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Transformation products of pharmaceuticals occurring in wastewater effluents: molecular to population level toxicity for zebrafish embryos and larvae

Pedro Miguel Carneiro Rodrigues

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Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar;
Faculdade de Ciências da Universidade do Porto e
Faculdade de Farmácia da Universidade do Porto)

Orientador: Professor Doutor Luís Oliva Teles
Categoria: Professor Auxiliar
Afiliação: Faculdade de Ciências da Universidade do Porto (FCUP)

Coorientadora: Doutora Laura Guimarães
Categoria: Investigadora Auxiliar
Afiliação: Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Coorientador: Professor Doutor António Paulo Carvalho
Categoria: Professor Auxiliar
Afiliação: Faculdade de Ciências da Universidade do Porto (FCUP)

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Resumo

Os contaminantes emergentes têm sido cada vez mais detetados a concentrações mais elevadas em águas naturais, sendo considerados um potencial risco para espécies não alvo. Entre estes contaminantes, incluem-se os pesticidas e os fármacos. Estes compostos são potencialmente perigosos uma vez que, entram continuamente nos sistemas aquáticos, e a infraestrutura existente para o tratamento de águas não foi concebida para remover eficazmente este tipo de compostos. Recentemente, foi também demonstrado que, além dos compostos parentais, vários produtos de transformação de pesticidas e fármacos têm sido cada vez mais detetados em amostras de água doce e efluentes de estações de tratamento de água residual (ETARs). Além disso, o risco ecotoxicológico destes produtos de transformação é ainda menos conhecido do que os seus respetivos compostos parentais. Inclusive, alguns destes produtos de transformação são detetados em concentrações mais altas e considerados potencialmente mais tóxicos que os seus compostos parentais. Tendo em conta estes dados, os principais objetivos deste trabalho passaram por investigar a toxicidade dos produtos de transformação mais frequentemente detetados nos efluentes de ETAR, e com isto gerar dados para melhor compreender os modos de ação destes compostos, tendo em vista a contribuição para a construção de modelos de vias de efeitos adversos (*adverse outcome pathways, AOP)*. Após a pesquisa bibliográfica realizada para este estudo, dado o grande número de compostos para os quais falta informação relevante, determinou-se que apenas fármacos e seus metabolitos seriam alvo de estudo mais pormenorizado. A escolha recaiu nos fármacos: carbamazepina, venlafaxina, fluoxetina e tramadol, assim como nos seus metabolitos mais representativos. Devido às inúmeras vantagens que este modelo dispõe para trabalhos de ecotoxicologia, o peixe zebra (*Danio rerio*) foi escolhido como modelo teste deste trabalho. Numa primeira abordagem, foi executada uma rotina de ensaios preliminares, que foi posteriormente refinada e

melhorada. Nessa primeira avaliação experimental, os embriões de peixe zebra foram expostos a diferentes concentrações de venlafaxina e fluoxetina (compostos parentais) e ao metabolito norfluoxetina durante as primeiras 80 horas pós-fertilização (hpf). Nas concentrações teste utilizadas encontravam-se valores encontrados em águas superficiais e de efluentes de ETAR. Mortalidade e vários parâmetros do desenvolvimento embrionário foram registados. Também foi realizado um ensaio sensoriomotor e determinada a expressão génica de 38 genes. Os genes avaliados estão envolvidos em processos neuro hormonais e de destoxificação deste tipo de compostos. Os resultados obtidos nestes ensaios preliminares permitiram levantar novas questões que levaram a alguns ajustes do desenho experimental definido inicialmente. Em seguida, foram avaliados os efeitos da exposição durante 168hpf de embriões e larvas de peixe zebra aos seguintes compostos parentais: carbamazepina, venlafaxina ou tramadol (compostos parentais), e os seus respetivos metabolitos: carbamazepina-10,11-epoxi, 10,11-dihidrocarbamazepina, O-desmetilvenlafaxina, N-desmetillvenlafaxina, O-desmetiltramadol e N-desmetiltramadol (0.1−100µg/L). Os resultados obtidos demonstraram que, para as malformações no desenvolvimento embrionário, existe uma relação de dose-resposta para a maioria dos compostos e parâmetros de desenvolvimento testados. A carbamazepine-10,11-epoxi, O-desmetilvenlafaxina e o tramadol foram os compostos em que se registou maior percentagem de malformações embrionárias. Em comparação com o grupo controlo, todos os compostos testados causaram um decréscimo significativo das respostas das larvas num ensaio sensoriomotor. Ao nível da toxicidade molecular, foram encontrados efeitos significativos para a maioria dos 32 genes alvo testados, de entre os quais se destacam os genes a*bcc1*, *abcc2*, *abcg2a*, *nrf2*, *pparg* e *raraa* como comuns para os três blocos (composto parental + metabolitos) de fármacos. Para cada bloco, os modelos de expressão génica indicaram diferenças entre os padrões de expressão obtidos para os compostos parentais e os seus respetivos metabolitos. Para os blocos da venlafaxina e carbamazepina foi possível determinar possíveis biomarcadores de exposição para estes blocos. A partir destes resultados, efetuou-se seguidamente um estudo de proteómica em larvas de peixe zebra expostas por 168h a estes três compostos parentais e um metabolito de cada, em duas concentrações de exposição diferentes, para investigar mais aprofundadamente os seus modos de ação e obter outra informação crucial para o refinamento dos seus AOPs. Os resultados obtidos mostraram que a exposição a carbamazepina, venlafaxina, tramadol ou os seus metabolitos principais (carbamazepina-10,11-epoxi, O-desmetilvenlafaxina, O-desmetiltramadol) pode alterar a expressão proteica das larvas expostas e vias metabólicas essenciais. Os resultados sugeriram também, que a este nível, os metabolitos poderão ser menos

potentes que os respetivos compostos parentais, dado que causam disrupção significativa da expressão proteica a concentração mais elevada que o composto parental. O trabalho permitiu identificar proteínas diferencialmente expressas para todos os tratamentos testados que indicam que a exposição a estes compostos pode interferir com funções vitais do peixe, mesmo em exposições a baixas concentrações, na gama encontrada em amostras de água ambientais. Por último, a integração de toda a informação obtida nos diferentes níveis de organização biológica permitiu contribuir para refinar os AOPs em desenvolvimento para estes compostos na AOP-Wiki (aopwiki.org), através da criação de novas versões preliminares mais completas, aqui apresentadas.

Globalmente, os resultados obtidos e aqui apresentados demonstram que não só os compostos parentais, mas também os metabolitos dos fármacos testados, têm um impacto negativo nos embriões e primeiros estádios larvares do peixe zebra. Este impacto traduz-se num aumento das anomalias embrionárias, na redução das respostas sensoriomotoras, diferentes padrões de expressão génica e diferenças significativas no proteoma dos animais expostos, que impactam vias metabólicas essenciais para a sobrevivência dos organismos. Além disso, novas questões podem ser colocadas para investigação futura como os potenciais efeitos negativos de misturas complexas de compostos parentais e diferentes metabolitos de fármacos; efeitos tardios da exposição precoce (embrião e larva) na natação e reprodução dos animais expostos; e descrever possíveis impactos da exposição a pesticidas e seus produtos de transformação, cuja inclusão neste estuda estava inicialmente programada. Resumindo, são necessários mais esforços na investigação sobre exposição a produtos de transformação de químicos de origem antropogénica, de forma a melhor compreender os efeitos destes compostos em organismos aquáticos e fornecer dados úteis para aplicação em novas metodologias, mais eficazes, para tratamentos de água; mais campanhas de monitorização; avaliação de risco ambiental e até para aplicação de nova legislação de definição de valores regulamentares para a contaminação de águas superficiais e efluentes de ETAR destes produtos de transformação.

Palavras-chave: fármacos; produtos de transformação; efluentes de ETAR; ómicas; embriotoxicidade, *adverse outcome pathways*

Abstract

Emerging contaminants have been increasingly detected at higher concentrations in natural waters and are considered a potential risk for non-target species. Among these contaminants are pesticides and pharmaceutical products. These compounds are

potentially hazardous once they continuously enter aquatic systems, and the existing infrastructures of water treatment were not designed to fully remove such compounds. Over recent years, research has also shown that besides the parental compounds, numerous transformation products (metabolites and/or degradation products of these chemicals) of pesticides and pharmaceuticals have been increasingly detected in freshwater samples and wastewater effluents. Moreover, their ecotoxicological risks are even more unknown than the ones described for parental compounds. Some of these transformation products are found at higher concentrations than their parental compounds and considered potentially more toxic to living organisms. Therefore, the aims of this work were to investigate the toxicity of transformation products most frequently detected in effluents of wastewater treatment plants (WWTPs), contributing with information about their toxicity and modes of action useful to build adverse outcome pathways (AOP). For that, the most relevant pesticide and pharmaceutical transformation products occurring in water systems were first identified through a literature review. From, the results obtained, given the large number of compounds for which relevant information is lacking, the work was focused on the most prevalent pharmaceutical compounds and their respective metabolites. These were carbamazepine, venlafaxine, fluoxetine and tramadol and their most representative metabolites. Zebrafish (*Danio rerio*) was selected as test species because of its unique advantages as animal model. A first approach to the ensuing investigation, where a main strategy was developed and tested, was performed. Here, zebrafish larvae were exposed to venlafaxine, fluoxetine or norfluoxetine (fluoxetine main metabolite) for 80 hours post-fertilisation (hpf), at test concentrations within the range found in superficial water and WWTP effluents. Survival and several developmental hallmarks were recorded. Moreover, a sensorimotor assay and the determination of the mRNA expression of 38 different genes were evaluated. The genes were involved in neurohormonal and detoxification processes. From the obtained results, new questions arose and led to adjustments of the initial methodology. Subsequently, the work focused on investigating the effects on zebrafish embryos and larvae of 168hpf exposures to carbamazepine, venlafaxine, tramadol (parental compounds) or their respective metabolites: carbamazepine-10,11-epoxide, 10,11-dihydrocarbamazepine, O-desmethylvenlafaxine, N-desmethylvenlafaxine, O-desmethyltramadol, and N-desmethyltramadol (0.1−100µg/L). For embryonic malformations, a dose-response relationship was found for most endpoints and compounds evaluated. Carbamazepine-10,11-epoxide, O-desmethylvenlafaxine and tramadol elicited the highest malformation rates. Comparing to the control, all compounds significantly decreased larvae responses on a sensorimotor assay. For molecular toxicity, among 32 tested genes, the responses

of a*bcc1*, *abcc2*, *abcg2a*, *nrf2*, *pparg* and *raraa* were common to the three drug blocks tested. For each block, the modelled gene expression patterns indicated differences in expression between parental compounds and metabolites. Also, for the venlafaxine and carbamazepine blocks it was possible to propose potential biomarkers of exposure. Based on these results, a proteomics study was then carried out on zebrafish larvae exposed for 168h to these three parent compounds and one metabolite of each, at two different exposure concentrations, to further investigate their modes of action and obtain other crucial information for the refinement of their AOPs. The results obtained showed that exposure to carbamazepine, venlafaxine, tramadol or their main metabolites (carbamazepine-10,11-epoxy, O-desmethylvenlafaxine, O-desmethyltramadol) can alter the protein expression of exposed larvae and essential metabolic pathways. The results also suggested that at this level, metabolites may be less potent than their parent compounds, as they cause significant disruption of protein expression at higher concentration than the parental compound. The work allowed identifying differentially expressed proteins for all treatments tested, indicating that exposure to these compounds can interfere with vital functions of the fish, even at low exposure concentrations, in the range found in environmental water samples. Finally, the integration of all the information obtained at the different levels of biological organisation made it possible to contribute to refining the AOPs underdeveloped for these compounds in the AOP-Wiki (aopwiki.org), through the creation of new drafts presented here.

Overall, the obtained results demonstrate that pharmaceutical products, not only the parental compounds but also some their metabolites, can have negative impact on zebrafish embryos and early larval stages. This impact was translated into increases of developmental anomalies, reduced sensorimotor responses, different patterns of gene expression, and significant alterations in proteome of the exposed animals, impacting pathways critical for their survival. Moreover, some questions arise for long-term investigation, like potential negative effects of complex mixtures of different metabolites and parental compounds; delayed effects of early exposure to these compounds on swimming behaviour and reproduction; and describe possible impacts from pesticides exposure, which were initially thought to be included in the present research. All in all, further developments about exposure to transformation products are needed for a better understanding of the effects these compounds may elicit on aquatic organisms and provide useful knowledge to be applied in new, more efficient water treatments, monitoring campaigns, risk assessment calculations and even to establish new legislation defining regulatory levels for transformation products in natural and effluent waters.

Keywords: pharmaceuticals; transformation products; WWTP effluents; omics; embryo toxicity, adverse outcome pathways

Supporting publications

Rodrigues, P., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. (2022). Occurrence of pharmaceutical and pesticide transformation products in freshwater: update on environmental levels, toxicological information and future challenges. Reviews of Environmental Contamination and Toxicology 260, 14. https://doi.org/10.1007/s44169-022-00014-w

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Rodrigues, Pedro and Guimarães, Laura and Carvalho, António Paulo and Oliva Teles, Luís, Carbamazepine, Venlafaxine, and Tramadol Metabolites: Toxicological Effects on Zebrafish Embryos and Larvae. Available at SSRN: https://ssrn.com/abstract=4236042 or http://dx.doi.org/10.2139/ssrn.4236042 (Preprint)

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Abbreviations

2-4-D – 2,4-Dichlorophenoxyacetic
5-HT – 5-hydroxytryptamine
5-ht2c - serotonin receptor 5-ht 2c
5-ht1a - serotonin receptor 5-ht 1a
ABC - ATP-binding cassette
abcb - P-glycoprotein
abcc - multidrug resistance associated protein
abcg2 - breast cancer resistance-associated protein
adra2b - noradrenaline receptor alpha-2b
adra2c - noradrenaline receptor alpha-2c
Ahr - Aryl hydrocarbon receptor
ALDOC – Aldolase, Fructose-Biphosphate C
AMPA – aminoethylphosphonic acid
ANOVA – analysis of variance
AOP's – Adverse Outcome Pathways
ATP – adenosine triphosphate
BCAA – branched-chain amino acid
CA – cluster analysis
cat - catalase
CBZ – carbamazepine
CBZep – carbamazepine-10,11-epoxide
CHRM1 – muscarinic cholinergic receptor M1
CHRM3 – muscarinic cholinergic receptor M3
CHRNA4 – neuronal acetylcholine receptor subunit alpha-4
cyp - Cytochrome P450
Ct – cycle threshold
Ctr – control group
Cu/Zn sod - superoxide dismutase
dat - dopamine transporter
DDE – dichlorodiphenyldichloroethylene
DDT – dichlorodiphenyltrichloroethane
DEP's – differentially expressed proteins
DHEAS – dehydroepiandostrone sulfate
diCBZ - 10,11-dihydrocarbamazepine
drd1b - dopamine receptor D1B

drd2b - dopamine receptor D2B

dpf - days post fertilization

DT_{50} – dissipation time

EC_{50} – median effect concentration

EC's – emerging contaminants

ECHA – European Chemicals Agency

ECOSAR – ecological structure-activity relationship

egr – early growth response

EI – electron ionization

EPA – United States Environmental Protection Agency

ER_{50} – median effect residue

EROD – ethoxyresorufin-O-deethylase

ESA – ethne sulfonic acid

ESI – electrospray ionization

EU – European Union

FASP – Filter-Aided sample preparation

FDR – false discovery rate

FL - fluoxetine

GC – gas chromatography

GC-MS – gas chromatography-mass spectrometry

GST - glutathione S-transferase

HPLC – high pressure liquid chromatography

HPLC-MS – high pressure liquid chromatography mass spectrometry

hpf - hours post fertilization

IC_{50} – median inhibition concentration

keap – Kelch-like ECH-associated protein

KEGG – Kyoto Encyclopaedia of Genes and Genomes

KIs – inhibitory constants

LC – liquid chromatography

LC_{50} – median lethal concentration

LC-MS/MS - liquid chromatography with tandem mass spectrometry

LLE – liquid-liquid extraction

LR_{50} – median lethal residue

MANOVA – multivariate analysis of variance

mao - monoamine oxidase

MoA – modes-of-action

MS – mass spectrometry

MXR - multixenobiotic resistance
NAD – nicotinamide adenine dinucleotide
NDUFS1 – NADH; ubiquinone oxirredutase core subunit S1
NDV – n-desmethylvenlafaxine
net - norepinephrine transporter
NF – norfluoxetine
NK1 – neurokinin 1 receptor
nrf2 – NF-E2-related transcription factor
NSAID – non-steroid anti-inflammatory
NTRA – n-desmethyltramadol
ODV – o-desmethylvenlafaxine
OPRD1 – δ-opioid receptor
OPRK1 – κ-opioid receptor
OPRM1 – μ-opioid receptor
OTRA – o-desmethyltramadol
OXA – oxalinic acid
PCP's – personal care products
PCR - polymerase chain reaction
PDK – pyruvate dehydrogenase kinase
PLS – partial least squares regression
ppars - peroxisome proliferator activated receptor
Ppharm – psycopharmaceuticals
PPI – protein-protein interactions
pxr -pregnane X receptor
qRT-PCR - quantitative real time PCR
QSAR – quantitative structure-activity relationship
Rpl3 – 60s ribosomal protein L3
RNAseq – high-throughput RNA sequencing
RT – room temperature
rxr- retinoid X receptor
SCN – sodium channel genes
sert - serotonin transporter
SNRI – serotonin and norepinephrine reuptake inhibitor
SPE – solid-phase extraction
SPME – solid-phase microextraction
SSRI - selective serotonin reuptake inhibitor
TRA – tramadol

TRPV1 – transient receptor potential cation channel subfamily V member 1

UPLC – ultra performance liquid chromatography

UV – ultraviolet light

VEN - venlafaxine

vmat2 - vesicular monoamine transporter

WWTP’s - Wastewater Treatment Plants

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3.2

Figure 2. Illustrative examples of pigmentation anomalies found at 32hpf for FL (fluoxetine) and at 80hpf for NF (norfluoxetine). Control embryos and larvae are shown in the left images; exposed embryos and larvae showing pigmentation anomalies (red arrows) are shown in the right.

Figure 3. Heatmap of normalised gene expression (fold change) obtained for zebrafish larvae exposed to NF and FL for 80hpf. The magnitude and direction of variation, relative to the solvent control, are indicated by the colour scale.

Figure 4. Relative expression of genes from cluster e. The small letters indicate the homogeneous subsets indicated by the Tukey HSD for the comparison of norfluoxetine based on MANOVA on for fold change (NF1, NF2) and fluoxetine treatments (FL1, FL2) ($p<0.05$). The stars indicate statistically significant differences ($p<0.05$) relative to the solvent control (from the results of the ANOVA on normalised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

Figure 5. Relative expression of genes from cluster g. The stars indicate statistically significant differences ($p<0.05$) relative to the solvent control (from the results of the ANOVA on normalised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

Figure 6. Relative expression of genes from cluster h. The small letters indicate the homogeneous subsets indicated by the Tukey HSD for the comparison of norfluoxetine based on MANOVA on for fold change (NF1, NF2) and fluoxetine treatments (FL1, FL2) ($p<0.05$). The stars indicate statistically significant differences ($p<0.05$) relative to the solvent control (from the results of the ANOVA on normalised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

3.3

Figure 1. Mortality and gross malformations. Cumulative rates of mortality and malformations recorded in assays with zebrafish embryos exposed to norfluoxetine, venlafaxine or a mixture of these substances. * indicates significant differences ($p<0.05$) relative to the control; # indicates significant differences relative to 8hpf (hour post fertilization).

Figure 2. Monoamine receptors and transporters involved in serotonergic, noradrenergic and dopaminergic neurotransmission. Expression of monoamine receptors and transporters in embryos exposed to norfluoxetine or venlafaxine for 80hpf. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test.

Figure 3. Nuclear receptors and ABC transporters. Expression of nuclear receptors and ABC transporters in embryos exposed to norfluoxetine or venlafaxine for 80hpf. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test.

Figure 4. Cluster analysis for norfluoxetine. The analysis was based on the weighted pair-group average (1-Pearson r) and performed on the gene expression data obtained for zebrafish larvae exposed to norfluoxetine for 80hpf.

Figure 5. Cluster analysis for venlafaxine. The analysis was based on the weighted pair-group average (1-Pearson r) performed on the gene expression data obtained for zebrafish larvae exposed to venlafaxine for 80hpf.

Figure 6. Sensorimotor reflexes. Effects of norfluoxetine (3.2ng/L), venlafaxine (2000ng/L) and the mixture of both substances on the sensorimotor responses of zebrafish larvae. Possible differences among treatments were identified using the Chi-square test. Small letters indicate the homogeneous groups.

Figure 7. Interaction effects at the molecular level. Effects of norfluoxetine (3.2ng/L), venlafaxine (2000ng/L) and the mixture of both substances on the expression of serotonin, dopamine and noradrenaline receptors, the vesicular monoamine transporter, PPAR nuclear receptors and ABC transporters. Data is presented in fold change, relative to the control groups. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test. Significant interactions between norfluoxetine and venlafaxine upon gene expression were found for the monoamine receptors and the vesicular monoamine transporter.

Figure 8. Cluster analysis for the mixture. The analysis was based on the weighted pair-group average (1-Pearson r) performed on the gene expression data obtained for zebrafish larvae exposed to norfluoxetine (3.2 ng/L), venlafaxine (2000 ng/L) or their mixture.

Chapter 4.

Figure 1. Hatching rates at 72hpf for each tested drug and its two main metabolites: carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZep) and 10,11-dihydrocarbamazepine (diCBZ); venlafaxine (VEN), O-desmethylvenlafaxine (ODV) and N-desmethylvenlafaxine (NDV); tramadol (TRA), O-desmethyltramadol (OTRA) or N-desmethyltramadol (NTRA).

Figure 2. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to carbamazepine or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). Statistical comparisons were made against the solvent control. † indicates significant differences relative to the negative control at $p<0.05$. Different letters indicate significant differences among groups at $p<0.05$.

Figure 3. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to venlafaxine or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). Different letters indicate significant differences among groups at $p<0.05$.

Figure 4. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to tramadol or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). Different letters indicate significant differences among groups at $p<0.05$.

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Chapter 5.

Figure 1. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; CBZ01: carbamazepine 0.1µg /L; CBZ100 carbamazepine 100µg /L; CBZep01 carbamazepine 10,11-epoxide 0.1µg /L, CBZep100 carbamazepine 10,11-epoxide 100µg /L.

Figure 2 Log_2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

Figure 3. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L carbamazepine (CBZ0.1). The red cluster was associated with KEGG terms glycolysis/gluconeogenesis; purine metabolism; glycine, serine and threonine metabolism; metabolic pathways; carbon metabolism; biosynthesis of amino acids; valine, leucine and isoleucine degradation; fatty acid degradation; and nucleocytoplasmic transport. The green cluster was associated to KEGG amino sugar

and nucleotide sugar metabolism. The blue cluster was associated to KEGG nucleocytoplasmic transport.

Figure 4. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L carbamazepine (CBZ100). This cluster was associated to oxidative phosphorylation; porphyrin metabolism; cardiac muscle contraction; and metabolic pathways.

Figure 5. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L carbamazepine 10,11-epoxide (CBZep01). This cluster was associated to glycolysis/gluconeogenesis; fructose and mannose metabolism; galactose metabolism; starch and sucrose metabolism; amino sugar and nucleotide sugar metabolism; carbon metabolism and metabolic pathways.

Figure 6. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L carbamazepine 10,11-epoxide (CBZep100). The red cluster was associated with KEGG terms oxidative phosphorylation, cardiac muscle contraction, porphyrin metabolism and metabolic pathways. The green cluster was associated to the four remaining KEGGs: purine metabolism, alanine, aspartate and glutamate metabolism, nucleocytoplasmic transport, and RNA polymerase.

Figure 7. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; VEN01: venlafaxine 0.1µg /L; VEN100: venlafaxine 100µg /L; ODV01: o-desmethylvenlafaxine 0.1µg /L, ODV100: o-desmethylvenlafaxine 100µg/L

Figure 8. Log2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

Figure 9. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L venlafaxine (VEN01). The red cluster was associated with KEGG terms carbon metabolism, arginine biosynthesis and oxidative phosphorylation. The green cluster was associated to the two remaining KEGGs: nucleocytoplasmic transport and metabolic pathways.

Figure 10. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L o-desmethylvenlafaxine (ODV01). The red cluster was associated with KEGG term: nucleocytoplasmic transport. The green cluster was associated with the two remaining KEGGs: oxidative phosphorylation and metabolic pathways.

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Figure 12. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; TRA01: tramadol 0.1µg /L; TRA100: tramadol 100 µg/L; OTRA01: o-desmethyltramadol 0.1µg/L, OTRA100: o-desmethyltramadol 100µg/L

Figure 13 Log_2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

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Figure 15. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L tramadol (TRA100). The red cluster was associated with KEGG terms: carbon metabolism and metabolic pathways.

Figure 16. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L o-desmethyltramadol (OTRA01). The red cluster was associated with KEGG terms: oxidative phosphorylation and metabolic pathways. The green cluster was associated to two KEGG terms: arginine and proline metabolism and metabolic pathways. The blue cluster 3 was associated to the remaining KEGG term: glycolysis/gluconeogenesis.

Figure 17. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L o-desmethyltramadol (OTRA100). The red cluster was associated with KEGG terms: pentose phosphate pathway; fructose and mannose metabolism; starch and sucrose metabolism; arginine and proline metabolism; glycolysis/gluconeogenesis; biosynthesis of amino acids; oxidative phosphorylation; carbon metabolism; metabolic pathways. The green cluster was associated with the remaining KEGG term: protein processing endoplasmic reticulum.

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Chapter 1: General introduction and objectives

Chapter 1: General introduction and objectives

Water is the most precious resource from our planet and the main factor for the existence of life as we know it. However, worldwide, the human population is increasing at high rates, and our economic activity and technological advances have catalysed our role on the modification of the environment, in particular the aquatic ecosystems. These human demands oblige a continuously higher need for clean and safe water, making its quality a priority for the protection of all forms of life. However, these same demands contributed to the increasing pollution levels over the past decades, as well as the degradation of several water bodies (Cosgrove and Loucks, 2015; Pattel et al., 2020).

Scientific advances on the detection of chemicals in water samples unveiled a wide range of compounds, continuously identified and quantified in aquatic ecosystems. These compounds were later described as emerging contaminants or emerging contaminants of concern (ECs). Emerging contaminants of concern are natural or synthetic chemicals or materials, which have been increasingly detected at higher concentrations, or that are suspected to be present in different ecosystems (including aquatic), and which persistence and potential toxicity are capable of significantly interfere with the metabolism of different species, posing a threat to human and environmental health (Sauvé and Desrosiers, 2014). A wide range of compounds with different origin and purpose have been considered ECs. Among others, the most known ECs are: pharmaceuticals, pesticides, personal care products (PCPs), surfactants and plasticizers. These compounds share a common characteristic that makes them contaminants of concern. Their removal from the environment in the next years is an almost impossible task, since they continuously enter aquatic systems and the existing infrastructures of water treatment were not designed to fully eliminate them (Wilkinson et al., 2017). Over recent years, research has also shown that besides the parental compounds, numerous metabolites and degradation products (collectively referred to as transformation products) have been detected in environmental samples. These compounds are increasingly detected in freshwater samples and wastewater effluents. Though studies have indicated the potential of various of them to cause severe toxicological effects, and recognise them as ECs, the information available is still sparse and the data generated over the latest years were in need for systematised interpretation.

In this study, we started to focus on two of the main classes of ECs: pesticides and pharmaceutical products. These two classes were chosen because they are the main classes of compounds recurrently included in the watch list of substances for wide monitoring of the EU Water Framework Directive. Currently, together they represent over 88% of the listed compounds (European Union, 2022). Pesticides include a wide group

of compounds such as herbicides, insecticides, fungicides, and nematicides that are classified according to their chemical composition and/or mode of action (Arias-Estevez et al., 2007). Pesticides are applied in the soil, but they reach aquatic ecosystems through different pathways by diffuse or point-source pollution sites (Vryzas, 2018). Pesticides transport is made through different media, implying several ways to pollute water bodies. Groundwater is heavily impacted by pesticides by leaching of landfill and septic tanks or industrial leakage, while run-off from landfills after heavy rains is the main source of pesticides to surface waters (McKnight et al., 2015). Pharmaceuticals are bioactive substances of known chemical structure designed to have pharmacological properties set to the diagnose, treatment or prevention of human or animal diseases. Pharmaceutical products are continuously released into the environment, although in small quantities. After consumption by humans or animals, these compounds suffer a series of transformations on living organisms and are excreted in two different forms: unchanged (small proportion) or as active or inactive metabolites (Jjemba, 2006; Brown et al., 2015). The presence of both pharmaceuticals and pesticides in effluents from WWTPs (Wastewater Treatment Plants) is described as a main source of contamination of water bodies (Celiz et al., 2009; Wittmer et al., 2010). As previously mentioned, the treatment processes implemented in WWTPs show incomplete or low removal efficiency for several ECs, as many pharmaceuticals and pesticides. Also, in some cases, after the treatments the total burden of pharmaceutical or pesticide compounds or metabolites in the effluent can be higher than the one measured in the influent (Wittmer et al., 2010; Luo et al., 2014). Indeed, the treatments cause degradation of parental compounds into several transformation products, but conversion of metabolites back into the original compounds can also happen (Luo et al., 2014). All this originates the discharge into the environment of parental compounds and many more transformation products (Santos et al., 2010; Luo et al., 2014). Nevertheless, investigation about occurrence and fate of these products in the aquatic systems has been neglected. Moreover, for many of them, information about the detrimental effects and toxicological risk they may cause is sparse and they are not included in regulatory monitoring frameworks (Murray et al., 2010; Evgenidou et al., 2015; NORMAN network, www.norman-network.net). Despite this, harmful effects of these contaminants have been described in several reports and are recognised by the scientific community. For aquatic species, exposure to these compounds were reported to have impact in species survival, behaviour, embryonic development, reproduction, growth, endocrine disruption, disturbance of different organs function, and disruption of molecular biomarkers, among other more specific toxic effects (Atzei et al., 2021; Gao et al., 2021; Huerta et al., 2020; Rozmankova et al., 2020; Rodrigues et al., 2020; Isidori et al., 2005). Additionally, some metabolites are found at

higher concentrations than their parental compounds and, most importantly, may be more toxic and consequently more dangerous for the wildlife (Wittmer et al., 2010; Celiz et al., 2009). While such reports raise high concern, most of the existing literature is still focused on toxicity caused by exposure to the parental compounds. Investigation of the toxic effects and modes of action of transformation products in non-target species, like aquatic ones, has been mostly neglected. From the above it comes clear their potential for environmental impact, high contribution to the toxicity of wastewater effluents and the urgent need to investigate their effects on non-target aquatic species.

The initial main objective of this thesis was therefore to investigate the toxicity of transformation products from pharmaceuticals and pesticides most frequently detected in effluents of wastewater treatment plants, contributing with information about their modes of action useful to build adverse outcome pathways (AOP). However, from the data collected on the literature review (Chapter 2) and given the high number of compounds for which information is lacking, only pharmaceutical compounds were studied in more detail herein. As previously mentioned, demographic, economic and technological advances increased the need for clean water, but also the pollution levels on aquatic ecosystems. Therefore, an effort to reduce these pollution levels, promoting conditions for ecosystem conservation or restoration/recovery, should be a focus in the near future. Moreover, there is a need to develop relevant and sensitive toxicological information and tools useful to assess the toxicological efficiency of new wastewater treatment technologies, targeting the removal of such complex chemicals. On the other hand, if a chemical can cause hazardous effects, working within the AOP framework and contributing to its development can enhance risk characterisation. An AOP describes a biological pathway linking molecular alterations to apical adverse outcomes (Ankley et al., 2010). It involves a characterisation of various levels of biological organisation, identifying the sequence of molecular events and the subsequent cascade of cellular and higher-level events that will cause ultimately a toxic effect in an organism exposed to a chemical substance. Owing to this, AOPs have been recognised as providing a relevant and consistent basis to organise toxicological information and integrate dose-response with exposure assessment (Perkins et al., 2019). They are considered important for regulatory purposes and advised for use in different stages of environmental risk assessment (e.g., hazard assessment to risk characterization) or to predict adverse effects of chemicals, for example (Marty et al., 2017). Hence, the specific aims of this work were: i) to identify relevant metabolites or degradation products from pharmaceuticals and pesticides occurring in water systems more frequently and at higher concentrations; ii) to assess their potential teratogenicity and early developmental effects in relation to their parental compounds; iii) to investigate their molecular modes-of-action

and metabolism; iv) to produce high throughput proteomic information for identification of other relevant toxicological biomarkers of exposure and/or effect; and v) to make a first integration of the data gathered in the AOP framework, producing new draft AOPs for the chemicals investigated whenever possible. It is important to notice that specific objectives ii) to v) were only applied to pharmaceutical transformation products.

The toxicological evaluation was based on the zebrafish embryotoxicity assay, which was extended up to 168 hours post-fertilisation (hpf) to allow investigation of early larval development and behaviour. Zebrafish (*Danio rerio*) was selected because of its unique advantages as model species. It can reproduce all year long, easily providing a high number of embryos for the toxicity assays, and these embryos are transparent, allowing effective monitoring of early developmental life stages (Prakash et al., 2019). Moreover, using zebrafish embryos and early larvae in research also complies with ethical guidelines on animal experimentation. For these reasons, it is a very successful model in the investigation of various human diseases, as well as in toxicological research to assess detrimental effects of chemicals and their modes of action.

To meet the objectives established, a review of the available literature was first carried out and used to plan the subsequent research. Therefore, after this general introduction, chapter 2 presents, as a state-of-the-art to the subject, the literature review about the occurrence of pesticides and pharmaceutical metabolites and degradation products (together often referred to as transformation products) in freshwater, as well as the environmental levels they occur, their known toxicological effects, and future challenges associated to this type of contaminants (Rodrigues et al., 2022). From this review and the conclusions obtained herein, we chose to study only pharmaceutical compounds. In fact, pharmaceutical transformation products identified in different water compartments have been less studied than pesticides and existing toxicological data are described for concentrations usually much higher than the environmentally relevant ones. Considering this scenario, subsequent work was focused on main metabolites of four pharmaceuticals: carbamazepine (an anticonvulsant medication indicated for the treatment of epilepsy and neuropathic pain), venlafaxine and fluoxetine (selective inhibitors of monoamine uptake prescribed to treat depression and obsessive-compulsive disorders) and tramadol (an opioid medication recommended to deal with moderate up to moderately severe pain). These parental compounds belong to three different therapeutic classes and are among the most representative ones found in environmental samples. Following this, the third chapter provides a first approach to the ensuing investigation where a main strategy was developed and refined. Here, zebrafish larvae were exposed to venlafaxine and fluoxetine (parental compounds) and norfluoxetine (fluoxetine metabolite) for 80hpf at different test concentrations within the

range found for superficial water and WWTP effluents. Two different works were then performed and included in this chapter (Rodrigues et al., 2020; 2022). The first one had the purpose of comparing the toxicity of a parental compound (fluoxetine) and its metabolite norfluoxetine. The second had the purpose to compare the toxicity of venlafaxine and norfluoxetine, as well as identify toxic effects of the mixture of both compounds. Survival and several developmental hallmarks were registered, and the determination of the mRNA expression of 38 different genes were evaluated in both studies. Moreover, a sensorimotor assay was included in the second study. The selected genes were involved in neurohormonal and detoxification processes and were selected according to available knowledge on the mode of action of these substances in other vertebrates. The results (Rodrigues et al., 2020; 2022) allowed to draw information about the toxicity and mode of action of fluoxetine and norfluoxetine. It was also possible to describe potential interactions between norfluoxetine and venlafaxine. From the obtained results, new questions arose and led to adjustments of the initial methodology. The duration of the exposures was increased to understand if effects were influenced by presence of the chorion, and to avoid an analytical time-frame coincident with hatching, which could influence the molecular analysis, as well as to allow for limited influence of the yolk sac on the proteomic analysis. Some genes were substituted or left out of the final testing due to the absence of significant differences in the preliminary works. The proteomic study was added to the methodology to better depict which pathways were significantly impacted by pharmaceutical parental compounds and metabolites exposure and correlate with the results obtained in the other assays, and to contribute to the drafts of the AOP models. Chapter 4 was thus dedicated to a global characterisation, in relation to the parental compounds, of the effects of single exposures to three blocks of drugs selected from the results of Chapter 2: carbamazepine (CBZ) and its metabolites carbamazepine-10-11-epoxide (CBZep) and 10-11-dihydrocarbamazepine (diCBZ); venlafaxine (VEN) and its metabolites o-desmethylvenlafaxine (ODV) and n-desmethylvenlafaxine (NDV); tramadol and its metabolites o-desmethyltramadol (OTRA) and n-desmethyltramadol (NTRA). The assays tested environmental levels of these compounds in 168hpf exposures, evaluating their potential embryotoxicity. Moreover, behaviour was studied by means of a sensorimotor assay, and molecular toxicity was investigated through evaluation of mRNA expression, as previously indicated. From the results obtained in this study (Rodrigues et al., submitted), metabolites and concentrations of interest were selected for proteomic evaluation using shotgun proteomics methodology and the assays described for the previous chapter. Thus, chapter 5 presents the shotgun proteomics assays for the selected concentrations and compounds; CBZ, CBZep, VEN, OVD, TRA and OTRA. Results from this chapter

provided further insight about the effects of the tested compounds on the expression of the total proteome in 168h zebrafish larvae and allowed the identification of all pathways impacted by exposure to these compounds, thus producing essential information for the drafting of new AOPs for these three groups of drugs. Chapter 6 is dedicated to the main discussion of the results obtained throughout this investigation and presents the new AOP drafts for each of the three groups of chemicals (parental compound and respective metabolites). In Chapter 7 the main conclusions are presented, and future challenges and new promising investigation paths are addressed.

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Chapter 2: Occurrence of pharmaceutical and pesticide transformation products in freshwater: update on environmental levels, toxicological information and future challenges

Chapter 2. Occurrence of pharmaceutical and pesticide transformation products in freshwater: update on environmental levels, toxicological information and future challenges

2.1 Pesticide and pharmaceutical transformation products as environmental contaminants

Over the past decades, scientists produced a wealth of information about the toxic effects of pesticides and pharmaceuticals on freshwater species. Both groups of compounds are widely used in the world, with recognised benefits for human health and welfare (Santos et al., 2010; Mcknight et al., 2015). Moreover, given today's social habits, together with the increase in life expectancy, the human population growth and the consequent increase in food demand, the use of both pharmaceuticals and pesticides is globally escalating. More so under the actual pandemics caused by the SARS-CoV-2 coronavirus. Despite their benefits for humankind, these classes of chemicals have also been associated with severe human and environmental health risks and are common micropollutants of freshwater systems (Corcoran et al., 2010; Santos et al., 2013; Reemtsma et al., 2013; Ortiz de García et al., 2014; Mcknight et al., 2015). Pesticides and pharmaceuticals are designed to have specific biological activity, exerting the desired effect before undergoing excretion and/or degradation. These same characteristics also make them persistent in the environment, ultimately causing toxicity to non-target fauna and flora (Fent et al., 2006; McKnight et al., 2015). Owing to these characteristics, and the still limited available information, they are the most representative classes included in the watch list of substances for Union-wide monitoring of the Water Framework Directive. Currently, they represent over 88% of the listed compounds (European Union, 2022).

Pesticides occur in natural water, mainly by runoff from agricultural fields where they are applied and through industrial wastewater. Although soils can store a good amount of pesticides due to the high affinity of these compounds to organic matter, surface water and groundwater are susceptible to pesticide contamination because of the existing soil-water interconnections (Sharma et al., 2019). Due to their increased use, detection of pesticides in different water compartments is becoming more and more frequent (Corcoran et al., 2010; Reemtsma et al., 2013; Ortiz de García et al., 2014; Evgenidou et al., 2015; Vryzas, 2018). On the other hand, the distribution of pharmaceutical substances in the environment is predominantly made by aqueous transport of compounds contained in discharged wastewater effluents, which persisted through the

conventional treatment processes (Khan et al., 2020). Contamination by pharmaceutical compounds may also occur by terrestrial runoff from agricultural fields, and aquaculture activities (Hong et al., 2018).

Once pesticides and pharmaceuticals reach the aquatic environment, they undergo a series of abiotic and biotic transformation and degradation processes. Hydrolysis, photodegradation and biodegradation are considered the most important mechanisms involved in their transformation or degradation (Syafrudin et al., 2021; Khan et al., 2020). Hydrolysis is an abiotic degradation process that creates products more polar than the parental compounds. These reactions are mainly catalysed by hydrogen or hydroxide molecules (Bavumiragira et al., 2022). Photolysis or photochemical degradation of pesticides and pharmaceuticals occurs by decomposition of these compounds in the presence of ultraviolet (UV) light. In water compartments exposed to sunlight, pesticides and pharmaceuticals containing chemical functional groups able to absorb solar radiation are prone to photolysis. The reaction transforms parental compounds into transformation products that are usually more biodegradable and hydrolysable (Wilkinson et al., 2017; Bavumiragira et al., 2022). Biodegradation is a biotic process that can result in the partial or complete transformation of pesticides and pharmaceuticals. by microorganisms. These microorganisms are present in wastewater treatment plants (WWTPs) or occur naturally in suspended solids, sediments, or animals microbioma (Wilkinson et al., 2017). Microbial degradation is recognised in the literature as having an important role in the degradation of several pharmaceuticals in a wide range of water compartments (Christensen and Li, 2014). For pesticides, microbial degradation includes the mineralisation process, which consists in the break of a parental pesticide into carbon dioxide and co-metabolisation where microbial-catalysed reactions break pesticides into other chemical forms (Syafrudin et al., 2021). Surface waters receiving wastewater effluents rich in microorganisms are usually prone to show higher biodegradation effectiveness. High rates of biodegradation are typically observed along the sediment-water interface in water bodies and wetlands (Li et al., 2016). Moreover, many pesticides and pharmaceuticals are hydrophobic, easily sorbing onto suspended solids. Therefore, concentration of compounds sorbed to sediments are influenced by the water dynamics (Syafrudin et al., 2021; Bavumiragira et al., 2022). However, the effectiveness of these degradation processes is also dependent on secondary conditions. Low turbidity, small depth, low total organic carbon content and sandy sediments favour the degradation of pesticides and pharmaceuticals (Baena-Nogueras et al., 2017). On the other hand, different physicochemical characteristics of both the water system and the type of compound can lower the degradation efficiency (Syafrudin et al., 2021; Bavumiragira et al., 2022). Nevertheless, the described processes originate transformation products that

enter in natural water by a panoply of different sources. In recent years, several works have reported the detection of these transformation products in the range of ng to µg/L, sometimes at concentrations even higher than those found for the parental compounds (le Cor et al., 2021). However, the focus of reports was primarily on the detection and quantification of the parental compounds. Concern about their transformation products, with the involvement of more groups in this research, took off mostly in the last decade, especially for pharmaceutical transformation products.

Investigation about the occurrence and fate of transformation products in the aquatic environment skyrocketed in recent years mainly due to advances reached in the chemical analytical methods (Fent et al., 2006; Valls-Cantenys et al., 2016). New instruments and methods with higher separation efficiencies, ability to find more polar compounds and deal with confounding matrix effects, appeared allowing scientists to detect trace concentrations in environmental compartments (Fent et al., 2006; Celiz et al., 2009; Valls-Cantenys et al., 2016). Previous excellent reviews have been dedicated to this topic, though mostly to pharmaceutical and personal care products, or emerging contaminants of concern, and less so to pesticides (La Farre et al., 2008; Celiz et al., 2009; Mompelat et al., 2009; Evgenidou et al., 2015; Picó & Barceló, 2015; le Cor et al., 2021; Ibanez et al., 2021; Mosekiemang et al., 2021; Madikizela et al., 2022). Furthermore, the number of works produced about this theme suffered a remarkable increase in recent years. Many of these compounds, parental or transformation products, are however little known in terms of potential detrimental effects and not included in the regulatory monitoring frameworks. Hence, they are nowadays recognised as emerging contaminants of concern (Murray et al., 2010; Evgenidou et al., 2015; NORMAN network, www.norman-network.net). Pesticides and pharmaceuticals, in particular, are the two main classes of chemicals continuously represented in the watch list of the Water Framework Directive and are thus the focus of this review.

The aim of this literature review was to identify ecotoxicological knowledge gaps limiting the risk assessment of transformation products of pesticides and pharmaceuticals found in aquatic samples. We present and discuss updated information about quantification methods, occurrence, fate, and the effects of transformation products of these two classes of chemicals. Over recent years, information has been published that needed to be systematised and appraised to bring understanding about their potential impacts on human health and aquatic biota. An important aspect, still enigmatic, is whether these transformation products are more harmful to non-target organisms than their parental compounds and which other factors may influence their potential toxicity. Another problem is the concern raised by transformation products not only as sole compounds per se but also in complex mixtures; mixtures of different metabolites of the same

substance and mixtures of different substances, including parental compounds and transformation products.

2.2 Applied Methodology

The literature review carried out focused on the global occurrence and fate of the target contaminants in different freshwater systems (i.e., surface-, ground- and influent/effluent wastewater), as well as on the available toxicological and ecotoxicological data. It covers articles published between 1997 and 2022, which have been searched using SCOPUS, Web of Science, Pubmed, and Google Scholar databases. The terms “pesticides” or “pharmaceuticals” were searched for in combination with “transformation products” or “degradation products”, “metabolites”, “freshwater”, “quantification”, “human health” or “aquatic species”. The search fields were the “article title”, “abstract” and “keywords”. Criteria for inclusion of articles in the review were related to the detail provided by the studies (e.g. quantification of the transformation products identified, suitable information about the species employed in the biotests, the age of the exposed organisms, relevant exposure design and endpoints assessed), as well as authors' awareness and control of essential experimental conditions that may bias the test results. All analytical methods of quantification have been included, rather than focusing on the most widespread quantification techniques. Adding to this, most articles available in the literature are directed to parental compounds. Some of these works identify a few metabolites. Others do not include terms related to transformation products in the search fields, and so they may not have been detected. Some articles identify different transformation products but do not quantify them, preventing prediction of their concentration in environmental samples (e.g., Mosekiemang et al., 2021; Madikizela et al., 2022). Articles about degradation experiments of pesticides and pharmaceuticals under controlled conditions have also been included, since such transformation processes can occur in natural conditions.

2.3. Sources and fate of environmental contamination

2.3.1 Transformation products of pesticides

Pesticides have been used since ancient times. Most of them were mainly inorganic compounds or substances of natural origin. However, the development and synthesis of organic pesticides after the second world war increased exponentially its use, making pesticides the second most used group of substances in the environment, only behind

fertilizers (Davis, 2014; Stokstad and Grullon, 2013). Millions of tons of pesticides are applied each year, predominantly in agriculture, which is the main activity responsible for the leaking of pesticides and their sub-products into freshwater ecosystems (Fenner et al., 2013). Although applied in the soil, pesticides can reach aquatic ecosystems through non-point source/diffuse or point-source pollution sites (Vryzas, 2018; Figure 1).

