	274/6716	2.79E-30	4.51E-28	ENSSSCG0	COX7C/NI	ssc:10003	126
ssc00190	Oxidative phosphorylati	83/1142	144/6716	8.08E-29	8.72E-27	ENSSSCG0	COX7C/NI	ssc:10003	83
ssc05016	Huntington disease	129/1142	302/6716	3.90E-27	3.16E-25	ENSSSCG0	COX7C/NI	ssc:10003	129
ssc05020	Prion disease	116/1142	264/6716	9.94E-26	6.44E-24	ENSSSCG0	COX7C/NI	ssc:10003	116
ssc05014	Amyotrophic lateral scle	136/1142	354/6716	3.25E-23	1.76E-21	ENSSSCG0	COX7C/NI	ssc:10003	136
ssc05208	Chemical carcinogenesis	100/1142	223/6716	4.44E-23	2.05E-21	ENSSSCG0	HMOX1/EI	ssc:44551	100
ssc05415	Diabetic cardiomyopathy	85/1142	198/6716	2.89E-18	1.17E-16	ENSSSCG0	COX7C/NI	ssc:10003	85
ssc04714	Thermogenesis	92/1142	231/6716	4.27E-17	1.54E-15	ENSSSCG0	COX7C/RP	ssc:10003	92
ssc05022	Pathways of neurodeger	147/1142	454/6716	6.64E-17	2.15E-15	ENSSSCG0	COX7C/NI	ssc:10003	147
ssc05171	Coronavirus disease - CO	98/1142	256/6716	9.03E-17	2.66E-15	ENSSSCG0	ENSSSCG0	ssc:10062	98
ssc04932	Non-alcoholic fatty liver	69/1142	152/6716	1.45E-16	3.92E-15	ENSSSCG0	COX7C/NI	ssc:10003	69
ssc05010	Alzheimer disease	124/1142	371/6716	1.61E-15	4.02E-14	ENSSSCG0	COX7C/NI	ssc:10003	124
ssc03050	Proteasome	29/1142	45/6716	1.41E-12	3.26E-11	ENSSSCG0	ENSSSCG0	ssc:10015	29
ssc00983	Drug metabolism - other	29/1142	63/6716	7.11E-08	1.54E-06	ENSSSCG0	ENSSSCG0	ssc:10052	29
ssc03040	Spliceosome	48/1142	136/6716	1.57E-07	3.18E-06	ENSSSCG0	SNRPG/M	ssc:10052	48
ssc04723	Retrograde endocannabi	42/1142	124/6716	3.22E-06	6.13E-05	ENSSSCG0	NDUFB5/N	ssc:10052	42
ssc00790	Folate biosynthesis	13/1142	25/6716	6.41E-05	0.001154	ENSSSCG0	AKR1C8/P	ssc:65440	13
ssc01240	Biosynthesis of cofactors	41/1142	135/6716	7.84E-05	0.001337	ENSSSCG0	NQO1/DH	ssc:10028	41
ssc03060	Protein export	12/1142	23/6716	0.000118	0.001832	ENSSSCG0	SEC11C/SE	ssc:10052	12
ssc04141	Protein processing in en	47/1142	164/6716	0.000119	0.001832	ENSSSCG0	ERP29/SE	ssc:10015	47
ssc00480	Glutathione metabolism	21/1142	55/6716	0.000139	0.002051	ENSSSCG0	ENSSSCG0	ssc:10052	21
ssc05204	Chemical carcinogenesis	19/1142	48/6716	0.000165	0.002322	ENSSSCG0	ENSSSCG0	ssc:10012	19
ssc00980	Metabolism of xenobioti	18/1142	50/6716	0.000937	0.012655	ENSSSCG0	ENSSSCG0	ssc:10052	18
ssc00280	Valine, leucine and isole	18/1142	51/6716	0.001223	0.015845	ENSSSCG0	ACADM/A	ssc:39710	18
ssc05017	Spinocerebellar ataxia	37/1142	136/6716	0.001742	0.02171	ENSSSCG0	ENSSSCG0	ssc:10015	37