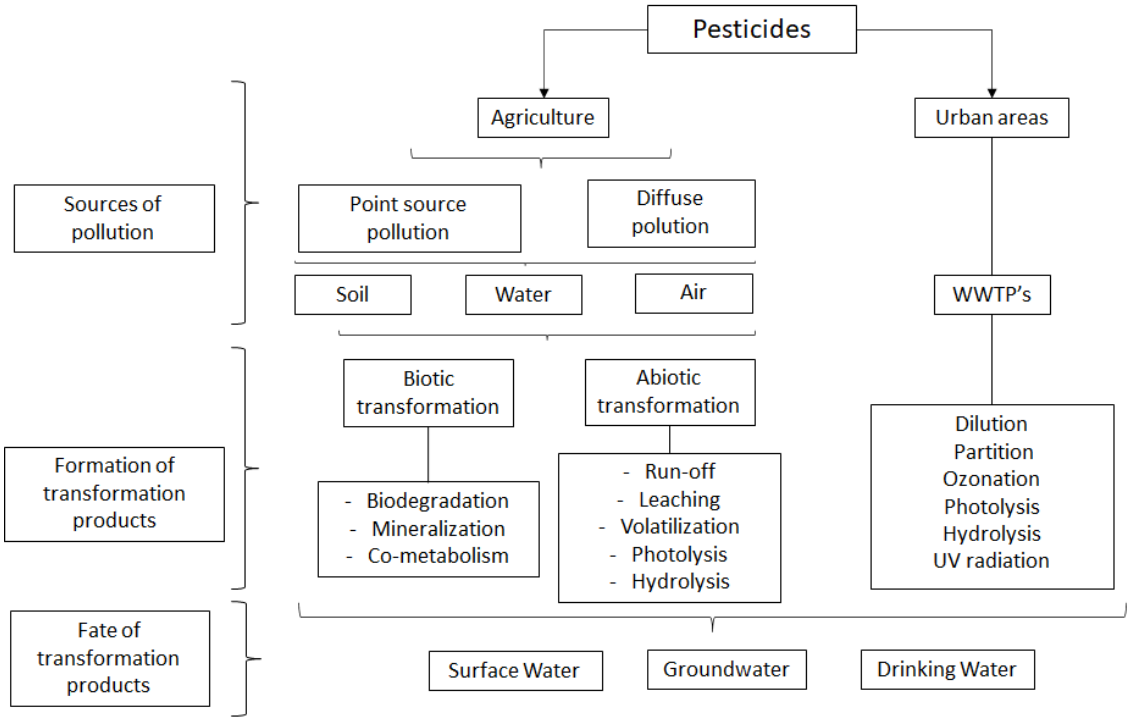

Figure 1. Sources of pollution, as well as formation and fate of transformation products deriving from pesticides.

Diffuse pollution sources are responsible for low concentrations of pesticides in the environment, while point-source pollution is responsible for their large accumulation (Vryzas, 2018). Diffuse or non-point-source pollution is related with the movement of pesticides from large areas across the watersheds that reach the aquatic environment. Point-source pollution is related to a specific identifiable source which can include chemical run-off during storage, loading, disposal, as well as the misapplication of pesticides to water bodies (Syafrudin et al. 2021). Groundwater is heavily impacted by pesticides, and their metabolites or degradation products (e.g. N,N-dimethyl-sulfamide; aminomethylphosphonic acid; 2,6-Dichlorobenzamide), mainly resulting from point-source pollution (Postigo and Barceló, 2015). Leaching of landfill and septic tanks or industrial leakage are among the main sources of groundwater contamination by pesticides and metabolites (Postigo and Barceló, 2015). Pesticide characteristics are responsible for higher or lower rates of leaching to groundwaters. Also, they play an essential influence on the degradation of pesticides and the formation of transformation products, including active metabolites. Sorption–desorption, volatilization, chemical and

biological degradation, uptake by plants, soil infiltration and leaching are some processes responsible for the appearance of new metabolites and their transportation into groundwater (Arias-Estevez et al., 2007). Groundwater tends, however, to be less affected by contamination than other water bodies, due to the natural attenuation capacity of aquifers and their large capacities (Postigo and Barceló, 2015). Nonetheless, recent monitoring studies show that pesticides, mainly herbicides, and their metabolites/degradation products (e.g. desethylatrazine; cyanazine amide) are present in aquifers.

Surface waters are mainly contaminated by diffuse pollution sources. Run-off of pesticides and metabolites from landfills after heavy rains are the main pathways of transportation of these substances to surface waters (Vryzas, 2018). Moreover, during rainfall pesticides and degradation products imprisoned in soil or sediments can reach surface waters due to movements of those sediments (Vryzas, 2018). Application of pesticides using sprays or even the plantation of seeds can be a source of surface water contamination, thanks to wind dispersal (Vryzas, 2018). In these waters, photodegradation is the main process responsible for the degradation of pesticides. The formation of such metabolites can reach higher concentrations and show higher toxicity than the parental compounds (Reddy and Kim, 2015). Wastewater treatment plants are also among the main sources of point-source pollution. Pesticides applied in urban areas (e.g., in maintenance of green areas or ponds) tend to finish in WWTPs, where traditional wastewater treatment methods are ineffective for the removal of these compounds (Rousis et al., 2017; Munze et al., 2017). Moreover, WWTPs effluents show in some cases higher concentrations of pesticides and their transformation products, as well as more toxicity, than the influents. Owing to all this, pesticides and their metabolites can ultimately reach drinking water, exposing humans, as indicated by their detection in the serum and blood of some patients in clinical and scientific studies (Chau et al., 2015; Tyagi et al., 2015).

2.3.2 Transformation products of pharmaceuticals

According to Daughton (2016), the first studies regarding the presence of pharmaceuticals in the environment date back to the 1940s. Later on, between the 60s and 70s, several works were produced about the possibility of contamination of drinking and surface water by pharmaceuticals, through the discharge of wastewater effluents (e.g., Stumm-Zollinger and Fair, 1965; Hignite and Azarnoff, 1977). Nowadays, pharmaceutical products are continuously released into the environment, although in small quantities (Figure 2).

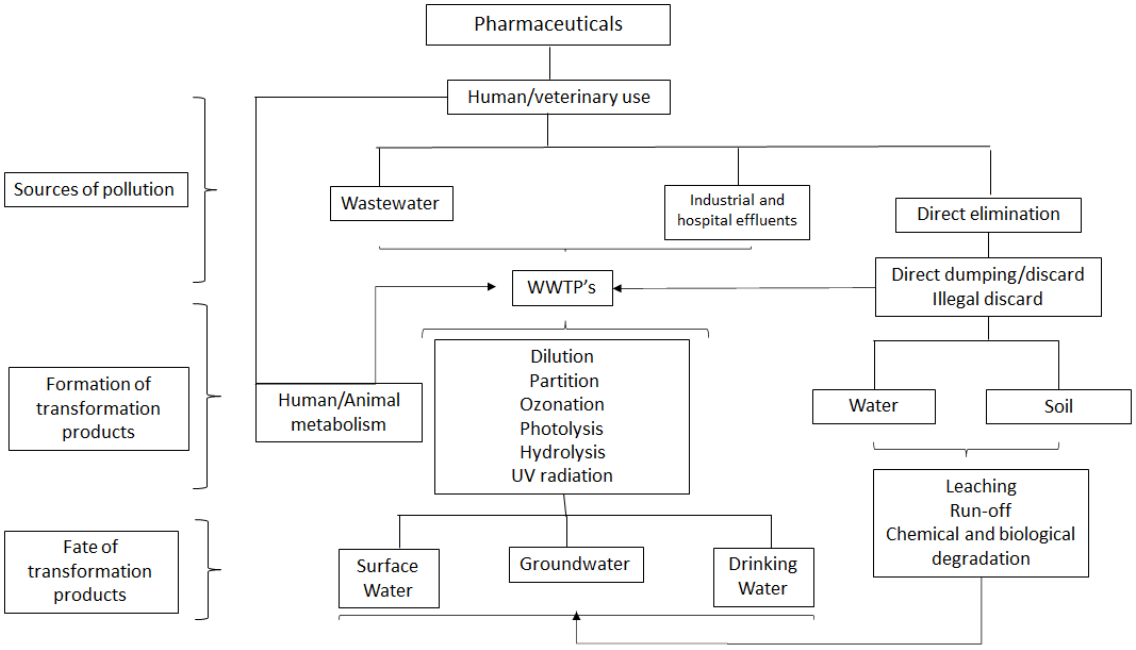

Figure 2. Sources of pollution, as well as formation and fate of transformation products deriving from pharmaceuticals.

After consumption by humans, pharmaceuticals pass through the liver where they are directly effluxed from the organism (phase 0) or enter phase I and phase II of drug metabolism (Figure 3). In phase I, more polar metabolites, often still active, are produced through oxidation, reduction, or hydrolysis reactions. These reactions are commonly mediated by different CYP450 genes (e.g., CYP1A; CYP2B; CYP3A). Many of these transformation products become substrates of phase II, where endogenous hydrophilic groups are added through methylation, glucuronidation, acetylation, sulfation or conjugation with glutathione or amino acids such as glycine, taurine, and glutamic acid to form water-soluble inactive compounds that can be excreted by the body in phase III (Figure 3). Phase III excretion is mediated by ABC transporters and different solute carriers. Due to such reactions, pharmaceuticals can thus be excreted by humans in different forms: unchanged (small proportion) or as active or inactive metabolites (Jjemba, 2006; Brown et al., 2015).

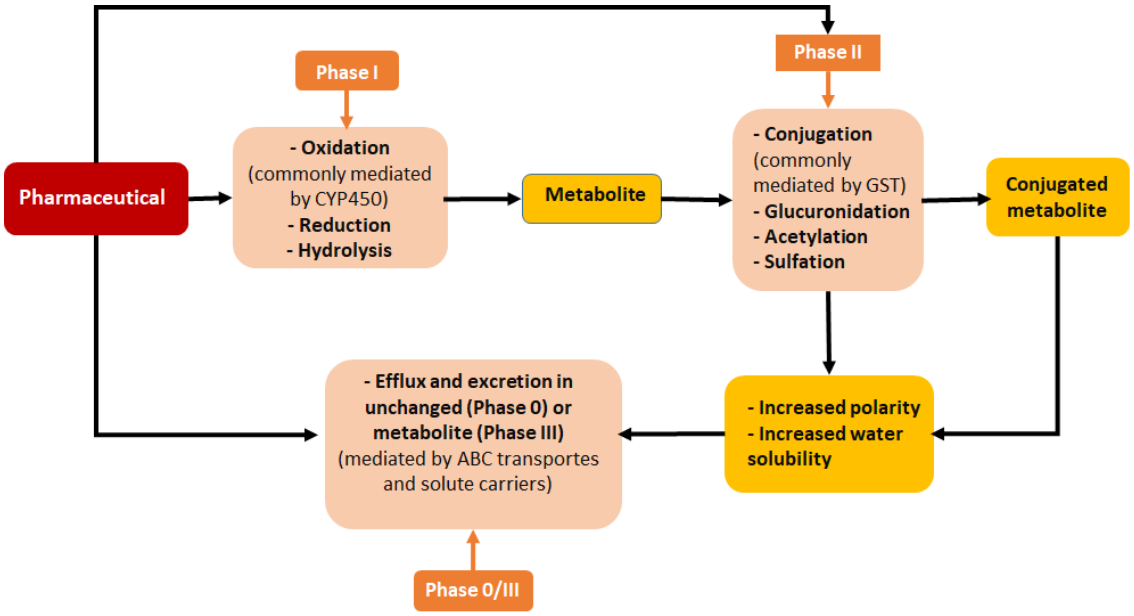

Figure 3. Schematic representation of pharmaceutical's biotransformation and excretion.

Hospital effluents and direct elimination (e.g., through inadequate sanitary disposal) of unused pharmaceuticals in sewage are therefore among the most important sources of water contamination (Santos et al., 2010) by pharmaceutical transformation products. Influents are treated in WWTPs (Wastewater Treatment Plants) by three main processes of pollutant removal. In the first treatment, the removal of suspended solids occurs. This treatment has a low degree of efficiency in the removal of micropollutants like pharmaceuticals (parental compounds or metabolites). In the second treatment, several types of reactions occur, such as dilution, partition, biotic and abiotic transformation (Luo et al., 2014). In this treatment, the level of efficiency is variable depending on the substance or metabolite in question, as well as their physicochemical properties (Luo et al., 2014). The third treatment is related to health questions to humans or specific uses of the treated water. It consists in further removal of substances like nitrogen or phosphorus, and it is not mandatory, in general (Guardabassi et al., 2002; Luo et al., 2014). After this processing, in some cases, the total load of pharmaceutical compounds or metabolites in the effluent can be higher than that in the influent (Luo et al., 2014). This can be explained by the degradation of parental compounds into several metabolites or degradation products and the transformation of metabolites back into the parental compounds that can occur during the biological treatment in the WWTPs (Luo et al., 2014). Parental compounds and metabolites can also be imprisoned in faecal matter and released into the water during the biological treatment, thus increasing the overall concentration of those substances (Luo et al., 2014). This shows that the treatments available in WWTPs are still not fully efficient in the removal of these micropollutants. Hence, discharge of contaminated effluents introduces into natural

waters the parental compounds and many more metabolites or transformation products (e.g., venlafaxine, tramadol, O-desmethyltramadol) (Santos et al., 2010; Luo et al., 2014). Additionally, some of these pharmaceutical metabolites are expected to be more toxic than parental compounds and consequently more dangerous to the wildlife (Celiz et al., 2009). The use of medicines is not exclusive to humans. These are also used in agriculture and aquaculture to treat diseased animals. As in humans, they are also excreted mostly as metabolites in the urine and faeces of animals or through adsorption in dirt pounds and after tanks cleaning, thus entering the environment without any kind of treatment and contaminating the soil and water (Santos et al., 2010). This contamination contributes to further input of transformation products into natural waters via run-off and leaching from the affected soils (Kemper, 2008). Other anthropological activities also act as sources of contamination. Industry discharges (sometimes illegally), the use of WWTPs sludge contaminated with all kinds of pharmaceutical compounds as fertilizer, or leakage of septic tanks from households still not connected to the sewage systems, are examples of these (Carrara et al., 2008; Santos et al., 2010).

2.4. Detection of pesticide and pharmaceutical transformation products in water compartments

2.4.1. Analytical methods of pesticide and pharmaceutical transformation products

As previously mentioned, knowledge about contamination of the aquatic environment by pesticides and pharmaceutical transformation products has increased, mainly due to advances reached in analytical methods (Fent et al., 2006; Valls-Cantenys et al., 2016). New methods, with higher separation efficiencies and the ability to find more polar compounds, appeared. This allowed scientists to detect concentrations in environmental compartments in the order of ng/L and µg/L and consequently raised awareness and concern about their potential hazardousness (Fent et al., 2006; Santos et al., 2010; Valls-Cantenys et al., 2016). However, these advances in analytical methods, are not efficient if the correct sample preparation is not performed. The extraction of the analytes from an environmental water sample is a crucial step before the instrumental analysis. Extraction techniques are based on the passage of an analyte by different solvents, which must be the most suitable for the type of analytical tool to be employed (Rutkowska et al., 2019; Campanale et al., 2021). This step can highly influence the analytical process, mainly for quantitative analysis, since the analyte volume must be increased, while any interferences must be eliminated (Campanale et al., 2021). Several sample

preparation techniques are already described in the literature. For analysis of water samples, SPE (solid-phase extraction) is the most extensively used technique (Dimpe et al., 2016; Campanale et al., 2021). This method uses columns or disks able to retain the active compounds present in water samples and posteriorly release them by washing with small quantities of suitable solvents (Dimpe et al., 2016; Campanale et al., 2021). This provides an extract with few interferences, suitable for different analytical methodologies, such as High-Pressure Liquid Chromatography Mass Spectrometry (HPLC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS). More recently, a new SPE-based approach has been tested: Solid-Phase-MicroExtraction (SPME). This newer method is faster and requires fewer quantity of solvents and is well described as suitable for Gas chromatography (GC) analysis (Campanale et al., 2021). Liquid-liquid extraction (LLE) is a simple method widely used for water samples, that is also applied in the analysis of pesticide and pharmaceuticals (Dimpe et al., 2016; Campanale et al., 2021). This method has the advantage to be well established among different governmental agencies, but it also is time-consuming and requires the use of organic solvents that are harmful to the environment and even the handler (Dimpe et al., 2016; Campanale et al., 2021). Though the techniques described are the most well established for pharmaceuticals and pesticides, they have some disadvantages. One is the loss of more volatile analytes during the extraction process, which can affect the result of the analysis; another is the use of toxic solvents (Dimpe et al., 2016; Campanale et al., 2021). Much more methods are available in the literature, although less widespread. The development of new cost-effective and green methodologies for fast extraction is the next challenge in need to be addressed to improve and analytical determination. Regarding the analytical methods and instrumentation, GC and/or liquid chromatography (LC) coupled to mass spectrometry (MS) are, nowadays, the most applied methods to detect pesticides and pharmaceutical compounds. For LC, some variations to this method are well established in the literature, such as high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) (Gumustas et al., 2013). Gas chromatography is the most suitable method to separate non-polar parental compounds and their transformation products (Sparkman et al., 2011). This happens because of the inclusion of a derivatization process during GC, that increases volatility and sensitivity, but also increases the duration of the procedure (Subramaniam et al., 2013). Coupling the GC with the MS, has the advantage to offer a specific mass spectrum for a certain compound when an electron ionization (EI) is also performed (Foltz et al., 2016). Polar pharmaceutical or pesticide and their transformation products are mainly separated by LC (Martín-Pozo et al., 2019). Most of the pesticides and pharmaceuticals in their unchanged form or as transformation products are usually

quantified at low concentrations in environmental water samples (ng to µg/L). This makes liquid chromatography with tandem mass spectroscopy (LC-MS/MS) a widely used method for determination of these compounds. This is due to its higher discrimination between the analyte and matrix signal, coupled to robustness and relative ease of use (Kaufmann et al., 2012). However, the selectivity and sensitivity of the MS varies with the selected ionization. Electrospray ionization (ESI) is the most chosen technique for detecting pharmaceuticals, since it is the most potent ionization method for the target compounds (Huang et al., 2019).

2.4.2 Levels in different water compartments

Transformation products of pesticides

As mentioned above, advances in the detection techniques led to an increase in knowledge about the occurrence of pharmaceutical and pesticide transformation products in different water compartments. Nevertheless, the quantification of transformation products is still not a focus in scientific investigation, as often studies only present new methods of detection and their validation, but not the concentrations found in real samples, even for parental compounds (Wode et al., 2015; Boix et al., 2016). Overall, the search carried out in the scientific databases returned 87 articles providing concentrations of pesticide and pharmaceutical transformation products in environmental water samples. Only one article quantified both pesticide and pharmaceutical transformation products (Huntscha et al., 2012). Of these, 29 articles were dedicated to pesticides, presenting concentrations obtained for 92 transformation products resulting from 43 parental compounds (Figure 4, Table S1 in the Appendix A1.). The most assessed pesticide transformation products (69%) belonged to three functional classes: organochlorine and chloroacetanilide herbicides/pesticides; triazine herbicides and organophosphate and carbamate pesticides (Figure 4, Appendix A1. Table S1). Data about the quantification of pesticide transformation products found in different water samples, and respective detection methods, are presented in Figure 5 and Table S1 (Appendix A1.). Transformation products of triazine herbicides were frequently reported in different studies, with a special focus on atrazine and terbuthylazine (Figure 5, Appendix A1. Table S1). Concentrations of these varied widely from 0.046µg/L for desethylatrazine to 124,01µg/L for hydroxy-terbuthylazine. The high concentration found for hydroxy-terbuthylazine resulted from an experiment where the parental compound was applied in a constructed wetland planted with *Typha latifolia* (Papadopoulos et al., 2007). According to the authors, the maximum concentration of the metabolite was within

the highest concentration range found for the parental compound. This is of main concern, given that these concentrations are in the order of µg/L. Though knowledge about the environmental impact of this transformation product is sparse, recent works highlighted negative effects on the early developmental stages of fish species even at concentrations found in natural water samples (Velisek et al., 2014).

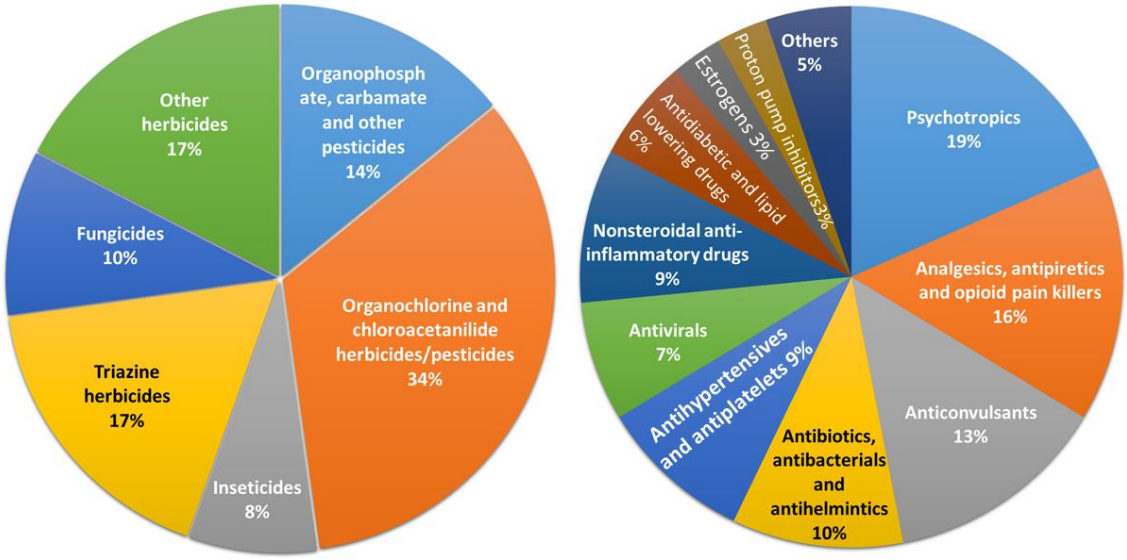

Figure 4. Relative frequency of transformation products quantified in environmental water samples per functional class of pesticides or pharmaceuticals. Concentrations for 92 pesticide transformation products derived from 43 parental compounds, and 98 pharmaceutical transformation products derived from 64 parental compounds, were found in the scientific literature published between 2000-2020.

Chloroacetanilide herbicides are widely used for grass control in several crops. Compounds of this class are structurally similar and were extensively used from the mid-1990s until recently (Elsayed et al., 2015). The main transformation products ethane sulfonic acid (ESA) and oxalinic acid (OXA), alongside the parental compound, are easily transported to water bodies and usually detected in both surface and groundwater (Appendix A1. Table S1), contributing to the degradation of water quality (Baran and Gourcy, 2013). Another interesting observation is the concentration level of metolachlor OXA and ESA in relation to the parental compound. Studies reported that metabolites of metolachlor, mainly ESA, were found in groundwater at higher concentrations than the parental compound (White et al., 2009; Baran and Gourcy, 2013). This may occur because metabolites adsorb less to soil particles, compared to the parental compound, and are thus more prone to infiltration to aquifer recharge (Baran and Gourcy, 2013). This highlights the importance of monitoring programs not only for pesticides alone but also for their transformation products.

One of the first mass-produced pesticides in the world was DDT (Dichlorodiphenyltrichloroethane). It is an inexpensive and highly efficient short-term insecticide, but in the long term, it is problematic to human and animal health (Kezios et al., 2013). This pesticide was systematically banned in developed countries since the 1970s and a global ban of DDT, for non-vector control use, was exerted in the Stockholm Convention on Persistent Organic Pollutants, which took effect in 2004. However, this substance, as well as several of its transformation products are still found in natural water bodies (Appendix A1. Table S1) and tissues of different organisms (Veljanoska-Sarafiloska et al., 2013). A study conducted in African lakes showed that 4,4-DDE, a DDT metabolite, was biomagnified in fish species of the lake (Deribe et al., 2013). This was worrying, as those fish were consumed by local populations, possibly impacting human health. It is also of high concern the fact that metabolites of DDT, as well as the parental compound, are still commonly found in the environment even after an almost total ban worldwide, showing the great persistence of this substance and its transformation products in the ecosystems.

Carbofuran is one of the carbamate pesticides most toxic to vertebrates, including humans but knowledge about its main transformation products is still sparse. Otieno and colleagues (2010) reported the presence of very high concentrations of 3-ketocarbofuran and carbofuran-3-hydroxy (Appendix A1. Table S1) in surface waters highly impacted by agrochemical procedures. Concentrations found (>890µg/L) were well above the standard water concentrations allowed by the USA and European authorities for safe drinking and human use (Otieno et al., 2010). Also, these two compounds appeared to be more persistent and were detected at higher concentrations than the parental compound (Otieno et al., 2010). Considering this, it would be crucial to gain a higher level of knowledge about the possible effects of these substances on non-target organisms, including humans, that could help infer about the need for more strict monitoring routines aiming at minimizing potential impacts on water quality and populations' health.

Figure 5. Maximum concentrations of pesticide (A) and pharmaceutical (B) transformation products found in different water compartments. The heatmaps were done with the log-transformed values (pg/L) (Appendix A1. Tables S1 and S2). Grey squares represent situations for which no information could be found.

Transformation products of pharmaceuticals

Fifty-eight articles were dedicated to pharmaceuticals, presenting concentrations obtained for 98 transformation products resulting from 64 parental compounds (Figure 4, Table S2, Appendix A1.). The most investigated transformation products belonged also

to three functional classes: psychotropic drugs; analgesics, antipyretics and opioid painkillers; and anticonvulsants (Figure 4, Appendix A1. Table S2). Carbamazepine transformation products, alongside metabolites of selective monoamine reuptake inhibitors and of ibuprofen, were the ones most reported in the literature (Figure 5). The range of concentrations found varied from <0.50 ng/L to 462000 ng/L, showing that very high concentration values of pharmaceutical metabolites are already found in the natural environment. Acetaminophen metabolites were the ones with higher reported concentrations. Sunkara and Wells (2011) reported concentrations higher than 400000 ng/L for acetaminophen glucuronide and sulphate in WWTP effluents. Those values were obtained in samples collected after conventional treatment processes in WWTP, pointing out the inefficiency of these treatments for the removal of micropollutants. Moreover, the authors refer that sometimes, metabolite concentrations were higher in the effluent than in the influent and one of the reasons for that was the bioconversion that may occur during the biological treatment, as mentioned previously. However, following UV treatment, none of the metabolites was found. This could be soothing, but the UV treatment is not always applied in WWTP; it is an optional treatment used mainly in water for human consumption (Luo et al., 2014; Guardabassi et al., 2002). Water without UV treatment loaded with transformation products can thus re-enter the water cycle, potentially risking aquatic fauna and flora. Also, it can be reused in agricultural practices and therefore contaminate crops, making metabolites enter the food chain with risk to human health. Carboxy ibuprofen was also reported at a very high concentration, higher than 100000 ng/L, in WWTP influents according to Paíga and colleagues (2016). Samples were collected in a relatively small WWTP designed to serve a little less than 50000 people. Receiving wastewaters were mainly domestic and conventional treatments with activated sludge were applied (Paíga et al., 2016). Carboxy ibuprofen is one of the most representative ibuprofen metabolites. Ibuprofen is a commonly used non-steroidal anti-inflammatory (NSAID) drug and in 2016 it was the most used NSAID in Portugal, where the study was conducted (Monteiro et al., 2017). Therefore, it is important to have a stricter monitoring routine for these substances to better evaluate the possible effects of metabolites in human and non-human health. Also, carbamazepine-10,11-epoxide was reported to occur at concentrations higher than 10000 ng/L in WWTP influents (Gros et al., 2012) and municipal wastewater (Petrovic et al., 2014). This is one of the main carbamazepine metabolites and one of the most detected in natural water samples (Appendix A1. Table S2). An interesting fact in the study of Petrovic and colleagues (2014), is that carbamazepine-10,11-epoxide was found in a much higher concentration than the parental compound. This was also reported previously by Lopez-Serna (2012) in a study conducted in the Ebro River in Spain. Those

data reinforce the necessity of an extensive assessment and monitoring routine for metabolites, once they can be more prevalent in water compartments, compared to their parental compounds.

2.5. Risks of pharmaceutical and pesticide transformation products

2.5.1 Human health

Although the available data is sparse, freshwater contamination does not affect only organisms living in those systems. Ultimately, humans can also suffer negative effects from exposure to transformation products. Humans are exposed to pesticide and pharmaceutical transformation products in different ways. Data presented in Tables S1 and S2 show levels of those transformation products detected in drinking water and groundwater as well, which is a common source of drinking water in cities around the world (Guimarães et al., 2019). As previously mentioned, exposure can occur via contaminated recreational water and/or consumption of contaminated freshwater organisms or other food produced with water originating from contaminated sites. Knowledge about human health risks caused by transformation products of pesticides and pharmaceuticals is still sparse, compared to parental compounds. Studies available in the scientific literature are presented in Table 1.

Table 1. Toxicological studies about the human health risks of pesticide and pharmaceutical transformation products.

Transformation product [Parental compound] Reference	Concentrations	Sample	Exposure duration	Endpoints	Effects
Pesticides					
Chloroacetanilide, aniline; hydroxychloroacetanilide and diethylquinoneimin [Alachlor] (Hill et al., 1997)	0; 0.03; 0.1 and 0.3µM	Lymphocyte cells	72 hours	Oncogenicity	Induction of chromatid exchange at 0.1µM for hydroxychloroacetanilide, 0.3µM for chloroacetanilide and aniline
Mitotane [DDT] (Daffara et al., 2008)	Distinct values for each sample	Blood cells and saliva	not appliable	Homonal levels and organ toxicity	Inhibition of cortisol and DHEAS. Indution of thyroid function perturbations, Inhibition of testosterone secretion.
2-4-dichlorophenol [2-4-D] (Bukowska, 2003)	10 to 500 ppm	Blood cells	1 hour	Antioxidant enzymes	Increase of superoxide dismutase and increase of gluthathione peroxidase activities
DDE [DDT] (Perez-Maldonado et al., 2006)	Distinct values for each sample	Blood cells	not appliable	Genotoxicity	Induction of peripheral blood mononuclear cells
p-p' DDE [DDT] (Geric et al., 2012)	4.1µg/ml	Lymphocyte cells	1; 6 and 24hours	Genotoxicity	Induction of DNA damage

p-p' DDE [DDT] (Geric et al., 2012)	3.9µg/ml	Lymphocyte cells	1; 6 and 24hours	Genotoxicity	Induction of DNA damage
Pharmaceuticals					
gemfibrozil 1-O-β-glucoronide [gemfibrozil] (Ogilvie et al., 2006)	0.25 to 64µM	Liver microssoms	2 to 40 minutes	CYP2C8 activity	Potent inhibitor of CYP2C8
2-hidroxyestrone and 16-α hydroxyestrone [strogens] (Eliassen et al., 2008)	not appliable	Blood cells	not appliable	Genotoxicity and mitogenicity	Levels of 2-hydroxyestrone, and the ratio between 2-hydroxyestrone and 16-α hydroxyestrone were linked with certain types of breast cancer tumours in woman

The adverse effects that pesticides can cause on human health are a long-known problem. This discussion gained bigger attention and impact since the publication of the book Silent Spring in 1962. In this publication, Rachel Carson described not only the environmental impacts coinciding with the widespread use of DDT in agriculture in the United States, but also the potential of DDT to cause cancer in exposed workers. In the book, other pesticides were also surveyed, such as 2,4-D (2,4-Dichlorophenoxyacetic acid), chlordane and heptachlor. More recently different environmental agencies, including EPA (United States Environmental Protection Agency) and ECHA (European Chemicals Agency), or international conventions are banning the use of some pesticides that were described as hazardous to human health. Among the pesticide metabolites that can elicit problems, mitotane was proven to be a selective toxicant to humans and is used as an adjuvant drug to treat adrenocortical tumours (Wajchenberg et al., 2000). Mitotane or o,p'-dichlorodiphenyldichloroethane (o-p'-DDD) is a DDT metabolite, and apparently the only chemical able to inhibit corticoid synthesis and at the same time destroy cortical cells (Wajchenberg et al., 2000). However, despite the therapeutic use, mitotane was already reported in the literature to have side effects at hormonal levels in patients who were treated with this compound (Daffara et al., 2008). The authors analysed the blood cells and the saliva of the patients and found that mitotane treatment was linked to the inhibition of cortisol and DHEAS (Dehydroepiandrosterone sulphate). Also, perturbations of the thyroid function were described. Moreover, for males, an inhibition of testosterone secretion was also found. However, these side effects were usually reversible with the adequate treatment. Another DDT metabolite, DDE (dichlorodiphenyldichloroethylene) was reported to induce apoptosis of human peripheral blood mononuclear cells, both in vitro and in vivo (Perez-Maldonado et al., 2006). The authors studied blood collected from 61 healthy children during the year 2004 and from 57 children from southern Mexico. Exposure to both DDT, DDD and DDE was found in the tested children. However, significant correlations between apoptosis and exposure to pesticides were only found for DDE blood levels, ($p= 0.010$ and 0.040 for 2003 and 2004, respectively). This causes great concern since DDE is the most

persistent DDT metabolite, and thus exposure tends to be chronic, and apoptosis of the cells could result in an impairment of the immune system (Perez-Maldonado et al., 2006). Both p,p′-DDE chloroethane and p,p′-DDD (dichlorodiphenyldichloroethane), were reported to induce DNA damage in human lymphocytes, even at low concentrations (Geric et al., 2012). In this study, in vitro human lymphocytes were exposed for 1, 6 and 24 hours to p,p'-DDE (4.1 µg/mL) or p,p'-DDD (3.9 µg/mL) and genotoxic effects were assessed using the cytokinesis-block micronucleus assay and the comet assay. Results showed an increase in the number of cells containing micronucleus, in relation to the control, in the 24h exposures. Also, according to the comet assay, the percentage of DNA damages increased, in relation to the control. It is important to notice that the concentrations used are in the range found in human fluids, suggesting that these effects are already occurring in humans exposed to the metabolites (Geric et al., 2012).

The metabolite 2,4-dichlorophenol, from the herbicide 2,4-D, was reported to cause effects on antioxidant enzymes and glutathione levels in human erythrocytes in vitro (Bukowska, 2003): the activity of superoxide dismutase decreased while that of glutathione peroxidase increased in a dose-dependent (10 to 500 ppm) manner. Moreover, exposure to 250 ppm 2,4-dichlorophenol also decreased the level of reduced glutathione in erythrocytes by 32%, in relation to the control. These effects are similar, though more pronounced, to those resulting from exposure to the parental compound 2,4-D, pointing to a major need for monitoring pesticide metabolites in natural samples.

Dialkylquinoneimine metabolites of chloroacetanilide herbicides like alachlor and acetochlor were reported to induce in vitro sister chromatid exchanges in human lymphocytes (Hill et al., 1997). This study was performed to test the hypothesis that the oncogenicity of chloroacetanilide herbicides previously described was caused by genotoxic intermediates like diethylbenzoquinoneimine, an alachlor metabolite. The investigation was done with cultured human peripheral lymphocytes, mostly T cells. At 0.3 µM high variability was observed, with effects elicited by N-dealkyl-alachlor, aniline metabolites and their 4-hydroxy derivatives, and diethylbenzoquinone, in only half of the cases. At 0.1–0.3 µM the ratio between treated and control cells for sister chromatid exchange was always higher in exposures to diethylbenzoquinoneimine than to dimethyl- and ethylmethylbenzoquinoneimines. The study showed that all the compounds assessed were toxic to lymphocytes and provided the first evidence that metabolites of chloroacetanilide herbicides were genotoxic to humans and could significantly affect the immune system (Hill et al., 1997).

Pharmaceutical metabolites are not usually expected to represent an exposure concern to humans. However, biotransformation and detoxification reactions can lead to the formation of active pharmaceutical metabolites potentially more toxic than the respective

parental compounds (Celiz et al., 2009). For example, gemfibrozil 1-O-β-glucuronide, the major gemfibrozil metabolite, was found to be a more potent inhibitor of CYP2C8 than the parental compound in human liver microsomes (Ogilvie et al., 2006). Also, Ogilvie and colleagues found that gemfibrozil glucuronide, contrarily to the parental compound gemfibrozil, was found to be a CYP2C8 selective inhibitor acting in a metabolism-dependent way. To depict such differences, the authors evaluated both the parental compound and its main metabolites as inhibitors of the main drug metabolizing CYP450 enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) in human liver microsomes. Compounds inhibiting the activity of the CYP450 complex can affect the metabolism of other drugs and lead to accumulation and potential toxic effects, exerting an undesired effect in the exposed person (Ogilvie et al., 2006). In fact, the chemical reactivity of glucuronide metabolites has been linked with toxic properties. These metabolites can reach appreciable concentrations in human tissues and blood. They can also undergo hydrolysis and pH-dependent intramolecular acyl migration, irreversibly reacting with human tissues. This can cause chemical alterations leading to drug toxicity expressed by alterations in functional properties of the modified molecules or hypersensitivity and other immunotoxic reactions (Shipkova et al., 2003).

Pharmaceutical endocrine disruptors have been linked to several adverse effects on human health (Safe et al., 2000). A wide range of parental compounds have been associated with hazardous effects on human reproduction and cancer development, among others, and metabolites are not excluded. Estrogen metabolites are reported as possible mitogenic and genotoxic substances. Furthermore, a significant association (positive) with plasma levels of 2-hydroxyestrone, and the ratio between 2-hydroxyestrone and 16-α hydroxyestrone, were found in women with certain types of breast cancer tumours (Eliassen et al., 2008). To investigate that, the authors analysed blood samples collected between 1989 and 1990 and tested them against controls not taking oestrogens. The authors, nevertheless, recognised the need for replicating the study and increasing research about the relationship between estrogen metabolites and estrogen receptors and progesterone receptors related to breast tumours in women.

2.5.2. Aquatic biota

Transformation products of pesticides

Knowledge about toxic effects caused by pesticide transformation products is still sparse, compared to parental compounds. Studies available in the scientific literature are presented in Table 2.

Table 2. Ecotoxicological studies about the effects of pesticide transformation products on aquatic species.

Transformation product [parental compound] Reference	*Species*	Concentrations	Exposure duration	Endpoints	Effects
o-p' DDT [DDT] (Donohoe and Curtis, 1996)	*Oncorhyncus mykiss*	0, 0.1, 1, 5, 10 and 30 mg/kg	42 days	Determination of vitellogenin levels	Increased levels of vitellogenin in plasma and interaction with hepatic estrogenic binding sites in vivo
o-p' DDE [DDT] (Donohoe and Curtis, 1996)	*Oncorhyncus mykiss*	0, 0.1, 1, 5, 10 and 30 mg/kg	42 days	Determination of vitellogenin levels	Increased levels of vitellogenin in plasma and interaction with hepatic estrogenic binding sites in vivo
p-p' DDE [DDT] (Donohoe and Curtis, 1996)	*Oncorhyncus mykiss*	0, 0.1, 1, 5, 10 and 30 mg/kg	42 days	Determination of vitellogenin levels	No differences found in vitellogenin levels, relative to controls
DDD [DDT] (Lotufo et al., 2000)	*Hyaella azteca*	0.095, 0.178, 0.366, 0.692 and 1.381 µg/L	10 days	Mortality and lethal residues in tissues	DDD was less lethal than the parental compound (DDT) but its lethality was higher than that of the control at >0.69 µg/L
	Diporeia spp.	0.944, 2.791, 7.420 and 17.056 µg/L	28 days		No significant effects found
DDE [DDT] (Lotufo et al., 2000)	*Hyaella azteca*	1.117, 2.258, 4.947, 8.208 and 22.021 µg/L	10 days	Mortality and lethal residues in tissues	DDE was less lethal than the parental compound (DDT) but its lethality was higher than that of the control at >2.258 µg/L
	Diporeia spp.	2.293, 4.726, 9.141 and 20.194 µg/L	28 days		No significant effects found
o-p' DDE [DDT] (Davis et al., 2009)	*Oreochromis mossambicus*	5 µg/g	35 days	Determination of Vitellogenin levels and hormone/insuline like growth factor i-axis	Increase in plasma levels of insuline growth factor
		100 µg/g	5 days		Increase in expression of both vitellogenin A and B, estrogen receptors α and β and also in insuline growth factor
3-4 dichloroaniline [diuron] (Scheil et al., 2009)	*Danio rerio*	0.005, 0.01, 0.1 0.25, 0.5 and 1 mg/L	8 and 11 days	Mortality and locomotor activity	Locomotor activity and mortality were impaired at ≥0.5 mg/l
		0.05, 0.1, 0.15, 0.2 and 0.2 5mg/L	168 hours	Hsp70 levels	A significant increase in relation to control was found at 0.25 mg/L
		0.5, 0.7, 1, 1.5 and 2 mg/L	11 days	Embryonic and larval development	Byproduct caused larvae deformations at ≥0.25 mg/l
3-4 dichloroaniline [diuron] (Felício et al., 2018)	*Oreochromis niloticus*	40 and 200 ng/L	7 days	Antioxidant and biotransformation biomarkers	Byproduct caused significant alterations in antioxidant and biotransformation biomarkers, with ethoxyresorufin-O-deethylase (EROD) activity showing a dose-dependent response
Deethylatrazine [atrazine] (Ralston-Hooper et al., 2009)	*Hyaella azteca*	550, 1000, 2500, 5000, 10000, 15000 µg/L	96 hours, 21 and 42 days	Mortality and sex ratio	LC50 values were 5100 µg/L at 96h and higher than 3000 µg/L at 21 days; no change in the sex ratio was found
	Diporeia spp.	0.03, 0.3, 3, 30, 300, 3000 µg/L	96 hours, 21 and 42 days	Mortality and sex ratio	LC50 values were 7200 µg/L at 96h and higher than 3000 µg/L at 21 days; no change in the sex ratio was found
	Pseudokirchneriella subcapitata	No reported	96 hours	Growth inhibition	Growth inhibition occurred at concentrations >2000 µg/L
Deisopropylatrazine [atrazine] (Ralston-Hooper et al., 2009)	*Hyaella azteca*	550, 1000, 2500, 5000, 10000, 15000 µg/L	96 hours, 21 and 42 days	Mortality and sex ratio	LC50 values were >3000 µg/L at 96h and 330 µg/L at 21 days; no change in the sex ratio was found
	Diporeia spp.	0.03, 0.3, 3, 30, 300, 3000 µg/L	96 hours, 21 and 42 days	Mortality and sex ratio	LC50 values were >3000 µg/L at 96h and 300ug/L at 21 days; no change in the sex ratio was found
	Pseudokirchneriella subcapitata	No reported	96 hours	Growth inhibition	Growth inhibition occured for concentrations higher than 3000 µg/L.
Therbuthylazine-2-hydroxy [therbuthylazine] (Koutnik et al., 2016)	*Procambarus fallax f. virginalis*	0.75, 75, 375 and 750 µg/L	62 days	Mortality, growth, oxidative balance, antioxidant defences, ontogeny and histology	Lower weight at 75 µg/L; delayed ontogenic development and lowered antioxidant defences in exposed animals
Desethyl-terbuthylazine [therbuthylazine] (Velisek et al., 2016)	*Cyprinus carpio*	1.80, 180, 900 and 1800 µg/L	7, 14, 20, 27 and 31 days	Growth, LC50, histology, oxidative stress, mortality	LC50 of 441.6 µg/L at 31 days; lower weight and length in fish exposed to 1800 µg/L for 7 days and 900 µg/L for 20 days; delayed ontogenetic development at >1.8 µg/L; decreased antioxidant enzyme activity in all concentrations
AMPA [glyphosate] (Guilherme et al., 2014)	*Anguilla anguilla*	11.8 and 23.6 µg/L	1 and 3 days	DNA and chromossome damage	Significant genotoxic effect in relation to control group

Fiprunil sulphide and sulfone [fipronil] (Weston and Ludy, 2014)	*14 macroinvertebrate species*	4–7 concentration steps separated by a factor of 2	48 and 96 hours	Mortality and ability to swim, cling or crawl, depending on the species	Mean 96-h EC50 of 7–10 ng/L
Fiprunil sulphide [fipronil] (Gong et al., 2021)	*Danio rerio* *Chlorella pyrenoidosa*	0.1 to 10 mg/L	72 hours	Mortality and oxidative stress Algae growth inhibition rate; content of pigment	LC50= 0.36mg/L. Significant decreased of SOD activity at 5mg/L EC50: 0.10mg/L; chlorphyll content significantly decreased in dose response realationship;
Fiprunil sulfone [fipronil] (Gong et al., 2021)	*Danio rerio* *Chlorella pyrenoidosa*	0.1 to 10 mg/L	72 hours	Mortality and oxidative stress Algae growth inhibition rate; content of pigment	LC50= 0.21mg/L. Significant decreased of SOD activity at 5mg/L EC50: 0.13mg/L; chlorphyll content significantly decreased in dose response realationship;
Fiprunil desulfinyl [fipronil] (Gong et al., 2021)	*Danio rerio* *Chlorella pyrenoidosa*	0.1 to 10 mg/L	72 hours	Mortality and oxidative stress Algae growth inhibition rate; content of pigment	LC50= 1.13 mg/L. Significant decreased of SOD activity at 5mg/L EC50: 0.43 mg/L; chlorphyll content significantly decreased in dose response realationship;
Metolachlor OXA [metolachlor] (Velisek et al., 2018)	*Procambarus fallax f. virginalis*	4.2, 42 and 420 µg/L	45 days	Growth rate, behaviour, oxidative stress, histology and mortality	Decreased growth and activity of antioxidant enzymes in all tested concentrations; delayed ontogenetic development and lower levels of reduced glutathione and lipid peroxidation
Metolachlor OXA [metolachlor] (Rozmankova et al., 2020)	*Danio rerio*	1, 30, 100 and 300 µg/L (single exposure); 1 and 30 µg/L (mixture)	120 hours	Mortality, hatching success, embryonic malformations, locomotion, spontaneous movements, heartbeat and gene expression	Increased craniofacial, non-inflated gas bladder and yolk sac malformations at 100 µg/L or higher. Induction of *p53* gene at 100 µg/L.
Metolachlor ESA [metolachlor] (Rozmankova et al., 2020)	*Danio rerio*	1, 30, 100 and 300 µg/L (single exposure); 1 and 30 µg/L (mixture)	120 hours	Mortality, hatching success, embryonic malformations, locomotion, spontaneous movements, heartbeat and gene expression	Increased craniofacial, non-inflated gas bladder and yolk sac malformations at 100 µg/L or higher. Induction of p53 gene at 100 µg/L. Induction of *p53 and thyroid system regulation (dio2, thra, thrb)* at 30 and 1µg/L, respectively.
3-trifluoromethyl-4-aminophenol [3-trifluoromethyl-4-nitrophenol] Huerta et al., 2020)	*Petromyzon marinus*	0, 5, 50 and 200 µM	Undefined exposure time	Respiratory control ratio, mitochondrial oxygen consumption and mitochondrial transmembrane potential	No significant effects found
4-nitro-3-methyl-phenol [3-trifluoromethyl-4-nitrophenol] (Huerta et al., 2020)	*Petromyzon marinus*	0, 5, 50 and 200 µM	Undefined exposure time	Respiratory control ratio, mitochondrial oxygen consumption and mitochondrial transmembrane potential	No significant effects found
4-amino-3-methylphenol [3-trifluoromethyl-4-nitrophenol] (Huerta et al., 2020)	*Petromyzon marinus*	0, 5, 50 and 200 µM	Undefined exposure time	Respiratory control ratio, mitochondrial oxygen consumption and mitochondrial transmembrane potential	Decreased respiratory control ratio at 50 µM; decreased oxygen consumption at 200 µM
4-nitroso-3-methyl-phenol [3-trifluoromethyl-4-nitrophenol] (Huerta et al., 2020)	*Petromyzon marinus*	0, 5, 50 and 200 µM	Undefined exposure time	Respiratory control ratio, mitochondrial oxygen consumption and mitochondrial transmembrane potential	No significant effects found
3-phenoxybenzyl alcohol [permethrin] (Hernandez-Moreno et al., 2022)	*Oncorhynchus mykiss*	0.78, 3.15, 12.5, 50 and 100 mg/L	96 hours	Mortality	Moderately toxic (LC50= 1.93 mg/L)
Benzenesulfonamide [asulam] (Hernandez-Moreno et al., 2022)	*Oncorhynchus mykiss*	0.78, 3.15, 12.5, 50 and 100 mg/L	96 hours	Mortality	Non toxic (LC50>100 mg/L)

benzimidazol [carbendazim] (Hernandez-Moreno et al., 2022)	*Oncorhynchus mykiss*	0.78, 3.15, 12.5, 50 and 100 mg/L	96 hours	Mortality	Slightly toxic (LC50= 66.19 mg/L)
cyanoacetamide [DBNPA] (Hernandez-Moreno et al., 2022)	*Oncorhynchus mykiss*	0.78, 3.15, 12.5, 50 and 100 mg/L	96 hours	Mortality	Slightly toxic (LC50= 68 mg/L)
cis-2,6-dimethylmorpholine [fenpropimorph] (Hernandez-Moreno et al., 2022)	*Oncorhynchus mykiss*	0.78, 3.15, 12.5, 50 and 100 mg/L	96 hours	Mortality	Non toxic (LC50>100 mg/L)
ethiprole sulfone [ethiprole] (Gao et al., 2021)	*Danio rerio*	100, 300, 800, 2000, 5000 µg/L	4 days	Mortality; oxidative stress; development	LC50 value was 1750 µg/L; induction of antioxidant enzymes and the developmental anomalies at 100 µg/L
ethiprole sulfide [ethiprole] (Gao et al., 2021)	*Danio rerio*	100, 110, 120, 150, 180 µg/L	4 days	Mortality; oxidative stress; development	LC50 value was 111 µg/L; induction of antioxidant enzymes and the developmental anomalies at 10 µg/L or higher.
rac-ethiprole amide [ethiprole] (Gao et al., 2021)	*Danio rerio*	100, 500, 2500, 10,000, 50,000 µg/L	4 days	Mortality; oxidative stress; development	LC50 > 50,000 µg/L
ethiprole sulfone amide [ethiprole] (Gao et al., 2021)	*Danio rerio*	100, 500, 2500, 10000, 50000 µg/L	4 days	Mortality; oxidative stress; development	LC50 > 50,000 µg/L
desethylsulfinyl ethiprole [ethiprole] (Gao et al., 2021)	*Danio rerio*	500, 800, 1500, 2500, 5000 µg/L	4 days	Mortality; oxidative stress; development	LC50= 1728 µg/L

One of the most controversial pesticides is DDT, which was reported to cause health issues to humans and living organisms in general. Moreover, studies are available in the literature linking exposure to DDT metabolites to negative effects on the health of aquatic organisms. Donohoe and Curtis (1996) injected juvenile rainbow trout with o,p'-DDT, o,p'DDE or p,p'-DDE with doses ranging from 5 to 30 mg/kg at 0, 14 and 28 days and sampling was done at 14 and/or 42 days. They reported that o,p'-DDT and o,p'-DDE had estrogenic activity, because of the elevated plasma vitellogenin levels they can elicit in vivo and their interaction with hepatic estrogenic binding sites (Donohoe and Curtis, 1996). A study conducted in freshwater amphipods (*Hyalella azteca* and *Diporeia* spp.) reported that the metabolites DDD and DDE are less lethal than DDT (Lotufo et al., 2000). *Hyalella azteca* and *Diporeia* spp. were exposed to a wide range of concentrations of DDD for 10 days and DDT and DDE for 28 days. Besides mortality, median lethal residue (LR50), mean effect concentration (EC50) and mean effect residue (ER50) in tissues were also assessed. Although metabolites were less lethal, mortality of *H. azteca* was significantly higher in DDD and DDE treatments than in the control at 0.692 µg/L and 2.258 µg/L, respectively (Lotufo et al., 2000). This raises high concern, once concentrations of DDD in this range have already been reported in freshwater ecosystems. The endocrine-disrupting activity of o,p'-DDE was also evaluated more recently (Davis et al., 2009). In this study, the authors investigated the effects of this metabolite and other compounds on the expression of the vitellogenin gene from the tilapia *Oreochromis mossambicus* and the growth hormone insulin-like growth factor-I

axis. Injection of 100 µg/g o,p'-DDE in fish increased the expression of vitellogenin A and B, as well as the transcription of estrogen receptors α and β and the expression of the putative somatolactin receptor and insulin-like growth factor (Davis et al., 2009). This once again reinforces the potential endocrine disruption that DDT metabolites may cause in freshwater fish. As previously mentioned, metabolites of triazine herbicides, are among the most frequently found in freshwater systems. Moreover, there is evidence in the literature linking these substances to negative effects on living organisms. The main degradation product of diuron is 3,4-dichloroaniline for which the toxic potential towards freshwater organisms is described in the literature. In zebrafish, a subchronic exposure (11 days) to this metabolite caused deformations at ≥0.25 mg/l, while locomotor activity and mortality were impaired at ≥0.5 mg/l (Scheil et al., 2009). A recent work investigated the effects of 3,4-dichloroaniline on biotransformation enzymes and the oxidative stress response in the liver and gills of the Nile tilapia (*Oreochromis niloticus*) (Felício et al., 2018). The authors found that in fish exposed for seven days to 40 and 200 ng/L the levels of several biotransformation and antioxidant enzymes were altered often in a non-monotonic response, except for ethoxyresorufin-O-deethylase (EROD) activity that exhibited a dose-dependent increase. Moreover, the multixenobiotic resistance (MXR) activity and the activity of glutathione S-transferase (GST) enzymes were decreased in gills after exposure to 3-4 dichloroaniline. Because the MXR mechanism is crucial for the protection of aquatic organisms against xenobiotics aggression (Ferreira et al., 2014), this suggests that exposure to this metabolite is endangering the health of fish and the contaminated aquatic systems. A reduction in this mechanism can lead to higher susceptibility of animals to xenobiotics by impairing homeostatic processes.

The acute and chronic toxicity of deethylatrazine and deisopropylatrazine, metabolites of atrazine, were investigated in two amphipod species and in the microalgae *Pseudokirchneriella subcapitata* (Ralston-Hooper et al., 2009). *Hyalella azteca* and *Diporeia* spp. were exposed to concentrations ranging from 0.55 to 15 mg/L for 96h and from 0.03 to 3000 µg/L for 21 days. Results showed the median lethal concentrations (LC50), and median growth inhibition concentration (IC50) for algae, were ≥1.5 mg/L, i.e., higher than the levels found in the environment (Ralston-Hooper et al., 2009). In a recent study, marbled crayfish (*Procambarus fallax* f. *virginalis*) were exposed for 62 days to four concentrations of terbuthylazine-2-hydroxy: 0.75 µg/l (environmentally relevant), 75, 375 and 750 µg/l (Koutnik et al., 2017). Antioxidant defences, oxidative balance, histology, early ontogeny, growth and mortality were the parameters assessed to depict possible effects of this metabolite. Concentrations over 75 µg/l caused lower weight compared to the control group. The outcome of the study showed that terbuthylazine-2-hydroxy delayed ontogenetic development. Also, levels of thiobarbituric

acid and antioxidant enzymes were significantly ($p < 0.01$) lower in groups exposed to the metabolite. This shows the potential danger of this metabolite to freshwater species, although the alterations found occurred in the groups exposed to non-environmental concentrations (Koutnik et al., 2017). The toxicity of terbuthylazine-desethyl, another metabolite of triazine herbicides, was assessed in the early stages of development of the common carp (*Cyprinus carpio*) (Velisek et al., 2016). Carp embryos were exposed to 1.80 µg/L (environmentally relevant), 180 µg/L, 900 µg/L, and 1800 µg/L and samples were collected on days 7, 14, 20, 27, and 31. The 31d LC50 of terbuthylazine-desethyl was estimated to be 441.6 µg/L. Animals also exhibited lower weight and length at 7 (1800 µg/L) and 20 (900 µg/L) days of exposure. Terbuthylazine-desethyl at non-environmental concentrations also delayed the ontogenetic development, in relation to control. However, antioxidant enzyme activity was significantly lower in all test concentrations, including the environmentally relevant one, indicating that contamination by this metabolite should be compromising feral aquatic populations.

The main metabolite of glyphosate, AMPA (aminomethylphosphonic acid), is one of the most controversial pesticides nowadays, due to its potential hazard to wildlife and human populations. Moreover, AMPA by itself was reported as hazardous to *Anguilla anguilla* by Guilherme et al. (2014). The eels were exposed for 1 and 3 days to environmentally relevant concentrations (11.8 and 23.6 µg/L) and genotoxicity was investigated by assessing damage to DNA through the Comet assay and erythrocytic nuclear abnormalities. These results showed a genotoxic effect of AMPA at concentrations already found in aquatic systems. About organophosphates, a recent study was conducted with the parasitic sea lamprey (*Petromyzon marinus*) to address possible effects on cardiac mitochondrial bioenergetics of the lampricide 3-trifluoromethyl-4-nitrophenol and its metabolite 3-trifluoromethyl-4-aminophenol, as well as 4-nitro-3-methyl-phenol (Huerta et al., 2020). The latter has a similar molecular structure and is a known transformation product of fenitrothion and its metabolites 4-amino-3-methylphenol and 4-nitroso-3-methyl-phenol. Mitochondria were extracted from the hearts of animals captured on the great lakes and incubated with 0, 5 and 50 µM of the test compounds to assess the respiratory control ratio and mitochondrial oxygen consumption or with 0, 5, 50 and 200 µM to assess the mitochondrial transmembrane potential. Results showed that 4-amino-3-methylphenol significantly lowered the respiratory control ratio (88% at 50 µM) and oxygen consumption by 64% (at 200 µM and with the addition of high concentrations of ADP) and by 45% (at 200 µM and addition of substrate for complex II). At last, for mitochondrial transmembrane potential, none of the tested transformation products caused significant alterations.

Fipronil is a phenylpyrazole insecticide with crescent use in urban areas. The toxicity of its sulphide and sulfone metabolites was not recognised until 2014 when Weston and Ludy carried out a study determining EC50 values for 14 macroinvertebrate species. Results indicated a mean 96-h EC50 of 7−10 ng/L for fipronil metabolites in *Chironomus dilutus* (Weston and Ludy, 2014). The same study also reported that creeks receiving urban stormwater run-off in California contained metabolite concentrations twice the EC50 found for *C. dilutus* and approximately one-third of the EC50 found for other aquatic macroinvertebrates (Weston and Ludy, 2014). A recent study evaluated the toxicity of different fipronil metabolites: fipronil sulphide, fipronil sulphone and fipronil desulfinyl (Gong et al., 2021). In this work, the authors analysed the effects of 72h exposure to these metabolites at concentrations ranging from 0.1 to 10mg/L on zebrafish embryos and the green algae *Chlorella pyrenoidosa*. In zebrafish, LC50 values of 0.36, 0.31, and 1.13 mg/L were found for fipronil sulphide, sulfone and desulfinyl, respectively. Moreover, at 5mg/L all metabolites significantly increased SOD activity, in relation to control. In *C. pyrenoidosa* growth inhibition, EC50 values of 0.10, 0.13, and 0.43 mg/L were found for fipronil sulphide, sulfone and desulfinyl, respectively. The metabolites investigated caused also a significant decrease in chlorophyll content, in relation to control, in a dose-response manner (Gong et al., 2021).

Metabolites of chloroacetanilide herbicides are highly prevalent in aquatic ecosystems, mainly in oxalinic and endosulfonic acid forms. Metolachlor OXA was reported to negatively affect the early life stages of marbled crayfish (Velisek et al., 2018). Animals were exposed for 45 days to 4.2 µg/L (environmentally relevant), 42 µg/L and 420 µg/L and several endpoints were assessed. Metolachlor OXA caused significantly lower growth and decreased activity of antioxidant enzymes at all tested concentrations. The highest tested concentrations delayed ontogenetic development and decreased the levels of reduced glutathione and lipid peroxidation (Velisek et al., 2018). More recently, a study was performed to evaluate the impacts of single and combined exposure of metolachlor and its metabolites metolachlor ESA and metolachlor OXA on zebrafish embryos (Rozmankova et al., 2020). In this study, zebrafish embryos were exposed for 120 hours to 1, 30, 100 and 300 µg/L of the single compounds or to 1 and 30 µg/L of a compound mixture, and sublethal endpoints such as malformations, hatching rate, larval length, spontaneous movements, heartbeat, and locomotion, as well as expression levels of eight genes linked to different critical pathways, were monitored. Increased craniofacial, non-inflated gas bladder and yolk sac malformations at 100 µg/L or higher were reported for both metabolites. For metolachlor OXA, a significant induction of p53 gene was found at 100 µg/L, compared to control, while for metolachlor ESA, a significant induction of p53 gene at 30 and 100 µg/L, and thyroid system regulation (dio2, thra, thrb)

was observed at 1 µg/L, in comparison to the control group. The disruption of the thyroid system represented a plausible danger for population maintenance since it occurred at low environmental concentrations (Rozmankova et al., 2020). A recent study evaluated the acute toxicity of several biocide metabolites using the rainbow trout (*Oncorhynchus mykiss*) as a test model (Hernandez-Moreno et al., 2022). The author exposed juvenile trout according to OECD TG203, for 96 hours to 0.78, 3.15, 12.5, 50 and 100 mg/L of the following metabolites: 3-phenoxybenzyl alcohol, benzenesulfonamide, benzimidazole, cyanoacetamide and cis-2,6-dimethylmorpholine. The most toxic metabolite was 3-phenoxybenzyl alcohol, with an LC50 value of 1.93 mg/L, considered moderately toxic by the authors. Benzimidazole and cyanoacetamide with LC50 values of 66.19 and 68 mg/L respectively were reported as slightly toxic, while benzenesulfonamide and cis-2,6-dimethylmorpholine with LC50 values higher than 100 mg/L were considered non-toxic (Hernandez-Moreno et al., 2022).

Ethiprole is a non-systemic phenyl-pyrazole compound widely used as an insecticide. Recently, a study was performed to evaluate zebrafish embryotoxicity and effects on antioxidant enzymes (catalase, CAT, and superoxide dismutase, SOD, activities) and oxidative stress (lipid peroxidation) of its main metabolites, i.e. ethiprole sulfone, ethiprole sulfide, ethiprole amide, ethiprole sulfone amide, and desethylsulfinyl ethiprole (Gao et al., 2021). Results showed that only ethiprole sulfone and sulfide had effects on antioxidant defences and embryonic development. Ethiprole sulfone had an LC50 value of 1750 µg/L, induced antioxidant enzymes and increased developmental anomalies at 100 µg/L. Ethiprole sulfide had an LC50 value of 111 µg/L, induced antioxidant enzymes and increased developmental anomalies at 10 µg/L or higher. Rac-ethiprole amide and ethiprole sulfone amide had LC50 values higher than 5000 µg/L, while the LC50 value for desethylsulfinyl ethiprole was 1728 µg/L (Gao et al., 2021).

Transformation products of pharmaceuticals

Nowadays, one main challenge to the scientific community is to understand the effects of these substances on non-target organisms. There are, already, several reports about this topic. However, knowledge about the toxic effects caused by pharmaceutical transformation products is still scarce. A summary of the works found in the literature is shown in Table 3.

Table 3. Ecotoxicological studies about the effects of pharmaceutical transformation products on freshwater species.

Transformation product [parental compound] Reference	*Species*	Concentrations	Exposure duration	Endpoints	Effects
prednisone, dexamethasone and their undisclosed photodegradation products [prednisone, dexamethasone] (Della Greca et al., 2004)	*Brachionus calyciflorus*	5different test concentrations without known value. Results are reported as median effective concentrations in ppm	24 hours	Mortality	5-prednisone and 2-dexamethasone photoderivates had lower LC50 values than parent compounds but at levels not found in environmental samples (mg/L range)
	Thamnocephalus platyurus		24 hours	Mortality	All photoderivates had lower LC50 values than parental compounds (higher toxicity), but at non environmentally relevant concentrations (>710 ppm)
	Daphnia magna		24 hours	Mortality	All photoderivates had lower EC50 values than parental compounds (higher toxicity), but at non environmentally relevant concentrations (mg/L range)
	Pseudokircheneriella subcapitata		72 hours	Growth inhibition	Toxic effects similar to those found for the other species, except Ceriodaphnia dubia
	Ceriodaphnia dubia		7 days	Population growth	Both the photoderivatives of prednisolone and dexamethasone showed higher toxic effects on *C. dubia* growth after 7 days
naproxen and its undisclosed photodegradation products [naproxen](Isidori et al., 2005)	*Brachionus calyciflorus*	Concentratation values are not given. All test solutions were dissolved in DMSO (0.01% v/v). 5 different concentration were tested, as well as, a negative control	24 /48hours	Mortality and reproduction	All photoderivates had lower LC50 values than parental compounds, but at levels not found in environmental samples (mg/L range) for acute assay. In the chronic reproduction assay only one photoderivate was less toxic than the parental compound
	Thamnocephalus platyurus		24 hours	Mortality	All photoderivates had lower LC50 values than parental compounds, but at levels not found in environmental samples (mg/L range)
	Ceriodaphnia dubia		24hours and 7 days	Mortality and reproduction	ll photoderivates had lower LC50 values than parental compounds, but at levels not found in environmental samples (mg/L range). For reproduction, only one photoderivate was less toxic than the parental drug
	Pseudokircheneriella subcapitata		96 hours	Growth	All photoderivatives of naproxen showed higher toxic effects on *P.subcapitata* growth
diclofenac, ketoprofen, atenolol and their photodegradation products (undisclosed) [diclofenac, ketoprofen, atenolol](Diniz et al., 2015)	*Danio rerio*	1 mg/L	7days	Oxidative stress	Diclofenac metabolites formed through UV photolysis treatments were more toxic than their parental compounds. Activity of antioxidant enzymes and lipid peroxidation levels were higher for byproducts than the parental drugs. Overall, oxidative stress response causing toxicity was observed for all pharmaceuticals and byproducts
norfluoxetine [fluoxetine] (Stanley et al., 2007)	*Pimephales promelas*	1 to 250 µg/L	7days	survival and growh	The authors related higher toxicity in fish exposed to s-fluoxetine, which in mammals is expected to be more potent than R-norfluoxetine
	Daphnia magna	10 to 1000 µg/L	21 days	immobilization, reproduction and grazin rate	No observed effects
Norfluoxetine [fluoxetine] (Fong and Molnar, 2008)	*Dreissena polymorpha*	100nM to 50 µM	4 hours	spawning	Increased spawning in zebra mussels at 1–50 µM
	Mytilopsis leucophaeata	100nM to 50 µM	4 hours	spawning	Increased spawning in zebra mussels at 1–50 µM
	Sphaerium striatinum	100nM to 10 µM	4 hours	parturition	Significant increase in parturition induced at 10 µM
norfluoxetine [fluoxetine] (Rodrigues et al., 2020)	*Danio rerio*	0.64, 3.2, 16, 80 and 400 ng/L	80hours	Embryonic develoment, gene expression and sensorymotor responses	Increase of embryonic anomalies in relation to control, mainly for pigmentation. No effects found for gene expression and sensomotory response

Norfluoxetine [fluoxetine] (Aztei et al., 2021)	*Danio rerio*	0.03 to 10 µM	5 days	Embryonic develoment, gene expression and light/dark movement	Inhibition of light/dark, zebrafish locomotory activity, mainly in dark. Resposes followed a dose-response relationship
norfluoxetine [fluoxetine] (Rodrigues et al., 2022)	*Danio rerio*	400 ng/L	80hours	Embryonic develoment and gene expression	Increase in pigmentation anomalies of embryos and larvae, relative to the parental compound
n-desmethylsertraline [sertraline] (Lajeunesse et al., 2011)	*Salvelinus fontinalis*	WWTP water samples (undisclosed concentrations)	3months	Tissue bioaccumulation and Na/K-ATPase activity	Bioaccumulation in several tissues, including (brain and liver). Na/K-ATPase activity negatively correlated with brain bioaccumulation desmethylsertraline exposed brain tissue
o-desmethylvenlafaxine [venlafaxine] (Stropnicky, 2017)	*Orconectes obscurus*	0, 1 and 8 µg/L	14 days	Agressive behaviour	Increase in the number of attacks per minute at the highest concentration tested
	Procambarus clarkii	0, 1 and 8 µg/L	14 days	Agressive behaviour	Increase in the number of attacks per minute at the highest concentration tested
o-desmethylvenlafaxine [venlafaxine] (Aztei et al., 2021)	*Danio rerio*	0.03 to 300 µM	5 days	Embryonic develoment, gene expression and light/dark movement	Inhibition of light/dark, zebrafish locomotory activity, mainly in dark. Resposes followed a dose-response relationship
clofibric acid [clofibrate] (Nunes et al., 2008)	*Gambuzia holbrooki*	176.4, 211.6, 253.92, 304.71 and 365.65 mg/L	96 hours	Oxidative damage	Decrease in the amount of oxidised glutathione content in the liver and gills in exposed fish
n- and o-desmethyltramadol [tramadol] (Zhuo et al., 2012)	*Danio rerio*	Intraperitonial injection of tramadol (65 mg/kg)	1hour	Weight, mithocondrial changes and behaviour	Detection of n- (mostly) and o-desmethyltramadol in brain tissue. Fish exposed to tramadol exhibited weight loss, abnormal behaviour and mitochondrial structural changes, possibly mediated by its byproducts
oxazepam [temazepam] (Huerta et al., 2016b)	*Pimephales promelas*	0.8, 4.7 and 30.6 µg/L	28 days	behaviour and bioaccumulation	Brain was the tissue with higher accumulation rates; behavioral effects detected in the novel tank diving test were observed in fish exposed to 4.7 µg/L
oxazepam [temazepam] (Fahlman et al., 2021)	*Perca fluviatilis*	15 µg/ L	14 days	anti-predator behavior	Stimulation of anti-predator behavior (decreased activity, decreased distance to conspecifics, and increased littoral habitat use)
oxcarbamazepine [carbamazepine] (Desbiolles et al., 2020)	*Lemna minor*	27 ng/L	17 days	Phytometabolites	Increase in nitrogen compounds. Chlorophyll index was higher in relation to control
	Hydra circumcinta	900 ng/L	14 days	Reproduction, morphological changes and oxidative stress biomarkers	Single exposure impacted the total antioxidant capacity
acridine 9-carboxylic acid [oxcarbazepine] (Desbiolles et al., 2020)	*Lemna minor*	27 ng/L	17 days	Phytometabolites	Alterations of the nitrogen balance and chlorophyll indices at environmental concentrations
oseltamivir carboxylate [osetalmivir] (Chen et al., 2020)	Oryzias latipes	0, 0.06, 0.3, 90 and 300 µg/L	14, 21 and 56 days	median survival,groth, reproduction and hatchability	Long-term parental exposure to byproducts affected the embryonic development of fish hatchability at 300 µg/L and development 90 µg/L
oseltamivir ethyl ester [osetalmivir] (Chen et al., 2020)	*Oryzias latipes*	0, 0.06, 0.3, 90 and 300 µg/L	14, 21 and 56 days	median survival,groth, reproduction and hatchability	Long-term parental exposure to byproducts affected the embryonic development of fish hatchability at 300 µg/L and development 90 µg/L
fenofibric acid [fenofibrate] (Jung et al., 2021)	*Danio rerio*	5, 10, 20, 30 and 40 mg/L	72 hours	Mortality	LC_{50}= 53.32 mg/L
carbamazepine-10,11-epoxide [carbamazepine] (Bars et al., 2021)	*Danio rerio*	250 µg/L	120 hours	embryonic development	Delay in swim bladder inflation at 120 hpf

5-(4-hydroxyphenyl)-5-phenylhydantoin [phenytoin] (Bars et al., 2021)	*Danio rerio*	250 µg/L	120 hours	embryonic development	No effects found

As mentioned above, metabolites can be formed during wastewater treatment in WWTPs. In fact, this situation is reported for photodegradation products of both prednisone and dexamethasone (DellaGreca et al., 2004). In this study, photoproducts of both pharmaceuticals were isolated, from an initial solution of 100 mL of both compounds mixed with 500 mL of water, and their toxicity to different species was evaluated: the rotifer *Brachionus calyciflorus* and the crustaceans *Thamnocephalus platyurus* and *Daphnia magna* for acute toxicity; the microalgae *Pseudokircheneriella subcapitata* and the crustacean *Ceriodaphnia dubia* for chronic toxicity. Acute assays lasted for 24 hours and were based on mortality (LC50). In chronic assays, growth inhibition was the endpoint assessed for algae (72 hours duration) and population growth was the endpoint for *C. dubia* (7 days duration). Some photodegradation products of prednisone and dexamethasone were found to be more toxic than the parental compounds. However, the LC50 values obtained by the authors were considerably higher than the concentrations generally found in surface waters. The chronic exposures decreased the population growth in *C. dubia* (DellaGreca et al., 2004). A similar study was conducted for the non-steroidal anti-inflammatory drug naproxen and its photodegradation products (Isidori et al., 2005). In this work, acute toxicity tests were conducted with *B. calyciflorus*, *T. platyurus* and *C. dubia*. Chronic toxicity was assessed (reproduction and/or growth) in *B. calyciflorus*, *C. dubia* and the microalgae *P. subcapitata*. Results showed that photodegradation products were more acutely toxic than the parental compound, although at levels (mg/L range) well above those found in freshwater systems. Chronic exposure reduced the population growth in *C. dubia* at low concentrations (µg/L) for some photoproducts (Isidori et al., 2005). This situation warns of the need to improve treatment methodologies, for better removal of both the parental compounds and their transformation products. A more recent study also reported that diclofenac metabolites formed through UV photolysis treatments were more toxic than their parental compound (Diniz et al., 2015) (Table 3).

Lienert and colleagues (2007) developed a study where the ecotoxicological risk of 42 pharmaceuticals and their metabolites was evaluated. In the study, both parental compounds and their respective metabolites were treated as a mixture of toxicants of similar action. When relevant data were not available in the literature, the authors estimated them from quantitative structure-activity relationships (QSAR). Moreover, from their known pharmaceutical information, they figured out the removal efficiency of these contaminants from urine. The results of this evaluation showed that mixtures of ibuprofen

and its metabolites could represent an ecotoxicological risk for aquatic organisms. Likewise, acetylsalicylic acid, bezafibrate, carbamazepine, diclofenac, fenofibrate and paracetamol, in a mixture with their respective metabolites could be of potential risk for aquatic organisms, however, to a lesser extent than ibuprofen. In Table S2, ibuprofen metabolites detected in environmental samples reach concentrations >120 000 ng/l that, together with the results of Lienert et al. (2007), suggests that this contamination is jeopardizing affected aquatic ecosystems and their populations. While QSAR models have some limitations that may generate not fully accurate data, the information presented by those authors established a relevant basis for highly needed subsequent research and risk assessment studies.

Norfluoxetine, the main fluoxetine metabolite, was reported to cause enantiospecific sub-lethal effects in *Pimephales promelas* and *Daphnia magna* (Stanley et al., 2007). In this study, *P. promelas* juveniles were exposed for seven days to 1, 10, 50, 100 and 250 µg/L of R-, rac- and S-fluoxetine. The enantiomer S-fluoxetine showed higher toxicity to growth, survival, and feeding rate. The authors related their results to the fact that S-norflluoxetine is more potent to mammals than R-fluoxetine. But this pattern was not found for *D. magna*. For this microcrustacean, a 21-day toxicity test was performed to determine immobilization, reproduction, and grazing rate. Less than 24-hpf individuals were exposed to 10, 50, 100, 250, 500, and 1000 µg/L of R-, rac-, and S-fluoxetine. The results obtained were similar for the three compounds, and the taxa differences were attributed to the higher homology between fish and mammals than between crustaceans and mammals. Norfluoxetine was also reported to induce spawning and parturition in bivalves (Fong et al., 2008). The authors exposed zebra mussels to 100nM–50 µM, dark false mussels to 100nM–50 µM and finger-nail clams to 100nM–10 µM. Norfluoxetine increased spawning in both zebra mussels and dark false mussels, relative to the respective controls, at concentrations in the range of 1–50 µM. In fingernail clams, norfluoxetine induced significant parturition only at 10 µM, relative to controls. Recently, Rodrigues and colleagues (2022) found that norfluoxetine could affect the embryonic development of zebrafish larvae. In the study, newly hatched embryos were exposed for 80hpf to norfluoxetine (0.0014 µM) and fluoxetine (0.0015µM). Larvae exposed to norfluoxetine showed an increased frequency of pigmentation anomalies, in relation to the parental compound (Rodrigues et al., 2022).

Still concerning the SSRI (selective serotonin reuptake inhibitors) type of depressants, the primary metabolite of sertraline, n-desmethylsertraline, was found to affect Na/K-ATPase activity in the trout brain (Lajeunesse et al., 2011). The authors studied the distribution of selected SSRI in several tissues of brook trout, as well as the Na/K-dependent ATPase pump activity in the brain. Fish were exposed for 3 months to a

WWTP-treated effluent (primary treatment) before and after ozonation. The metabolite n-desmethylsertraline was one of the main substances found in various tissues. Also, Na/K-ATPase activity was negatively correlated with the accumulation of n-desmethylsertraline in the brain. Within the group of serotonin and norepinephrine reuptake inhibitors (SNRI), o-desmethylvenlafaxine (the active metabolite of venlafaxine) was implicated in behavioural changes of freshwater organisms (Stropnicky, 2017). The author exposed two species of crayfish, *Orconectes obscurus* and *Procambarus clarkii*, to 0, 1 or 8 µg/L of o-desmethylvenlafaxine. The aggression behaviour of the crayfish, measured by the number of attacks per minute of exposed animals, was the endpoint assessed. An increase in the number of attacks was found for both species at 8 µg/L (Stropnicky, 2017). A more recent study related o-desmethylvenlafaxine exposure to behavioural changes in freshwater species (Aztei et al., 2021) The authors exposed zebrafish embryos to this metabolite in a concentration range of 0.03 to 300 µM, for 5 days. Embryonic development was monitored, and a light/dark behavioural assay was performed. No significant developmental anomalies were elicited by o-desmethylvenlafaxine. However, a dose-response inhibition on locomotory function, mainly under dark conditions, was found (Aztei et al., 2021).

Clofibric acid, a metabolite of clofibrate, is another metabolite with reported negative effects on fish species. This compound caused modifications of biomarkers related to antioxidant defences and oxidative stress in *Gambusia holbrooki* (Nunes et al., 2008). In their work, the authors exposed the fish for 96 hours to 176.34, 211.60, 253.92, 304.71 and 365.65 mg/L of clofibric acid. This metabolite caused a decrease in the activity of several antioxidant enzymes and in particular the levels of oxidised glutathione, in both the liver and gills. The effects of chronic tramadol exposure were studied in the zebrafish brain (Zhuo et al., 2012). Following intramuscular injections (25 or 65 mg/kg), both n- and o-desmethyltramadol were detected in brain tissue, mainly n-desmethyltramadol. This is important, since fish chronically exposed to tramadol exhibited weight loss, abnormal behaviour and mitochondrial structural changes. Considering that the two metabolites were present in the brain tissue, it may be possible that both can exert their effects on the exposed animals. Nevertheless, further studies focused on their administration and specific effects are needed to support this.

Oxazepam is one of the main metabolites of diazepam, a widely used benzodiazepine that is prescribed as an anticonvulsant, among other functions. In a recent study, specimens of *Pimephales promelas* were exposed to 0.8, 4.7 and 30.6 µg/L oxazepam for 28 days and the relationship between its internal concentrations and effects on fish behaviour were investigated with two types of tests: novel tank diving test and shelter-seeking test (Huerta et al., 2016b). The authors concluded the brain was the tissue with

higher accumulation rates and significant behavioural effects in the novel tank diving test were observed in fish exposed to 4.7 µg/L. Although 4.7 µg/L is a concentration higher than found in freshwater bodies, it raises concern about the effects this metabolite can exert on fish behaviour and ultimately endanger populations impacted by this substance. Another study with the same compound revealed behavioural changes on *Perca fluvialis* (Fahlman et al., 2021). The results showed that anti-predation behaviour was stimulated in exposed animals, characterised by decreased activity and distance to conspecifics, as well as increased littoral habitat use (Fahlman et al., 2021).

Carbamazepine is one of the most used anticonvulsants worldwide. Recently, some of its transformation products were a matter of study by Desbiolles and colleagues (2020). Their study focused on the chronic effects of oxcarbamazepine and acridine 9-carboxylic acid, in single or combined exposure with carbamazepine, in two different models: the duckweed *Lemna minor* and the cnidarian *Hydra circumcinta*. Tested concentrations were the same for both models; 600, 27 and 900 ng/L for carbamazepine, oxcarbamazepine and acridine 9-carboxylic acid, respectively. For *L. minor*, exposure lasted 17 days and different phytometabolites were monitored. Exposure to the transformation products separately and in a mixture with the parental compound caused alterations of nitrogen balance, namely an increase in nitrogen compounds. The chlorophyll index was also higher in oxcarbamazepine groups than in the control. Nevertheless, the phenols index varied deeply without any specific trend or alteration relative to the control group. *Hydra circumcinta* individuals were exposed to the compounds for 14 days and different endpoints were assessed, such as reproduction, morphological changes and evaluation of antioxidant and oxidative stress biomarkers. The results showed that oxcarbamazepine exposure had implications in the total antioxidant capacity of *H. circumcincta* increasing two-fold in relation to control. Exposure to acridine 9-carboxylic acid affected all tested endpoints, except the reproduction. Combined exposure assays resulted in an increase in malformations on cnidarians and a decrease in the budding rate (Desbiolles et al., 2020). Another carbamazepine metabolite (carbamazepine-10,11-epoxide) was recently addressed for its possible effects on zebrafish embryonic development (Bars et al., 2021). The authors exposed zebrafish embryos from ~3 to 120hpf to a concentration of 250 µg/L of this metabolite, i.e., considerably higher than the maximum concentration found in the environment. Embryonic development was monitored through the exposure period and anomalies were registered. Results showed that swim bladder inflation was significantly delayed in carbamazepine-10,11-epoxide-exposed larvae, compared to the control (Bars et al., 2021). This is important since inflation of the swim bladder allows larvae to stay in the water column and have more chances of survival.

A recent study focused on the metabolites of the well-known antiviral oseltamivir (Tamiflu), and their chronic effects on medaka *Oryzias latipes* (Chen et al., 2020). Results showed that long-term parental exposure to both oseltamivir carboxylate and oseltamivir ethyl-ester affected embryonic development and fish hatchability at 300 µg/L, and embryonic development at 90 µg/L. Fenofibric acid, a metabolite of the anti-lipidemic agent fenofibrate, was also evaluated for its toxicity to zebrafish embryos (Jung et al., 2021). An LC50 value of 53.32 mg/L was found at 72h, which is considerably higher than the normally occurring concentration in the environment.

2.6 The way forward

This review gives an updated perspective on freshwater contamination by pharmaceuticals and pesticide transformation products and the available information about the toxicity of these substances. Detection of pharmaceuticals and pesticides is increasing in freshwater ecosystems, and concentrations in the range of ng to µg/L have been widely reported. Moreover, this same trend is described for their metabolites and transformation products. This occurrence made this field one of the most studied by the scientific community in the last years, with a number of published works addressing the potentially hazardous effects of such previously overlooked substances. The present research identified concentrations of 190 metabolites and transformation products (92 from pesticides and 98 from pharmaceuticals) in water bodies and wastewater effluents, none of them included in monitoring programmes set to achieve the good environmental status of freshwater ecosystems. Their formation processes, environmental fate in aquatic ecosystems and effects on humans and biota, summarised in Figure 6, are varied and a considerable cause of concern. Reported concentrations are mainly in the order of ng to µg/L. The concentration heatmap produced in this work allows us to easily spot the substances found at higher levels.

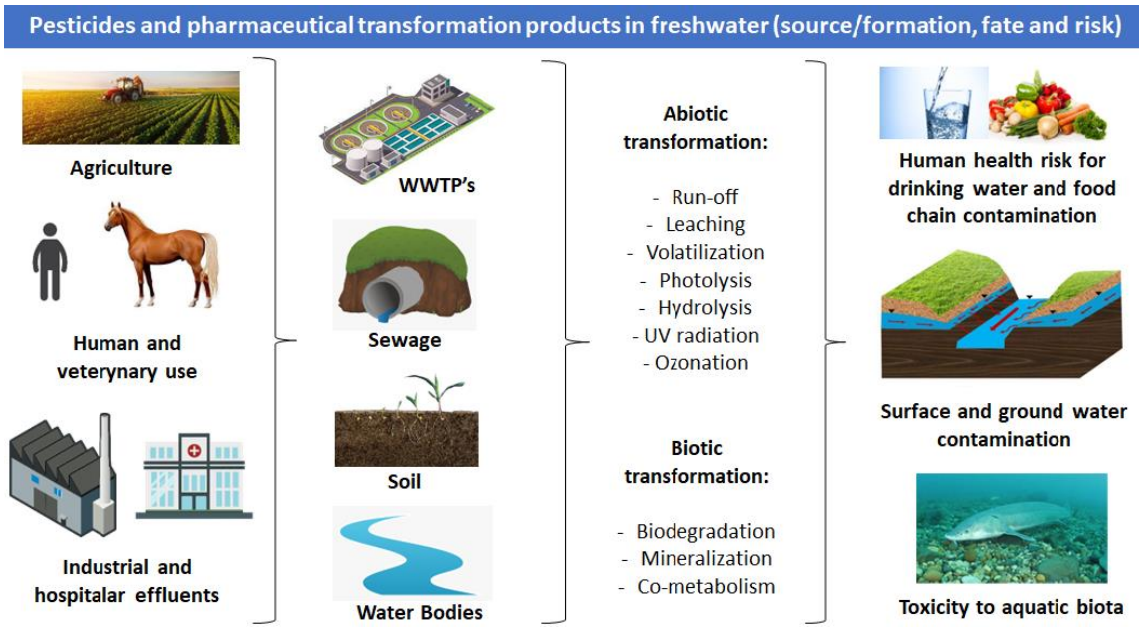

Figure 6. Overall representation of pesticides and pharmaceutical transformation products aquatic contamination and risks for human and aquatic species.

Although the information presented herein about the quantification of pesticides and pharmaceutical transformation products is extensive (almost 200 compounds), this may just represent the tip of the iceberg. Worldwide there are more than 1500 pesticides approved for use in agriculture and about 4000 pharmaceutical compounds approved for human consumption (aus der Beek et al., 2016; Anagnostopoulou et al., 2022). These parental compounds can have one or several transformation products, which brutally increases the potential number of these pollutants in the aquatic environment. Also, transformation products of pesticides banned for several decades now are still found in freshwater. Transformation products are in several cases more stable in the environment and consequently reach concentrations higher than their parental compounds (Schuhmann et al., 2019; Celiz et al., 2009). All these numbers and characteristics reinforce the need to increase the monitoring of these compounds in aquatic systems and evaluate their impact on human and environmental health.

The toxicological information available for the transformation products identified is very little and scattered, with no strategic approach underlying data collection for risk assessment and monitoring prioritisation. Concerning the risk to humans, less than ten metabolites (of the two groups combined) were investigated in in vitro studies. Various of these were found to elicit genotoxicity and effects on biotransformation and antioxidant processes. In aquatic organisms, only about 34% of the transformation products originating from pesticides and 14% of those originating from pharmaceuticals were evaluated for their potentially hazardous effects on biota. Most of these studies evaluated effects on only one (majority) or two trophic levels, and more than half of them on

vertebrates. Effects on plants and algae were rarely assessed. For pesticides, over 50% of the assessments were about acute and subacute toxicity effects, while for pharmaceuticals only about 20% of the assessments concerned chronic toxicity. Adding to this, for pharmaceutical metabolites various studies tested very high exposure levels, reporting effects at concentrations higher than those found in the environment. Nevertheless, for pesticide metabolites, several reports described a considerably wide range of negative effects on freshwater organisms, occurring at environmentally relevant concentrations. For pharmaceutical metabolites, different classes of drugs were proven to cause hazardous effects and jeopardise the homeostasis of freshwater species.

All in all, the data presented herein clearly demonstrate that pesticide and pharmaceutical transformation products pose a threat to aquatic fauna and flora. Concerning the relative toxicity of transformation products, compared to the parental compounds, the available data prevents a clear global conclusion. In some cases, the transformation products are in fact less toxic. In other cases, some transformation products can be more active and toxic than the parental substance. Nowadays, there is increasing evidence that pesticide transformation products can be more toxic and persistent than their parental compounds (Iwafune et al., 2018). In silico assays, performed with the ECOSAR (Ecological Structure Activity Relationships) software, which predicts the toxicity of different compounds, showed that the transformation products of several pesticides have a high toxicity potential to aquatic fauna and flora (Anagnostopoulou et al., 2022). Transformation products resulting from penoxsulam, pyrimethanil, imidacloprid, acetamiprid, thiacloprid and carbendazim were predicted to be more toxic than their parental compounds. In contrast, transformation products of fipronil present equal levels of toxicity, relative to fipronil itself (Anagnostopoulou et al., 2022). For pharmaceutical transformation products, there is a general idea that these compounds are less active and, consequently, less toxic than their parental compounds. However, there is evidence that some transformation products may be more toxic than the parental compounds. In humans, metabolites such as morphine and o-desmethyltramadol are more active than the parental compound (codeine and tramadol, respectively) (Rodieux et al., 2018). There are also reports of potential toxic effects elicited in patients, i.e., pethidine and dextroptopoxyphene (Coller et al., 2009). On the other hand, photodegradation products of prednisone, dexamethasone, naproxen, diclofenac, ketoprofen and atenolol formed in watercourses or even in WWTPs were reported to be toxic to different aquatic species at higher magnitude than their parental compounds (Della Greca et al., 2004; Isidori et al., 2005; Diniz et al., 2015). Nonetheless, for most of the transformation products identified, the information is still scarce to draw sound conclusions.

Something that is still not accounted for in most of the ecotoxicological works is the metabolism of parental substances in the test media. During exposure, parental compounds are metabolised and transformed by the exposed organisms. This is a process, influenced by media abiotic factors, which originates different transformation products. Such compounds can cause negative effects on the organisms, by themselves or in mixture with the respective parental compound. A previous study reported that fish exposed to tramadol exhibited weight loss, abnormal behaviour and structural mitochondrial changes that could be linked to the metabolites formed during the exposure, which accumulated in the animals' brains and muscular tissue (Zhuo et al., 2012). The possibility that several negative impacts reported on aquatic species exposed to pharmaceuticals may derive not only from those compounds, but also from the mixture with their metabolites, or even exclusively from the metabolites needs to be addressed in the near future.

Overall, the results warn of the need to continue improving treatment methodologies, for better removal of transformation products, not only to avoid their discharge to the aquatic environment but also to assure a better quality for water reuse. From a toxicological viewpoint, it is also striking the lack of mechanistic information useful to improve predictive toxicology and the risk assessment of these chemicals. Most works focused on assessing classical apical endpoints employing standard testing approaches. While this is always fruitful to obtain a quick grasp of the severity of a contamination scenario, more studies investigating the modes of action of these compounds are urgently needed. Also, the limited availability of reference standards for several transformation products makes it difficult to test the toxicity of these compounds to living organisms (Anagnostopoulou et al., 2022). However, this obstacle can be surpassed using in silico approaches, which reduce the need for animals and chemicals and can be valuable tools for toxicity and risk assessment.

Future toxicological investigations should be based on the framework of Adverse Outcome Pathways (AOP) (Ankley et al., 2010). This concept identifies various key events and relationships between them, linking a molecular initiating event to an adverse outcome of significance to risk assessment. The adverse outcome is usually considered at the organ level or higher, preferably the ecological level. It indicates a morphological or physiological alteration occurring in an organism, or its systems, that elicits functional impairment, or impairs its ability to compensate for chemical stress and achieve homeostasis. The AOP framework is recognised as useful to support regulatory decision-making and the prioritisation of chemicals for risk assessment (Vinken et al., 2017; Perkins et al., 2019), a most important aspect for the contamination scenario described herein. Present-day high-throughput technologies (e.g., proteomic sequencing) allowing

for the rapid and cost-effective generation of data should be used to identify key events and key event relationships through which the initiating event(s) will reflect on adverse outcomes to apical endpoints. Guidance documents for the development of AOPs were made available (OECD, 2013; OECD, 2018), as well as supporting databases and tools, such as the e.AOP.portal (http://aopkb.org), the AOP Wiki (http://aopwiki.org), the Effectopedia (http://effectopedia.org) and the Wikipathways (https://www.wikipathways.org/index.php/WikiPathways), the Harmonized Template 201: Intermediate effects (https://www.oecd.org/ehs/templates/harmonised-templates-intermediate-effects.htm) and the AOP Xplorer (http://datasciburgoon.github.io/aopxplorer. Collaborative networks based on resource and knowledge sharing, and rational effort application, should be made at a global level to establish and implement a structured strategy rapidly allowing to fulfil these gaps while avoiding unnecessary experimental redundancy (Martens et al., 2018).

The present work emphasizes the need to reinforce the existing knowledge about contamination by pharmaceutical and pesticide transformation products in freshwater systems. This report compiled and analysed a significant amount of information linking exposure to transformation products to adverse outcomes in aquatic species and humans. Technological needs and knowledge gaps were identified and discussed, delineating future research steps on the topic, ultimately aiming at improving water management and monitoring programs.

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Chapter 3: Preliminary testing

Chapter 3: Preliminary testing

3.1. General introduction

This chapter 3 was focused on establishing a testing routine for the analysis of pharmaceuticals and their transformation products, selected from the research performed on chapter 2, to know: carbamazepine, venlafaxine and fluoxetine, and tramadol. These parental compounds belong to three different therapeutic classes and are among the most representative ones found in environmental samples. For the first testing approach, which is represented in this chapter, zebrafish larvae were exposed to venlafaxine and fluoxetine (parental compounds) and norfluoxetine (fluoxetine metabolite) for 80hpf at different test concentrations, including levels within the range found for superficial water and WWTP effluents. Two different works were then performed and included in this chapter (Rodrigues et al., 2020; 2022). The first one had the purpose of comparing the toxicity of a parental compound (fluoxetine) and its metabolite norfluoxetine. The second had the purpose of comparing the toxicity of venlafaxine and norfluoxetine, as well as identify toxic effects of the mixture of both compounds. Survival and several developmental hallmarks were registered, and the determination of the mRNA expression of 38 different genes were evaluated in both studies. Moreover, a sensorimotor assay was included in the second study. The selected genes were involved in neurohormonal and detoxification processes and were selected according to available knowledge on the mode of action of these substances in other vertebrates. The results of these exposures are presented in the next two sub-chapters.

3.2. Differential molecular responses of zebrafish larvae to fluoxetine and norfluoxetine

3.2.1 Introduction

The detection of psychopharmaceuticals in aquatic ecosystems is a recognised problem of concern among the public and the scientific community. This is related to the increasing concentrations detected in water and sediments as a consequence of their growing consumption, linked with the massive increase in depression occurring worldwide (Santomauro et al., 2021). Among these psychopharmaceuticals are selective serotonin reuptake inhibitors (SSRI) antidepressants. Despite the increasing number of studies published in the last decade, the effects of SSRIs on non-target organisms, are still not fully understood (Salahinejad et al., 2022). Fluoxetine (FL) is an antidepressant

of the SSRI class (Mennigen et al., 2011) prescribed to treat obsessive-compulsive disorders, moderate to severe depression, anxiety, food and panic disturbances (Dulawa et al., 2004). Very recently, in-vitro studies have shown that FL may have antiviral effects on SARS-CoV-2, including in human lung tissue (Brunotte et al., 2021; Zimniak et al., 2021). A significant relationship between its use and a reduced risk of intubation or death in patients hospitalised for COVID-19 was also found in an observational study (Hoertal et al., 2021). This SSRI is used as FL-hydrochloride, which is the active ingredient of Prozac. The marketed drug is a racemate of s-fluoxetine and r-fluoxetine, two equipotent enantiomers (Baumann et al., 2002). It is also among the most prescribed and consumed antidepressants in the world (Menningen et al., 2011; Winder et al., 2012). Concentrations of FL found in environmental samples can reach 120ng.L-1 in surface waters and 540ng.L-1 in wastewaters (Fent et al., 2006; Kreke and Dietrich 2008; Connors et al., 2014).