ssc00520	Amino sugar and nucleot	17/1142	50/6716	0.002627	0.03102	ENSSSCG0	HEXB/AMI	ssc:39695	17
ssc00620	Pyruvate metabolism	15/1142	42/6716	0.002681	0.03102	ENSSSCG0	ACAT1/AC	ssc:10062	15
ssc03420	Nucleotide excision repa	19/1142	60/6716	0.003789	0.042329	ENSSSCG0	GTF2H5/M	ssc:10073	19
Down-regulated									
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc04919	Thyroid hormone signali	34/780	118/6716	2.61E-07	5.80E-05	ENSSSCG0	MED16/PL	ssc:10052	34
ssc05165	Human papillomavirus ir	65/780	303/6716	3.97E-07	5.80E-05	ENSSSCG0	LAMA5/C(	ssc:11025	65
ssc04360	Axon guidance	40/780	166/6716	3.91E-06	0.000272	ENSSSCG0	PLXNA3/S	ssc:10062	40
ssc04520	Adherens junction	26/780	88/6716	4.03E-06	0.000272	ENSSSCG0	FARP2/CR	ssc:10051	26
ssc04510	Focal adhesion	42/780	179/6716	4.66E-06	0.000272	ENSSSCG0	LAMA5/C(	ssc:11025	42
ssc04512	ECM-receptor interactior	21/780	75/6716	8.12E-05	0.003726	ENSSSCG0	LAMA5/HS	ssc:11025	21
ssc05206	MicroRNAs in cancer	35/780	157/6716	8.93E-05	0.003726	ENSSSCG0	SOX4/CCN	ssc:10051	35
ssc04015	Rap1 signaling pathway	40/780	195/6716	0.000199	0.006769	ENSSSCG0	FARP2/LP/	ssc:10051	40
ssc04330	Notch signaling pathway	17/780	58/6716	0.000209	0.006769	ENSSSCG0	NOTCH1/[	ssc:11025	17
ssc04070	Phosphatidylinositol sigr	23/780	94/6716	0.000348	0.010176	ENSSSCG0	PLCE1/PI4	ssc:10015	23
ssc05146	Amoebiasis	21/780	84/6716	0.000457	0.012119	ENSSSCG0	LAMA5/M	ssc:11025	21
ssc04310	Wnt signaling pathway	32/780	154/6716	0.00067	0.0163	ENSSSCG0	CCND2/D\	ssc:39716	32
ssc00562	Inositol phosphate meta	18/780	70/6716	0.000799	0.017957	ENSSSCG0	PLCE1/PI4	ssc:10015	18
ssc03013	Nucleocytoplasmic trans	24/780	108/6716	0.001162	0.024229	ENSSSCG0	ENSSSCG0	ssc:10051	24
ssc00310	Lysine degradation	16/780	62/6716	0.001475	0.027486	ENSSSCG0	KMT2C/M	ssc:10051	16
ssc04810	Regulation of actin cytos	39/780	208/6716	0.001506	0.027486	ENSSSCG0	LPAR5/IQ(	ssc:10216	39
ssc04530	Tight junction	30/780	149/6716	0.001653	0.0284	ENSSSCG0	ARHGAP1	ssc:10052	30
ssc05224	Breast cancer	27/780	132/6716	0.002182	0.035401	ENSSSCG0	NOTCH1/[	ssc:11025	27
ssc04151	PI3K-Akt signaling pathw	51/780	297/6716	0.002356	0.036211	ENSSSCG0	LAMA5/LP	ssc:11025	51
ssc01522	Endocrine resistance	20/780	90/6716	0.002904	0.042394	ENSSSCG0	NOTCH1/N	ssc:11025	20
ssc05100	Bacterial invasion of epit	17/780	73/6716	0.003497	0.04697	ENSSSCG0	ENSSSCG0	ssc:10014	17
ssc05225	Hepatocellular carcinom	31/780	163/6716	0.003539	0.04697	ENSSSCG0	DVL3/LRP!	ssc:10062	31

ileum organoids).