The SSRI antidepressants act by inhibiting presynaptic receptors involved in serotonin (5-hydroxytryptamine, 5-HT) reuptake. This results in an increase in the concentration of serotonin in the synaptic cleft, which potentiates serotonergic neurotransmission (Kreke and Dietrich 2008; Menningen et al., 2011). Serotonin is known to be involved in neurohormonal mechanisms regulating endocrine functions. Altered levels of serotonin can thus elicit changes in various physiological processes and animal behaviour, including aggression, feeding, locomotion and reproduction through interference with the hypothalamic-pituitary-interrenal axis and the hypothalamic-pituitary-gonadal axis (Fent et al., 2006; Connors et al., 2014). Various serotonin receptors have been described in invertebrate and vertebrate animals, which are highly evolutionarily conserved neurotransmission (Kreke and Dietrich 2008). Nevertheless, their specific physiological role is still unknown and poorly characterised in many species. This increases the challenges to evaluating the environmental impact and risks of these substances to aquatic systems within the conceptual framework of Advanced Outcome Pathways proposed for environmental risk assessment (Marty et al., 2017).

In zebrafish (Danio rerio), a widely used model in toxicology, serotonin receptors are present from early embryo development, with the serotonergic system becoming active between 24 and 96hpf (hours post-fertilisation) (Kastenhuber et al., 2010). Moreover, several studies have shown that FL exposure can cause different effects on zebrafish early development. Among them are decreased larval swimming activity (Huang et al., 2019; Atzei et al., 2021) and reduced exploratory behaviour (Vera-Chang et al., 2018). In transgenerational studies, exposure of embryos to FL led to reduced cortisol levels in their offspring (Martinez et al., 2019). Developmental exposure to FL was also reported to affect the egg stress axis and the abundance of transcripts linked

to epigenetic pathways (Martinez et al., 2019). Increased frequency of embryonic malformations, disruption of antioxidant enzymes, and altered transcription levels of genes involved in detoxification was also found (Cunha et al., 2016; 2018)

Apart from FL, another cause of great concern related to the occurrence of psychopharmaceuticals in the aquatic environment is norfluoxetine (NF), the main metabolite of FL. Norfluoxetine has been detected in surface waters (up to 8. 25ng.L-1) and in wastewaters (63ng.L-1) (Baker and Kasprzik-Hodern, 2008; Fernandes et al., 2020). In humans, about 90% of ingested FL suffers a series of demethylation reactions in the liver, catabolised by genes of the cyp (Cytochrome P450) family, which originate the formation of NF (Ring et al., 2001; Fong and Molnar, 2008). Like the parental compound, NF presents two enantiomers, r-NF and s-NF. However, r-NF is 20 times less potent than s-NF (Stanley et al., 2007). A previous study comparing the MoA of FL and NF in rats using acute subcutaneous administration has shown that NF significantly augmented the extracellular level of 5-HT in the plasma and the brain, and prolonged its retention time, contributing to inhibiting the 5-HT transporter and extending the efficacy of FL (Qu et al., 2009). In aquatic animals, the first published study assessing the effects of NF showed this compound can induce bivalve spawning (Fong and Molnar, 2008). Despite this, and its reported occurrence in the environment, only a few studies addressed the effects of NF on fish. For zebrafish early stages of development, NF was reported to inhibit larvae locomotor activity, in a direct dose-response relationship (Atzei et al., 2021). Increased frequency of pigmentation anomalies (Rodrigues et al., 2020) and impaired stress-related behaviour (Zindler et al., 2020a) was also reported. Moreover, some authors suggest the effects of low FL concentrations can, in part, be linked to NF (Zindler et al., 2020a). Recently, heart malformations (including abnormal circulation, thrombosis and pericardial edema) were also observed after developmental exposure of zebrafish embryos to NF (Chai et al., 2021). This highlights the need for studies on the mode-of-action (MoA) of this active metabolite, given its high frequency of occurrence in aquatic systems and potential to accumulate in zebrafish embryos at concentrations within the range found in environmental samples, with bioaccumulation factors higher than those found for fluoxetine (Chen et al., 2017; Zindler et al., 2020).

This study investigated and compared the effects of exposure to FL or NF on zebrafish embryotoxicity and gene expression levels of newly hatched larvae. This model was chosen due to the transparency of its eggs that allows easy monitoring of embryonic development, as well as the availability of the full genome in public databases. Thirty-four target genes coding for proteins with different functions in detoxification and several neurohormonal receptors involved in the MoA of both FL and NF were assessed. The outcome of this work adds knowledge about the MoA and toxic effects of both

compounds in aquatic organisms, that are still not fully known, and identifies possible molecular biomarkers of exposure to these compounds in fish.

3.2.2 Materials and Methods

Tested chemicals

In this study, two pharmaceuticals were used: fluoxetine (CAS#56296-78-7) from Sigma-Aldrich (Germany) and norfluoxetine (CAS#57226-68-3), in solid crystals containing racemic mixtures of their R and S enantiomers, from Cayman Chemical Company® (USA).

Zebrafish rearing and reproduction

Zebrafish breeders (Wildtype AB) were maintained in in-house certified facilities at CIIMAR – Interdisciplinary Centre of Marine and Environmental Research (Matosinhos, Portugal), in 70L aquaria with water circulation and continuous aeration. The temperature was kept at 27 ± 1ºC and the photoperiod was 14 hours light:10 hours dark. The animals were fed with Tetramin XL Flakes two times a day, in a total of 2.5g. For reproduction, breeders were placed in a breeding aquarium (1 female:2 male) bearing a bottom net covered with glass marbles.

Zebrafish embryo toxicity test

The assays were carried out as described in Cunha et al. (2016). Briefly, the tests were done in 24-well plates containing 10 embryos in each well. The embryos were exposed from 1hpf (hour post-fertilisation) until 80hpf to 0.0015 and 0.05µM of FL (FL1 and FL2, respectively) and 0.00006 and 0.0014µM of NF (NF1 and NF2, respectively). Tested concentrations were selected to include one concentration in the range found in environmental samples (FL1 and NF1) and a higher one for each chemical. The FL stock solution was in DMSO and that of NF was prepared in water. The experimental design included two control groups: Water and DMSO (DMSO 0.004%). The assays were performed in duplicate each time and repeated three times, each with a different batch of embryos. A total of 40 embryos were exposed per treatment in each assay. Observations were made at 8, 32 and 80hpf using a Nikon Eclipse TS100 inverted microscope to check for embryo lethality and abnormal development by assessing and

recording a list of endpoints defined according to the existing literature (Kimmel et al., 1995). Table 1 presents the list of endpoints recorded in each developmental stage.

Table 1. Endpoints observed in embryotoxicity assays at three post-fertilisation time points.

Endpoint	8hpf	32hpf	80hpf
Cumulative mortality	X	X	X
Delay/pause in development	X	X	X
Abnormal growth	X	X	X
75% epiboly	X		
Anomalies in the eyes		X	X
Anomalies in the head		X	X
Anomalies in the spine/tail		X	X
Anomalies in the yolk sac		X	X
Anomalies in pigmentation		X	X
Pericardium edema		X	X
Unhatched embryos			X

The day before each assay, the microplate wells were filled with the respective test solution, to prevent decreasing bioavailability of the compound during the assay due to adsorption to the testing plates. The test solutions were renewed daily to avoid microbial contamination. At the end of the assays, the embryos were preserved in RNAlater and kept at -80ºC until the quantification of gene expression levels.

RNA extraction and cDNA synthesis

Total RNA was extracted with the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), following the manufacturer instructions. A BioTek microplate spectrophotometer, equipped with Take3 (2µL volume), was used to quantify the extracted RNA. DNA digestion of total RNA was done with deoxyribonuclease I Amplification Grade (Invitrogen). The cDNA synthesis was carried out with the qScript cDNA Synthesis Kit (Quantabio), according to the manufacturer instructions.

Gene expression

The levels of 34 gene transcripts selected for their potential involvement in the responses to these psychopharmaceuticals were measured by qPCR using primers previously described in the literature (Cunha et al., 2016; 2018) (Table S1, Supplementary material). The tar-get genes were selected from the available literature as monoamine receptors and trans-porters, as well as nuclear receptors and proteins, involved in FL and NF action and metabolism (Table S1, Appendix A2.). Namely, ABC transporters (abcc1, abcc2, abcb4 and abcg2); biotransformation and antioxidant enzymes (cyp1a1, cyp3a65, gst, sod and cat); serotonin, dopamine and noradrenaline receptors and transporters (5-ht1a,

5-htc2, drd1b, drd2b, adra2a, adra2b, adra2c, vmat 2, mao, serta, dat, net; and the nuclear receptors (pxr, ppara, pparb, pparg, rxraa, rxrab, rxrbb, rxrga, rxrgb, raraa, rarab, rarga, ahr. The full names of the genes and respective accession numbers are indicated in Table S1. Three reference genes were also assessed: elongation factor 1 (ef1), actin β1 (actb1) and ribosomal protein-like 8 (rpl8).

The identities of the amplicons obtained were confirmed through cloning and sequencing of the fragments of DNA, as described previously (Costa et al., 2012) (Appendix A2. Protocol A1). Efficiency curves were determined using 8 dilutions ranging from 0.05 to 50ng/μL of cDNA. qRT-PCR was done in an Eppendorf Mastercycler realplex 4 (Hamburg, Germany) using PerfeCTa SYBR® Green SuperMix from QuantaBio and 20μL reaction volume (Appendix A2. Protocol A2.). Duplicated were run for all reactions, according to the following cycles: 1 cycle at 95ºC for 3min followed by 40 cycles at 95ºC for 10sec; 51ºC (vmat2), 55ºC (5-ht2c and drdb1) or 54ºC (remaining genes) for 30sec; and 72ºC for 30sec. Blank samples and melting curves were run for each gene. Gene expression was quantified by normalisation to reference genes using the algorithm Normfinder (Urbatzka et al., 2013), which indicated actb1 and rpl8 as the most suitable reference gene combination. The method of Pfaffl, which accounts for the efficiency of the primers, was used to calculate the relative expression (Pffafl, 2001).

Statistical analysis

Differences among experimental conditions in embryotoxicity endpoints were analysed with the Chi-square test. Data were plotted as means and respective standard errors (SE). Considering that no significant differences were found between the water and the solvent controls, for any of the endpoints or expression levels analysed, all statistical comparisons were made against the solvent control, which for simplicity was indicated as control (ctr). Cluster analysis was used to investigate groups of genes with similar behaviour across substances and test concentrations. The elements used for the cluster analysis were the replicate expression values (in fold change), and the analysis was based on Pearson correlation values. Multivariate Analysis of Variance (MANOVA; based on the Wilks' Lambda criterion) was used to investigate differences among treatments for the most relevant gene clusters (fold change) identified. ANOVA followed by the Tukey HSD was used to identify homogeneous subsets. Statistical analysis was performed on TIBCO® Statistica software. The significance level was set at 5% for all statistical tests. Raw data is available at Rodrigues et al. (2021).

3.2.3 Results

Zebrafish embryo toxicity test

No statistically significant differences were found between the two control groups for any of the parameters analysed throughout the work. Cumulative mortality in the water control was 14%, while the solvent control had cumulative mortality of 16%. For all tested concentrations, mortality occurred between 8 and 32hpf (Figure 1). After that period, no mortality was observed. Differences in mortality in relation to ctr ($X^2(2,720) = 8.22$, $p = 0.016$) were found at 8hpf for NF1 and at 32hpf for FL1 and FL2 ($X^2(2,720) = 15.0$, $p = 0.0006$). For NF, a significant reduction in the mortality rate was found; for FL mortality was significantly increased, compared to the control group. For gross malformations, significant differences were found ($p<0.05$, compared to the control) for both compounds (Figure 1). A significant increase in gross malformations (~3-fold) was found at 32hpf for FL2 ($X^2(2,240) = 23.3$, $p < 0.00001$), while for NF a significant increase in gross malformations in relation to control was found at 80hpf for both tested concentrations (2.5-fold for NF1 and ~3-fold for NF2, $X^2(2,656) = 26.6$, $p < 0.00001$). Significant differences in gross malformations were mainly due to the appearance of anomalies in pigmentation in FL- (~8% for FL2 vs 0% for ctr) and NF-exposed larvae (~7% for NF1 and ~9% for NF2 vs 0% for ctr at 80hpf) (Figure 2); skeletal abnormalities were also observed in exposed embryos though at a much lower frequency (<5% vs 2% for ctr).

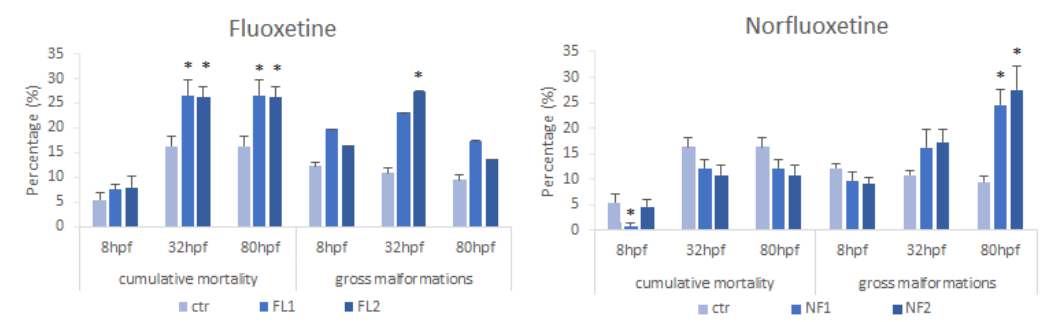

Figure 1. Cumulative mortality and rates of gross malformations recorded in embryos and larvae of zebrafish exposed to 0.0015 and 0.05μM fluoxetine (FL1 and FL2, respectively) (left graph) or 0.00006 and 0.0014μM norfluoxetine (NF1 and NF2, respectively). Results are expressed as mean±SE. * indicates statistically significant differences in relation to the solvent control (ctr) ($p<0.05$).

Gene expression

The Cluster Analysis identified eight groups of related genes (clusters a to h, Figure 3). Analysis of the heatmap for gene expression showed that clusters e and h were the most

relevant to distinguish between NF and FL (Figure 3). In contrast, cluster g was useful to distinguish the two FL concentrations evaluated. MANOVA confirmed that the treatment significantly influenced the expression of these groups of genes (cluster e: $F(15,23) = 6.36$, $p < 0.0001$; cluster g: $F(12,24) = 2.63$, $p = 0.02$; cluster h: $F(27,12) = 3.95$, $p = 0.007$).

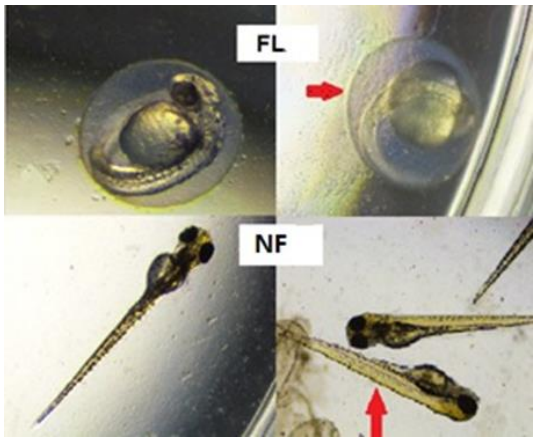

Figure 2. Illustrative examples of pigmentation anomalies found at 32hpf for FL (fluoxetine) and at 80hpf for NF (norfluoxetine). Control embryos and larvae are shown in the left images; exposed embryos and larvae showing pigmentation anomalies (red arrows) are shown in the right.

Cluster e was composed of the genes drd2b, 5-ht1a, dat, mao, cyp3a65. Of these, dat, mao and 5-ht1a were the most indicative genes, in terms of expression patterns and differences among group treatments, as indicated by the ANOVA results ($p < 0.05$, Table S2). Three separated homogeneous subsets were found for the expression of dat, distinguishing the effects of NF and FL, and the two concentrations tested for NF (Figure 3 and Figure 4). NF exposure caused upregulation of dat expression, which was more intense in the highest test concentration. FL exposure elicited downregulation of dat expression. The expression of mao was clearly different under the effect of NF (upregulated) and that of FL (downregulated) (Figure 3 and Figure 4). In addition, 5-ht1a expression was significantly different in FL2, compared to the remaining groups, for which clear downregulation was noted. Also, in larvae exposed to FL significant differences in expression were detected for all genes ($p < 0.05$, compared to the solvent control, Figure 4). The most relevant changes were the decreased expression observed for 5-ht1a (~2-folds), dat (3-folds) and mao (~2-folds) in the FL1 group, relative to the solvent control.

Cluster g comprised the genes net, serta, pparb and ahr2 (Figure 3 and Figure 5). Globally, the two FL concentrations had opposing effects on the expression of these genes; downregulation was found for FL1, and upregulation was observed in larvae exposed to FL2 (Figure 3 and Figure 5). No significant differences relative to the solvent

control were found for these genes, in larvae exposed to NF. For FL, significant downregulation of pparb and serta (~1-folds for both genes) was found for FL1 treatment, while significant upregulation was found for ahr2 in the FL2 treatment (1-fold) (Table S2, Appendix A2.).

Cluster h was composed of genes raraa, rxrga, gst, 5-ht2c, rxrab, rxraa, rarga, rarab, drd1b and abcc2. Of these, rarga, rarab and rxraa were the most indicative genes, characterising the expression patterns and differences among group treatments of the whole group. Three homogeneous subsets were found for the expression of rarga, completely separating the effects of NF and FL, and the two concentrations tested for FL (Figure 3 and Figure 6). NF exposure caused upregulation of rarga expression, while FL2 elicited its downregulation at 80hpf. For rarab two homogeneous subsets were identified, separating the larvae exposed to NF from those exposed to FL2. NF exposure also caused upregulation of rarab expression, compared to the downregulation found in larvae exposed to FL2. For rxraa, two homogeneous subsets were found, also pointing to upregulation of its expression, particularly in larvae exposed to NF2, compared to downregulation in larvae exposed to FL2 (Figure 4). These larvae also exhibited decreased expression compared to solvent control (Table S2, Appendix A2.). Compared to solvent control larvae, both FL concentrations elicited inhibition of rarab, rarga, 5-ht2c and abcc2 expression (Figure 6, Table S2, Appendix A2.).

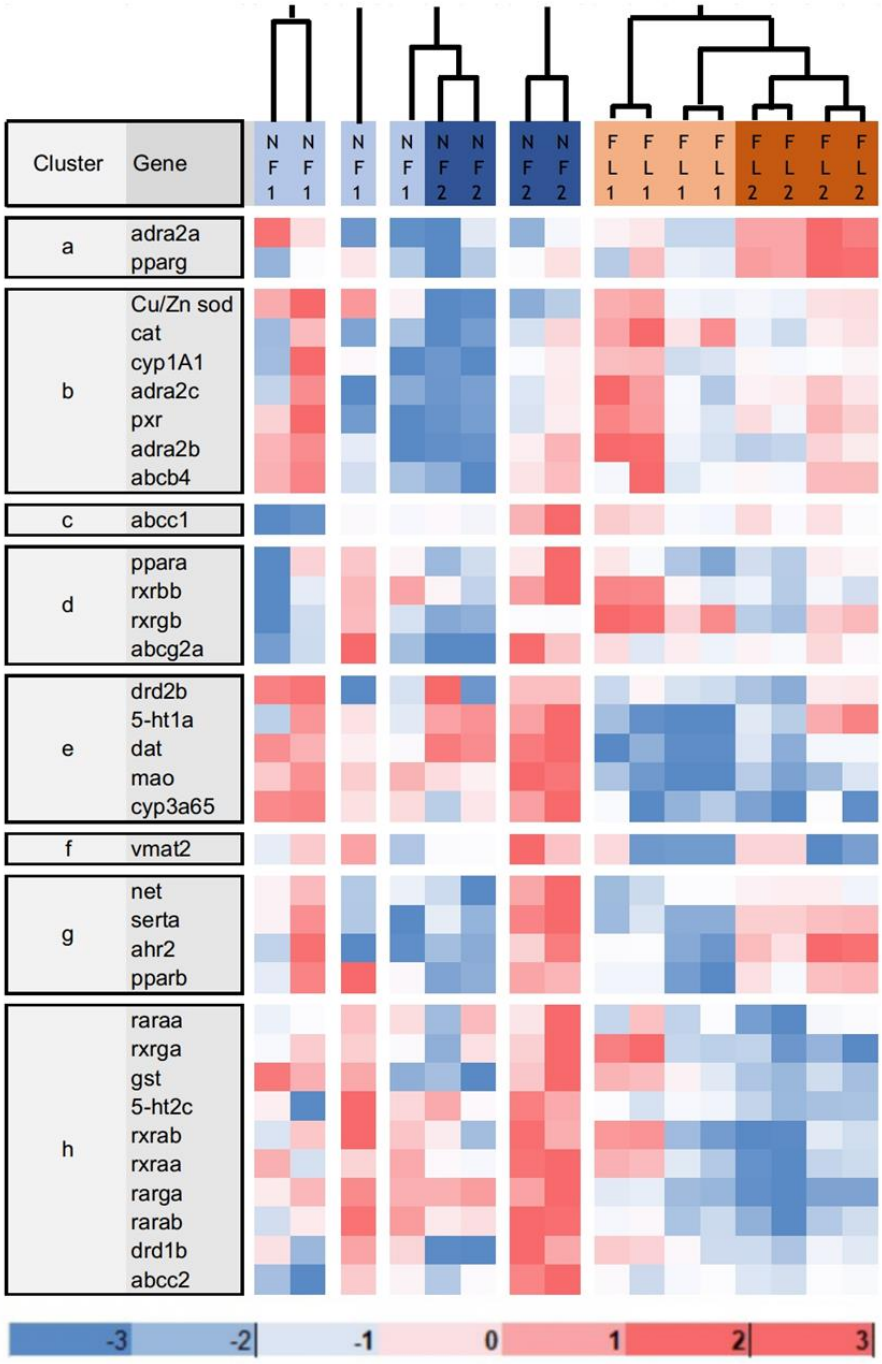

Figure 3. Heatmap of normalised gene expression (fold change) obtained for zebrafish larvae exposed to NF and FL for 80hpf. The magnitude and direction of variation, relative to the solvent control, are indicated by the colour scale.

3.2.4 Discussion

Embryo assays are very relevant to investigate potential hazardous effects of chemicals on aquatic organisms because animals are commonly more sensitive in their early

developmental stages than juveniles or adults (Lewin and Weiner, 2004; Celiz et al 2009; van der Ven et al., 2006; Cunha et al., 2016; 2018; Fonseka et al., 2016; Zindler et al., 2020a; Huang et al., 2019; Rodrigues et al., 2020; Atzei et al., 2021; Chai et al., 2021). Furthermore, they allow monitoring effects with impact on population maintenance that would otherwise be impossible to analyse later in life (i.e., teratogenic effects) (Cunha et al., 2016; 2018). This study evaluated the effects on zebrafish embryos and larvae of an antidepressant (FL) and its active metabolite (NF). These compounds were selected since more information about their MoA in non-target organisms is urgently needed. They are frequently detected in environmental samples (Evgenidou et al., 2015; Santos et al., 2016; Fernandes et al., 2020) and FL consumption is expected to increase given the predicted trends of incidence of psychiatric disorders. Whilst they represented already a very high burden of disease, the incidence of mental health problems is also increasing significantly with the COVID-19 pandemic (Santomauro et al., 2021). The study of pharmaceutical metabolites, such as NF, is also very pertinent since they are often active, exhibiting pharmacological properties and can also be more dangerous to aquatic fish than their parent compounds (Celiz et al., 2009). However, until very recently (Zindler et al., 2020a; Rodrigues et al., 2020; Chai et al., 2021), their investigation has been neglected in scientific studies, hampering the assessment of their risk to aquatic animals due to lack of toxicity as also highlighted by other authors for NF and other pharmaceutical metabolites (Kosma et al., 2020).

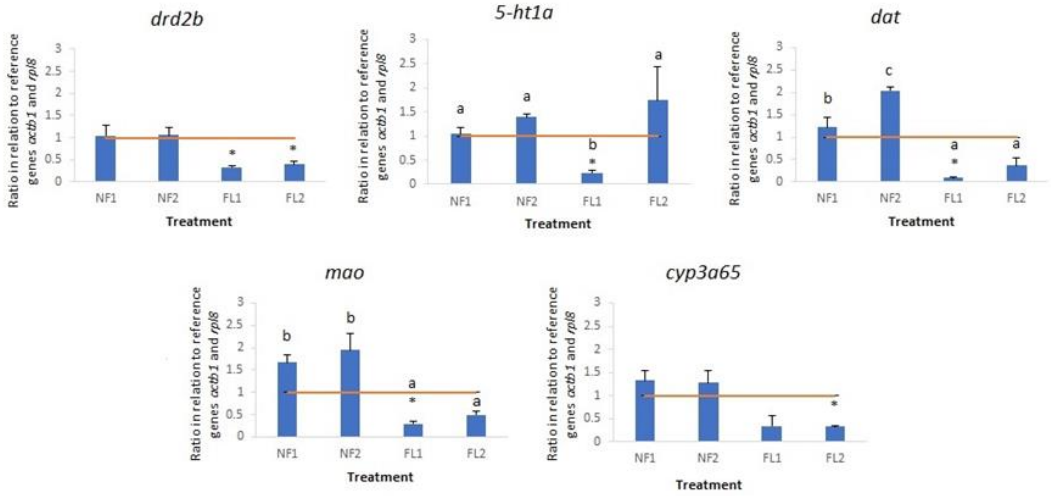

Figure 4. Relative expression of genes from cluster e. The small letters indicate the homogeneous subsets indicated by the Tukey HSD for the comparison of norfluoxetine based on MANOVA on for fold change (NF1, NF2) and fluoxetine treatments (FL1, FL2) ($p<0.05$). The stars indicate statistically significant differences ($p<0.05$) relative to the solvent control (from the results of the ANOVA on normalised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

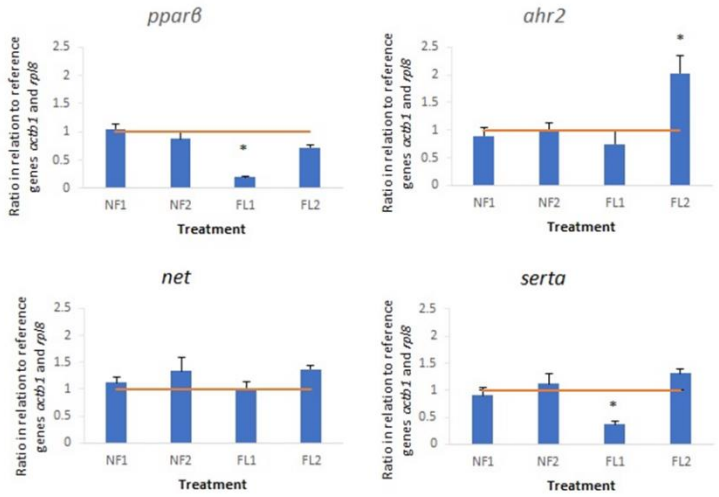

Figure 5. Relative expression of genes from cluster g. The stars indicate statistically signifi-cant differences ($p<0.05$) relative to the solvent control (from the results of the ANOVA on nor-malised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

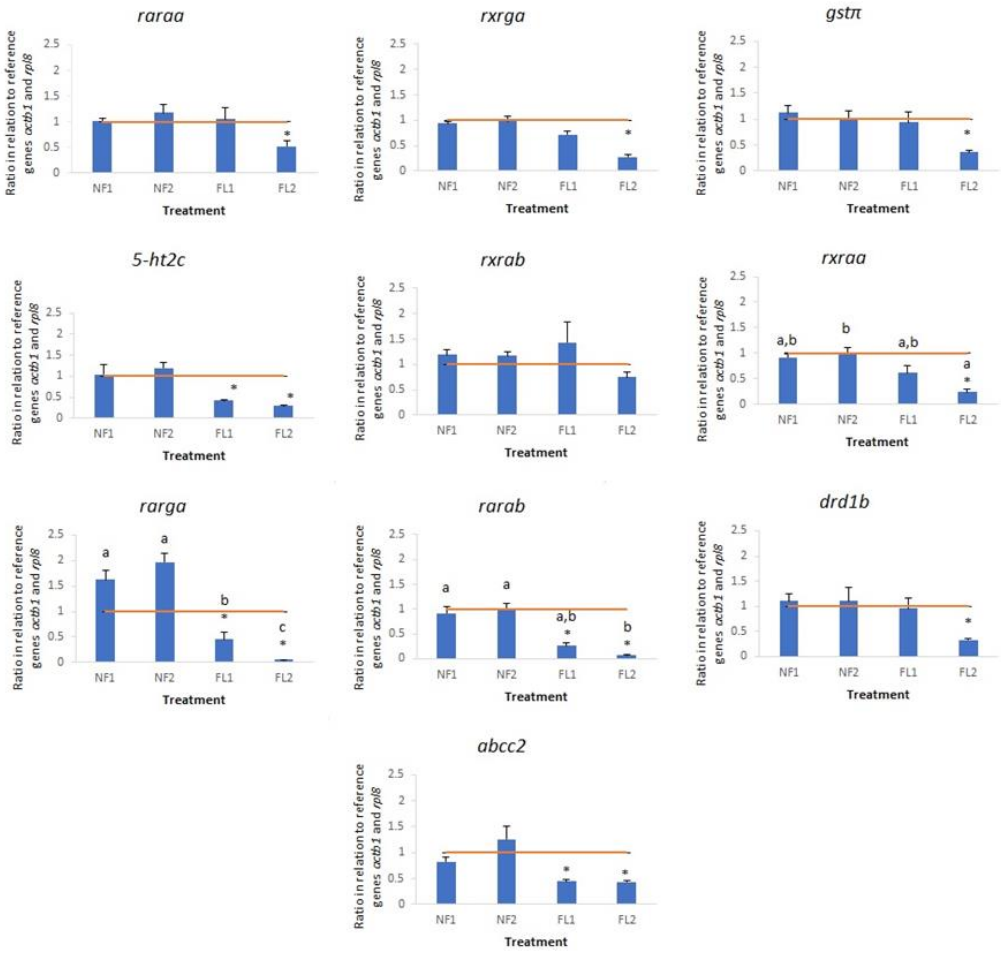

Figure 6. Relative expression of genes from cluster h. The small letters indicate the homogeneous subsets indicated by the Tukey HSD for the comparison of norfluoxetine based on MANOVA on for fold change (NF1, NF2) and fluoxetine treatments (FL1, FL2) ($p<0.05$). The stars indicate statistically significant differences

($p<0.05$) relative to the solvent control (from the results of the ANOVA on normalised expression, Table S2, Supplementary material). The horizontal line (x=1) indicates the baseline expression for the solvent control.

Zebrafish embryo toxicity test

The zebrafish embryotoxicity assays carried out fulfilled the validation criterium of OECD guidelines 212 and 236, which indicates as requirement hatching rates $\geqslant$80% for control groups. Few implications of SSRI exposure on survival were found at the tested concentrations. The mortality rate was low at 8hpf but increased at 32hpf in the FL and NF exposed embryos. This could be related to the fact that the monoaminergic systems through which the test drugs exert their therapeutic action were not active at 8hpf. The formation of these systems is known to start at 24hpf in zebrafish embryos (Kastenhuber et al., 2010; Airhart et al., 2012). This is supported by the results of our preliminary assays, where similar mortality levels were obtained at 32hpf when the exposures were started with 24hpf embryos, compared to the present work. A recent study using zebrafish (Atzei et al., 2021), reported that both FL and NF exposure caused a dose-dependent inhibition of locomotor activity in larvae, and increased the embryo malformation rates. High mortality rates for both compounds were also observed at the highest tested concentrations (89.9µM for FL and 60µM for NF) (Atzei et al., 2021). However, it is important to notice that the concentrations associated with high mortality rates in (Atzei et al., 2021) are much higher than the ones tested in the present work.

The antidepressant drugs investigated in this study elicited an augmentation of embryonic malformations at 32hpf for FL and 80hpf for NF. This is of concern for NF, once that a significant increase in relation to solvent control was found for an environmentally relevant concentration (NF1) at 80hpf. The main anomalies found for both compounds were in pigmentation. This may be due to the interaction of FL and NF with the embryonic adrenergic receptors of melanophores (Xu and Xie, 2011). The melanophores are pigment cells that contain eumelanin. They confer black or dark-brown pigmentation to larvae through the tyro-sine kinase signalling pathway. Because these cells originate from neural crest tissue, and somites are crucial for patterning neural crest migration, these results highlight the need for future investigation of possible somite defects in FL- and NF-treated embryos (Svetic et al., 2007). Pigmentation anomalies were previously detected in zebrafish embryos and larvae treated with venlafaxine (Rodrigues et al., 2020). An interesting result of the present work is also the time frame for the appearance of pigmentation anomalies, which was different for both compounds, 32hpf for FL and 80hpf for NF. Such an outcome in the developmental window could be related to FL and NF metabolism and/or to differences in potency and ratio of their

enantiomers (Hiemke and Hartter, 2000). According to the literature (Chu and Metcalfe, 2007), fish can accumulate norfluoxetine over time, reaching values equal or even higher than those of the parent compound (Zindler et al., 2020b). Differences in bioaccumulation and/or MoA could explain the higher frequency of anomalies observed in later phases of development for norfluoxetine exposed larvae. A recent study investigating the effects of fluoxetine and norfluoxetine (racemate and its S-enantiomer) in the visual motor response of zebrafish larvae, also showed that fluoxetine caused impairments at lower concentrations than the metabolite (both when tested as racemate or S-enantiomer) (Zindler et al., 2020a). Moreover, the authors reported additive effects of FL and NF on the embryonic visual motor response. The effects they observed were dependent on the concentrations of the tested equimolar mixtures and appear to be in agreement with the results of the investigations previously done in rats (Qu et al., 2009). In contrast, the investigation of Chai et al. (2021)], indicated that in zebrafish larvae racemic NF elicited more severe alterations in the cardiac structure, and cardiac fibrosis, than racemic FL. However, racemic FL induced a more severe immune cell infiltration in cardiac tissue than the racemic NF. These authors also showed that racemic FL is biotransformed into NF, with a stronger enrichment in S-norfluoxetine than R-norfluoxetine (Chai et al., 2021). Overall, the results reported are complex and dependent on the effects measured, in addition to the test concentrations and interactive effects of FL and NF.

Gene expression

Gene expression analysis revealed differing global patterns of response to the two SSRIs. Some variability among replicates was found, probably owing to slight differences in the genetic background as a wildtype strain animal was used, but overall, the results were in line with previous literature reports on the effects of FL and NF on the expression of these genes in zebrafish larvae (Cunha et al., 2016; Martinez et al., 2019; Rodrigues et al., 2020). Despite this, significant differences among the two SSRI were revealed by MANOVA. Mainly significant downregulation was observed in FL exposed larvae, against a tendency for no change or upregulation relative to the solvent control in those exposed to NF. Nevertheless, for NF larvae, significant differences (downregulation) were only observed for Cu/Zn sod (~1-fold, compared to the solvent control). Obtained results also denoted that the variation in gene expression was globally more accentuated in FL larvae, compared to NF. Data from the literature (Hiemke and Hartter, 2000) indicates that NF is more potent than its parent compound. Moreover, it was reported that NF is more toxic (up to 10 times) than FL to aquatic crustaceans and protists like *Tetrahymena thermophila*, *Spirostomum ambiguum* and *Thamnocephalus platyurus*

(Nalecz-Jawecki, 2007; Andrés-Costa et al., 2017). However, our results contradict those findings, at least for zebrafish in the early stages of development. The results of this study indicate that at similar exposure levels (NF2 and FL1) NF larvae experienced lesser alterations in gene expression and lower mortality than FL larvae. Comparing these two treatments, it is observable that both caused significant anomalies in the embryonic development of zebrafish. At the molecular level, however, for FL more significant differences were detected in the expression of detoxification genes and of those involved in SSRI MoA, than for NF.

Results from the cluster analysis identified eight groups of related genes. Of these, clusters e and h were the most relevant to distinguish NF and FL at the molecular level, while cluster g was useful to distinguish the two fluoxetine concentrations evaluated. From cluster e, the genes dat, mao and 5-ht1a that were the most indicative genes of differences among group treatments, are involved in the mechanism of action of both compounds. The genes dat, mao and 5-ht1a are present in monoaminergic systems where both SSRI compounds act. The action of NF and FL in these genes is, however, completely opposite to each other: downregulation for FL and upregulation for NF. The SSRI MoA occurs through inhibition of reuptake by the monoamine presynaptic receptors, mainly the serotonin receptors (Kreke and Dietrich, 2008; Mennigen et al., 2011). Considering this, we can hypothesise FL is exerting an expected effect on genes present in monoaminergic systems, causing their downregulation. In contrast, NF-exposed larvae showed a distinct response, with a predominance of upregulation or mild effects. This once again is in agreement with the previous findings about the potency of norfluoxetine, compared to fluoxetine, in the visual motor response of zebrafish larvae (Zindler et al., 2020a)

Cluster g comprised the genes net, serta, pparb and ahr2. Globally, we observed down-regulation for the lowest FL concentration and upregulation for the highest one. FL acts by inhibiting monoaminergic systems, which leads to an expected inhibition of net and serta transporters. In the present data, such expected inhibition occurred for serta in FL1-exposed larvae. The opposite expression levels elicited by exposure to FL1 and FL2 also point out a non-monotonic response of this cluster of genes. This kind of response was already described for non-target organisms exposed to SSRI compounds, supporting the above-mentioned assumption (Rodrigues et al., 2014;2015; Martinez et al., 2019). Furthermore, in a recent study conducted with human intestine cellular lines, the authors found serotonin could induce cyp1a1 ex-pression via the ahr through a process involving its uptake into the cell by sert (serotonin transporter) (Manzella et al., 2018). Although no evidence of this was described for fish, once both serta and ahr have similar patterns of expression and were comprised in the same cluster, it is possible that FL1 could have

caused some disruption in the serotonin-mediated CYP pathway of drug metabolism. However, in-depth follow-up studies would be needed to elucidate this question.

Cluster h was formed by the genes raraa, rxrga, gst, 5-ht2c, rxrab, rxraa, rarga, rarab, drd1b and abcc2. Of these, rarga, rarab and rxraa were the most indicative, characterising the expression patterns and differences among group treatments of the whole group. Those 3 genes belong to the families of retinoid x and retinoid acid nuclear receptors. Retinoid x receptors have the particularity of forming homodimers, as well as heterodimers with other nuclear receptors (e.g., retinoic acid receptors), to regulate various physiological pro-cesses (Xu et a., 2005). Differences between the compounds could lead to distinct impact on pathways where those genes are involved, creating potential opposing responses, including expression levels.

Overall, not many studies about effects of NF in fish species are available in the literature, making comparisons difficult. Nevertheless, the results reported herein are generally consistent with previous findings, showing similar up- or downregulation patterns were elicited by these substances (Cunha et al., 2016; Martinez et al., 2019; Parolini et al., 2019; Rodrigues et al., 2020).

3.2.5 Molecular biomarkers of exposure to psychopharmaceuticals in fish

Molecular biomarkers can be defined as a group of biomarkers that can be discovered using standard genomics and proteomics methodologies, as is the case of qPCR (Lewin and Weiner, 2004). Zebrafish is a well-known test model for the study of behavioural neuroscience, depression, and several mental diseases (Fonseka et al., 2016). During the past few years, research about new molecular biomarkers of exposure to psychopharmaceuticals has been increasing, mainly for antidepressants (Table 2).

Mianserin, a tetracyclic antidepressant that has antihistaminic and hypnosedative effects in humans, was reported to exert endocrine disruption on zebrafish (van der Ven., et al 2006). Treatment with this antidepressant induced estrogenicity biomarkers, like vitellogenin 1 and zona pellucida proteins. These could be defined as biomarkers of exposure to mianserin. Expo-sure of zebrafish to fluoxetine and sertraline produced a specific expression profile for both antidepressants (Park et al., 2012). However, the authors also reported a set of 8 genes that showed similar patterns of expression for both compounds and mentioned the expression pattern of the gene encoding for FK506 binding protein 5 as a possible biomarker of exposure to SSRI expression. Amitriptyline, a tetracyclic antidepressant, was reported to cause changes in the adrenocorticotropic hormone, oxidative stress enzymes, antioxidant defences, nitric oxide production and the total activity of nitric oxide synthase (Yang et al., 2014). It is important to notice that,

like in our study, the authors reported U-shaped dose-response relationships of the studied endpoints.

Table 2. Putative molecular and biochemical biomarkers of exposure to psycopharmaceuticals (PPharm).* identified through match with other organisms with sequences available in databases.

PPharm	Fish species	Stage / Duration of exposure	Exposure concentration ($\mu g/L^{-1}$)	Relevant altered genes or biochemical parameters	Reference
Mianserin	*Danio rerio*	Adults / 14 days	25 and 250	- *vitellogenin 1* - *zona pellucida glycoproteins*	van der Ven et al.,2006
Fluoxetine Sertraline	*Danio rerio*	Larvae / 96 hours	25 and 250	- *FK506 binding protein 5*	Park et al., 2012
Amitriptyline	*Danio rerio*	Larvae / 120 hours	0.001, 0.01, 0.1, 1, 10, 100 and 1000	- adrenocorticotropic hormone - nitric oxide synthase activity	Yang et al., 2014
Amitriptyline Fluoxetine Mianserin	*Danio rerio*	Larvae / 120 hours	0.1, 1 and 10	- *dual-specificity phosphatase 5* - *prostaglandin D2 synthase b* - *early growth response 1* - *early growth response 4*	Wu et al., 2017
Venlafaxine	*Danio rerio*	Larvae / 80 hours	0.016, 0.08, 0.4, 2.0 and 10	- *norepinephrine receptor 2b* - *hydroxytryptamine receptor 2c* - *dopamine receptor 1b* - *vesicular monoamine transporter 2*	Rodrigues et al., 2020
Fluoxetine	*Danio rerio*	Larvae / 96 hours	0.05 and 0.5	- *solute carrier family 6 member 4a* - *solute carrier family 6 member 4b* - *solute carrier family 6 member 11* - *cortisol levels* - *connective tissue growth factor a* - *cytochrome P450 family 11 subfamily A polypeptide 1* - *7-dehydrocholesterol reductase* - *lecithin-cholesterol acyltransferase* - *lanosterol synthase* *-nicotinamide phosphoribosyltransferase b* - *phosphomevalonate kinase* - *retinoblastoma 1* - *steroidogenic acute regulatory protein* - *steroid sulfatase (microsomal), isozyme S* - *uncoupling protein 2* - *FK506 binding protein 5* - *forkhead box k1* - *6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 4b* - *serine/threonine kinas 35* - *GATA Binding Protein 4* - *T-box transcription factor 5* - *transforming growth factor beta* - *connective tissue growth factor* - *cytochrome P450 (CYP) 2K6* - *hydroxytryptamine receptor 3A* - *cytochrome c oxidase subunit 2 ** - *Na- and Cl-dependent GABA transporter 3 ** - *C1a6 major histocompatibility class I receptor **	Parolini et al., 2019
Fluoxetine	*Danio rerio*	Larvae / 144 hours	0.54 and 54		Atzei et al., 2021
Fluoxetine Paroxetine	*Danio rerio*	Larvae / 144 hours	100		Huang et al., 2020
Fluoxetine Norfluoxetine	*Danio rerio*	Larvae / 9 days	0.1		Chai et al., 2021
Fluoxetine	*Dicentrarchrus labrax*	juveniles / 21 days	0.5 and 50		Costa et al., 2021
Sertraline			0.01 and 1		

				- *hydroxytryptamine receptor 3A* - *vitamin D 25-hydroxylase ** - *uridine phosphorylase ** - *hexokinase **	
Fluoxetine	*Danio rerio*	Larvae / 80 hours	0.5 and 17	- *drd2b* - *5ht2c* - *abcc2*	Present work

In a previous study, Wu and colleagues (2017) performed a complete transcriptomic analysis of zebrafish to evaluate embryonic exposure effects of amitriptyline, fluoxetine and mianserin. For that, the authors performed RNAseq (high-throughput RNA sequencing) analysis, followed by qPCR for the most promising set of genes resulting from sequencing. The genes egr1 and egr4 (early growth response 1 and 4) were under-expressed for all tested antidepressants, making it a possible molecular biomarker of exposure. Later on, exposure of zebrafish to FL for 96hpf indicated the upregulation of serotonin and GABA transporters (slc6a4a, slc6a4b, slc6a11) as of interest as biomarkers of exposure (Parolini et al., 2019). A transgenerational study investigating the effects of early developmental exposure to FL (Atzei et al., 2021) reported decreased cortisol levels associated with reduced exploratory behaviour of adult males for three generations. The authors concluded that FL exposure of 3hpf embryos for 6d caused disruption of the stress axis as manifested by a reduction of the basal and stress-induced cortisol levels, following an acute net handling stressor. The authors also found altered expression levels of genes controlling cortisol synthesis and the pathways of cholesterol or steroid synthesis. This FL-induced low-cortisol levels were more prominent in males and was associated with significantly reduced exploratory behaviours for two generations. Comparative transcriptomic analysis of zebrafish larvae exposed to fluoxetine or paroxetine identified upregulation of genes involved in mitochondrial and neurodevelopmental processes as also of interest as markers of exposure (Huang et al., 2020). Besides the identified malformations in cardiac development, Chai and colleagues (2021), also detected abnormal expression (mostly upregulation) of five genes (gata4, tbx5, tgf-β, ctgf and cyp2k6) in 9d larvae exposed to racemic FL or NF, and S-norfluoxetine, that may provide useful biomarkers of exposure. In a transcriptomic study with sea bass juveniles exposed chronically (21 days) to antidepressants FL and venlafaxine, Costa et al. (2021) also identified the serotonin receptor 5-ht3a as a promising molecular biomarker of exposure to the studied compounds, in addition to other genes involved in metabolism and neurotransmission (Table 2).

Our study analysed a vast set of genes using qPCR, unveiling drd2b, 5-ht2c and abcc2 as markers of exposure to FL. Further, different profiles of expression for low and high FL concentrations were identified, which could be used as molecular biomarkers of exposure in zebrafish. On the other hand, although the results obtained in this study

show differences in gene expression patterns between FL and NF, no clear differences could be depicted between NF-exposed larvae and controls. This makes it difficult to determine a possible molecular profile that could be used as biomarker of exposure to the metabolite. Future research should focus on other types of methodologies and endpoints (e.g., bio-chemical analysis, transcriptomics, proteomic studies) to discover other promising molecular biomarkers of exposure to these compounds.

3.2.6 Conclusions

In conclusion, FL caused higher mortality levels than NF. In contrast, both compounds increased the frequency of abnormalities during zebrafish embryonic development; pigmentation anomalies were the most frequently found. At the molecular level, two clusters of genes with altered expression were useful to distinguish the two SSRIs. One cluster was linked to the adrenergic pathway eliciting pigmentation anomalies. The other was associated mostly with nuclear receptors. Interestingly, NF and FL were mostly found to elicit opposing genomic expression in early zebrafish larvae, possibly originating from differences in their intrinsic pharmacokinetic properties that could lead to different MoA. Overall, embryo pigmentation and the expression of several genes appear as promising biomarkers of fish exposure to antidepressant drugs. Future research should focus on conducting further in-depth investigations comparing the toxicity of parent psychopharmaceuticals and their metabolites, single and in mixtures as detected in wastewater effluents, to improve monitoring and risk assessment routines.

3.2.7 References

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3.3 Norfluoxetine and venlafaxine in zebrafish larvae: single and combined toxicity of two pharmaceutical products relevant for risk assessment

3.3.1 Introduction

The occurrence of pharmaceuticals in aquatic systems is a continuously growing problem. Psychoactive drugs, especially, are frequently detected in the water and sediments due to their increasing prescription and consequent consumption (Paíga et al., 2016). This high consumption is related to the overwhelming appearance in the market of selective serotonin reuptake inhibitors (SSRI) and lately with the serotonin–norepinephrine reuptake inhibitors (SNRI) (Gusmão et al., 2013; Melnyk-Lamont et al., 2014). Of these, norfluoxetine (SSRI) and venlafaxine (SNRI) are two of the pharmaceutical residues most frequently detected in natural and waste waters, and at higher concentrations, compared to other psychoactive substances (Paíga et al., 2016). Norfllluoxetine is the active metabolite of the SSRI fluoxetine, which ranks high in consumption worldwide (Mennigen et al., 2011; Winder et al., 2012). Norfluoxetine has been detected in fish tissues, in the ng/g range (Brooks et al., 2005), and in different environmental water samples at concentrations up to 8.25 and 13 ng/L (Petrie et al., 2015; Fernandes et al., 2020). Despite this, the number of studies available about the effects of norfluoxetine in aquatic non-target organisms is very limited. Only as late as 2008, Fong and colleagues published the first study showing that exposure to norfluoxetine can induce spawning in clam and mussel species. After consumption, fluoxetine is metabolised in the liver, where demethylation reactions lead to the formation of norfluoxetine (Hiemke *et al*., 2000). In humans, different genes from the Cyp (Cytochrome P450) family catalyse such reactions (Ring *et al*., 2001). The SSRI mode-of-action (MoA), in which norfluoxetine is included, encompasses the inhibition of serotonin (5-hydroxytryptamine, 5-HT) reuptake at presynaptic receptors. This action leads to an increased concentration of active 5-HT in the synaptic cleft, potentiating its effects (Mennigen *et al*., 2011).

Venlafaxine is commercialised under the trade name Effexor®. At present it ranks very high among antidepressant prescriptions (Sansone and Sansone, 2014; Melnyk-Lamont *et al*., 2014). Venlafaxine was detected in environmental water samples at levels up to 1.31 and 2.19 µg/L (Schultz and Furlong, 2008; Schultz *et al*., 2010; Gonzalez Alonso *et al*., 2010). Similar mechanisms of action have been described for SNRIs and SSRIs. The main difference is that, in addition to inhibition of 5-HT reuptake at presynaptic receptors, SNRIs further inhibit the reuptake of norepinephrine. They can also inhibit dopamine

reuptake, although this is a recognised weaker effect of SNRIs, compared to their action on the serotonergic and noradrenergic pathways. Overall, SNRIs cause increased concentration of the three monoamines in the synaptic cleft, enhancing their physiological action (Gutierrez *et al*., 2003; Mennigen *et al*., 2011). Various studies about the effects of venlafaxine in fish showed this drug can cause reproduction and behaviour alterations (Schultz *et al*., 2011; Gaulus *et al*., 2013; Thompson *et al.*, 2017). Venlafaxine can also affect the stress response in fish by blocking the epinephrine-induced production of glucose (Ings *et al*., 2012).

Serotonin and norepinephrine receptors have been found in vertebrates and invertebrates. From an evolutionary viewpoint, they show very high conservation, including in various areas of the fish brain and during early stages of the embryonic development (Kreke and Dietrich., 2008; Kastenhuber *et al*., 2010). In zebrafish, for example, the serotonergic system develops between 1-4 dpf (days post fertilization). Adrenergic neurons start appearing after 24 hpf, while the whole adrenergic system is complete at 5dpf (Kastenhuber *et al*., 2010). The specific physiological role and MoA of these monoaminergic receptors is, however, little known in many species. This hampers the evaluation of SSRIs and SNRIs impact on aquatic ecosystems, or the assessment of remediation technologies set to decrease their presence in waste waters. The aims of this study were therefore to increase knowledge about the effects and MoA of single and combined exposures to norfluoxetine and venlafaxine during embryonic development, identifying potential biomarkers that could be useful to trace the exposure. The zebrafish embryotoxicity assay was employed to evaluate mortality, and embryonic malformations elicited by the exposure, as well as alterations on the expression of 34 genes involved in the MoA and metabolism of norfluoxetine and venlafaxine. The target genes evaluated encode for: i) the serotonin, dopamine and norepinephrine neurohormonal receptors and transporters; ii) the monoamine oxidase (involved in oxidative deamination of monoamines) and the vesicular monoamine transporter (acting on the uptake of monoamines into storage vesicles and their release at synapses); iii) several ligand-binding nuclear receptors regulating drug metabolism (such as retinoid X receptors, retinoic acid receptors and peroxisome proliferator-activated receptors); iv genes involved in detoxification (phase 0 and phase III ABC transporters (ATP-binding cassette) and antioxidant processes. Rates of mortality and malformations, stimulated swimming behaviour and gene expression were also evaluated in embryos exposed to a mixture of norfluoxetine and venlafaxine at concentrations found in natural aquatic systems.

3.3.2 Material and methods

Chemicals

Norfluoxetine (CAS Number 57226-68-3) and venlafaxine (CAS Number 99300-78-4) were obtained from Cayman Chemical Company® and the European Pharmacopoeia Reference Standard®, respectively. All other chemicals used were obtained from local suppliers.

Zebrafish embryotoxicity test

Zebrafish (*Danio rerio*) breeders were maintained under standard culture conditions and used to obtain test embryos. Assays were performed following the methodology adopted by Cunha *et al*. (2016). Briefly, all exposures were repeated three times, each with a different batch of embryos. Two replicate assays were conducted in each repetition/batch. Each replicate was carried out in a 24-well plate. A total of 40 embryos (10 embryos/well) were exposed per test condition in each replicate. Each assay plate was loaded with the test solutions for 24h before the embryo assays were done, to minimise losses of the test chemicals in the media by adsorption to the plates. For each assay, the test solutions were completely renewed the day after and ~1hpf embryos were exposed. The concentrations of norfluoxetine evaluated ranged from 0.64 to 400 ng/L; those of venlafaxine ranged from 16 to 10000 ng/L. The tested concentrations include levels found in aquatic systems and above, because of possible differences in responses to low and high exposure concentrations (Rodrigues et al. 2015, Cunha et al. 2016, Fong and Ford 2016). Effects of a mixture of norfluoxetine (3.2 ng/L) and venlafaxine (2000 ng/L) were also assessed. Concentrations in the mixture were selected to represent levels found in environmental water samples. Control and antidepressant test media were completely renewed daily in all experiments.

Embryological development was evaluated at 8, 32 and 80hpf using an inverted microscope (Nikon Eclipse TS100). Endpoints recorded were mortality, delay/arrest of cell division, developmental delay, 75% epiboly, pigmentation (presence or absence), formation of abnormal cell masses, eye, head and spinal abnormalities, pericardial edema and hatching rate, as described by Kimmel *et al.* (1995). At 80hpf live embryos were isolated into RNA*later* until analysis of gene expression by qPCR.

Sensorimotor reflexes

Sensorimotor reflexes of 80hpf larvae were assessed on the mixture experiment as described by Cunha et al. (2018). In summary, gentle touches were applied to the head and the tail of the embryos using a micropipette tip. Each animal was touched 10 times on the head and 10 times on the tail (head and tail touches were alternated), and a 30s resting period was allowed between each individual touch. Immediate swimming (*i.e.,* positive) or no movement (negative) responses exhibited upon stimulation were recorded for each touch.

RNA isolation and cDNA synthesis

Isolation of the total RNA was done with a GE Healthcare kit (Illustra RNAspin Mini RNA Isolation kit, following the protocol of the manufacturer. The quality of the RNA extracted was checked by agarose gel electrophoresis. RNA was quantified in a BioTek microplate spectrophotometer with a Take3 micro-volume adapter. The Invitrogen Deoxyribonuclease I Amplification Grade kit was used to digest genomic DNA; the Biorad iScript cDNA Synthesis Kit was employed for cDNA synthesis. All reactions were set according to the respective manufacturer protocols.

Gene expression

SybrGreen-based quantitative real time PCR (qRT-PCR) was employed to assess the expression of thirty-four genes in larvae obtained from single exposures to norfluoxetine or venlafaxine. The expression of each gene was done in at least four independent exposure replicates. Following exposure to the single compounds, a subset of ten genes showing strong alterations (at least 50%) in expression relative to controls was subsequently selected for evaluation in the mixture assays. Detailed information on the genes, their function and the primers employed is provided in the Appendix A3., Table S1. The reference genes assessed were *actb1*, *ef1* and *rpl8* (Appendix A3. Table S1). The identities of the amplicons obtained were previously confirmed through the cloning and sequencing of DNA fragments following the description of Costa and colleagues (2012). The highest fluorescence signal obtained for the shortest Cycle threshold (Ct) was used to determine the primer concentrations for qRT-PCR. Primer efficiency was assessed by a series of 8 cDNA dilutions ranging from 0.05 to 50 ng/µL. The qRT-PCR reactions (20 µL reaction volume) were run in an Eppendorf Mastercycler realplex 4. The SybrGreen was from Biorad. Each reaction was run in duplicate. The reaction

parameters were set as follows: a first cycle at 94ºC for 2min; 40 cycles for 30sec at 94ºC for denaturation, for 30sec at respective annealing temperatures, and for another 30 sec at 72ºC for extension. Primer annealing temperatures were 51ºC for *vmat2*, 55ºC for *5-ht2c* and *drdb1,* 54ºC for the remaining genes. To check for the formation of non-specific products, blank samples as well as melting curves were run for each of the genes assessed. Normalisation for quantification of the gene expression was done using *actb1* and *rpl8* as reference genes for norfluoxetine, and *ef1* and *rpl8* as reference genes for venlafaxine, according to the results of Normfinder (Andersen *et al*. 2004). The mathematical template of Pfaffl (2001), which incorporates the primer efficiencies was used to calculate the relative expression.

Statistical analysis

Differences between treatments regarding gross embryonic malformations were evaluated with the Chi-square test; embryos from three replicates were analysed and data is presented as relative frequencies. Behaviour data was also analysed with the Chi-square test to investigate differences in the frequency of response to stimulus among test conditions and between the stimulated regions (head or tail) of the larvae. Analysis of variance (ANOVA) was used to investigate differences in gene expression among treatments, for each drug evaluated. When ANOVA indicated significant differences, the Tukey HSD test was employed to identify homogeneous groups. Investigation of possible interaction between norfluoxetine and venlafaxine when in mixture were investigated using dummy variables in ANOVA, followed by the Tukey HSD. Cluster analysis, a multivariate agglomerative method, was used to identify the genes acting similar across treatments. The cluster analysis was based on Pearson correlation values and was done with the replicate fold change values. All statistical analysis were performed with Statistica and significant differences were accepted when p was lower than 0.05.

3.3.3 Results and discussion

In recent years it has been show that metabolites of psychoactive drugs are frequently found in natural and waste waters. In some cases, they may have higher affinity to monoamine receptors than their parent drugs (Hiemke and Hartter, 2000), possibly causing stronger or different detrimental impact on non-target aquatic animals. Nevertheless, investigation of their effects, single and in mixture with other occurring drugs, has been overlooked, with limited information available for the risk assessment of these substances.

Exposure and population-level effects

The assays presented herein were conducted, under tight controlled conditions, with very stable pharmaceutical compounds. Though very limited information is available for norfluoxetine a seven-day half-life was determined for this compound (Merlob & Schaefer 2015). Venlafaxine was found to be stable in natural water for 60 days with low dissipation percentage (Li & McLachlan, 2019) and very low direct photo and microbial degradation (Rúa-Gómez & Püttmann 2013). In addition to this, the 24-well plates used to perform the embryo assays were bathed with the test solutions for 24h before the embryos were exposed. The aim was to minimise losses of the chemicals by adsorption to the test recipient. The test solutions were also completely renewed every 24h. Previous works have also shown great consistency between nominal and exposure test concentrations over seven days for venlafaxine and three days for norfluoxetine, with high recovery rates mostly above 90% (Appendix A3., Table S5 (Melnyk-Lamont et al. 2014; Ribeiro et al. 2014; Hodkovicova et al. 2020). In face of these data and under the experimental conditions employed, our exposure concentrations are expected to be very close to the nominal concentrations.

Rates of mortality and malformations

The assays carried out fulfil the valid criterium of the OECD guidelines 212 and 236, with a hatching rate ≥80% observed in the control groups. The mortality rates recorded in the norfluoxetine, or venlafaxine treatments were at control levels throughout the 80hpf (Figure 1). For the mixture of norfluoxetine and venlafaxine an increased mortality rate was found (~13% at 8hpf and 20% at 32hpf, $p<0.05$ relative to controls), possibly related to interaction of the two drugs upon the development of the adrenergic and serotonergic systems, which in zebrafish start to form at 24hpf (Kastenhuber *et al.,* 2010; Airhart *et al.,* 2012).

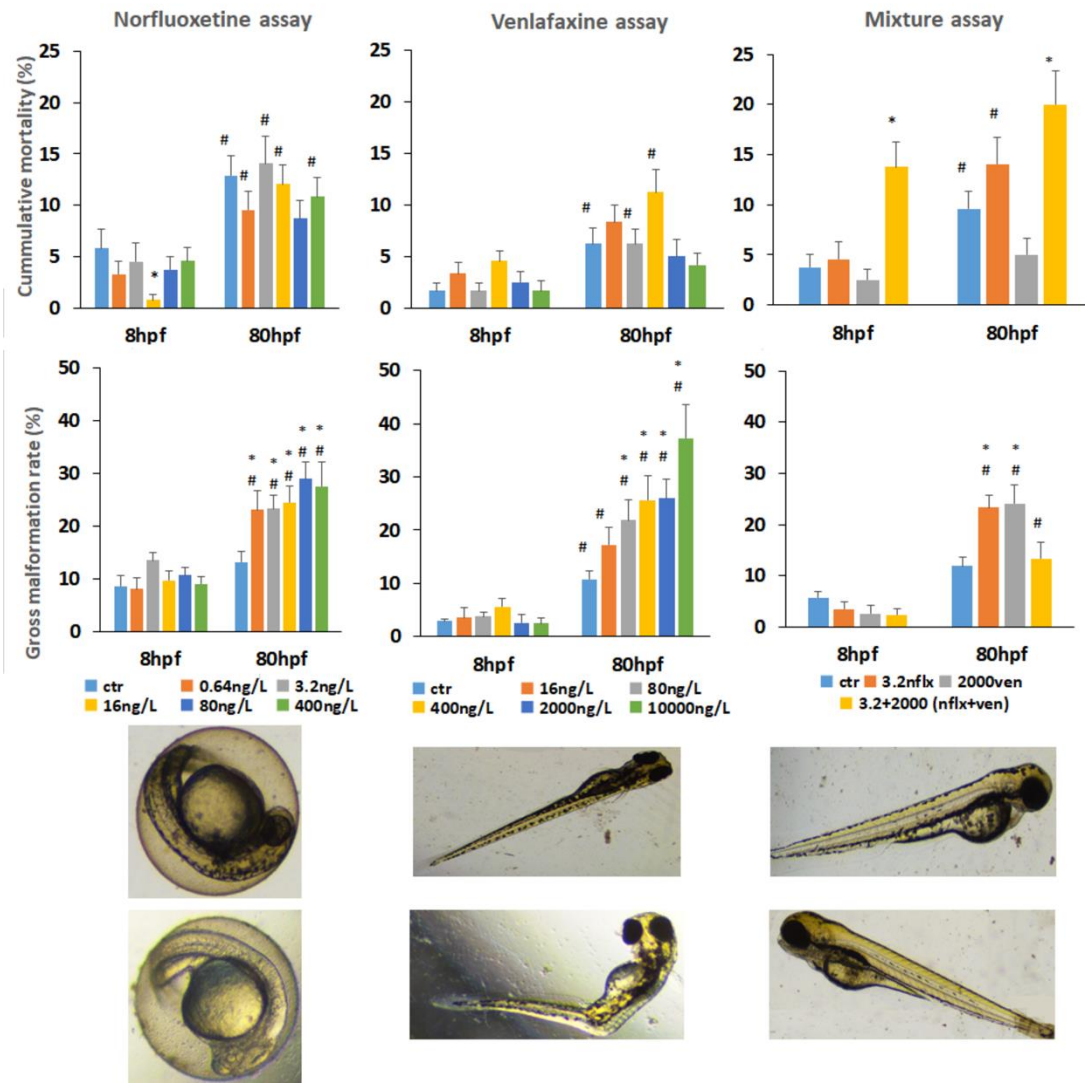

Figure 1. Mortality and gross malformations. Cumulative rates of mortality (top) and embryonic malformations (middle) recorded in assays with zebrafish embryos exposed to norfluoxetine, venlafaxine or a mixture of these substances. * indicates significant differences ($p<0.05$) relative to the control; # indicates significant differences relative to 8hpf (hour post fertilization), as indicated by the Chi-square test. Images of normal embryos (upper tier) and embryos exposed to the mixture, showing the most representative anomalies found (bottom tier), are also shown; amplification 40x.

An increased (~2-fold) rate of embryo malformations was found for all norfluoxetine concentrations, relative to the control ($p<0.05$, Figure 1). Altered dark pigmentation in embryos and larvae, related to melanocytes, was the most frequent anomaly found. Venlafaxine (>80 ng/L) caused increases in the rate of embryo malformations ranging from two to four folds ($p<0.05$, relative to controls; Figure 1); namely abnormal embryo pigmentation and yolk egg and spinal deformities. For the mixture, 32hpf surviving embryos also exhibited a higher rate of morphological abnormalities (mainly pigmentation) than controls and the single drugs ($p<0.05$, Figure 1). To the best of our knowledge, reports describing the occurrence of embryonic malformations in fish due to

exposure to environmental levels of norfluoxetine or venlafaxine are not available in the literature. Findings of a previous study suggest exposure of embryos of this same species to 500 ng/L venlafaxine would produce a very low rate of abnormalities (Galus et al., 2013). In our study, most malformations detected in the venlafaxine exposures may be related to a developmental delay (Figure 1), with only the abnormal pigmentation persisting at 80hpf. The abnormal pigmentation found may have occurred through loss of melanocytes, abnormal melanocyte morphology or reduced melanocyte pigmentation (Colanesi et al., 2012). According to investigations in zebrafish embryos by Colanesi and colleagues (2012), pale pigmentation can be associated with developmental delay and/or reduced fitness. Alterations in melanocytes may be linked to an action of norfluoxetine and venlafaxine upon adrenergic pathways (Kreke and Dietrch, 2008; Kastenhuber et al., 2010; Airhart et al., 2012). On the one hand, adrenergic receptors are known to be present in the melanophores, responsible for the dark pigmentation (Xu and Xie, 2011). On the other hand, increased expression of adrenergic receptors (*e.g., adra2b*) was found in our embryos exposed to venlafaxine (at ≥400 ng/L) and the mixture (see below), while inhibition of the norepinephrine transporter (*net*) was observed at 10 µg/L venlafaxine. Our results also suggest an influence of these compounds on the somitogenesis or the neural crest formation. Previous investigation found that, in a highly conserved process, transport of norepinephrine promotes differentiation of neural crest stem cells (Hu et al., 2009). Moreover, loss of function of this transporter during the mouse embryonic development has been shown to deregulate signalling pathways critically involved in neural crest formation, with consequences at chromatophore level as well as noradrenergic cell differentiation (Hu et al., 2009). Not only a relation between neural crest tissue and somites has been identified in the zebrafish (Svetic et al. 2007) but also correct somite development is vital for patterning neural crest migration. The finding reported herein, thus, point to the need for further investigation on a possible interference with somitogenesis in embryos subjected to these drugs and their mixture to better understand the mortality and malformations ratio observed.

Sensorimotor response

To further elucidate about the impact of the combined exposure to both drugs, a behavioural sensorimotor test was done in larvae at the end of the exposure period. The results revealed no differences between responses to stimuli applied either to the head or the tail. Thus, for the purpose of statistical comparison the whole number of stimuli applied (head plus tail) per larvae and treatment was considered. Significant differences among treatments were found (Pearson Chi-square=25, df=3, $p<0.001$; Figure 6), with

two homogeneous subsets identified; one comprising the control and the norfluoxetine treatment, and another composed by venlafaxine and the mixture, which showed comparatively lower rate of response to stimulation.

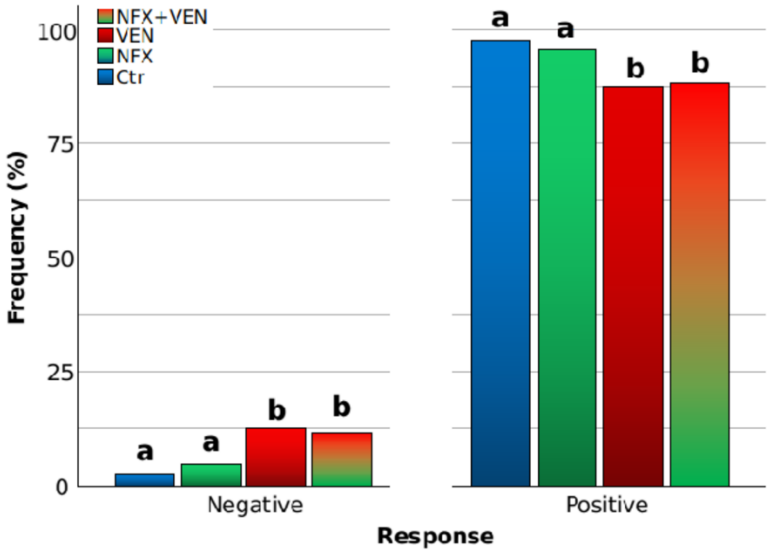

Figure 2. Sensorimotor reflexes. Effects of norfluoxetine (3.2ng/L), venlafaxine (2000ng/L) and the mixture of both substances on the sensorimotor responses of zebrafish larvae. Possible differences among treatments were identified using the Chi-square test. Small letters indicate the homogeneous groups.

This may possibly occur through an effect of venlafaxine on the serotonergic pathway during larvae development. Decreased positive responses, mainly after tail stimulation, was also observed in zebrafish larvae exposed to fluoxetine (Cunha et al., 2018). In zebrafish embryos, brief swimming episodes in response to tail touch start to occur first at 27 hpf, as shown by work in dechorionated embryos (Saint-Amant and Drapeau, 1998). At this stage, head touches seem to activate trigeminal sensory neurons, while tail touches appear to stimulate spinal Rohon–Beard sensory neurons, both of which innervate the descending reticulospinal Mauthner neurons, in the hindbrain (Brustein et al., 2003 and references therein). Previous works also indicated that serotonergic neuromodulation plays an important role in the early functional organisation of the locomotory neural network (Brustein et al., 2003), with serotonin exerting a different role early in larval development relative to more mature zebrafish (Gabriel et al, 2009).

Molecular effects

No significant differences among treatments were identified for the expression of any of the 34 target genes quantified in larvae exposed to norfluoxetine (Figures 3 and 4; Figure S1 and Table S3, Appendix A3.; Rodrigues et al. 2020). A cluster analysis was performed

to identify genes with similar patterns of expression. Eight gene clusters were found (Figure 5, clusters a to h).

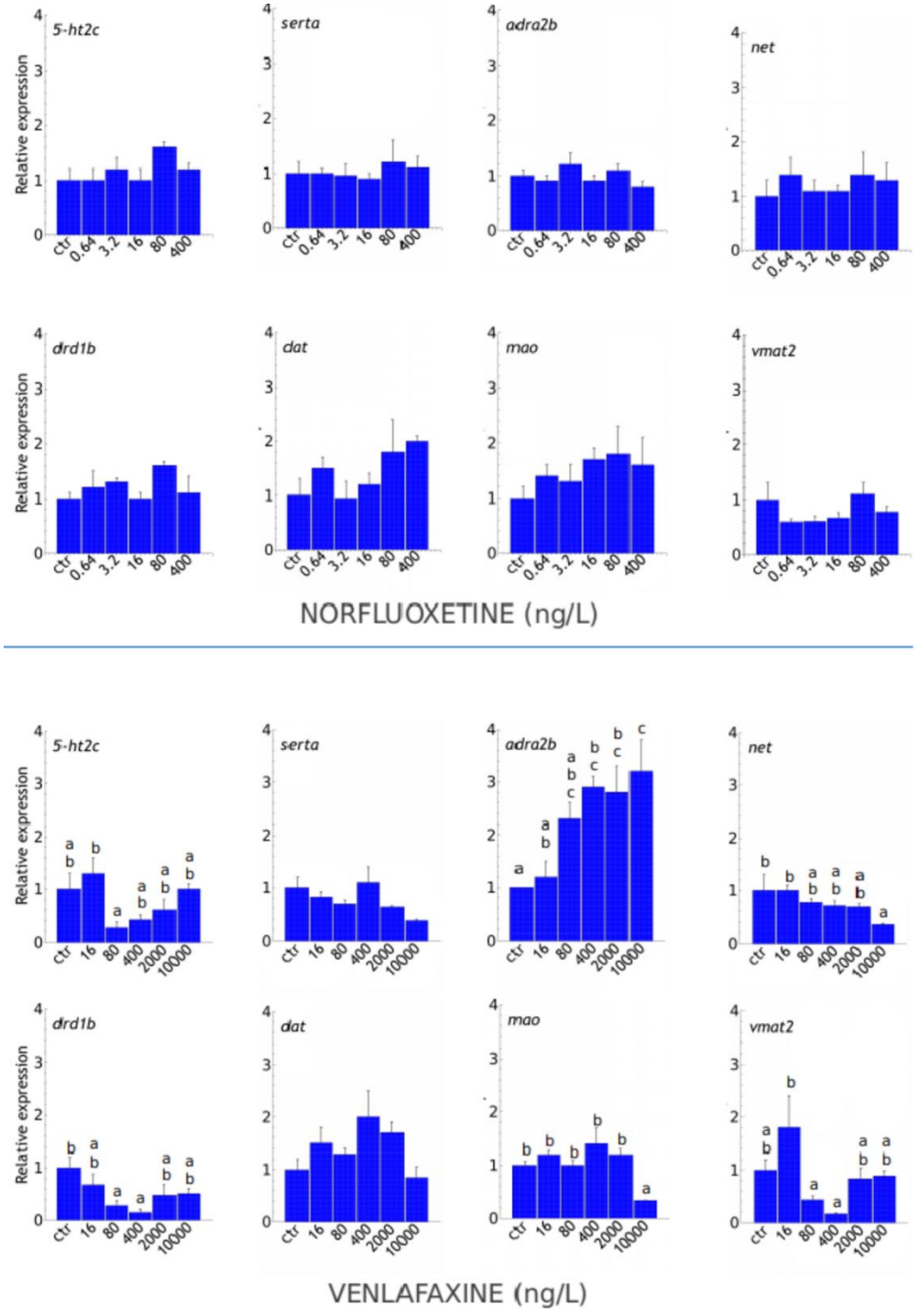

Figure 3. Monoamine receptors and transporters involved in serotonergic, noradrenergic and dopaminergic neurotransmission. Expression of monoamine receptors and transporters in embryos exposed to norfluoxetine or venlafaxine for 80hpf. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test.

Within clusters d, f and h smaller subsets of genes, showing very strong correlations ($r \geq 0.8$) to each other, were identified. For cluster d, this subset comprised the genes *mao*,

net, *serta* and *cyp3a65*, which showed a slight tendency for upregulation at higher norfluoxetine concentrations. The result suggests the transport and metabolism of serotonin and norepinephrine are coupled. For cluster f, the subset comprised only *pxr* and *abcb4*, showing a tendency for slight downregulation in embryos exposed to 0.64 ng/L. A correlation between *pxr* and *abcb4* was previously found by Hodkovicova *et al.* (2020) in control zebrafish larvae at 96 and 144hpf. The subset in cluster h comprised retinoix X receptors and retinoic acid recpetors (*rxraa*, *rxrgb*, *rxrbb*, *rxrab*, *rarab*, *rxrga*, *raraa*), which expression was at control levels.

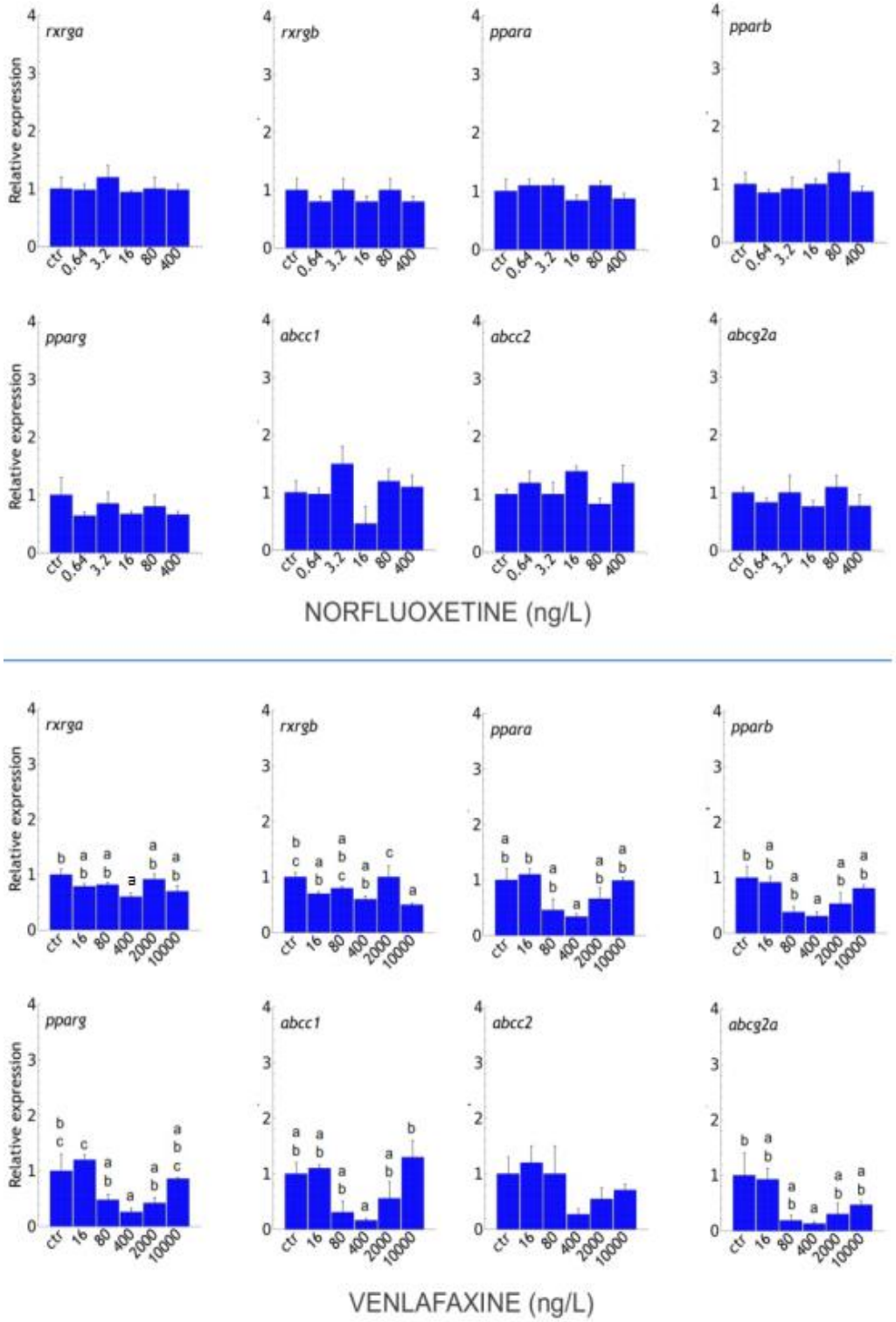

Figure 4. Nuclear receptors and ABC transporters. Expression of nuclear receptors and ABC transporters in embryos exposed to norfluoxetine or venlafaxine for 80hpf. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test.

Venlafaxine exposure led to significant changes in the expression of several genes, compared to controls (Figures 3 and 4, Figure 3 and Table 4). Regarding the monoaminergic genes, downregulation was found at 80 and 400ng/L for *5-ht2c* (-70%

and -60%, respectively), *drd1b* and *vmat2*, and at 10000ng/L for *5-ht1a* (-80%), *serta* (-60%) and *mao*. Induction was observed for the expression of *adra2b* (at ≥400ng/L). Alterations found for the *5-ht1a* and *adra2b* genes could be related to inhibition of the G protein-coupled receptors in the Gi/Go pathway (Kelder *et al.,* 2012; Kutmon *et al.,* 2016). The results also revealed an opposite trend of expression of *5-ht2c* between 16 ng/L (induction) and the 80 and 400ng/L (inhibition) venlafaxine. This was unexpected once that, as SNRI, this drug would act by inhibiting serotonin receptors (Mennigen *et al.,* 2011). However, during the first weeks of human treatment, when doses ingested are still low, several antidepressants can cause over-activity of *5-ht2c* receptors, originating mild side effects (Milan, 2005). A similar action on serotonin receptors appears to take place also in zebrafish embryos upon exposure to a low venlafaxine concentration.

According to the literature, decreased reuptake of monoamines can reshape both postsynaptic and presynaptic responses originating a depletion of vesicular stores in the long-term (Kristensen *et al.,* 2011). Such a mechanism could also cause downregulation of *vmat2*, as seen herein in zebrafish larvae, leading to increased extracellular levels of catecholamines, such as norepinephrine and serotonin (Wimalasena, 2011). The cluster analysis performed for venlafaxine expression data identified eleven different groups of genes showing common response patterns (Figure 7). Within these, genes showing the strongest correlations ($r \geq 0.8$) were *rxrga/rarga* and *adra2c/ahr2* (cluster d), *raraa* and *gst* (cluster j) and the subset of *pparb*, *ppara*, *abcg2a* and *abcc2* (cluster k). The correlated subset of cluster d (*mao*, *net*, *serta* and *cyp3a65*) further support coupling of the transport and metabolism of serotonin and norepinephrine.

Among the nuclear receptors investigated in our work, the most affected by venlafaxine were PPAR and RXR, namely: *pparα* and *rxrga* (at 400ng/L), *pparβ* (at 80 and 400ng/L), *pparγ* (at 80, 40 and 2000ng/L) and *rxrgb* (at 10000ng/L), all downregulated relative to controls. Nuclear receptors play a role in the regulation of genes involved in xenobiotic metabolism (Xu *et al.,* 2005). Retinoid nuclear receptors are known to form heterodimers with other nuclear receptors (*e.g.*, *rar*, *ppar*) for regulation of their main targets (Xu *et al,* 2005). This binding ability is responsible for the role played by *rxr* in the regulation of numerous drug metabolising enzymes (Xu *et al.*, 2005). The reduced expression reported here can compromise the regulation of such enzymes, impairing the organisms' ability to achieve homeostasis. Additionally, earlier work in fish has shown *ppar* receptors play a crucial role in lipid and xenobiotic metabolism (Wang *et al.,* 2008; Maradonna *et al.,* 2015). Hence, zebrafish ability to metabolise lipids and xenobiotics seems to be impaired by low venlafaxine concentrations. The *ppar* nuclear receptors also have anti-inflammatory and neuroprotective roles in the central nervous system. In particular, *pparα* and *pparγ* agonists can influence dopaminergic neurons linked to drug addiction.

Such agonists can reduce drug self-intake in mammals, and the stimulation of dopaminergic neurons belonging to the mesolimbic dopaminergic pathway of the brain reward system (Panlilio *et al.,* 2012; Melis *et al.,* 2008). The *ppar* downregulation elicited by venlafaxine in this study suggests drug dependence may occur upon continued intake, and sheds light on the *dat* induction found. This aspect deserves further investigation since the lack of physiological dependence is considered an advantage to the use of venlafaxine by humans (Kelsey, 2000).

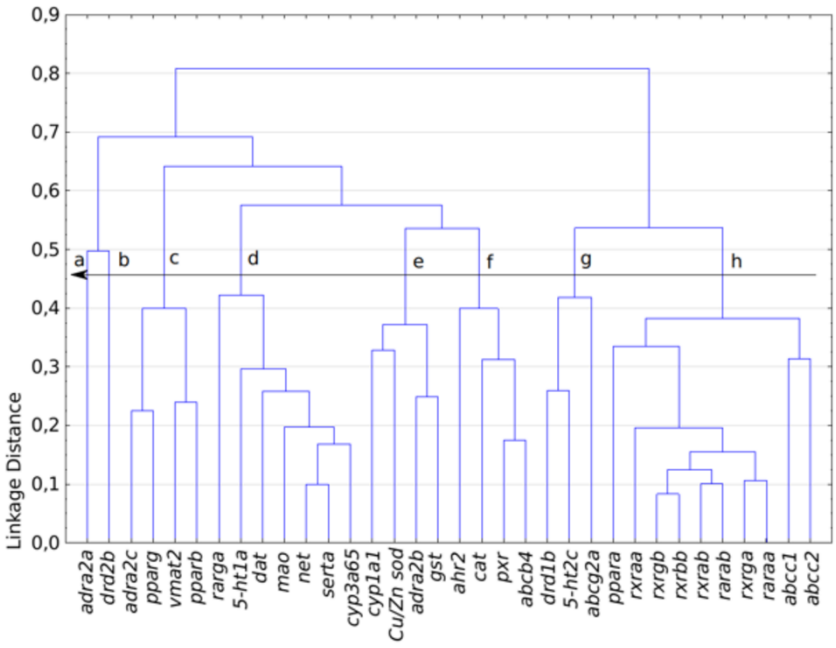

Figure 5. Cluster analysis for norfluoxetine. The analysis was based on the weighted pair-group average (1-Pearson r) and performed on the gene expression data obtained for zebrafish larvae exposed to norfluoxetine for 80hpf.

For ABC transporters, downregulation of *abcc1* (at 400ng/L) and *abcg2a* (at 80 and 400ng/L) was observed. These genes act on the efflux of metabolites outside the cell, on phase III of biotransformation (Ferreira *et al*., 2014). Low levels of venlafaxine thus appear to impair the ability of cells to efflux metabolites, potentially affecting homeostasis. Downregulation of *abcc1* (at 300 and 30000ng/L) and *abcb4* (at 300ng/L) was also reported for zebrafish larvae exposed to venlafaxine for 96hpf (Hodkovicova *et al*., 2020), though at the end of the exposure (144hpf) no differences were found relative to the controls. In our case, 80hpf was decided because we wanted to investigate effects elicited by exposure during the embryonic development, as the chorion limits entry of certain molecules into the embryo. The results of Hodkovicova and colleagues (2020) point out the ability of venlafaxine to elicit different effects in embryos and larvae, as after hatching the organisms are directly exposed to the drug, without the chorion protection.

Noteworthy are also the high positive correlations found between *ppar* receptors and *abc* transporters, which further support an effect of venlafaxine on the lipidic metabolism of zebrafish larvae and the involvement of ABC transporters in this process. For their action, *ppar* receptors form heterodimers with retinoid X receptors (*rxr*). The binding to their ligands triggers the synthesis of three classes of proteins, namely ATP-binding cassette (ABC) transporters, Cyp member proteins and lipid binding proteins, which catalyse the anabolism, metabolism and elimination of lipids (Lynch and Price, 2007). In this pathway, the *abcc2* gene is involved in lipid metabolism, particularly of steroids originating from cholesterol metabolism.

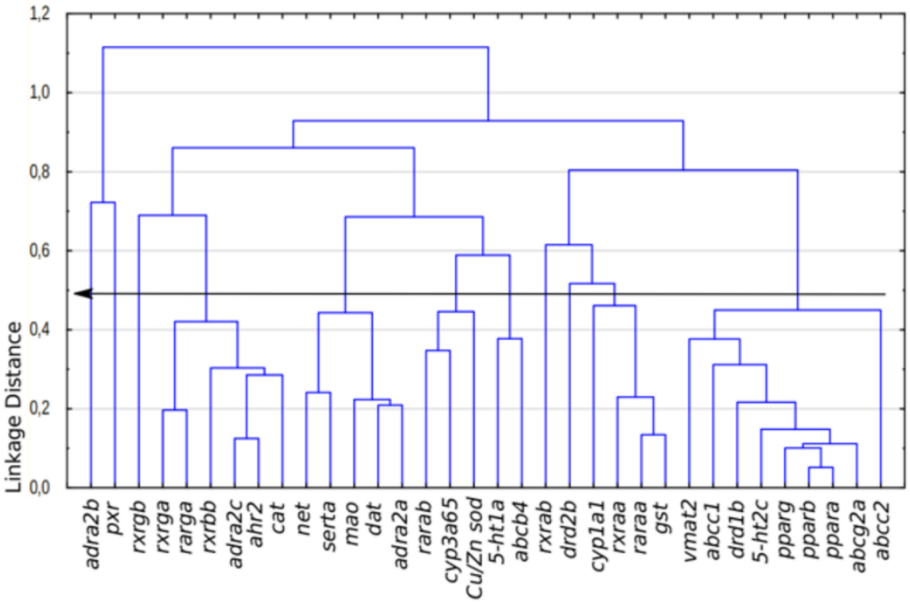

Figure 6. Cluster analysis for venlafaxine. The analysis was based on the weighted pair-group average (1-Pearson r) performed on the gene expression data obtained for zebrafish larvae exposed to venlafaxine for 80 hpf.

Ten genes showing the strongest alterations (relative to controls, ≥50%) in venlafaxine-exposed larvae were selected for evaluation in the mixture assays. The results showed the neurohormonal genes were the most affected. Significant differences in expression among treatments were found for *5-ht2c*, *drd1b*, *adra2b*, *vmat2*, *pparg*, *abcc1*, and *abcc2* (Figure 7; Table 5, Rodrigues et al., Data in Brief).

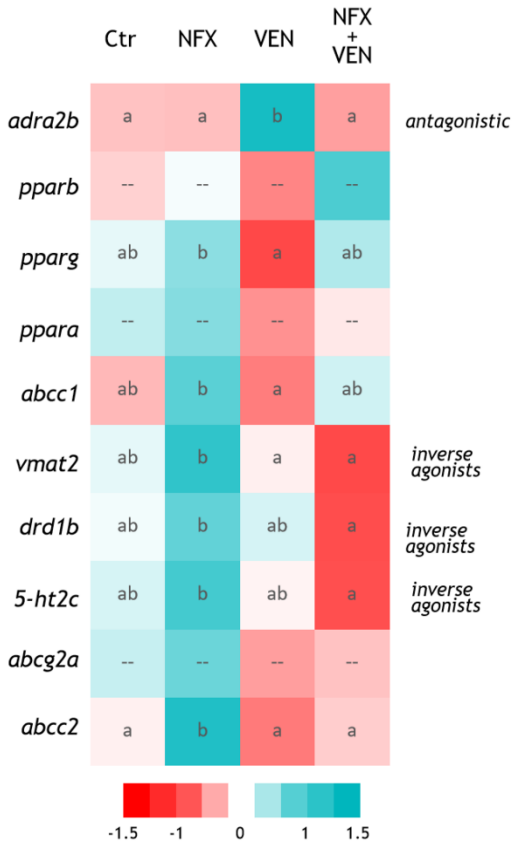

Figure 7. Interaction effects at the molecular level. Effects of norfluoxetine (3.2ng/L), venlafaxine (2000ng/L) and the mixture of both substances on the expression of serotonin (5-ht2c), dopamine (drd1b) and noradrenaline (adra2b) receptors, the vesicular monoamine transporter (vmat2), peroxisome proliferator-activated receptors subtype alpha (ppara), subtype beta 1 (pparb) and subtype gamma (pparg), and ABC transporters (abcc1, Multidrug resistance protein 1 or MRP1; abcg2a, ATP-binding cassette transporter sub-family G, member 2a; abcc2, Multidrug resistance protein 2 or MRP2). Data is presented in fold change, relative to the control groups. Homogeneous subsets are identified by small letters, according to the results of the Tukey HSD test. Significant interactions between norfluoxetine and venlafaxine upon gene expression were found for the monoamine receptors and the vesicular monoamine transporter.

Among those, larvae exposed to the mixture showed a different *drd1b*, *5-ht2c* and *vmat2* relative to embryos exposed to norfluoxetine ($p<0.05$). Significant interactions between the two drugs were also found for the monoamine receptors and the vesicular monoamine transporter. Namely, for *adra2b*, suggesting an antagonistic action, in which when in mixture the effect of venlafaxine is neutralised by norfluoxetine. For *5-ht2c*, *drd1b* and *vmat2*, the results suggest an action between the two drugs as inverse agonists (Figure 7), in which the effect of norfluoxetine is reversed by venlafaxine. The cluster analysis spotted five groups of genes with similar behaviour (a to e, Figure 8).

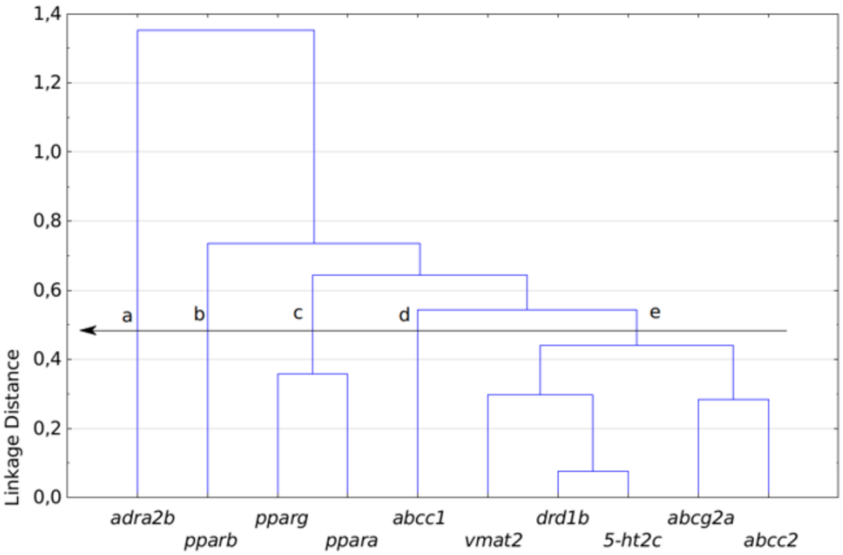

Figure 8. Cluster analysis for the mixture. The analysis was based on the weighted pair-group average (1-Pearson r) performed on the gene expression data obtained for zebrafish larvae exposed to norfluoxetine (3.2 ng/L), venlafaxine (2000 ng/L) or their mixture.

Significant strong correlations were found between *pparg* and *ppara* ($r > 0.6$), *vmat2* and drd1b or 5-ht2c ($0.6 < r > 0.8$), and abcg2a and abcc2($r > 0.7$) (Figures 7 and 8). A very strong high correlation was found between *drd1b* and *5-ht2c* ($r > 0.9$). These two genes tended to be upregulated upon exposure to norfluoxetine and downregulated upon exposure to the mixture, a pattern consistent with an interaction of inverse agonism between the two drugs. Inverse agonism can occur when a ligand binding to the same receptor-binding site of an agonist, more than antagonising the effects of that agonist, causes the reverse effect by suppressing spontaneous receptor signalling. The mixture effects observed appear to be an indication of the complex interactions that may occur between the two drugs during embryo development, possibly leading to increased mortality and malformations rates in somewhat more susceptible individuals.