Table A2.5. *Effects of Fludioxinil on ileum organoids (up-regulated denotes increased pathway expression and down-regulated denotes decreased pathway expression in PPP-treated ileum organoids).*

Up-regulated								
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc04141	Protein processing in en	78/1144	165/6744	5.73E-20	1.89E-17	ENSSSCG0	PDIA4/RPI	ssc:100522	78
ssc03050	Proteasome	31/1144	45/6744	1.33E-14	2.20E-12	ENSSSCG0	PSMC1/PS	ssc:100155	31
ssc05016	Huntington disease	99/1144	302/6744	4.72E-12	5.19E-10	ENSSSCG0	TUBA4A/C	ssc:100151	99
ssc05012	Parkinson disease	90/1144	273/6744	3.34E-11	2.75E-09	ENSSSCG0	KEAP1/TU	ssc:100136	90
ssc05010	Alzheimer disease	109/1144	369/6744	5.01E-10	3.31E-08	ENSSSCG0	TUBA4A/S	ssc:100151	109
ssc05014	Amyotrophic lateral scle	103/1144	357/6744	6.45E-09	3.55E-07	ENSSSCG0	TUBA4A/V	ssc:100151	103
ssc05017	Spinocerebellar ataxia	50/1144	137/6744	2.30E-08	1.08E-06	ENSSSCG0	PSMC1/PS	ssc:100155	50
ssc05022	Pathways of neurodegen	121/1144	453/6744	4.29E-08	1.77E-06	ENSSSCG0	TUBA4A/V	ssc:100151	121
ssc00980	Metabolism of xenobioti	25/1144	52/6744	1.91E-07	6.99E-06	ENSSSCG0	CYP1A1/C	ssc:403103	25
ssc03060	Protein export	15/1144	23/6744	3.21E-07	1.06E-05	ENSSSCG0	SEC61A1/S	ssc:100155	15
ssc05020	Prion disease	77/1144	266/6744	5.00E-07	1.50E-05	ENSSSCG0	TUBA4A/H	ssc:100151	77
ssc05204	Chemical carcinogenesis	23/1144	50/6744	1.57E-06	4.32E-05	ENSSSCG0	CYP1A1/C	ssc:403103	23
ssc01240	Biosynthesis of cofactors	45/1144	134/6744	1.76E-06	4.46E-05	ENSSSCG0	NQO1/GC	ssc:100286	45
ssc00510	N-Glycan biosynthesis	21/1144	52/6744	5.13E-05	0.001209	ENSSSCG0	RPN1/ALG	ssc:397606	21
ssc00983	Drug metabolism - other	24/1144	64/6744	6.39E-05	0.001406	ENSSSCG0	ENSSSCG0	ssc:397682	24
ssc00140	Steroid hormone biosyn	20/1144	51/6744	0.000126	0.00259	ENSSSCG0	CYP1A1/EI	ssc:403103	20
ssc05208	Chemical carcinogenesis	60/1144	227/6744	0.000171	0.003321	ENSSSCG0	NQO1/CYI	ssc:100286	60
ssc00982	Drug metabolism - cytocl	18/1144	45/6744	0.000202	0.003707	ENSSSCG0	ENSSSCG0	ssc:397682	18
ssc00860	Porphyrin metabolism	15/1144	35/6744	0.000279	0.00484	ENSSSCG0	ENSSSCG0	ssc:100624	15
ssc00040	Pentose and glucuronate	12/1144	25/6744	0.000319	0.00526	ENSSSCG0	UGDH/EN	ssc:100627	12
ssc00970	Aminoacyl-tRNA biosynt	17/1144	46/6744	0.000892	0.014023	ENSSSCG0	AARS1/FA	ssc:100525	17
ssc00480	Glutathione metabolism	19/1144	55/6744	0.001179	0.016336	ENSSSCG0	GCLC/ENS	ssc:100522	19
ssc02010	ABC transporters	17/1144	47/6744	0.001181	0.016336	ENSSSCG0	ABCC3/EN	ssc:100513	17
ssc00520	Amino sugar and nucleot	18/1144	51/6744	0.001188	0.016336	ENSSSCG0	UGDH/GN	ssc:100627	18