Relevance for risk assessment

Larvae exposed to venlafaxine exhibited U-shaped response curves for several genes (Figures 3 and 4), with stronger alterations at low rather than high concentrations, as previously identified by other authors for selective serotonin reuptake inhibitors (Rodrigues *et al.,* 2014, 2015; Cunha *et al.,* 2016; Fong and Ford, 2016). These should be typically expected from compounds exhibiting endocrine-disrupting activity (Vandenberg *et al.,* 2012) and pose recognised challenges to their risk assessment. The distinct effects may result from the various receptors, and their subtypes, and their abundance in different cell types. The presence of distinct coregulators (such as specific

ions) may further add to the differing behaviour of target genes. It is well known, for example, that some SNRIs and SSRIs act on more than one receptor/receptor subtype, which may be expressed differently in various cell types and organs, thus exerting varying influence on gene expression or cellular phenomena (Garcia-Colunga *et al.,* 1997; Govorunova *et al.,* 2010). Our data further support that risk estimation for these compounds needs to take advantage of methods incorporating hormesis and non-monotonic responses (Vandenberg et al., 2012; Rodrigues et al., 2014, 2015; Cunha et al., 2016; Fong and Ford, 2016).

A new finding of this study comes from the interactive effects observed for norfluoxetine when in combination with venlafaxine, which increased the mortality rate relative to the single compounds. Besides pigmentation anomalies, few significant effects were detected in norfluoxetine larvae. The work of Hiemke and Hartter (2000), indicated that norfluoxetine is more potent than its parental compound in inhibiting the neurotransmitter uptake. Nevertheless, the effects of norfluoxetine over the zebrafish embryonic development were less severe than those of fluoxetine (Cunha *et al.,* 2016; Rodrigues et al., 2020). While differential effects of norfluoxetine and fluoxetine upon developmental and adult stages cannot be excluded, this can be related to the potency of the two norfluoxetine enantiomers. Likewise, the parent drug, norfluoxetine is composed of an *r* and an *s* enantiomer. However, unlike fluoxetine, norfluoxetine enantiomers have different potency; *r*-norfluoxetine is 20-fold less potent than *s*-norfluoxetine (Stanley et al., 2007). The formulation used in the present study to prepare the test solutions is a racemic mixture in the form of solid crystals. The less active *r* metabolite may have contributed to the decreased toxicity observed, relative to fluoxetine. Thus, the interaction effects could not be predicted from the single compound responses and strongly highlight the need for evaluating the potential toxicity of antidepressant metabolites and carry out risk assessments of these compounds, especially in mixture with other bioactive drugs.

3.3.4 Conclusions

In the present work exposure of zebrafish embryos to the active metabolite norfluoxetine caused only mild alterations, mainly abnormal pigmentation of embryos and newly hatched larvae. Venlafaxine increased the malformation rate (depigmentation and spinal deformities mainly) and caused impaired sensorimotor reflexes and alterations in the expression of genes belonging to the serotonergic, noradrenergic and dopaminergic pathways, as well as nuclear receptors related to lipid and drug metabolism. The mixture of norfluoxetine and venlafaxine elicited different interaction effects, not predictable from

the results of the single exposures, depending on the level of biological organisation and the signalling pathways possibly affected (e.g., serotonergic, noradrenergic and/or dopaminergic). Of concern are in particular the increased mortality and embryo malformation rates observed, as well as the antagonism and inverse agonism at the molecular level. Pigmentation and the expression of *adra2b*, *5-ht2c*, *drd1b*, *vmat2* were identified as promising biomarkers of exposure to selective monoamine reuptake inhibitors during embryogenesis. The results obtained further suggest that populations of aquatic fish may be already facing increased mortality, among other negative consequences, in systems where both these psychotropic drugs have been detected. The results call for urgent investigation of the hazardous effects of pharmaceutical metabolites and their risk assessment.

3.3.5 References

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Chapter 4: Carbamazepine, venlafaxine, tramadol and their metabolites: toxicological effects on zebrafish embryos and larvae

Chapter 4: Carbamazepine, venlafaxine, and tramadol metabolites: toxicological effects on zebrafish embryos and larvae

4.1 Introduction

Occurrence of pharmaceutical compounds in aquatic ecosystems is a growing problem. Among those most detected are carbamazepine (CBZ), venlafaxine (VEN) and tramadol (TRA). In recent years, their main metabolites have also been detected in surface water and effluents of wastewater treatment plants in the ng/L to µg/L range. While relevant toxicological information for these three main compounds has been produced over the last decade, information about the effects of their metabolites is scarce or inexistent. (Paíga et al., 2016; Hai et al., 2018)

Carbamazepine is a well-known anticonvulsant prescribed to treat epilepsy, trigeminal neuralgia and several psychiatric diseases like schizophrenia and bipolar disorders (Hirschfeld and Kasper, 2004; Pearce et al., 2008; Leucht et al., 2014). Carbamazepine has been detected in natural water samples in concentrations ranging from a few ng/L to 6.3µg/L (Ternes, 1998). This drug is mainly described as a sodium channel blocker; it binds to inactive voltage-gated sodium channels keeping them in the inactivated state and thus inhibiting the generation of action potentials. Moreover, action of CBZ results in the confinement of calcium entry to the interior of synaptic membranes (Tolou-Ghamarie et al., 2013; Rogawski et al., 2016). Diminished polysynaptic nerve responses and decreased post-tetanic potentiation are the mainly accepted mechanisms exerting therapeutic effect. Carbamazepine administration increases GABA levels and interferes with glutaminergic and dopaminergic neurotransmission (Tolou-Ghamarie et al., 2013; Ayano, 2016; Rogawski et al., 2016). Studies also point out an action of CBZ as a serotonin reuptake inhibitor and releasing agent, as well as inhibitor of norepinephrine release (Kawata et al., 2001; Ayano, 2016). Reported negative effects of CBZ on fish include disruption of locomotion, feeding and growth (Nassef et al., 2010; Van den Brandhof and Montforts, 2010). A recent study demonstrated that exposure to CBZ also induced hepatic DNA damage, apoptosis, and caspase activities (Yan et al., 2021). In humans CBZ is highly metabolised in the liver (95% of the compound) (Puranik et al., 2013). The main pathway of CBZ metabolism is the transformation into carbamazepine-10,11-epoxide (CBZep) (Pearce et al., 2008), catalysed by CYP enzymes, mainly by CYP3A4, but also by CYP2C8 and CYP3A5 (Kerr et al., 1994; Pearce et al., 2008). This is an active metabolite and equipotent in relation to the parental compound (Bourgeois and Wad, 1984). This metabolite can in turn be metabolized into the inactive metabolite

10,11-dihydrocarbamazepine (diCBZ) by the action of EPHX1 (Puranik et al., 2013). Both CBZep and diCBZ were already quantified in environmental water samples in maximum concentrations of 16.2µg/L and 4µg/L, respectively (Bahlmann et al., 2014; Petrovic et al., 2016). Data about CBZep and diCBZ toxicity to aquatic species is still sparse. However, CBZep was already found in different fish tissues like gills and muscle (Valdes et al., 2016). The same study reported that CBZ is biotransformed by fish, probably in a metabolic pathway similar to humans. More recently, a study demonstrated that ozonation treatment increased carbamazepine toxicity to fish embryos and linked the increase in embryotoxicity to the formation of both CBZep and diCBZ during the treatment (Pohl et al., 2019).

Venlafaxine is a serotonin and norepinephrine reuptake inhibitor (SNRI). It is one of the most prescribed antidepressants worldwide to treat major depressive and anxiety disorders, chronic pain syndrome and phobia (Melnyk-Lamont et al., 2014; Sansone and Sansone, 2014). It has been detected in environmental water samples from a few ng/L up to 1.31 and 2.19µg/L (Schultz and Furlong, 2008; Schultz et al., 2010; Gonzalez Alonso et al., 2010). It acts by inhibiting presynaptic receptors of both serotonin and norepinephrine reuptake, leading to an increase in the overall concentration of both monoamines in the synaptic cleft, potentiating their action (Mennigen et al., 2011). It can also inhibit dopamine reuptake, although this inhibition is weaker, compared to the action on the serotonergic and noradrenergic pathways. Several studies showed that VEN can cause alterations in fish reproduction and behaviour (Schultz et al., 2010; Gaulus et al., 2013; Ziegler et al., 2021). It can also alter pigmentation of zebrafish embryos and adults (Ruuskanen et al., 2005; Xu and Xie, 2011; Rodrigues et al., 2020), and block epinephrine-induced glucose production affecting the fish stress response. Recently it was also shown to impact the developmental programming of female fish (Thompson et al., 2022). Venlafaxine is metabolised at high rates in the human liver (>90%) (Howell et al., 1993; Klamerus et al., 1992). Demethylation of venlafaxine to its main active metabolite, O-desmethylvenlafaxine (ODV), is the primary metabolism route, occurring through the action of Cytochrome P450 (CYP2D6) enzymes (Magalhães et al., 2014). This metabolite, also known as desvenlafaxine, acts as a SNRI and is approved for the treatment of major depressive disorder (Lieberman and Massey, 2009). N-demethylation of VEN into N-desmethylvenlafaxine (NDV) is considered a minor metabolic route in humans, which is catalysed also by CYP enzymes (CYP3A4 and to a lesser extent by CYP2C9 and CYP2C19) (Fogelman et al., 1999). These metabolites have been found in environmental samples at concentrations as high as 10µg/L in WWTP activate sludge for ODV (Writer et al., 2013) and 200ng/L in WWTP samples for NDV (Metcalfe et al., 2010). Nevertheless, knowledge about their effects on aquatic species is scarce. Recent

works demonstrated an increase in aggressive behaviour (attacks to opponents per minute) of crayfish exposed to 8µg/L of ODV, in relation to the control, and reduced locomotory behaviour in fish exposed to ODV in dark conditions (Stropnicky, 2017; Atzei et al., 2021).

Tramadol belongs to the opioid class of pharmaceuticals. It is prescribed to treat acute or chronic pain of different severity degrees and fibromyalgia. This drug has been detected in environmental water samples in concentrations ranging from a few ng/L to 6µg/L (Kasprzyk-Holdern et al., 2008), reaching a maximum concentration of 50µg/L in river sediments (Kusari et al., 2016). Tramadol exerts its therapeutic effects on a variety of receptors in the noradrenergic, serotoninergic, dopaminergic, and opioid systems. Tramadol is marketed as a racemic mixture (Miotto et al., 2017). The positive enantiomer (+) acts as a strong inhibitor of serotonin reuptake and as a serotonin releasing agent. This enantiomer also has affinity to opioid receptors. On the other hand, the negative enantiomer (-) inhibits noradrenaline re-uptake (Vazzana et al., 2015) and is also an agonist of the µ-opioid receptor, and agonist of the δ-opioid and κ-opioid receptors. Its action extends to the serotonin receptor 2c (5HT2C), M1 and M3 muscarinic acetylcholine receptor and α7 nicotinic acetylcholine receptors. Tramadol was also reported to inhibit the uptake of dopamine by rat synaptosomes *in vitro* (Driessen et al., 1993) and to interfere with dopamine receptors and uptake in the rat brain *in vivo*, though with less magnitude compared to noradrenaline (Faron-Górecka et al., 2004; Ezzeldin et al., 2014). The toxicological effects of tramadol in fish include disruption of hatching and early ontogeny, as well as alteration of antioxidant enzyme activity, behaviour, weight, and mitochondrial structure (Zhuo et al., 2012; Sehonova et al., 2016). In humans, tramadol is mainly metabolized in the liver by demethylation and conjugation reactions. The main metabolism pathway is the o-demethylation of tramadol into o-desmethyltramadol (OTRA), its main active metabolite. The pathway is catalysed by Cytochrome P450 (CYP2D6), and the metabolite produced has 6 times more potency and 700-fold more affinity for the opioid receptor than the parental compound. Another step of the metabolization pathway is the n-demethylation to N-desmethyltramadol (NTRA), an inactive metabolite. This reaction is mainly catalysed by CYP2B6 and CYP3A4. Both metabolites have been detected in concentrations as high as 8.7 µg/L (OTRA) and ~7ug/L (NTRA) in wastewater samples (Baker and Kasprzyk-Hordern, 2013; Goswami et al., 2021). However, no reports dedicated to the effects of OTRA or NTRA on aquatic species could be found in the literature. Nevertheless, there are some interesting data available; some authors (Zhuo et al., 2012; Tanoue et al., 2017) found that both OTRA and NTRA can be detected in fish brain after exposure to the parental compound, with NTRA at significantly higher concentrations than OTRA.

Serotonergic, noradrenergic, and dopaminergic systems are conserved in fish and start to form in zebrafish as early as 24hpf (hours post-fertilisation) (Kastenhuber *et al*., 2010). Furthermore, they have been linked to different aspects of fish physiology and behaviour, such as reproduction, locomotion, feeding and aggression (Kreke and Dietrich, 2008; Fontaine et al., 2013, Melnyk-Lamont *et al*., 2014). Despite the levels of TRA, CBZ and VEN metabolites detected in environmental water samples and their potential to interfere with these monoaminergic systems, toxicological information about their effects on non-target species is scarce. This limits our understanding of their impact and estimation of their risk to aquatic ecosystems because some of these metabolites have pharmacological properties similar to their parental compounds, and even higher affinity to the receptors where they act (e.g., ODV and OTRA). Also, metabolites are often quantified at concentrations higher than those of their parental compounds. The main aims of this work were, therefore, to investigate the effects of carbamazepine, venlafaxine, tramadol and their main metabolites on zebrafish development. The working hypotheses were that: (i) the tested metabolites would have toxicity similar to or higher than their respective parental compound, as reflected in altered developmental malformations and sensorimotor behaviour; (ii) the expression profiles of genes related to the monoaminergic and detoxification systems would be altered by exposure to the drug metabolites, possibly eliciting molecular toxicity.

4.2. Material and methods

4.2.1 Chemicals

Tramadol (CAS Number 36282-47-0) and venlafaxine (CAS Number 99300-78-4) were obtained from the European Pharmacopoeia Reference Standards®. o-desmethyltramadol (CAS Number 80456-81-1), o-desmethylvenlafaxine (CAS Number 93413-62-8) and 10,11-dihydrocarbamazepine (CAS Number 3564-73-6) were purchased from Merck. Carbamazepine (CAS Number 298-46-4) was obtained from Alfa Aesar. Carbamazepine-10,11-epoxide (CAS Number 36507-30-9) was obtained from Green Pharma Ambinter. N-desmethyltramadol (CAS Number 73806-55-0) was obtained from Akos Consulting & Solutions GmbH and n-desmethylvenlafaxine (CAS Number 93413-90-2) from Syncom.

4.2.2 Zebrafish embryotoxicity assays

Mature zebrafish (*Danio rerio*, AB line) adults were maintained under standard culture conditions and used to produce embryos for the toxicological assays. Assays were performed according to the following protocol: 50 embryos (~1hpf) were placed in 6-well microplates and exposed to 0.1, 1, 10 and 100µg/L of each parental compound or its metabolites. The inclusion of at least one environmentally relevant concentration for each compound was mandatory. Stock solutions of CBZ, CBZep, diCBZ and NTRA were prepared in DMSO (0.01%). For the remaining chemicals, stock solutions were prepared in ultra-pure water. A negative control group (dechlorinated water) and a DMSO (0.01%) solvent control (when applied) were also included in the experimental design. All exposure assays were repeated four times each, with a different batch of embryos.

At 24hpf, 40 embryos of each test condition were transferred to 24-well microplates in order to monitor their development and register the occurrence of embryonic malformations, as described by Kimmel et al. (1995). Observations were made at 24, 48, 72, 96, 120, 144 and 168hpf for mortality, developmental abnormalities (somites number and formation, tail detachment, yolk sac deformities, otoliths absence or shape, deformities in eyes, irregular heartbeat, blood circulation defects, skeletal deformities and existence of side-wise position) and hatching rate, using an inverted microscope (Nikon Eclipse TS100). At 168hpf larvae were collected into microtubes and preserved in RNAlater. They were subsequently used to assess the expression of target and reference genes.

4.2.3 Sensorimotor assays

Based on the results of survival and embryonic malformations which showed no significant differences for NDV, NTRA and diCBZ, sensorimotor reflexes of 168hpf larvae were determined for 0.1 and 100µg/L treatments of CBZ, CBZep, VEN, ODV, TRA and OTRA, following the protocol described in Rodrigues et al. (2020). Briefly, a micropipette tip was used to apply gentle touches to the larvae's head and tail. Ten touches per region were applied to each animal, alternating the part where the touch was applied; a mandatory 30s resting period was observed between each touch. Positive (*i.e.,* immediate swimming) or negative (*i.e.,* no/retarded movement) responses exhibited by the larvae after stimulation were registered for every touch. Preliminary analysis revealed no significant differences between responses to touches applied either to the head or the tail for any of the compounds and concentrations tested. Thus, the statistical treatment was done using the whole number of touches (head plus tail) per larvae and per treatment.

4.2.4 Gene expression

Total RNA content was isolated with the Illustra RNAspin Mini Isolation kit (GE Healthcare). The quality of the extracted RNA was assessed through agarose gel electrophoresis. Obtained RNA was quantified in a microplate spectrophotometer (BioTek) using a micro-volume adapter (Take3). Digestion of genomic DNA was done with PerfeCta DNase I (RNase free; Quantabio); qScript cDNA Synthesis Kit (Quantabio) was used for cDNA synthesis. All reactions were set according to the respective manufacturer protocols.

Gene expression was assessed through qPCR. This technique was employed to determine the expression of thirty two genes of interest in larvae exposed to CBZ, CBZep, diCBZ, VEN, ODV, NDV, TRA, OTRA and NTRA: serotonin transporter and receptors (*sert/ slc6a4aI, 5-ht1a*, *5-ht2c*), norepinephrine transporter and receptors (*net*, *adra2b*, *adra2c*), dopamine receptors and transporters (*dat*, *drd1b*, *drd2b*), vesicular monoamine transporter (*vmat2*) and monoamine oxidase (*mao*), retinoic acid nuclear receptors (*raraa*, *rarab*, *rarga*), retinoid X receptors (*rxraa*, *rxrab*, *rxrbb*, *rxrga*, *rxrgb*), peroxisome proliferator activated nuclear receptors (*pparα*, *pparβ*, *pparγ*), pregnane X nuclear receptor (*pxr*), aryl hydrocarbon nuclear receptor (*ahr2*), ABC transporters (*abcb4*, *abcc1*, *abcc2*, *abcg2a*), biotransformation enzymes (*cyp1a1*, *cyp3a65*, *gstpi*), antioxidant enzymes (*nrf2*, *keap*). The expression of each target gene was done at least in four independent replicates. Detailed information on gene function, accession number, designed primers and assay conditions is provided in the Appendix A4. (Table S1.). Three reference genes were assessed: actb1, ef1 and rpl8. All qPCR reactions were run in a Mastercycler realplex 4 (Eppendorf, Hamburg, Germany) using PerfeCTa SYBR® Green SuperMix from QuantaBio in a 20 µL reaction volume. Each reaction was run in duplicate. The reaction parameters were set as follows: an initial cycle at 94ºC for 2min; 40 cycles of 30sec at 94ºC for denaturation, 30sec at respective annealing temperatures, and a final period of 30sec at 72ºC for extension. Annealing temperatures for primers were: 51ºC for *vmat2*, 55ºC for *5-ht2c* and *drdb1*, 54ºC for the remaining genes. Blank samples, and melting curves, were run for every gene, to determine if non-specific products were formed. Normalisation of target gene expression was done according to the results of Normfinder (Andersen et al. 2004). Relative gene expression was calculated with the template of Pfaffl (2001), which integrates primer efficiencies.

4.2.5 Statistical analysis

Data were analysed by block of chemicals, *i.e.,* each parent compound and its respective metabolites, to investigate their comparative toxicity. Given that no statistically significant differences were found between the water and the solvent controls, for any of the parameters assessed, all statistical comparisons were made against the solvent control, herein indicated as control (ctr) for simplicity. Frequency data concerning mortality, hatching rates and sensorimotor responses were analysed with the Chi-square test followed by pairwise comparisons with Bonferroni adjustment. Effects of the experimental factors (chemical species and test concentrations) on embryotoxicity endpoints were investigated using a Correspondence Analysis followed by the Chi-square test with Bonferroni adjustment. The data were analysed at the time points showing the highest cumulative frequency of malformations. Molecular data were analysed by means of a saturated orthogonal multiple linear regression analysis to assess the effects of the single experimental factors and their interactions on the expression of each gene (Box et al., 1978; Tomassone et al., 1983). Fourteen regressors were established for each block of chemicals to account for the chemical species (two regressors of 1^{st} and 2^{nd} order), the concentrations tested (four regressors of 1^{st} to 4^{th} order) and their interactions (eight regressors) to adequately describe the response of each gene. To investigate patterns of gene expression, a partial least squares regression (PLS) was then carried out using the orthogonal regressors (X, independent variables) and the variation in gene expression (Y, dependent variables). The significant Y components extracted by the PLS were entered in a Cluster Analysis (CA) to identify groups of genes exhibiting similar responses. The information obtained was used to estimate the average model response of each gene cluster. A model gene expression for each cluster and toxicant was derived using the equation of the saturated orthogonal multiple linear regression. Each predicted model was determined using the average of the regression coefficients characteristic of the expression of the genes in the cluster. Identification of the characteristic regressors in each cluster was obtained from an integrated interpretation of the PLS components as described in Eder et al. (2021) and Pinto et al. (2022). For the correspondence analysis, the multiple regression analysis and the PLS the significance level used was 0.01. For the remaining tests it was 0.05. The statistical tests were carried out with Statistica 14 (TIBCO).

4.3 Results and discussion

4.3.1 Zebrafish embryotoxicity assays

Embryotoxicity assays were performed under maximum controlled conditions. All three parental drugs were reported to be stable in water for at least 60 days, showing low dissipation values (Li and McLachlan. 2019) and very low UV or microbial degradation (Rua-Gomez and Puttmann, 2013; Kruglova et al., 2014; Andreozzi et al., 2002). For the metabolites, OTRA and ODV were reported to have a half-life of 21h and 18h respectively, undergoing both photo and biodegradation (Rua-Gomez and Puttmann, 2013). Also, CBZep was found to be highly stable to chlorination conditions (Soufan et al., 2013) and diCBZ was reported to have a DT50 (dissipation time of 50% from an initial concentration) of 8 days (Loffler et al., 2005). Concentrations of CBZep and diCBZ also remained stable after ozonation treatment, which is described as the most efficient for CBZ degradation (Pohl et al., 2020). The 24 well-plates used on the assays were bathed with the respective test solution for 24 hours prior to the beginning of the assays to assure that losses of chemicals by adsorption to the microplate material were minimal. Test solutions were renewed every 24 hours to avoid proliferation of microorganisms and degradation of chemicals. The literature also shows great consistency between nominal and exposure concentrations for these compounds with recovery rates of about 70% for NTRA and about higher than 80% for the remaining compounds (Melnyk-Lamont et al., 2014; Guruje et al., 2019; Chen et al., 2020; Hodkovicova et al., 2020). In addition, all assays fulfilled the validity criteria of OECD Guidelines 212 and 236 of hatching rates above 80% in control groups.

The results of the toxicity tests showed no significant differences among experimental conditions regarding cumulative mortality at 168hpf for any of the investigated parental drugs or their metabolites. These results agree with the literature available for the three drugs. For CBZ, 72hpf median lethal concentrations (LC_{50}) were >245mg/L (Van den Brandhof and Montforts, 2010) and >500µM (Weigt et al., 2011). Interestingly, Gaulus et al. (2013) reported increased mortality of zebrafish exposed for 96hpf to 0.5µg/L, whilst significant decrease (about 70% relative to the control) was found following exposure for 96hpf to 10µg/L CBZ. Pohl et al. (2020) also found increased mortality after 144hpf exposure to CBZ, but at very high exposure concentrations of 50mg/L (~38% mortality) and 100mg/L (~88%), while testing for the efficiency of ozonation treatment to degrade this drug. For VEN, significantly increased mortality was elicited by 96hpf exposure of zebrafish embryos to 0.5µg/L (about 30% relative to the control), but no differences in mortality were observed in embryos exposed to 10µg/L (Gaulus et al., 2013). In a recent work, Rodrigues and colleagues (2020) found no significant differences in mortality, compared to controls, in zebrafish embryos exposed for 80hpf to 16ng/L to 10µg/L VEN. In line with these results, safety data sheets from ThermoFischer Scientific report a 96h LC_{50} of 9.4mg/L ODV for the freshwater fish *Pimephales promelas*. Median lethal

concentrations for zebrafish exposed to TRA for 144hpf were reported to be >200µg/L by Sehonova et al. (2016) and >6.25mg/L by Bachour et al. (2019). Very recently, Kirla et al. (2021) found 120hpf LC_{50} values well above those tested herein, of 2.22 and 19.37µM for TRA and OTRA, respectively.

No significant differences among experimental conditions could be found for the hatching rate of embryos exposed to most of the test compounds, except for 1µg/L diCBZ at 72hpf that elicited a 41% decrease comparing to the control (Fig. 1); since at 96hpf the hatching rate for this condition was similar to that of the control (data not shown), this should be interpreted as a transient hatching delay.

Previous embryotoxicity evaluations with zebrafish found that exposure to 122mg/L CBZ for 72hpf caused retarded growth and no hatching (Van den Brandhof and Montforts, 2010). Another study reported non-hatching 72hpf EC_{10} (10% effective concentration) and EC_{50} of 35mg/L and 46mg/L CBZ, respectively (Bekker Van Woudenberg et al., 2014). Recently, Chen et al. (2020) found no significant differences in the hatching rate of 4hpf zebrafish embryos exposed to 1, 2 and 5µg/L CBZ (within the range of concentrations tested herein) for 6 days. Similar results were obtained for CBZ, CBZep and diCBZ at concentrations in the mg/L range (Pohl et al., 2020). In contrast, hatching delays were found at 48 and 72hpf in embryos exposed to 85µM and 250µM CBZ, respectively, which in some replicates was later recovered (Bars et al., 2021). For VEN, the results were in accordance with those reported by Sehonova et al. (2019) and Rodrigues et al. (2020). Thompson et al. (2017) observed a significant 15 and 32% increase in the hatching rate at 48hpf of zebrafish embryos exposed via microinjection to 1 and 10 ng/L VEN, respectively, comparing to the control group. Altogether, the results suggest chorion is acting as a protective barrier against exposure to VEN, decreasing the amount of contaminant that reaches the embryos. Atzei et al. (2021) also found no alterations in the hatching time upon exposure of zebrafish embryos to CBZ (5-250µM), CBZep (0.003-35µM), VEN (.03-319µM) and ODV (0.03-334µM). For TRA, previous authors reported a significant hatching delay at 96hpf in groups exposed to the range of 1µg/L to 5g/L (Sehonova et al., 2016); at 120hpf all embryos were completely hatched. Very recently, Kirla et al. (2021) noticed occasional delayed hatching at low TRA concentrations (<50µM), though at 120hpf all larvae were hatched. Differences in embryo/strain sensitivity or exposure methodology (e.g., test media, frequency of media renewal) may account for the results observed in the two studies. Kirla and colleagues (2021) also tested OTRA but reported no effects on the hatching rate at exposure concentrations >50µM.

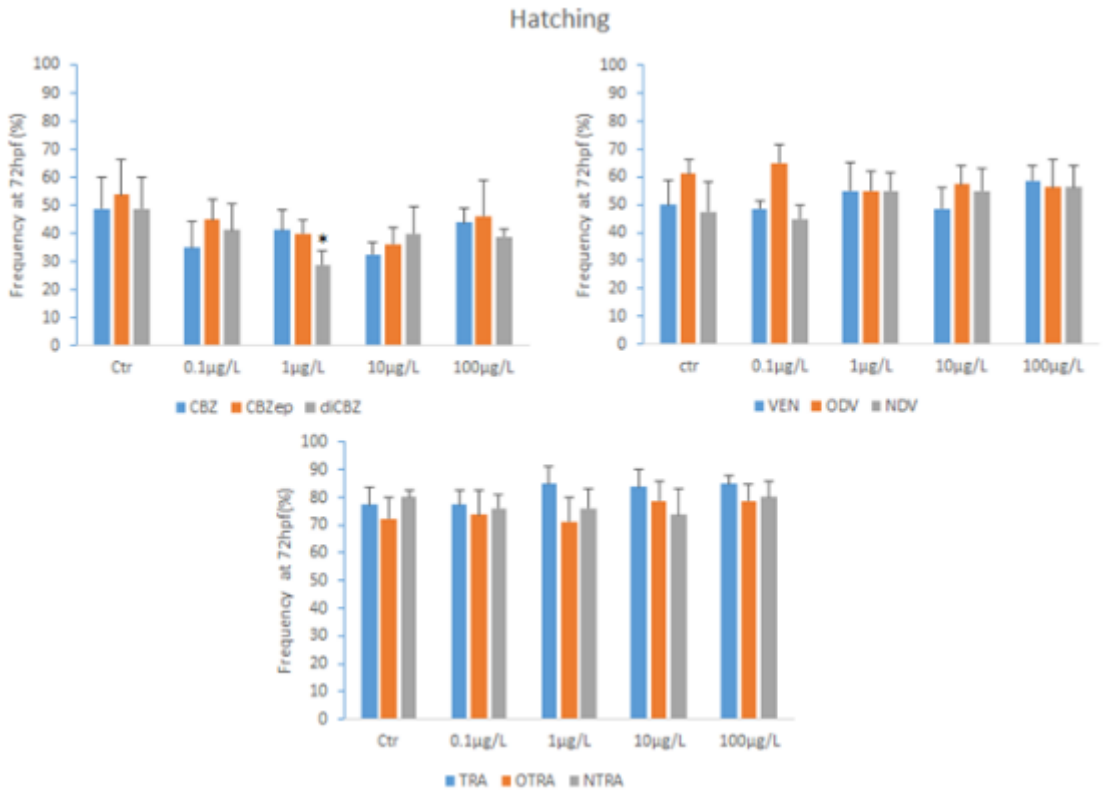
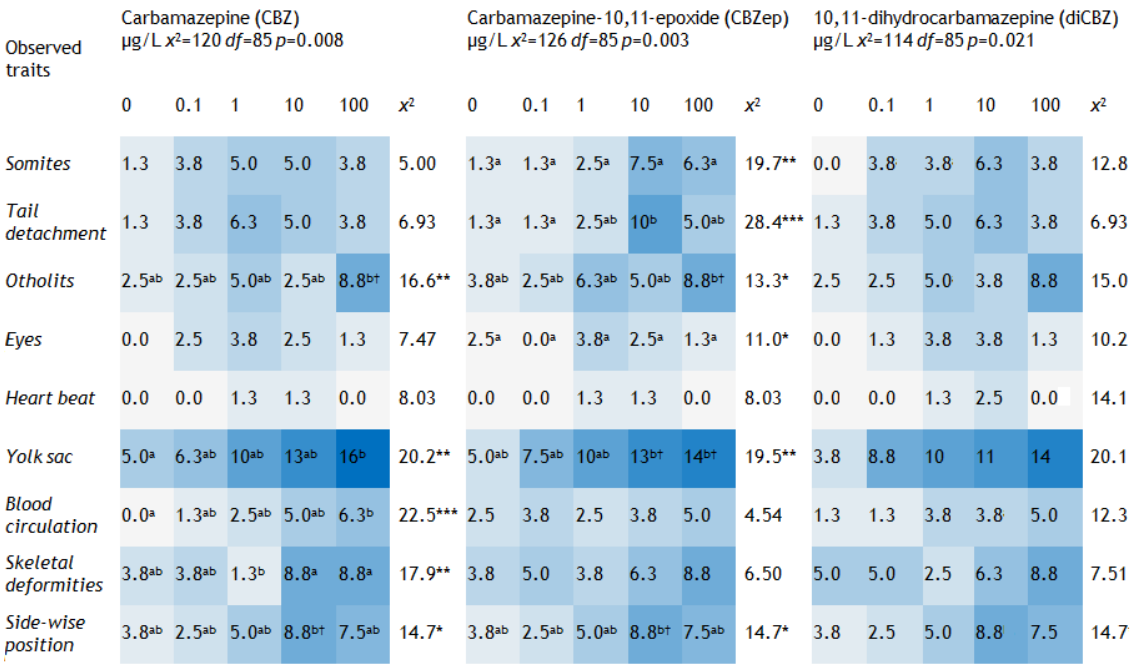


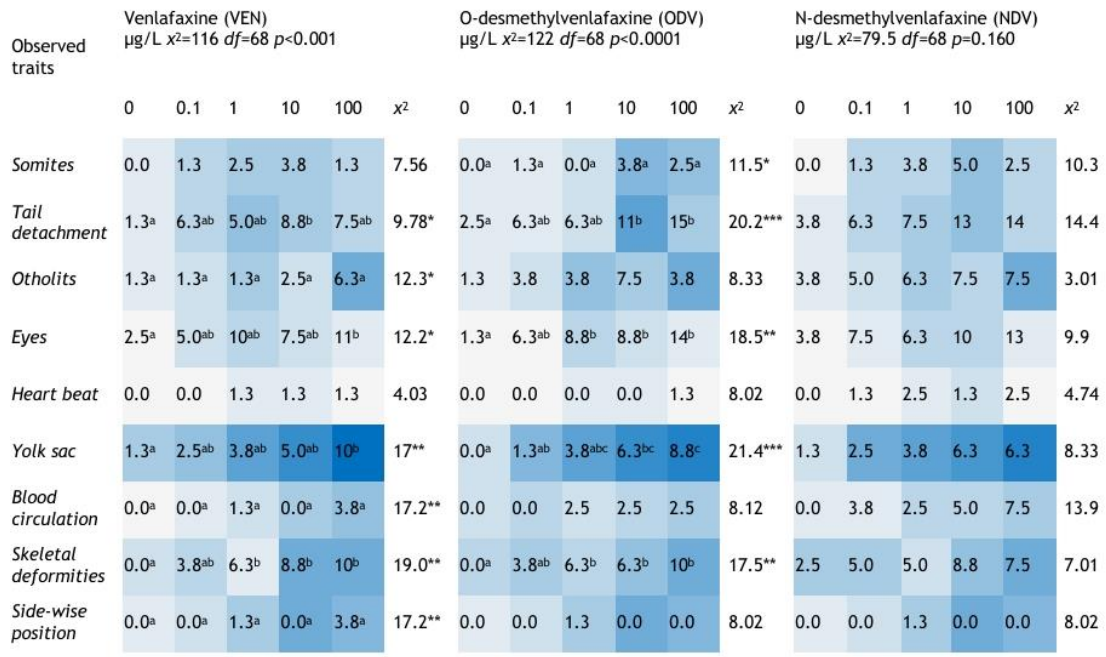
Figure 1. Hatching rates at 72hpf for each tested drug and its two main metabolites: carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZep) and 10,11-dihydrocarbamazepine (diCBZ); venlafaxine (VEN), O-desmethylvenlafaxine (ODV) and N-desmethylvenlafaxine (NDV); tramadol (TRA), O-desmethyltramadol (OTRA) or N-desmethyltramadol (NTRA).

A clear concentration-response relationship was found for several developmental malformations and tested compounds. Figures 2 to 4 show the rates recorded and allow a clear comparison of the compounds and their malformation profile. The CBZ, CBZep, ODV and TRA were the compounds eliciting the highest malformation rates, compared to the control. The rates were not very high (up to 10-15%) but were often statistically significant, especially for the higher test concentrations. Yolk sac malformations were the most frequent and significant alterations induced by CBZ and CBZep at fairly similar levels. Significant abnormalities in blood circulation were also found in CBZ embryos and tail detachment in CBZep (Fig. 2). Also, significant levels of malformations were elicited by VEN and ODV exposure, namely in tail detachment, eyes, yolk sac/oedemas and skeleton. The most relevant were the deformities in tail detachment and the eyes, which were more frequent in ODV embryos exposed to 10 and 100µg/L than in VEN embryos exposed to the same levels (Fig. 3).



Observed traits	Carbamazepine (CBZ) µg/L x^2=120 *df*=85 *p*=0.008						Carbamazepine-10,11-epoxide (CBZep) µg/L x^2=126 *df*=85 *p*=0.003						10,11-dihydrocarbamazepine (diCBZ) µg/L x^2=114 *df*=85 *p*=0.021					
	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2
Somites	1.3	3.8	5.0	5.0	3.8	5.00	1.3^{a}	1.3^{a}	2.5^{a}	7.5^{a}	6.3^{a}	19.7**	0.0	3.8	3.8	6.3	3.8	12.8
Tail detachment	1.3	3.8	6.3	5.0	3.8	6.93	1.3^{a}	1.3^{a}	2.5^{ab}	10^{b}	5.0^{ab}	28.4***	1.3	3.8	5.0	6.3	3.8	6.93
Otholits	2.5^{ab}	2.5^{ab}	5.0^{ab}	2.5^{ab}	8.8^{b†}	16.6**	3.8^{ab}	2.5^{ab}	6.3^{ab}	5.0^{ab}	8.8^{b†}	13.3*	2.5	2.5	5.0	3.8	8.8	15.0
Eyes	0.0	2.5	3.8	2.5	1.3	7.47	2.5^{a}	0.0^{a}	3.8^{a}	2.5^{a}	1.3^{a}	11.0*	0.0	1.3	3.8	3.8	1.3	10.2
Heart beat	0.0	0.0	1.3	1.3	0.0	8.03	0.0	0.0	1.3	1.3	0.0	8.03	0.0	0.0	1.3	2.5	0.0	14.1
Yolk sac	5.0^{a}	6.3^{ab}	10^{ab}	13^{ab}	16^{b}	20.2**	5.0^{ab}	7.5^{ab}	10^{ab}	13^{b†}	14^{b†}	19.5**	3.8	8.8	10	11	14	20.1
Blood circulation	0.0^{a}	1.3^{ab}	2.5^{ab}	5.0^{ab}	6.3^{b}	22.5***	2.5	3.8	2.5	3.8	5.0	4.54	1.3	1.3	3.8	3.8	5.0	12.3
Skeletal deformities	3.8^{ab}	3.8^{ab}	1.3^{b}	8.8^{a}	8.8^{a}	17.9**	3.8	5.0	3.8	6.3	8.8	6.50	5.0	5.0	2.5	6.3	8.8	7.51
Side-wise position	3.8^{ab}	2.5^{ab}	5.0^{ab}	8.8^{b†}	7.5^{ab}	14.7*	3.8^{ab}	2.5^{ab}	5.0^{ab}	8.8^{b†}	7.5^{ab}	14.7*	3.8	2.5	5.0	8.8	7.5	14.7

Figure 2. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to carbamazepine or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*p<0.05, **p<0.001, ***p<0.0001). Statistical comparisons were made against the solvent control. † indicates significant differences relative to the negative control at p<0.05. Different letters indicate significant differences among groups at p<0.05.



Observed traits	Venlafaxine (VEN) µg/L x^2=116 *df*=68 *p*<0.001						O-desmethylvenlafaxine (ODV) µg/L x^2=122 *df*=68 *p*<0.0001						N-desmethylvenlafaxine (NDV) µg/L x^2=79.5 *df*=68 *p*=0.160					
	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2
Somites	0.0	1.3	2.5	3.8	1.3	7.56	0.0^{a}	1.3^{a}	0.0^{a}	3.8^{a}	2.5^{a}	11.5*	0.0	1.3	3.8	5.0	2.5	10.3
Tail detachment	1.3^{a}	6.3^{ab}	5.0^{ab}	8.8^{b}	7.5^{ab}	9.78*	2.5^{a}	6.3^{ab}	6.3^{ab}	11^{b}	15^{b}	20.2***	3.8	6.3	7.5	13	14	14.4
Otholits	1.3^{a}	1.3^{a}	1.3^{a}	2.5^{a}	6.3^{a}	12.3*	1.3	3.8	3.8	7.5	3.8	8.33	3.8	5.0	6.3	7.5	7.5	3.01
Eyes	2.5^{a}	5.0^{ab}	10^{ab}	7.5^{ab}	11^{b}	12.2*	1.3^{a}	6.3^{ab}	8.8^{b}	8.8^{b}	14^{b}	18.5**	3.8	7.5	6.3	10	13	9.9
Heart beat	0.0	0.0	1.3	1.3	1.3	4.03	0.0	0.0	0.0	0.0	1.3	8.02	0.0	1.3	2.5	1.3	2.5	4.74
Yolk sac	1.3^{a}	2.5^{ab}	3.8^{ab}	5.0^{ab}	10^{b}	17**	0.0^{a}	1.3^{ab}	3.8^{abc}	6.3^{bc}	8.8^{c}	21.4***	1.3	2.5	3.8	6.3	6.3	8.33
Blood circulation	0.0^{a}	0.0^{a}	1.3^{a}	0.0^{a}	3.8^{a}	17.2**	0.0	0.0	2.5	2.5	2.5	8.12	0.0	3.8	2.5	5.0	7.5	13.9
Skeletal deformities	0.0^{a}	3.8^{ab}	6.3^{b}	8.8^{b}	10^{b}	19.0**	0.0^{a}	3.8^{ab}	6.3^{b}	6.3^{b}	10^{b}	17.5**	2.5	5.0	5.0	8.8	7.5	7.01
Side-wise position	0.0^{a}	0.0^{a}	1.3^{a}	0.0^{a}	3.8^{a}	17.2**	0.0	0.0	1.3	0.0	0.0	8.02	0.0	0.0	1.3	0.0	0.0	8.02

Figure 3. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to venlafaxine or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*p<0.05, **p<0.001, ***p<0.0001). Different letters indicate significant differences among groups at p<0.05.

Observed traits	Tramadol (TRA) µg/L x^2=123 *df*=68 *p*<0.00001						O-desmethyltramadol (OTRA) µg/L x^2=108 *df*=68 *p*=0.001						N-desmethyltramadol (NTRA) µg/L x^2=106 *df*=85 *p*=0.058					
	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2	0	0.1	1	10	100	x^2
Somites	0.0	0.0	2.5	2.5	1.3	8.10	0.0^{a}	0.0^{a}	2.5^{a}	3.8^{a}	3.8^{a}	11.7*	1.3	2.5	1.3	1.3	2.5	4.92
Tail detachment	2.5	1.3	6.3	6.3	6.3	8.84	0.0^{a}	1.3^{ab}	5.0^{bc}	8.8^{c}	10^{c}	26.3	2.5	3.8	3.8	3.8	7.5	9.70
Otholits	0.0^{a}	1.3^{ab}	6.3^{b}	6.3^{b}	5.0^{b}	15.2**	0.0^{a}	0.0^{a}	6.3^{b}	5.0^{b}	5.0^{b}	18.5**	0.0	3.8	3.8	5.0	5.0	15.3
Eyes	0.0^{a}	1.3^{ab}	0.0^{a}	5.0^{b}	2.5^{ab}	16.3**	0.0	1.3	1.3	3.8	1.3	8.12	1.3	2.5	1.3	1.3	1.3	1.45
Heart beat	2.5^{a}	5.0^{a}	7.5^{a}	10^{a}	10^{a}	10.4*	0.0	0.0	0.0	1.3	0.0	8.02	3.8	10	7.5	10	10	10.8
Yolk sac	2.5^{a}	5.0^{ab}	7.5^{ab}	10^{ab}	14^{b}	17.1**	2.5^{a}	7.5^{ab}	8.8^{ab}	11^{b}	11^{b}	11.0*	3.8	10	8.8	10	15	18.8
Blood circulation	0.0^{a}	2.5^{ab}	7.5^{bc}	7.5^{bc}	11^{c}	23.6***	1.3	1.3	5.0	5.0	5.0	8.00	2.5	5.0	6.3	7.5	10	20.6
Skeletal deformities	0.0^{a}	2.5^{ab}	7.5^{bc}	7.5^{bc}	11^{c}	23.6***	1.3^{a}	5.0^{ab}	5.0^{ab}	7.5^{ab}	10^{b}	12.6*	2.5	5.0	6.3	7.5	10	20.7
Side-wise position	0.0	0.0	0.0	0.0	0.0	-	0.0	1.3	0.0	1.3	1.3	4.03	1.3	1.3	1.3	0.0	1.3	4.03

Figure 4. Frequency of malformations (%) observed after single exposure of ~1hpf zebrafish embryos to tramadol or its main metabolites. Data for each tested substance were analysed through Correspondence analysis (CA); global chi-square values are indicated near the name of the substance. Partial chi-square values and respective significance (with the Bonferroni correction) indicate the observed traits that significantly contributed to the overall significance detected in the CA (*p<0.05, **p<0.001, ***p<0.0001). Different letters indicate significant differences among groups at p<0.05.

As to TRA and OTRA, these drugs were found to cause significant increases in embryo anomalies; specifically in the yolk sac (100µg/L TRA; ⩾10µg/L OTRA), skeletal (100µg/L TRA and OTRA) and in the otoliths (⩾1µg/L TRA and OTRA) (Fig. 4). In addition, TRA embryos also exhibited eye (10µg/L) and blood circulation (100µg/L) problems, while OTRA embryos showed lack of tail detachment (⩾10µg/L). Yolk sac deformation, often with oedema, was the most frequent anomaly observed (CBZ, CBZep VEN, ODV, TRA at 100µg/L; OTRA ⩾10µg/L). The literature indicates the yolk sac functions as a "backpack" for chemicals. Different chemicals were found to accumulate at high percentages >49% in the yolk, causing deformities or oedema, including CBZ (Halbach et al., 2020). Retardation of tail detachment, as found for CBZep (10µg/L), VEN (10µg/L), ODV (⩾10µg/L) and OTRA (⩾1µg/L), has been recognised as a sign of developmental delay at gastrulation (Hallare et al., 2006). Herein, tails remained non-detached after 24hpf, indicating retarded embryo development at early stages. This result is in contrast with the previous report showing that VEN treatment can accelerate neurogenesis and the overall development of zebrafish embryos (Thompson et al., 2017). However, the administration method was through microinjection of the embryos, rather than waterborne exposure as in our work, which represents a real exposure scenario. Eye

malformations with the emergence of non-pigmented areas were observed for VEN (100µg/L), ODV (⩾1µg/L) and TRA (10µg/L). The altered development of the eyes can disrupt the visual performance of the animals, leading to increased difficulties to find food and identify possible predators, diminishing the probability of survival. Low VEN levels (1µg/L) were described to increase the eye area in zebrafish embryos; however, no data was provided on the presence of deformities or non-pigmented areas (Tang et al., 2021). In humans, TRA can cause visual disturbance, miosis, blurred vision and lacrimation disorders in extreme cases (Subedi et al., 2019). It is possible that some of these effects can also occur in TRA-exposed fish, contributing to increased eye anomalies. The skeletal deformities observed for VEN, ODV and TRA (⩾1µg/L) raise concern since they occur at levels found in environmental samples. Moreover, they can affect the swimming capacity of larvae and, consequently, hamper escaping from predators and food search, threatening animal survival. Otoliths are structures mainly formed by calcium carbonate, with a crucial role in body balance, hearing, and vestibular function in fish (Han et al., 2019). The absence of concomitant differences in side wise position of exposed larvae suggests the otolith malformations detected at ⩾1ug/L TRA and OTRA may be impairing vestibular and hearing functions. Future studies should focus on deeper investigations of these malformations, as well as the blood circulation anomalies detected for TRA (100µg/L). In humans, recent studies reported that 15% of chronic TRA users have developed some type of cardiovascular disease (Musich et al., 2020). Also, exposure of mice and crayfish to TRA caused cardiac inflammation and alteration of cardiac activity (Bakr et al., 2021; Lozek et al., 2019). Tramadol was reported to have a low risk of cardiovascular effects by itself; however, its administration can result in the life-threatening serotonin syndrome, which causes blood conduction defects (Chen and Ashburn, 2015). Overall, our work shows for the first time that the active metabolite ODV can be more harmful than the parental compound since it produced higher frequency of anomalies than VEN. Future testing will help clarify the mechanisms underlying these findings and increase knowledge about endpoints like larvae length, spontaneous movement, and pigmentation. The present study is also, to the best of our knowledge, the first indicating an increase in malformations during zebrafish embryonic development and early larval stages upon exposure to TRA. A previous study about the exposure of zebrafish embryos to TRA, at similar levels, showed no statistically significant differences in developmental traits, in relation to the control (Sehonova et al., 2016). Differences in testing methodologies, data analysis and/or metabolism of tramadol between different aquatic organisms or fish lines may be of interest to account for in future testing (Tanoue et al., 2019).

4.3.2 Sensorimotor assays

The results of the sensorimotor test showed that exposure to 100µg/L of CBZ, CBZep, VEN and ODV significantly decreased the positive responses to head or tail stimulation of zebrafish larvae (Fig. 3). Likewise, decreased responses were observed for exposure to 0.1 and 100µg/L of TRA and OTRA. Zebrafish larvae exhibit brief swimming responses to mechanic stimulation as early as 27hpf (Saint-Amant and Drapeau, 1998). In this developmental window, head touches are associated to the activation of trigeminal sensory neurons, while tail touches are linked to the stimulation of Rohon-Beard sensory neurons. These two events are connected to the innervation of the descending reticulospinal Mauthner neurons, which mediate stimulated escape responses (Brunstein et al., 2003). Escape responses presuppose the coordination of a cascade of neural signalling processes (Brunstein et al., 2003), including the activation of voltage-gated sodium channels, upon which CBZ acts. Overexpression of voltage-gated sodium channel SCN5 leads to the sensitization of Mauthner cells, increasing escape responses to electric pulses (Tabor et al., 2014). However, CBZ can block these sodium channels, inhibiting their action (Jo and Bean, 2014), which can possibly lead to the decreased behavioural responses observed. Qiang et al. (2016) reported that CBZ-exposed zebrafish embryos were more sensitive to light and touch than the controls, though they attributed such effects to the action of CBZ on GABA (gamma-aminobutyric acid) receptors.

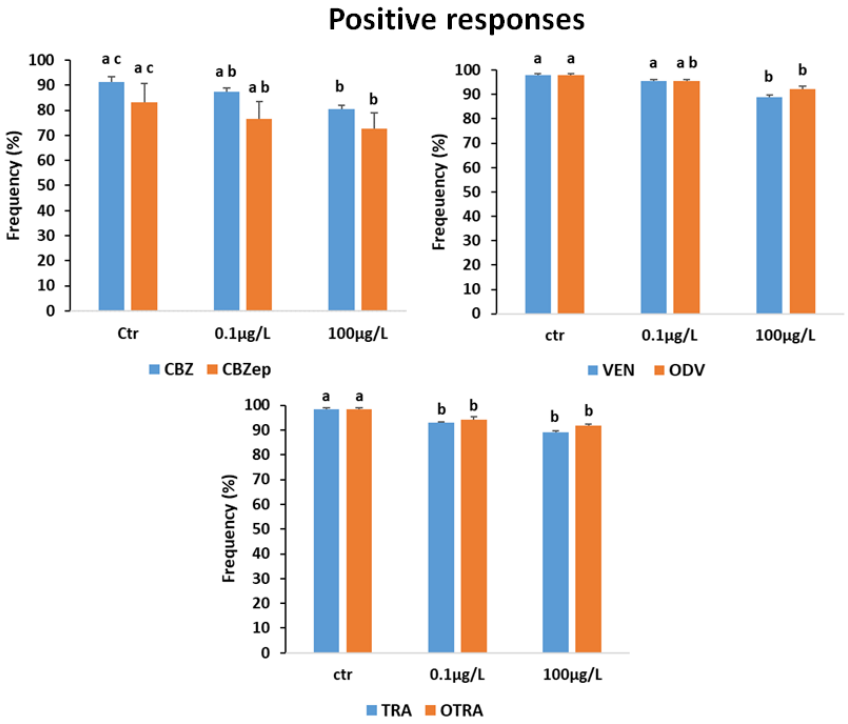


Figure 5. Results of the behavioural sensorimotor assay performed on zebrafish larvae exposed for 168hpf to carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZep), venlafaxine (VEN), O-

desmethylvenlafaxine (ODV), tramadol (TRA) or O-desmethyltramadol (OTRA). Different letters indicate statisitcally significant differences at $p<0.05$.

A recent study found that exposure to CBZ inhibited spontaneous movement of zebrafish embryos at 28/29hpf (Chen et al., 2020). Furthermore, hypoactivity of zebrafish larvae in dark periods upon exposure to CBZ was also described (van Woudenberg et al., 2014; Pohl et al., 2019). Recently, Aztei and colleagues (2021), reported inhibition of locomotor activity of zebrafish larvae exposed to CBZ in the range tested in our study, but no differences were found for CBZep exposure. The monoaminergic system is also known to play a major role on the functional organisation of locomotion at early developmental stages (Brunstein et al., 2003), possibly influencing the locomotor responses obtained for these blocks of drugs. Compounds with modes of action linked to neurotransmitter modulation, as the ones tested herein, would all be expected to interfere with the cross talking between sensory receptors and motor neurons, resulting in reduced behavioural responses after stimulation (Little and Brewer, 2001). In their microinjection experiments, Thompson et al. (2017) also found an effect of VEN on larvae neurogenesis and behaviour. Exposure of zebrafish embryos to 2µg/L VEN for 80hpf led to decreased response to stimulation in relation to the control (Rodrigues et al., 2020). In a recent study, Aztei and colleagues (2021) observed reduced locomotor behaviour of zebrafish larvae exposed in the dark to 0.3 to 300µM VEN or ODV for 120 hpf, in a light-dark transition test; the results were typical of a dose-response relationship. In the case of TRA, exposure for 144hpf resulted in a significant anxiolytic effect of zebrafish larvae in relation to control (LOEC of 320µg/L), with decreased swimming distance in the dark (Bachour et al., 2020). Exposure to TRA (~1µg/L) also reduced the time spent in locomotion of crayfish; animal covered shorter distances following a stress stimulus (Lozek et al., 2019). Kirla and colleagues (2021) found decreased locomotor activity in zebrafish larvae exposed for 120hpf to TRA (at concentrations ≥1µM) but not to OTRA.

4.3.3 Molecular toxicity

To characterise and compare the mechanisms underlying fish responses to the test drugs and their metabolites a saturated orthogonal multiple linear regression analysis was first carried out. For all three drug blocks, significant effects of the chemical species and/or test concentrations were found at $p<0.0001$ for most of the genes investigated (Tables S2 to S4 in Appendix A4.), with the models explaining a strong proportion of the fitted data (>65%). For the CBZ block, the genes *sert*, *abcg2a*, *rarab*, *rarga*, *mao*, *rxrgb*, *pparg*, *adra2c*, *rxrbb*, *nrf2*, *rxrab*, *abcc1*, *ppara*, *abcc2*, *drd2b* and *raraa* were in the 50th percentile of higher explained variance (R^2≥79%, Appendix A4. Table S2). For the VEN

block, those were the genes *ahr2*, *gst*, *rarab*, *raraa*, *abcc1*, *nrf2*, *drd1b*, *abcg2a*, *abcc2*, *pparg*, *dat*, *rxrab*, *rxraa*, *mao*, *rxrbb* and *abcb4* (R^2≥81%, Appendix A4. Table S3). For the TRA block, genes in the 50th percentile of highest explained variance were *nrf2*, *dat*, *keap*, *rarga*, *abcc1*, *abcc2*, *raraa*, *rxrga*, *pxr*, *ahr2*, *abcb4*, *abcg2a*, *pparg*, *sert*, *drd2b*, *rxraa* (R^2≥91%, Appendix A4. Table S4). Among those with better fitted models the genes *abcc1*, *abcc2*, *abcg2a*, *nrf2*, *pparg* and *raraa* were common to the three blocks (Fig. 4). Three of these genes belong to the large superfamily of ATP-binding cassette (ABC) drug transporters, which are membrane proteins with diverse functions (Holland et al. 2003). They convert the energy gained from ATP hydrolysis into trans-bilayer movement of substrates either into the cytoplasm (import) or out of the cytoplasm (export). They are membrane transporters, mediating the cellular efflux of a wide range of organic chemicals; some of them also transport certain metals (Luckenbach et al., 2014). Moreover, their enhanced expression can confer multixenobiotic resistance to organisms inhibiting polluted habitats; *abcc1* and *abcc2* are drug efflux transporters and *abcg2a* acts in the disposition and response to certain drugs. Previous studies have shown that CBZ can induce the expression of ABCB1, ABCC2 and ABCG2 in HepG2 and Caco2 cells, at transcript and protein levels, and increase their functional activity (Grewal et al., 2017). Venlafaxine and desvenlafaxine were also shown to induce the expression of drug efflux transporters (Bachmeier et al., 2013). For the remaining drugs no other reports of interaction with these transporters could be found in the literature. The *nrf2* gene is known to regulate cellular antioxidant responses. At baseline, *nrf2* is coupled to *keap1*, with the latter promoting proteasomal degradation of *nrf2*, i.e., repressing its expression. Upon stimulation, *nrf2* uncouples from *Keap1* and is translocated to the nucleus where it can activate the expression of antioxidant enzymes (Hahn et al., 2015). On the other hand, *pparg* is a regulator of adipocyte differentiation, which has been implicated in adipogenesis and the regulation of lipid metabolism in humans and fish (Wafer et al., 2017). Obesity is often reported in CBZ patients and *in vitro* studies have shown that both CBZ and VEN can affect adipogenesis (Kruk et al., 2018; Im et al., 2019). For CBZ, this effect is probably mediated through the *pparg/ß-catenin* system; *pparg* expression increasing adipogenesis and *β-catenin* acting as its negative regulator (Im et al., 2019). The gene *raraa* is a subunit of the retinoid acid in zebrafish. Retinoid acid has a vital role for vertebrate development, especially for the specification of pancreas (Lopez-Perez et al., 2021). However, no data was found for the disruption of this function after exposure to the tested drugs.

Altogether, our results suggest that exposure to these drugs may increase cellular efflux, antioxidant defences and adipogenesis in zebrafish.

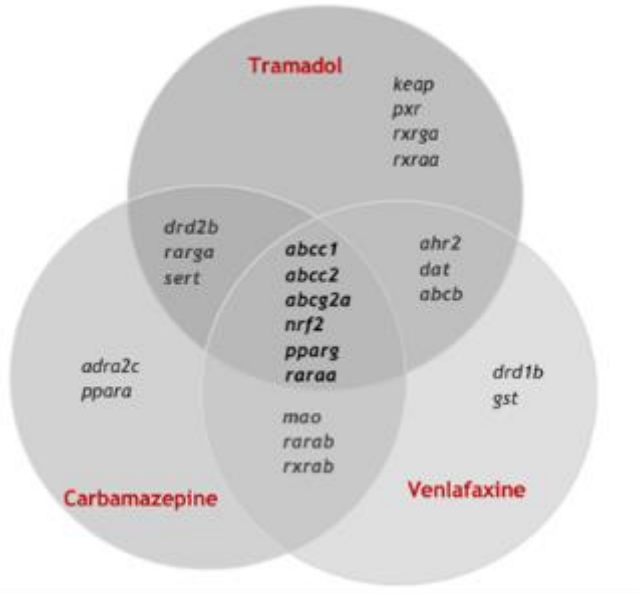


Figure 6. Common genes in the 50th percentile of higher explained variance as indicated by the multiple regression analyses for the drugs blocks investigated.

To investigate the patterns of gene expression and compare the responses of the parental compounds with their respective metabolites, molecular data and the orthogonal regressors were analysed through PLS followed by a cluster analysis. The PLS extracted five significant components explaining 52% of the Y inertia for the CBZ block, five significant components explaining 59% of the Y inertia for the VEN block and seven significant components explaining 76% of the Y inertia for the TRA block (Fig. S1, Appendix A4.). The CA identified 11, 10 and 8 clusters of interest for CBZ, VEN and TRA blocks, respectively (Fig. S2, Appendix A4.). Based on the statistical results, model responses for the gene expression of each cluster and tested drug were built (Figs. 5 to 7). For the CBZ

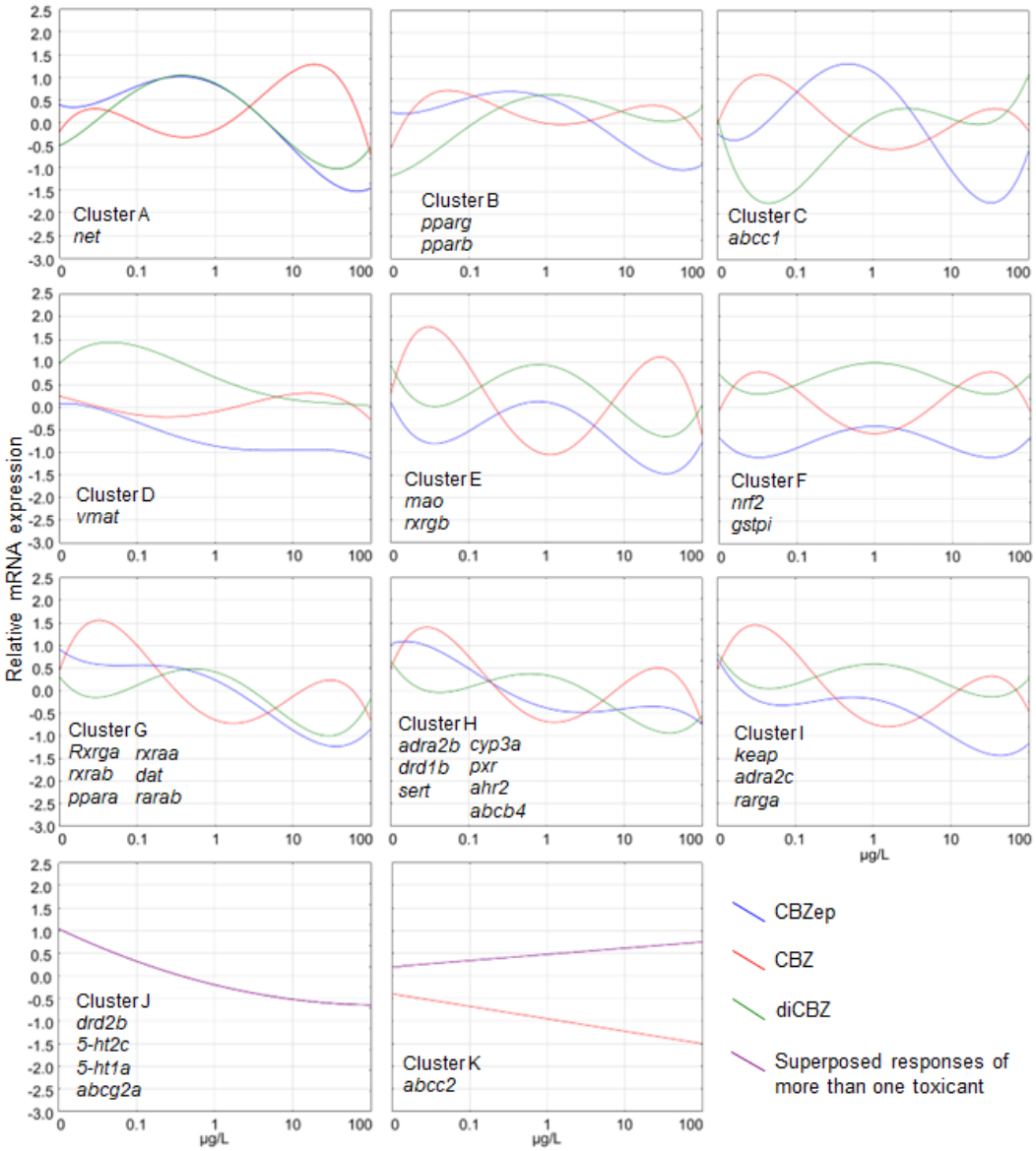


Figure 7. Model responses to 168hpf exposure to carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZep) or 10,11-dihydrocarbamazepine (diCBZ) derived using the equation of the saturated orthogonal multiple linear regression for the groups of genes identified through the Cluster Analysis.

block, clusters C, E and I showed the stronger changes in gene expression relative to controls (Fig. 5). Non-monotonic response patterns were observed for the three clusters and different responses were elicited by the parent compound and its metabolites. In cluster C (*abcc1*), a tendency for up-regulation at low and intermediate exposure levels was elicited by CBZ and CBZep, respectively, with slightly stronger effects of CBZep relative to CBZ. Tendency for down-regulation was found for diCBZ (at low exposure levels) and CBZep (at high exposure levels). For clusters E (*mao*, *rxrgb*) and I (*keap*, *adra2c*, *rarga*), both metabolites elicited fairly similar non-monotonic response trends, which differed in their magnitude and were opposite to those elicited by CBZ; CBZep showing a tendency to down-regulate the expression of these genes and diCBZ driving towards up-regulation. In contrast, the parent compound tended to cause marked over

expression of these gene clusters at low and high exposure levels and inhibition at intermediate levels (Fig. 5). Cluster F (*nrf2*, *gstpi*) showed response patterns very similar to cluster E, though the expression alterations caused by CBZ were weaker. The association of both genes in the same cluster was also very clear, given that *nrf2* mediates the induction of *gstpi*, which also has an antioxidant role (Hahn et al., 2015 and references therein). Of note were also the results obtained for clusters J (*drd2b*, *5-ht2c*, *5-ht1a*, *abcg2a*) and K (*abcc2*). For cluster J, all drugs caused the same pattern of expression, and the main driver was the exposure concentration, with decrease in the expression of the genes in this cluster with the increase in concentration. This identical pattern of response for both parental compound and metabolites makes the genes comprised in this cluster potential biomarkers of exposure for the block of CBZ, CBZep and diCBZ. For cluster K, the two metabolites tended to act similarly and in opposition to CBZ for which down-regulation of gene expression increased with the exposure concentration.

Different and non-monotonic responses to the parental drug and its metabolites were also found for most of the clusters in the VEN block (Fig. 6). The strongest changes in expression were found for cluster D (*drd1b*, *adra2b*, *cyp3a*, *pparg*, *abcc1*) upon exposure to ODV, which tends to cause overexpression of these genes at both low and high exposure levels. Interestingly, the responses of *adra2b*, *drd1b* and *pparg* were clustered together in this group. Human adipocytes express noradrenergic and dopaminergic receptors (including DRD1) that are involved in the stimulation of lipolysis and the regulation of lipid metabolism (Yu et al., 2022). The grouping with *pparg,* mediating adipogenesis and lipid regulation, suggests similar processes may occur in zebrafish. Cluster F comprises genes that are closely linked to the mode of action of these compounds. As a SNRI, VEN and metabolites act in monoaminergic systems, as it is the case *5-ht2c* and *5-ht1a* which are serotonin receptors, as well as *mao* and *vmat,* which act on monoamines degradation and transport. Likewise, for the CBZ block, grouping of *nrf2* and *gstpi* in the same cluster was also found here (Fig. 6). In addition, for cluster I (*rxrbb*, *rxraa*, abcb4) exposure to each of the three drugs led to the same pattern of expression, very close to a negative dose-response relationship, making the genes comprised in this cluster, potential biomarkers of exposure for this block. For cluster J (*keap*, *net*, *abcg2a*, *drd2b*, *abcc2*) the two metabolites elicited the same response on gene expression, opposed to that observed for VEN.

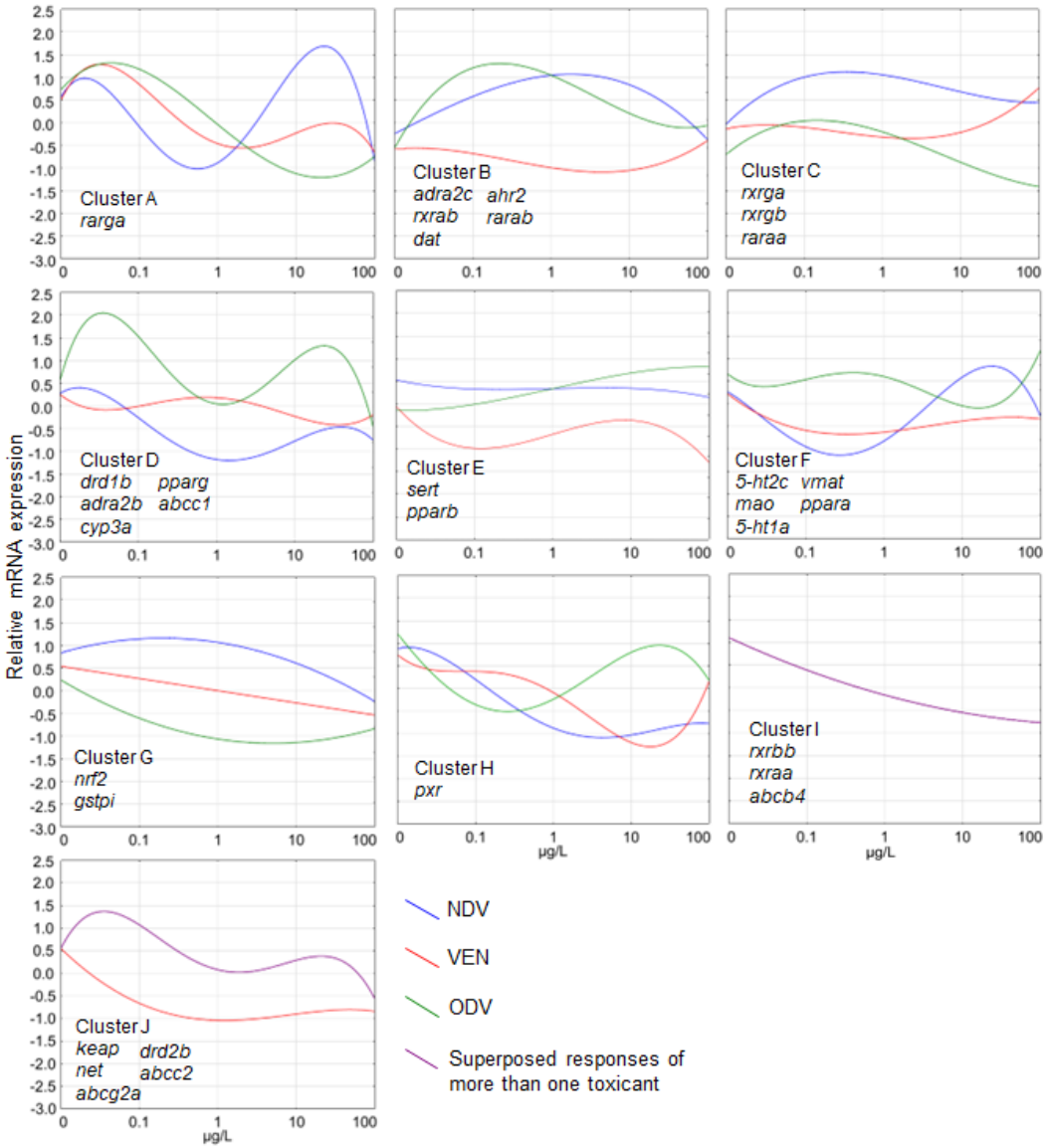


Figure 8. Model responses to 168hpf exposure to venlafaxine (VEN), O-desmethylvenlafaxine (ODV) or N-desmethylvenlafaxine (NDV) derived using the equation of the saturated orthogonal multiple linear regression for the groups of genes identified through the Cluster Analysis.

Likewise, for CBZ and VEN, non-linear responses were typically exhibited by gene clusters found for the TRA block (Fig. 7). The strongest alterations in expression were estimated for cluster A (*ppara*), with TRA and NTRA acting similarly on the expression of the gene (down-regulation at low and high exposure concentrations and up-regulation at intermediate levels) and OTRA eliciting an opposing pattern of expression (up-regulation at high exposure levels). Peroxisome proliferator-activated nuclear receptors have key roles in maintaining lipid homeostasis, their disruption often causing dysregulation of tissue differentiation and progression of metabolic disease. Among them, *ppara* is strongly expressed in tissues with high fatty acid oxidative activity and is crucial for the regulation of lipid metabolism and control of lipogenic genes in fish (Ning et al., 2019; Li et al., 2020). Alterations in *ppara* expression have also been linked to abnormal

neural development, highlighting an essential role in the regulation of the proliferation of neuronal and glial precursors (Hsieh et al., 2018). Given the widespread expression of *ppar* and their function, alterations in their signalling may possibly be at the genesis of the malformations found herein, especially those related to the yolk sac (Venezia et al., 2021). Cluster D (*sert*, *dat*, *nrf2*, *keap*, *rxrab*, *abcc1*, *rxrga*, *raraa*, *cyp3a*, *abcb4*) was very interesting in the sense that TRA tended to cause no alteration in the expression of its genes after 168hpf exposure at any of the concentrations tested.

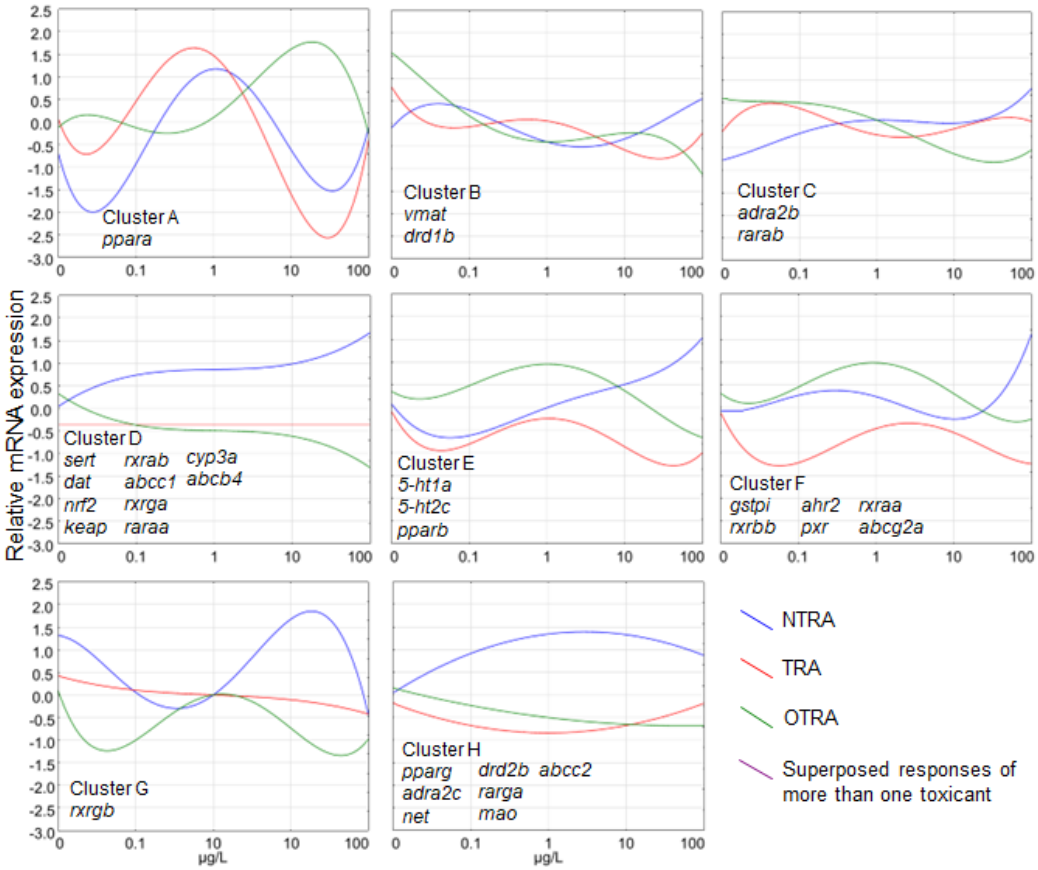


Figure 9. Model responses to 168hpf exposure to tramadol (TRA), O-desmethyltramadol (OTRA) and N-desmethyltramadol (NTRA) derived using the equation of the saturated orthogonal multiple linear regression for the groups of genes identified through the Cluster Analysis.

Genes in this cluster were related to the transport of serotonin and dopamine and to the detoxification response. Future work will allow confirming this for other exposure durations and developmental time windows. Also, in this cluster, OTRA and NTRA tended to elicit opposite responses, with clear up (NTRA) and down-regulation (OTRA), especially at high exposure concentrations (Fig. 7). Cluster E were mainly related with serotonin receptors, which can be explained by the affinity of TRA and, to a lesser extent, OTRA for serotonin receptors. In cluster H (*pparg*, *adra2c*, *net*, *drd2b*, *rarga*, *mao*, *abcc2*), similar responses were elicited by OTRA and TRA, tending to inhibition of expression at

intermediate exposure levels, in contrast with TRA that tended to induce expression at the same levels.

Overall, after 168hpf all metabolites tested were able to alter the expression of most of the genes assessed, several of them involved in monoamine signalling and metabolism. In several cases, very small or no alteration in the responses of the gene clusters was found for the parental compounds, in clear contrast to the respective metabolites; CBZ in cluster D (*vmat*), VEN in clusters B (*adra2c*, *rxrab*, *dat*, *ahr2*, *rarab*), D (*drd1b*, *adra2b*, *cyp3a*, *pparg*, *abcc1*) and F (*5-ht2c*, *mao*, *5-ht1a*, *vmat*, *ppara*), TRA in clusters C (*adra2b*, *rarab*) e D (*sert*, *dat*, *nrf2*, *keap*, *rxrab*, *abcc1*, *rxrga*, *raraa*, *cyp3a*, *abcb4*) and G (*rxrgb*). In other cases, what appears to be a different response may be indicative of a lower potency of one of the chemical species, which only at higher concentrations or possibly longer exposure times will result in an induction or inhibition of expression of similar intensity of that of the other(s) species in the block; CBZ and CBZep in cluster C, ODV and NDV in cluster B, VEN and NDV in cluster H, OTRA relative to TRA and NTRA in cluster A, OTRA and NTRA in cluster G.

4.3.4 Final remarks

Considering the main working hypothesis, this study showed that CBZep, ODV and OTRA metabolites increased the embryonic malformation rates at exposure concentrations found in environment samples. The most affected phenotypes were related to otolith malformations, tail detachment and yolk sac abnormalities. The latter were possibly related to altered expression of dopaminergic, noradrenergic and peroxisome proliferator-activated receptor pathways, as shown by the molecular genetics analysis. The rate of malformations elicited by ODV was higher than that found for the parental compound. The test compounds also increased the rate of abnormal sensorimotor responses with TRA and OTRA showing higher potency (effects elicited at $\geqslant$0.1 μg/L) than CBZ, CBZep, VEN and ODV ($\geqslant$100 μg/L). Further testing of other genes (e.g. encoding voltage-gated sodium channels or opioid receptors involved in CBZ or TRA action) and proteomic approaches will contribute to the construction of adverse outcome pathways for these drugs. The results obtained are worrying. They indicated that exposure to these drug metabolites in contaminated aquatic systems may put natural populations at significant risk. The data presented herein call for future studies on the transformation products of pharmaceuticals, as well as their consideration for inclusion in monitoring programmes and risk assessment evaluations.

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Chapter 5: Comparing the effects of pharmaceuticals and their metabolites on zebrafish larvae through shotgun proteomics

Chapter 5. Comparing the effects of pharmaceuticals and their metabolites on zebrafish larvae through shotgun proteomics

5.1 Introduction

The previous chapter indicated that exposure to CBZ, VEN or TRA, as well as their metabolites CBZep, ODV and OTRA, altered the malformation rates in exposed embryos and larvae. Gene expression alterations were also elicited by the exposure to these compounds. Nevertheless, information on the molecular events and changes in proteins involved in their toxicity is limited (Zhuo et al., 2012; Simmons et al., 2017; Yan et al., 2018; Navon et al., 2021), and not available for early fish development. This hampers our understanding of their mechanisms of action.

Proteomics is a potential useful tool to analyse the toxicity of pharmaceuticals and their active metabolites, in a perspective of organism integrity (Ronsein et al., 2015). The continuous development of quantitative proteomic techniques allows the comprehensive systematic study of changes in the proteome and overall expression of proteins. These methods are helpful to determine the contribution of stressors to the changes observed and to understand the toxicity mechanisms and the biological processes they alter (Ronsein et al., 2015).

Therefore, the main goals of this study were to understand the impact of CBZ, VEN, TRA and their main metabolites to zebrafish larvae at the proteomic level, namely changes on the expression profile of proteins from exposed animals, and their quantification, as well as identifying potential mechanisms underlying the toxic effects previously observed (Chapter 4). Based on the results obtained for the malformations and gene expression changes described in Chapter 4, two concentrations of each parental compound and the most active metabolite were selected for proteomics investigation. The experimental design adopted was the same and the animals were collected for the proteomic study at 168hpf. The results are compared and discussed for each block of chemicals.

5.2 Material and methods

5.2.1 Exposure

Zebrafish embryos (0-3hpf) were placed in 6 well plates and exposed to 0.1 (CBZ01; CBZep01; VEN01; ODV01; TRA01 and OTRA01) and 100µg/L (CBZ100; CBZep100;

VEN100; ODV100; TRA100 and OTRA100) of all compounds in a total volume of 10mL, per well, for 168 hours. A control group containing only dechlorinated water and a solvent control group of DMSO (0.01%) for CBZ and CBZep were added to the assays. A total of 300 embryos (50 embryos/well) were exposed per treatment in each replicate. The assay was repeated four times. To minimise losses of the test chemicals from the media by adsorption, the test plates were incubated with the test solutions for 24h prior to embryo exposure and media on the wells were renewed daily for the whole exposure period. Dead or abnormal embryos were removed daily to avoid interferences in the results. At the end of the assays, surviving larvae of each treatment were collected, frozen in liquid nitrogen and stored at -80ºC.

5.2.2 Protein Extraction

The samples (zebrafish 168hpf larvae) were defrosted in ice and incubated in SDT buffer (0.5 g fresh weight/mL) for 20 min at room temperature (RT). The SDT buffer contained 0.1MTris/HCl pH 7.6, 2% SDS, and 0.1 M dithiothreitol with 1:100 protease inhibitor (Halt PI Cocktail CAT #78429, Thermo Scientific, Waltham, MA, USA). Following the incubation, six sonication cycles of 3s each (potency of 60% 10 W, VC 50; Sonics & Materials Inc., Danbury, CT, USA) were performed. The samples were then incubated at room temperature for 2 h under light-protected conditions and subsequently heated for 3 minutes at 95ºC. Then, samples were centrifuged at 16,000 g at 21ºC for 20 min. The supernatant was collected and transferred to new collection tubes, and the protein content was quantified by absorbance measurement at 280 nm (A280 application, DeNovix, Ds-11 FX Spectrophotometer: Wilmington, DE, USA). Samples were stored at -20ºC until further processing.

5.2.3 Filter-Aided Sample Preparation (FASP)

Sample preparation for LC/MS analysis was performed according to the FASP method (Wiśniewski et al., 2009), with minor modifications. Protein samples (80 mg) were diluted in 200 µL of UA buffer (8 M urea, in 0.1 M Tris/HCl, pH 8.5). Samples were then transferred to filter units (30 kDa MWCO, MRCF0R030, Merck, Tullagreen, Ireland). Previously to the transference, the units were washed in water and UA buffer. Thereon, 100 µL of iodoacetamide (0.05 M) was pipetted to the filter units, and mixed at room temperature for 1 min in a Thermomixer (Eppendorf, Hamburg, Germany), and then incubated for 20 min. After this incubation, filter units were centrifuged for 10 min at 14,000 g (RT). Two washing cycles were performed with 100 µL of UA buffer, followed

by 15 min of centrifugation, as well as 100 µL of ammonium bicarbonate (0.05M) for the same time period. Digestion of peptides was performed by addition of trypsin proteomics grade (CAT #3708985001, Roche, Mannheim, Germany). A ratio of 1:100 enzyme-to-protein in ammonium bicarbonate (0.05M) was chosen to the digestion. Samples with trypsin were in first place mixed for 1 min in Thermomixer at RT (Hamburg, Germany) and then incubated overnight (~18h) in a wet chamber at 37 C. The resulting peptides were eluted to new collection tubes by centrifugation (14,000 g, 10 min). A second elution was performed with NaCl (0.5M) followed by centrifugation, and eluates collection. Peptide concentrations were measured at 280 nm (A280 application) and acidified using formic acid (0.1% v/v). A desalting step was performed with C18 columns (C18 UptiTip™ CAT# BI5280, Glygen, Interchim Innovations, Montluçon, France). Conditioning of columns was performed according to the manufacturer's protocol. Samples were then transferred to the columns and washed with formic acid (0.1%, v/v). Following step was the elution of peptides with acetonitrile (60% v/v) and formic acid (0.1%, v/v) into new tubes. The final peptide concentration was measured at 280 nm. The samples were at last, fully dried with a vacuum concentrator (CentriVap, Labconco, Kansas City, MO, USA) and stored at -20 C.

5.2.4 LC–MS/MS Analysis

LC–MS/MS analysis was performed using a nano-LC coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific,Waltham, MA, USA). Peptides separation was executed by reverse-phase chromatography with an EASY nLC 1200 system (Thermo Scientific). Peptides were spiked into a pre-column (Acclaim PepMap 100, 75 _x0016_m 2 cm, Thermo Scientific), and separated using an EASY-Spray C18 reversed-phase nano-LC column (PepMap RSLC C18, 2 _x0016_m, 100A 75 _x0016_m 25 cm, Thermo Scientific) by a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B) following this protocol: from 6% B to 40% B in 80 min; from 40% B to 100% B in 20 min at a flow rate of 0.3 _x0016_L/min. Obtained peptides were electrosprayed and analysed by a QExactive HF mass spectrometer (Thermo), in a data-dependent mode and positive polarity. Complete scans were performed on a 380–1400 m/z range at 120,000 resolution. The 15 multiple charged ions with higher strength were isolated (1.2 m/z isolation window) and fragmented with a dynamic exclusion of 30.0 s at a 30,000 resolution.

5.2.5 Protein Identification and Quantification

Scaffold (version Scaffold_4.11.1, Proteome Software Inc., Portland, Oregon) was used to confirm MS/MS-based peptide and protein identification. Results were valid if these criteria were fulfilled: proteins had to have >99.9% probability and also contain at least two unique identified peptides; peptides had to have >95.0% probability by the Scaffold local false discovery rate (FDR) algorithm. Protein probabilities were determined by the Protein Prophet algorithm. Proteins that contained similar peptides and, therefore, could not be differentiated by MS/MS analysis alone were grouped to fulfil the principles of parsimony. Only proteins sharing significant peptide evidence were used for analysis. MS and MS/MS tolerances were set to 0.6 Da 10 ppm. Trypsin was used for protein cleavage allowing for a single missed cleavage. Qualitative information was checked with Venn diagrams on Scaffold.

5.2.6 Determination of differentially expressed proteins

Normalized Average Precursor Intensity was used to report quantitative differences between methods. Proteins that were identified in a minimum of two of the three replicates in one of the treatments were considered representative. Protein expression data were analysed Molecular data were analysed by means of a saturated orthogonal multiple linear regression analysis to assess the effects of the single experimental factors and their interactions on the expression of each gene (Box et al., 1978; Tomassone et al., 1983). Five regressors were established for each block of chemicals to account for the chemical species (two regressors of 1^{st} and 2^{nd} order), the concentrations tested (two regressors of 1^{st} and 2^{th} order) to adequately describe the response of each protein. To investigate patterns of protein expression, a partial least squares regression (PLS) was then carried out using the orthogonal regressors (X, independent variables) and the variation in gene expression (Y, dependent variables). The significant Y components extracted by the PLS were entered in a Cluster Analysis (CA) to identify groups of proteins exhibiting similar responses. All statistical tests were carried out with Statistica 14 (TIBCO) with a 5% significance level. The heatmap and cluster analysis for each comparison was based on Pearson correlation values and was done with the treatment log2 fold change values on Morpheus by Broad Institute (RRID:SCR_017386).

5.2.7 Functional annotation

The informatics tool STRING: functional protein association networks (https://string-db.org/) was applied to search evidence of protein interaction and determine how these interactions between proteins and the potential molecular pathways were impacted in CBZ; CBZep; VEN; ODV; TRA and OTRA-exposed zebrafish. This evidence was obtained from several sources. Examples of this are: experiments, databases, text mining, co-expression, gene fusion, neighbourhood and co-occurrence. The outcome of Kyoto Encyclopaedia of Genes and Genomes (KEGG), obtained in STRING analysis, were used according to full networks with a medium confidence of 0.400 FDR and stringency of 5%. Significant p-values for the enrichment in protein-protein interactions (PPI) were obtained at 5% of significance. When p-values were higher than 5% artificial nodes were added to the networks to achieve significant p-values. The last step was grouping proteins into clusters in the software, using the k-means clustering method available on STRING.

5.3 Results

5.3.1 Carbamazepine and its main metabolite

Protein quantification

Shotgun proteomic analysis was performed for protein quantification in three different samples for each treatment. From the analysis, it was possible to quantify 3241 different proteins. From the total of quantified proteins, 2201 proteins were quantified in the control group (67.9% of the total quantified proteins); 2473 in CBZ01 (76.3% of the total quantified proteins); 2240 in CBZ100 (69.1% of the total quantified proteins); 2269 in CBZep01 (70% of the total quantified proteins) and 2233 for CBZep100 (68.9% of the total quantified proteins). Distribution of the quantified proteins by treatment is shown in Figure 1.

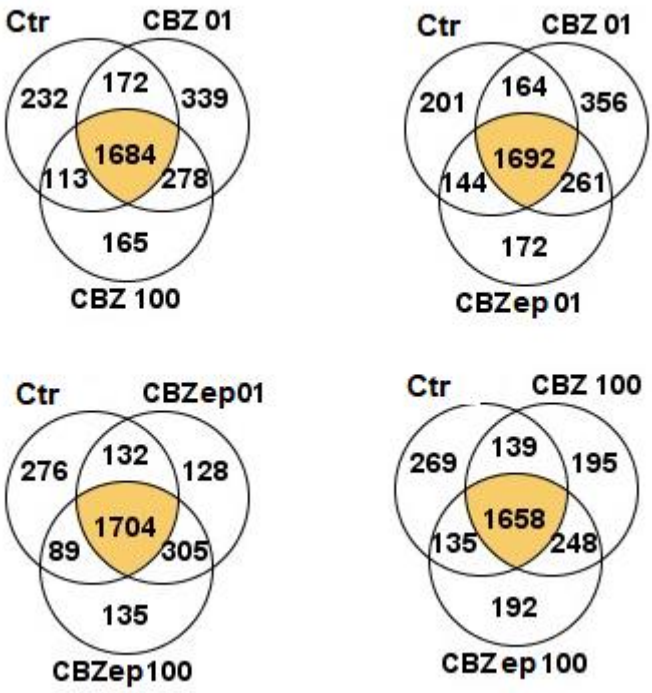

Figure 1. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; CBZ01: carbamazepine 0.1 µg /L; CBZ100 carbamazepine 100 µg /L; CBZep01 carbamazepine 10,11-epoxide 0.1 µg /L, CBZep100 carbamazepine 10,11-epoxide 100 µg /L.

The Venn diagrams on the left compare common and exclusive proteins of each compound and the control group. The Venn diagrams on the right side compare the test concentrations, i.e., proteins differentially expressed between the control, CBZ and its metabolite for similar exposure concentrations. In all cases, most quantified proteins were common to all the test conditions. At least 1658 common proteins were detected, which represent approximately 51% of the total quantified proteins. The number of proteins exclusive to just one treatment was about 20% or less of the proteins shared among the three groups.

Identification of differentially expressed proteins

From our results, it was possible to identify a total of 130 Differentially Expressed Proteins (DEPs) for all treatments. Different patterns of expression were observed for this set of proteins:

- 10 proteins were under-expressed while 120 were over-expressed for CBZ01
- 29 proteins were under-expressed, while 101 were over-expressed for CBZ100.
- 37 proteins were under-expressed, while 93 were over-expressed for CBZep01.
- 26 proteins were under-expressed, while 104 were over-expressed for CBZep100

Larvae from the 0.1µg/L concentration of the parental compound (CBZ) showed 95 DEPs; at the 100µg/L of CBZ there were 36 DEPs. Regarding the 0.1µg/L concentration of the active metabolite (CBZep), exposure of zebrafish larvae to this test substance produced 9 DEPs, while in the highest concentration of CBZep (100µg/L) there were 32 DEPs. Comparisons between CBZ and CBZep treatments and the control group in log2 fold change of the quantified proteins, as well as the similarity of the samples is given on Figure 2. The dendograms resulting from the hierarchical cluster analysis clearly separate the comparison CBZ01 vs CTR from the remaining ones. Looking to the heatmap this distinction is explained by the pattern of the log2 fold change values, which magnitude of response is more pronounced in this comparison. Although overexpression in comparison to control was observable for all treatments, for CBZ01 there were fewer proteins with values close to zero. The remaining comparisons were comprised in the same cluster. However, this cluster can be divided between CBZ100 and CBZep treatments against the control. This separation occurs due to an intermediate expression pattern of some blocks of proteins for CBZ100 vs CTR. The list of total significant proteins per treatment is available in the Appendix A5. (Table S1).

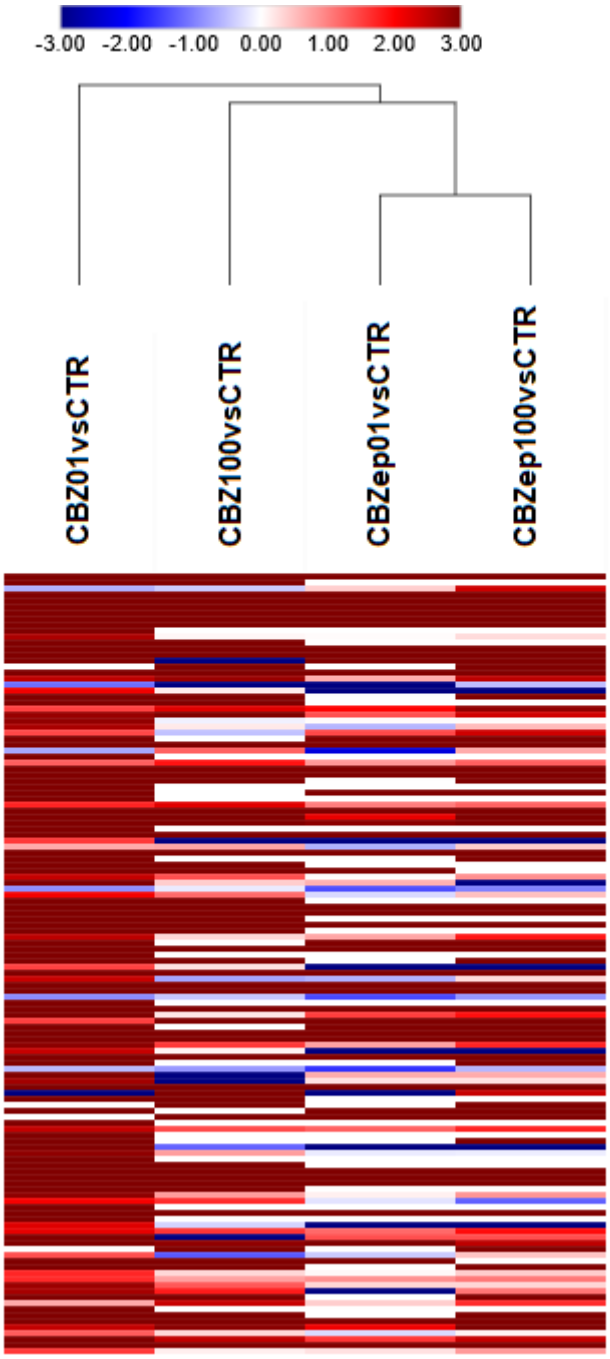

Figure 2 Log_2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

Functional analysis

Using DEPs, it is possible to perform a functional analysis on STRING-DB to evaluate possible impacts of the tested CBZ and CBZep concentrations on pathways with implications on different biological processes. For 0.1µg/L CBZ exposure, the obtained network had a significant PPI enrichment p-value (0.00274), allowing to observe relevant interactions between DEPs and therefore design observed networks. For this treatment

the DEPs were linked to 10 KEGG terms (Table 1), namely: glycolysis/gluconeogenesis; purine metabolism; glycine, serine and threonine metabolism; amino sugar and nucleotide sugar metabolism; biosynthesis of amino acids; valine, leucine and isoleucine degradation; fatty acids degradation; carbon metabolism, nucleocytoplasmic transport, and metabolic pathways. These KEGG terms had strength values between 0.61 (metabolic pathways) and 1.28 (glycine, serine and threonine metabolism). For 100µg/L CBZ exposure, the obtained network had a non-significant PPI enrichment p-value (0.793). Such values may indicate that either the number of DEPs is rather small, or that this is mostly a random group of proteins that are not greatly connected. It may also happen that these proteins were not sufficiently studied yet and thus their interactions may not be known to the software. Therefore, additional nodes were added to find possible connections with biological implications and obtain a significant PPI enrichment p-value (0.00766). For this treatment the significant proteins were linked to four different KEGG terms (Table 1), namely: oxidative phosphorylation; porphyrin metabolism; cardiac muscle contraction; and metabolic pathways. These KEGG terms had strength values varying between 0.62 (metabolic pathways) and 1.57 (porphyrin metabolism).