ssc00563	Glycosylphosphatidylino	11/1144	26/6744	0.002063	0.027228	ENSSSCG0	PIGV/ENS	ssc:100525	11
ssc00280	Valine, leucine and isole	17/1144	50/6744	0.002559	0.031452	ENSSSCG0	ACAT1/AL	ssc:100626	17
ssc00053	Ascorbate and aldarate n	10/1144	23/6744	0.002573	0.031452	ENSSSCG0	UGDH/EN	ssc:100627	10
ssc00190	Oxidative phosphorylati	38/1144	145/6744	0.002997	0.035324	ENSSSCG0	ATP6V0A2	ssc:100154	38
ssc01230	Biosynthesis of amino ac	20/1144	64/6744	0.003477	0.039564	ENSSSCG0	TKT/TALD	ssc:100127	20

Table A2.5 (continued)

Down-regulated									
KEGGID	**Description**	**GeneRatio**	**BgRatio**	**pvalue**	**padj**	**geneID**	**geneName**	**keggID**	**Count**
ssc04510	Focal adhesion	66/1265	179/6744	6.03E-09	1.83E-06	ENSSSCG0	ACTG1/EN	ssc:39765	66
ssc04520	Adherens junction	40/1265	89/6744	1.13E-08	1.83E-06	ENSSSCG0	ACTG1/VC	ssc:39765	40
ssc04810	Regulation of actin cytos	73/1265	210/6744	1.76E-08	1.92E-06	ENSSSCG0	ACTG1/C9	ssc:39765	73
ssc04530	Tight junction	56/1265	152/6744	8.97E-08	7.31E-06	ENSSSCG0	ACTG1/MS	ssc:39765	56
ssc04110	Cell cycle	58/1265	161/6744	1.31E-07	8.54E-06	ENSSSCG0	CDKN1C/C	ssc:11025	58
ssc05165	Human papillomavirus in	93/1265	302/6744	1.73E-07	9.42E-06	ENSSSCG0	FN1/CCNE	ssc:39762	93
ssc05206	MicroRNAs in cancer	54/1265	154/6744	9.57E-07	3.92E-05	ENSSSCG0	VIM/CD44	ssc:10052	54
ssc04512	ECM-receptor interactior	32/1265	74/6744	9.61E-07	3.92E-05	ENSSSCG0	FN1/AGRN	ssc:39762	32
ssc04390	Hippo signaling pathway	51/1265	144/6744	1.36E-06	4.93E-05	ENSSSCG0	ACTG1/CC	ssc:39765	51
ssc05205	Proteoglycans in cancer	62/1265	192/6744	3.77E-06	0.000123	ENSSSCG0	ACTG1/ITF	ssc:39765	62
ssc05203	Viral carcinogenesis	61/1265	191/6744	6.71E-06	0.000199	ENSSSCG0	CCND2/HE	ssc:39716	61
ssc04015	Rap1 signaling pathway	62/1265	196/6744	7.98E-06	0.000217	ENSSSCG0	ACTG1/FG	ssc:39765	62
ssc05100	Bacterial invasion of epit	30/1265	74/6744	1.02E-05	0.000257	ENSSSCG0	ACTG1/VC	ssc:39765	30
ssc04218	Cellular senescence	52/1265	159/6744	1.53E-05	0.000355	ENSSSCG0	ITPR3/CCN	ssc:10015	52
ssc04933	AGE-RAGE signaling path	35/1265	94/6744	1.80E-05	0.000391	ENSSSCG0	ICAM1/FN	ssc:39675	35
ssc05219	Bladder cancer	19/1265	40/6744	3.19E-05	0.00065	ENSSSCG0	CCND1/CD	ssc:10073	19
ssc05200	Pathways in cancer	125/1265	487/6744	5.70E-05	0.001094	ENSSSCG0	FN1/CCNE	ssc:39762	125
ssc04919	Thyroid hormone signali	39/1265	115/6744	7.17E-05	0.001299	ENSSSCG0	ACTG1/PL	ssc:39765	39
ssc04610	Complement and coagul	25/1265	63/6744	8.43E-05	0.001446	ENSSSCG0	C9/C6/EN	ssc:10003	25
ssc05146	Amoebiasis	30/1265	82/6744	0.000103	0.001671	ENSSSCG0	C9/VCL/FN	ssc:10003	30
ssc05135	Yersinia infection	43/1265	133/6744	0.000111	0.001716	ENSSSCG0	ACTG1/FN	ssc:39765	43
ssc04670	Leukocyte transendothel	34/1265	98/6744	0.000124	0.001834	ENSSSCG0	ACTG1/VC	ssc:39765	34
ssc05160	Hepatitis C	45/1265	143/6744	0.000157	0.00223	ENSSSCG0	OAS2/SOC	ssc:59512	45
ssc04625	C-type lectin receptor sig	32/1265	94/6744	0.000288	0.003915	ENSSSCG0	ITPR3/EGF	ssc:10015	32
ssc04151	PI3K-Akt signaling pathw	80/1265	303/6744	0.000509	0.006635	ENSSSCG0	FN1/CCNE	ssc:39762	80
ssc05133	Pertussis	25/1265	70/6744	0.000578	0.007248	ENSSSCG0	ENSSSCG0	ssc:10052	25
ssc05167	Kaposi sarcoma-associate	53/1265	186/6744	0.000687	0.008299	ENSSSCG0	ICAM1/ITF	ssc:39675	53
ssc05230	Central carbon metaboli	23/1265	64/6744	0.000858	0.009985	ENSSSCG0	IDH2/EGFI	ssc:39760	23
ssc00100	Steroid biosynthesis	10/1265	19/6744	0.000921	0.010359	ENSSSCG0	FDFT1/SQ	ssc:10031	10
ssc04668	TNF signaling pathway	34/1265	109/6744	0.001146	0.011936	ENSSSCG0	ICAM1/SO	ssc:39675	34
ssc05212	Pancreatic cancer	26/1265	77/6744	0.001191	0.011936	ENSSSCG0	CCND1/EG	ssc:10073	26
ssc05164	Influenza A	43/1265	147/6744	0.001206	0.011936	ENSSSCG0	ACTG1/OA	ssc:39765	43
ssc05222	Small cell lung cancer	30/1265	93/6744	0.001208	0.011936	ENSSSCG0	FN1/LAM	ssc:39762	30
ssc04360	Axon guidance	48/1265	169/6744	0.001302	0.012485	ENSSSCG0	RRAS/SEM	ssc:10051	48
ssc04115	p53 signaling pathway	25/1265	74/6744	0.001457	0.013567	ENSSSCG0	CCND2/CC	ssc:39716	25
ssc04392	Hippo signaling pathway	12/1265	27/6744	0.001921	0.017395	ENSSSCG0	FRMD6/LI	ssc:10015	12
ssc04621	NOD-like receptor signal	44/1265	155/6744	0.002056	0.018116	ENSSSCG0	OAS2/ITP	ssc:59512	44
ssc05412	Arrhythmogenic right ve	23/1265	68/6744	0.002182	0.018476	ENSSSCG0	ACTG1/AC	ssc:39765	23
ssc04010	MAPK signaling pathway	69/1265	267/6744	0.00221	0.018476	ENSSSCG0	FGFR4/RR	ssc:10012	69
ssc04061	Viral protein interaction	21/1265	61/6744	0.00262	0.021349	ENSSSCG0	ENSSSCG0	ssc:55395	21
ssc04380	Osteoclast differentiatio	31/1265	105/6744	0.004742	0.036834	ENSSSCG0	SOCS3/TN	ssc:49318	31
ssc04062	Chemokine signaling pat	43/1265	157/6744	0.004746	0.036834	ENSSSCG0	ENSSSCG0	ssc:55395	43
ssc05221	Acute myeloid leukemia	21/1265	64/6744	0.005005	0.037945	ENSSSCG0	CCND1/DL	ssc:10073	21
ssc04066	HIF-1 signaling pathway	30/1265	102/6744	0.005748	0.04259	ENSSSCG0	EGFR/GAP	ssc:39707	30
ssc04064	NF-kappa B signaling pat	28/1265	94/6744	0.006185	0.043835	ENSSSCG0	ICAM1/PL	ssc:39675	28
ssc04070	Phosphatidylinositol sigr	28/1265	94/6744	0.006185	0.043835	ENSSSCG0	ITPR3/PLC	ssc:10015	28