Regarding the active metabolite CBZep, exposure to 0.1µg/L resulted in a network which had a non-significant PPI enrichment p-value (1). Therefore, additional nodes were included to obtain a significant PPI enrichment p-value (0.0485). For this treatment the significant proteins were linked to seven different KEGG terms (Table 1), namely: glycolysis/gluconeogenesis; fructose and mannose metabolism; galactose metabolism; starch and sucrose metabolism; amino sugar and nucleotide sugar metabolism; carbon metabolism and metabolic pathways. These KEGG terms had strength values varying from 0.75 (metabolic pathways) to 2.32 (starch and sucrose metabolism). For 100µg/l CBZep, the network identified had a non-significant PPI enrichment p-value (0.6). Therefore, additional nodes were included to obtain a significant PPI enrichment p-value of 0.000286. For this treatment the significant proteins were linked to eight different KEGG terms (Table 1): alanine, aspartate, and glutamate metabolism; purine metabolism; oxidative phosphorylation; porphyrin metabolism; nucleocytoplasmic transport; cardiac muscle contraction; RNA polymerase and metabolic pathways. These KEGG terms had strength values varying from 0.69 for metabolic pathways to 1.68 for RNA polymerase. To summarize the affected KEGGs resulting from our analysis, a heatmap with all affected KEGGs per treatment, as well as the strength values was performed to better elucidate the most prominent changes as well as, describe the main KEGGSs altered by the compound (parental/metabolite) or the tested concentration (Table 1). A summary of the suggested impacted KEGGs per treatment, as well as the

count in the network, strength, false discovery rate values and involved proteins is given in the Appendix A5. (Table S2).

Table 1. Suggested KEGG terms, and respective strength values, based on DEP proteins of each CBZ and CBZep treatment when compared to the control group.

	CBZ		CBZep	
	CBZ01vsCtr	CBZ100vsCtr	CBZep01vsCtr	CBZep100vsCtr
dre00010 - Glycolysis / Gluconeogenesis	1.06		2.01	
dre00051 - Fructose and mannose metabolism			1.93	
dre00052 - Galactose metabolism			2.06	
dre00190 - Oxidative phosphorylation		1.47		1.42
dre00230 - Purine metabolism	1.1			1.21
dre00250 - Alanine, aspartate and glutamate metabolism				1.64
dre00260 - Glycine, serine and threonine metabolism	1.28			
dre00500 - Starch and sucrose metabolism			2.32	
dre00520 - Amino sugar and nucleotide sugar metabolism	1.15		2.2	
dre00860 - Porphyrin metabolism		1.57		1.52
dre01100 - Metabolic pathways	0.61	0.62	0.75	0.69
dre01200 - Carbon metabolism	1.12		1.65	
dre01230 - Biosynthesis of amino acids	1.23			
dre00280 - Valine, leucine and isoleucine degradation	1.19			
dre00071 - Fatty acid degradation	1.2			
dre03013 - Nucleocytoplasmatic transport	0.84			1.2
dre03020 - RNA polymerase				1.68
dre04260 - Cardiac muscle contraction		1.49		1.29

From the table it is possible to observe the range of strength values (between 0.61 and 2.32) and the diversity of the 18 suggested KEGGS and their number per treatment. Only one KEGG was common to all treatments: metabolic pathways (with strength values lower than 1). For the remaining suggested KEGGs, there are no pathways affected by more than two treatments. Furthermore, as mentioned previously, there are no KEGGs common to both concentrations of the same compound. However, it is possible to directly compare the same concentration for the two tested compounds. Glycolysis/gluconeogenesis; carbon metabolism; and amino sugar and nucleotide sugar metabolism were affected by the lowest concentration of both compounds. It can be observed that the overall strength values were higher for CBZep than for CBZ by about 23 to 91%. In fact, among all CBZep0.1 was the treatment exhibiting the highest strength

values. On the other hand, oxidative phosphorylation; cardiac muscle contraction; and porphyrin metabolism were affected by the highest concentration of both compounds, comparing to the control. However, in this case, strength values were fairly similar between the parental compound and the metabolite. Purine metabolism and nucleocytoplasmatic transport were the only KEGGs suggested by the two tested compounds but at different concentrations. The remaining nine KEGG terms were suggested only for one of the treatments.

To give a better perspective of the arrangement of the obtained networks, a k-means cluster analysis was performed to identify suitable protein clusters associated to the above mentioned KEGG terms. For the comparison between CBZ0.1 and the control, three different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 3). Cluster 1 (red) comprises 29 different proteins. This group of significant proteins was the one with most different KEGG terms described before, in a total of eight: glycolysis/gluconeogenesis; purine metabolism; glycine, serine and threonine metabolism; metabolic pathways; carbon metabolism; biosynthesis of amino acids; valine, leucine and isoleucine degradation; fatty acid degradation; and nucleocytoplasmic transport. Cluster 2 (green) grouped 38 different proteins and was the biggest cluster. It was associated to just one KEGG: amino sugar and nucleotide sugar metabolism. Cluster 3 (blue) was by residual margin the smallest cluster, grouping 27 different proteins. This group was associated to the remaining KEGG: nucleocytoplasmic transport. For the comparison between CBZ100 and the control, a single cluster was found, comprising the four KEGG terms previously described to be affected within this treatment (Figure 4): oxidative phosphorylation; porphyrin metabolism; cardiac muscle contraction; and metabolic pathways. This is indicative that the affected pathways are related with the totality of the network and not with parts of it. A similar result was obtained for the comparison between CBZep0.1 and the control, for which a single cluster was found, comprising the seven KEGG terms previously described to be affected within this treatment (Figure 5): glycolysis/gluconeogenesis; fructose and mannose metabolism; galactose metabolism; starch and sucrose metabolism; amino sugar and nucleotide sugar metabolism; carbon metabolism and metabolic pathways. Finally, for the comparison between CBZep100 and the control, two different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 6). Cluster 1 (red) comprises 35 different proteins and is the biggest of the two clusters. This group of significant proteins was associated with four different KEGG terms: oxidative phosphorylation, cardiac muscle contraction, porphyrin metabolism and metabolic pathways. Cluster 2 (green) grouped 17 different proteins and was the smallest cluster found. This group was associated to the four remaining KEGGs: purine metabolism,

alanine, aspartate and glutamate metabolism, nucleocytoplasmic transport, and RNA polymerase.

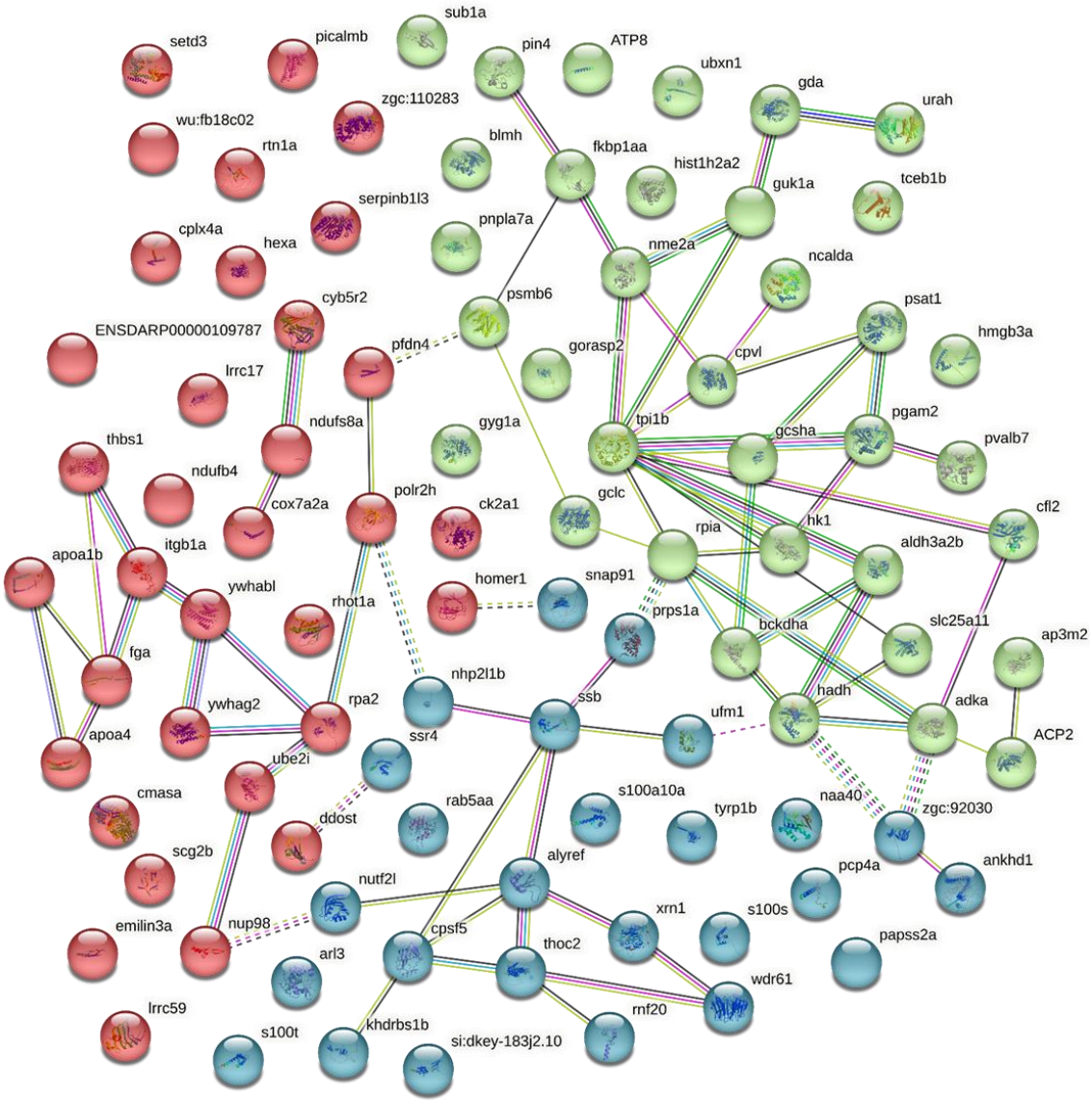

Figure 3. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L carbamazepine (CBZ0.1). The red cluster was associated with KEGG terms glycolysis/gluconeogenesis; purine metabolism; glycine, serine and threonine metabolism; metabolic pathways; carbon metabolism; biosynthesis of amino acids; valine, leucine and isoleucine degradation; fatty acid degradation; and nucleocytoplasmic transport. The green cluster was associated to KEGG amino sugar and nucleotide sugar metabolism. The blue cluster was associated to KEGG nucleocytoplasmic transport.

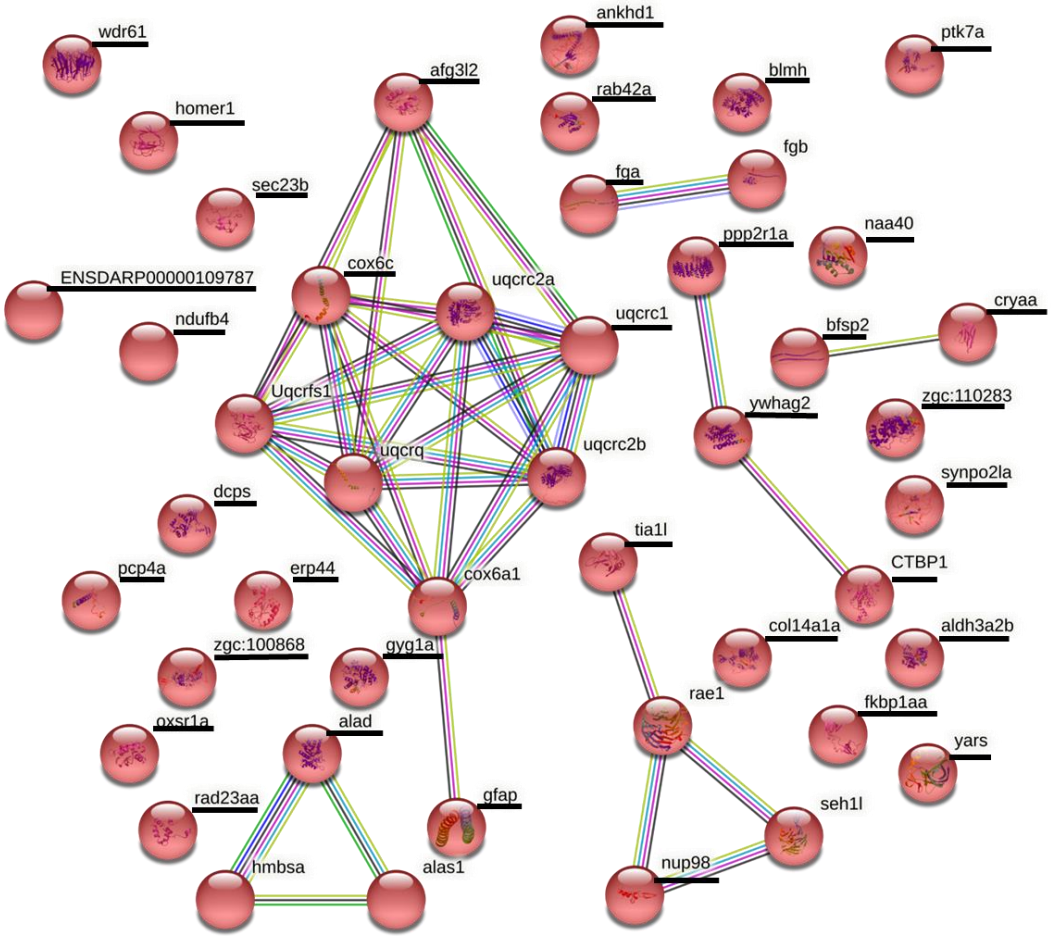

Figure 4. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L carbamazepine (CBZ100). This cluster was associated to oxidative phosphorylation; porphyrin metabolism; cardiac muscle contraction; and metabolic pathways.

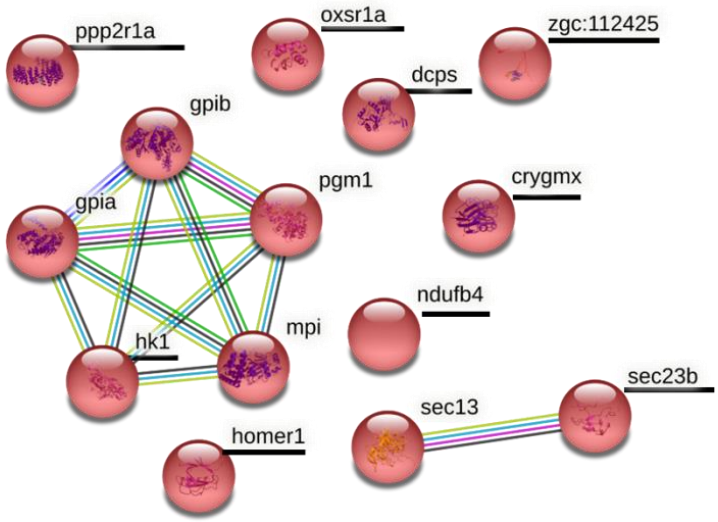

Figure 5. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L carbamazepine 10,11-epoxide (CBZep01). This cluster was associated to glycolysis/gluconeogenesis; fructose and mannose metabolism; galactose metabolism; starch and sucrose metabolism; amino sugar and nucleotide sugar metabolism; carbon metabolism and metabolic pathways.

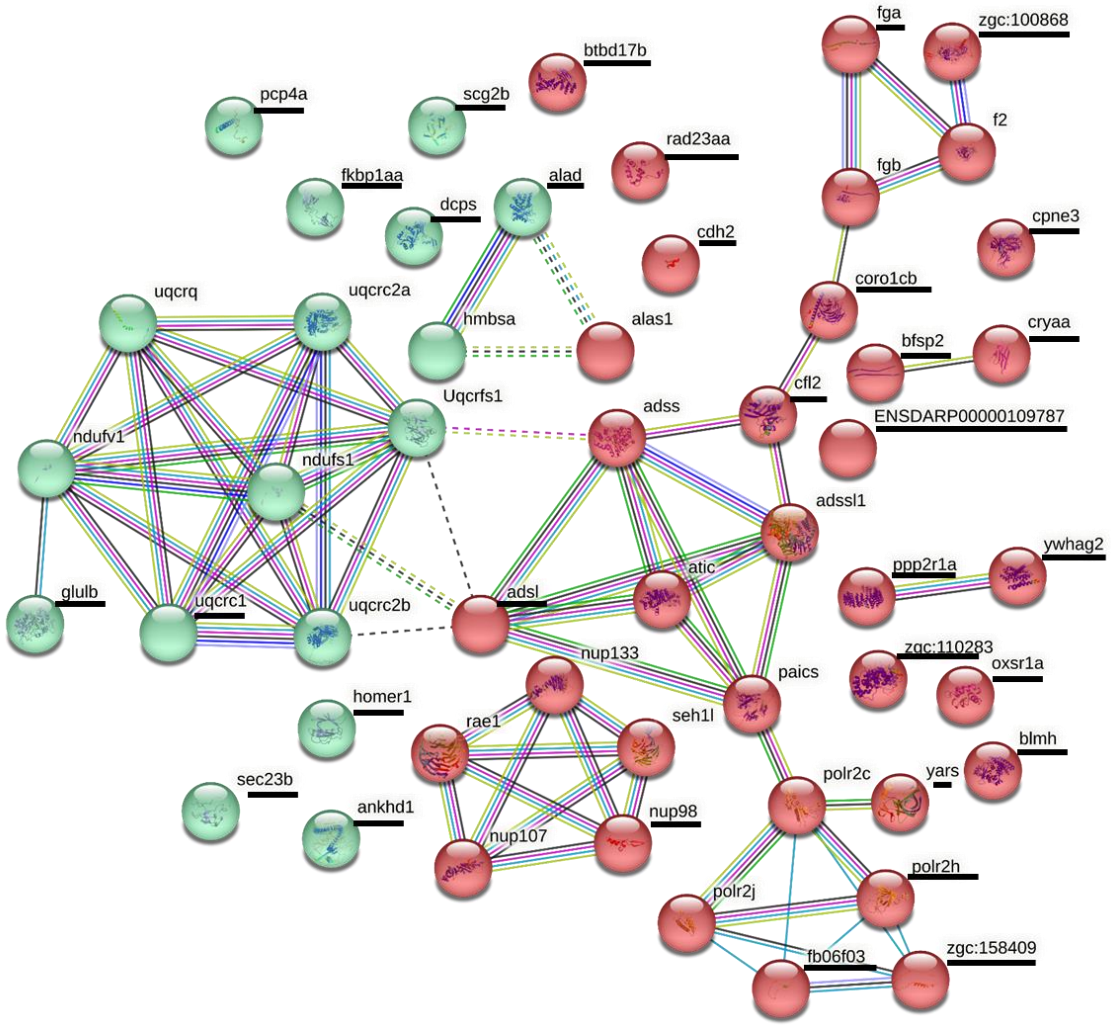

Figure 6. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L carbamazepine 10,11-epoxide (CBZep100). The red cluster was associated with KEGG terms oxidative phosphorylation, cardiac muscle contraction, porphyrin metabolism and metabolic pathways. The green cluster was associated to the four remaining KEGGs: purine metabolism, alanine, aspartate and glutamate metabolism, nucleocytoplasmic transport, and RNA polymerase.

5.3.2. Venlafaxine and its main metabolite

Protein quantification

Shotgun proteomic analysis was performed for protein quantification in three different samples for each treatment. From the analysis, it was possible to quantify 2048 different proteins. From the total proteins evaluated,1538 proteins were quantified for control group (75.1% of the total quantified proteins); 1359 for VEN0.1 (66.4% of the total quantified proteins); 1678 for VEN100 (81.9% of the total quantified proteins); 1478 for ODV0.1 (72.6% of the total quantified proteins) and 1478 for ODV100 (72.6% of the total

quantified proteins). Distribution of the quantified proteins by treatment is shown in Figure 7.

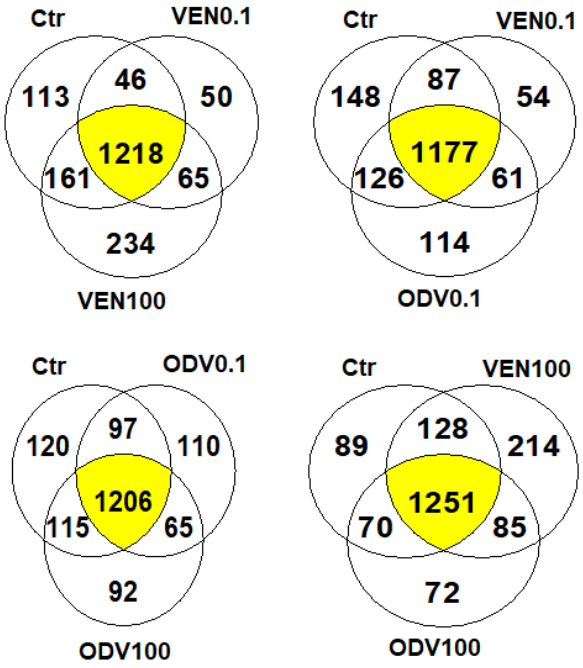

Figure 7. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; VEN01: venlafaxine 0.1µg /L; VEN100: venlafaxine 100µg /L; ODV01: o-desmethylvenlafaxine 0.1µg /L, ODV100: o-desmethylvenlafaxine 100µg/L

The Venn diagrams on the left compare common and exclusive proteins of each compound and the control group. The Venn diagrams on the right side compare the test concentrations, i.e., proteins differentially expressed between the control, VEN and its metabolite for similar exposure concentrations. In all cases, most quantified proteins were common to all the test conditions. At least 1177 common proteins were detected, which represent approximately 56% of the total quantified proteins. The number of proteins exclusive to just one treatment was about 20% or less than the proteins shared among the three groups.

Identification of differentially expressed proteins

From our results, it was possible to identify a total of 42 Differentially Expressed Proteins (DEPs) for all treatments. Different patterns of expression were observed for this set of proteins:

- 28 proteins were under-expressed while 14 were over-expressed for VEN01
- 18 proteins were under-expressed, while 24 were over-expressed for VEN100.

- 17 proteins were under-expressed while 25 were over-expressed for ODV01

- 27 proteins were under-expressed, while 15 were over-expressed for ODV100.

Larvae from the 0.1µg/L concentration of the parental compound (VEN) showed 13 DEPs; at the 100µg/L of VEN there were 5 DEPs. For the 0.1µg/L concentration of the active metabolite (ODV) zebrafish larvae showed 26 DEPs, while for the highest concentration of ODV (100µg/L) 23 DEPs were detected. Comparisons between VEN and ODV treatments and the control group in log2 fold change of the quantified proteins, as well as the similarity of the samples, is given in Figure 8.

The dendrograms resulting from the hierarchical cluster analysis clearly separate the VEN100vsCTR comparison from the other ones. Looking to the heatmap this distinction is explained by the pattern of log2 fold change values. These values are opposite from the other comparisons in a section where overexpression occurs, contrary to a predominance of underexpression for the other treatments against the control. Furthermore, the magnitude of response is less pronounced in this comparison. The remaining comparisons are linked to each other in the cluster. However, this cluster can be divided between VEN01 and ODV treatments against the control. These subclusters are somehow separated because of the pattern of response. VEN01vsCTR has a prominent underexpression pattern, without a characteristic block of proteins. ODV treatments were grouped together, although they have a distinct general pattern of expression. For ODV100 underexpression was prominent, while for ODV01 overexpression was the main obtained response. However, these two comparisons share a similarity of response for certain blocks of proteins. The list with the discrimination of the total significant proteins per treatment is available in Appendix A5. (Table S3).

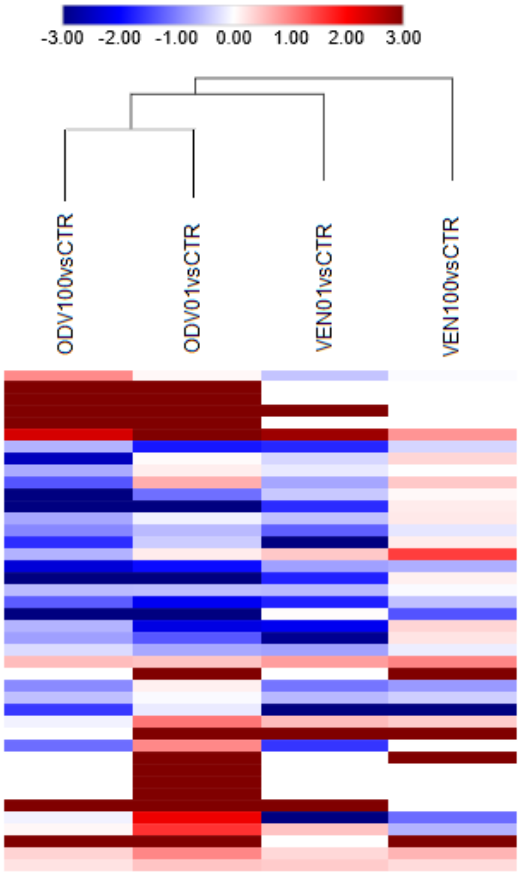

Figure 8. Log_2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

Functional analysis

Using DEPs, it is possible to perform a functional analysis on STRING-DB to evaluate possible impacts of the tested VEN and ODV concentrations on pathways with implications on different biological processes. For 0.1µg/L VEN exposure, the obtained network had a non-significant PPI enrichment p-value (0.24). Such values may indicate that either the number of DEPs is rather small, or that it is mostly a random group of proteins that are not strongly connected. It may also happen that these proteins were not sufficiently studied yet and thus their interactions may not be known to the software. Therefore, additional nodes were added to find possible connections with biological implications and obtain a significant PPI enrichment p-value (0.00415). For this treatment the significant proteins were linked to five different KEGG terms (Table 2): arginine biosynthesis, oxidative phosphorylation, carbon metabolism, nucleocytoplasmic transport, and metabolic pathways. These KEGG terms had strength values varying between 0.84 (metabolic pathways) and 1.9 (arginine biosynthesis). For 100µg/L of VEN

exposure, due to the low number of DEPs, it was not possible to obtain any significant pathway, even with additional nodes added to the initial list. Regarding the active metabolite ODV, exposure to 0.1µg/L resulted in a network which had a non-significant PPI enrichment p-value (0.711). Therefore, additional nodes were added to obtain a significant PPI enrichment p-value (0.0438). For this treatment the significant proteins were linked to three different KEGG terms (Table 2): oxidative phosphorylation, nucleocytoplasmic transport and metabolic pathways. These KEGG terms had strength values varying from 0.63 (metabolic pathways) to 1.5 (oxidative phosphorylation). For 100µg/L exposure, the obtained network had a non-significant PPI enrichment p-value (0.292). Therefore, additional nodes were added to obtain a significant PPI enrichment p-value ($1.53e^{-05}$). For this treatment the significant proteins were linked to five different KEGG terms (Table 2): arginine biosynthesis; alanine, aspartate and glutamate metabolism; carbon metabolism; oxidative phosphorylation and ribosome. These KEGG terms had strength values varying from 1.28 (carbon metabolism) to 1.74 (arginine biosynthesis). A summary of the suggested impacted KEGGs per treatment, as well as the count in the network, strength, false discovery rate values and involved proteins is given in the Appendix A5. (Table S4).

To summarize the affected KEGGs resulting from our analysis, a heatmap with all suggested KEGGs per treatment, as well as the strength values, was performed to better elucidate the most prominent changes, as well as describe the main KEGGs altered by the compound (parental/metabolite) or the tested concentration (Table 2)

Table 2. Suggested KEGG terms, and respective strength values, based on DEP proteins of each VEN and ODV treatment when compared to the control group.

	VEN		ODV	
	VEN01 vsCtr	VEN100 vs Ctr	ODV01 vs CTtr	ODV100 vs Ctr
dre00220 - Arginine biosynthesis	1.9			1.74
dre00190- Oxidative phosphorylation	1.71		1.5	1.38
dre01200 - Carbon metabolism	1.56			1.28
dre03013 - Nucleocytoplasmic transport	1.33		1.25	
dre01100 - Metabolic pathways	0.84		0.63	
dre03010 – Ribosome				1.7

dre00250 - Alanine, aspartate and glutamate metabolism				1.53

From the table it is possible to observe the range of strength values (between 0.63 and 1.9) and the diversity of the 7 suggested KEGGs and their number per treatment. The oxidative phosphorylation pathway was the KEGG term suggested by most of the treatments (except for VEN100). The strength values were mostly above 1 for all terms, except for “Metabolic pathways”. The strength values of the shared KEGG terms were higher in 0.1µ/l VEN than in the other treatments. Oxidative phosphorylation was also the only KEGG term to be suggested for the two ODV treatments. The RNA transport related term and metabolic pathways were suggested for the lowest concentrations of both compounds. The strength values between treatments for these biological processes were higher for the parental compound than the metabolite. However, these differences were mostly mild with values of ~6% for RNA transport and 25% for the metabolic pathways. For the KEGGS related to arginine biosynthesis and carbon metabolism, the parental compound had considerably higher strength values. In fact, the 1.9 value for arginine biosynthesis was the highest strength value found in the analysis. Ribosome and the alanine, aspartate and glutamate metabolism pathways were only impacted at 100µ/l ODV, with a relatively high strength value.

To give a better perspective of the arrangement of the obtained networks, a k-means cluster analysis was performed to identify suitable protein clusters associated to the above mentioned KEGG terms. For the comparison between VEN01 and the control, two different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 9). Cluster 1 (red) comprises 12 different proteins. This group of significant proteins was associated with three different KEGGs: carbon metabolism, arginine biosynthesis and oxidative phosphorylation. Cluster 2 (green) grouped 11 different proteins. It was associated to two different KEGGs: nucleocytoplasmic transport and metabolic pathways. For the comparison between ODV01 and the control, two different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 10). Cluster 1 (red) comprises 21 different proteins and is the biggest of the two clusters. It was associated to just one KEGG: nucleocytoplasmic transport. Cluster 2 (green), the smallest one, comprised 16 different proteins. It was associated to two different KEGGs: oxidative phosphorylation and metabolic pathways. For the comparison between ODV100 and the control, also two clusters were selected, each one related with proteins associated to different KEGGs (Figure 11). Cluster 1 (red) was the smallest cluster, comprising 13 different proteins. It was associated with three different KEGGs: carbon metabolism; arginine biosynthesis;

and alanine, aspartate and glutamate metabolism. Cluster 2 (green), the biggest of the two, grouped 20 different proteins. It was associated to the two remaining KEGGs: ribosome and oxidative phosphorylation.

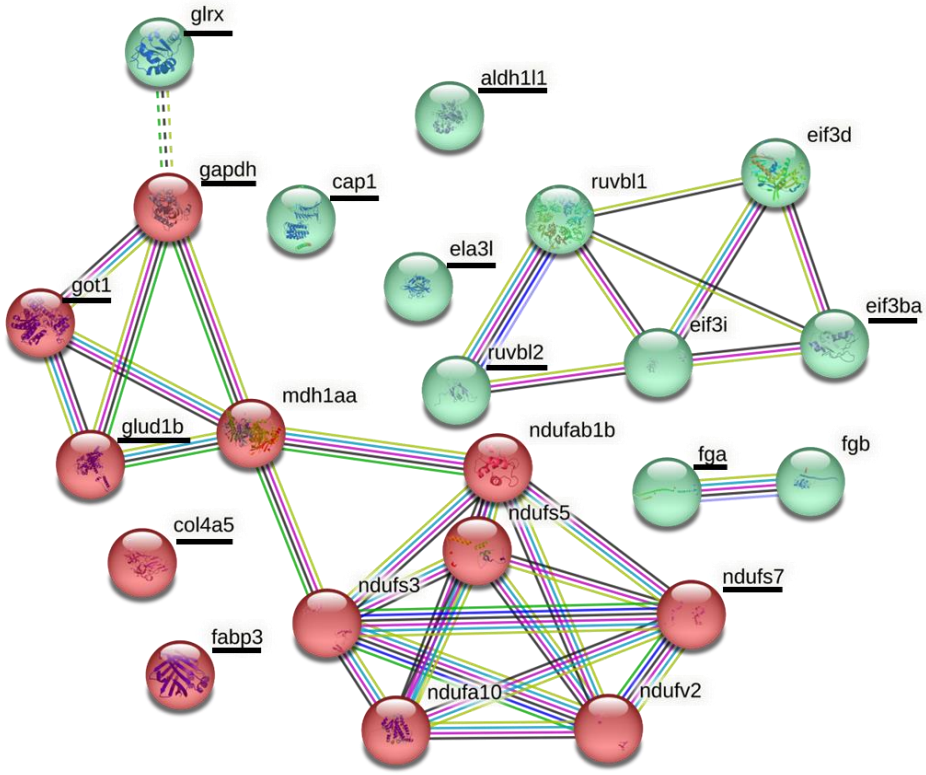

Figure 9. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L venlafaxine (VEN01). The red cluster was associated with KEGG terms carbon metabolism, arginine biosynthesis and oxidative phosphorylation. The green cluster was associated to the two remaining KEGGs: nucleocytoplasmic transport and metabolic pathways.

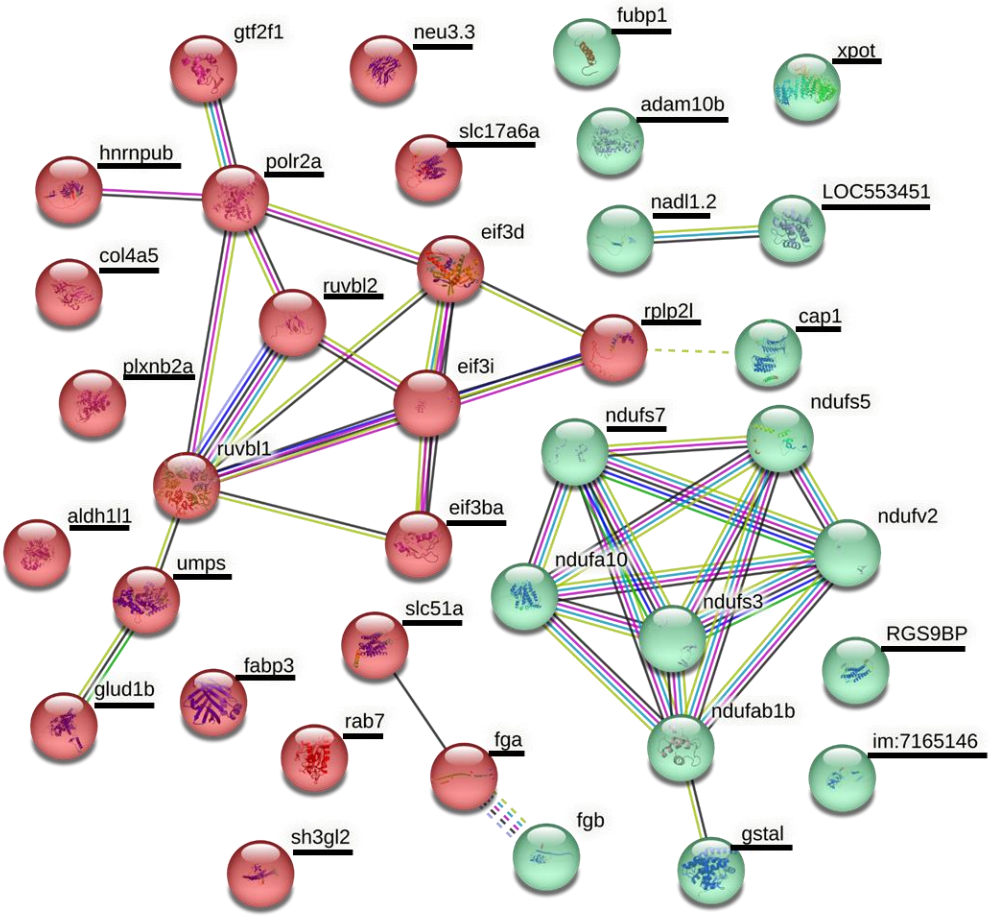

Figure 10. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L o-desmethylvenlafaxine (ODV01). The red cluster was associated with KEGG term: nucleocytoplasmic transport. The green cluster was associated with the two remaining KEGGs: oxidative phosphorylation and metabolic pathways.

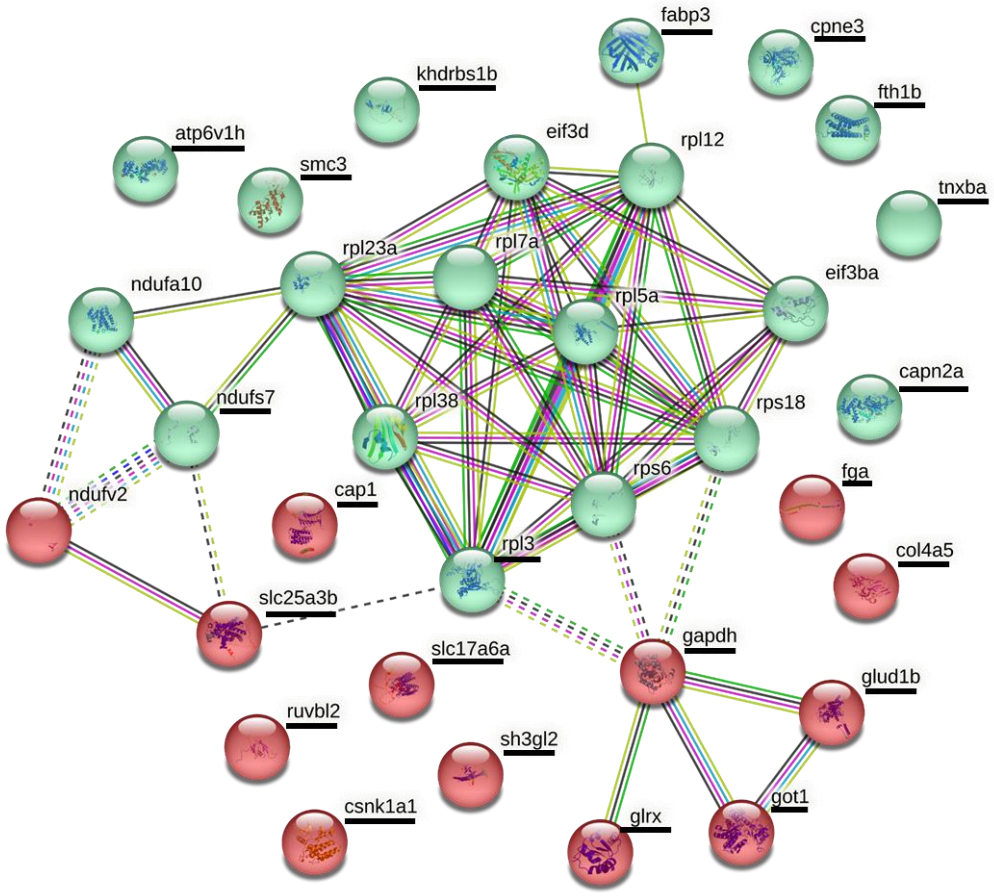

Figure 11. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L o-desmethylvenlafaxine (ODV100). The red cluster was associated with KEGG terms: carbon metabolism, arginine biosynthesis, as well as alanine, aspartate and glutamate metabolism. The green cluster was associated with the remaining two KEGGs: ribosome and oxidative phosphorylation.

5.3.3 Tramadol and its main metabolite

Protein quantification

Shotgun proteomic analysis was performed for protein quantification in three different samples for each treatment. From the analysis it was possible to quantify 1935 different proteins. From the total proteins assessed, 1561 proteins were quantified for the control group (80.7% of the total quantified proteins); 1259 for TRA01 (65.1% of the total quantified proteins); 1443 for TRA100 (74.6% of the total quantified proteins); 1446 for OTRA01 (74.7% of the total quantified proteins) and 1479 for OTRA100 (76.4% of the total quantified proteins). Distribution of the quantified proteins by treatment is shown in Figure 12.

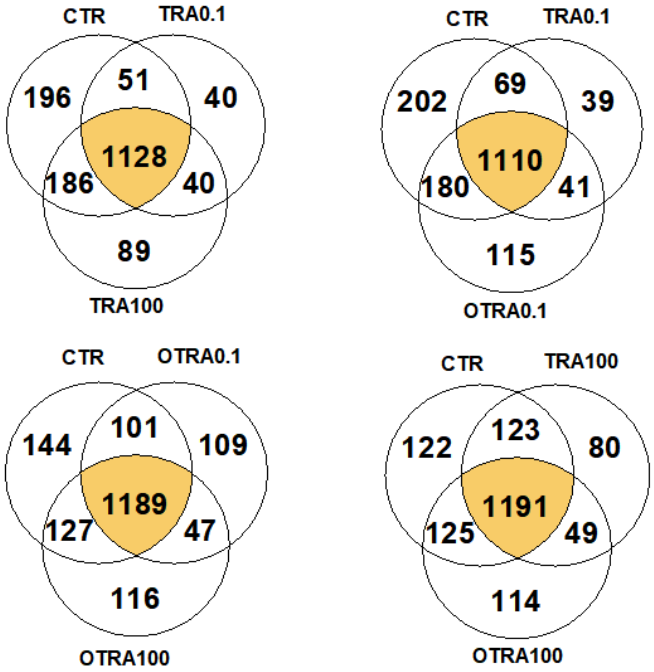

Figure 12. Venn diagrams representing the total number of shared and exclusive proteins per treatment. CTR: control; TRA01: tramadol 0.1µg /L; TRA100: tramadol 100µg /L; OTRA01: o-desmethyltramadol 0.1µg /L, OTRA100: o-desmethyltramadol 100µg/L

The Venn diagrams on the left compare common and exclusive proteins of each compound and control group. The Venn diagrams on the right side compare the test concentrations, i.e., proteins differentially expressed between the control, TRA and its metabolite for similar exposure concentrations. At least 1110 common proteins were detected, which represents approximately 57% of the total quantified proteins. The number of proteins exclusive to just one treatment was about 20% or less than the proteins shared among the three groups.

Identification of differentially expressed proteins

From the obtained results, it was possible to identify a total of 163 Differentially Expressed Proteins (DEPs), for all treatments. Different patterns of expression were observed for this set of proteins:

- 141 proteins were under-expressed while 22 were over-expressed for TRA01
- 133 proteins were under-expressed, while 30 were over-expressed for TRA100.
- 126 proteins were under-expressed, while 37 were over-expressed for OTRA01
- 143 proteins were under-expressed, while 20 were over-expressed For OTRA100

Larvae from the 0.1µg/L concentration of the parental compound (TRA) had 100 DEPs; at the 100µg/L of TRA there were 62 DEPs. Regarding the 0.1µg/L concentration of the active metabolite (OTRA), exposure of zebrafish larvae to this compound produced 83 DEPs, while for the highest concentration of OTRA (100µg/L) there were 89 DEPs. Comparisons between TRA and OTRA treatments and the control group in log2 fold change of the quantified proteins, as well as the similarity of the samples is given on Figure 13. The dendrograms resulting from the hierarchical cluster analysis clearly separate the TRA100vsCTR comparison from the remaining ones. Looking to the heatmap this distinction is explained by the pattern exhibited by the log2 fold change values, which magnitude of response is less pronounced in this comparison. Although underexpression in comparison to the control was observable for all treatments, for TRA100 there were more proteins with values close to zero. The remaining comparisons were comprised in the same cluster. However, this cluster can be divided between OTRA100 and the lowest concentration of both compounds against the control. This separation occurs due to an opposite pattern of expression of the upper part of the heatmap where, OTRA100vsCTR presents a predominance of underexpression while the other treatments have a predominance of overexpression. The list with the discrimination of the total significant proteins per treatment is available in Appendix A5. (Table S5).

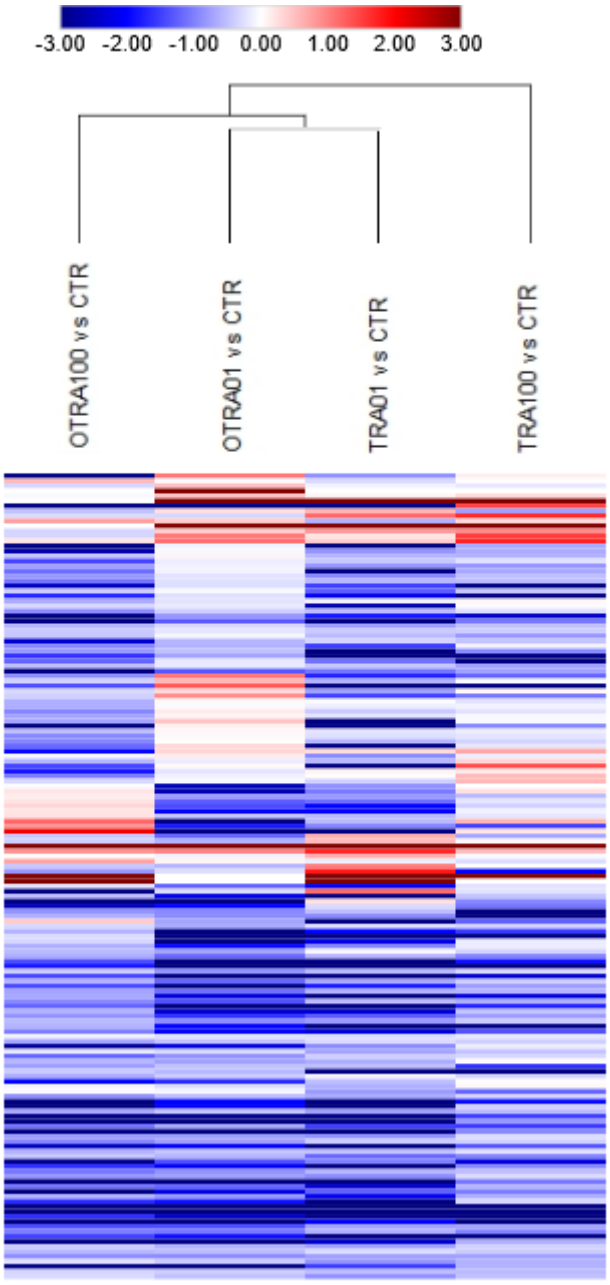

Figure 13 Log_2 fold-change heatmap and hierarchical cluster analysis from differentially expressed proteins (DEPs) for the comparison of each treatment to the control (Ctr).

Functional analysis

Using DEPs, it is possible to perform a functional analysis on STRING-DB to evaluate possible impacts of the tested TRA and OTRA concentrations on different pathways with implications on different biological processes. For 0.1µ/L TRA exposure, the obtained network had a PPI enrichment p-value (2.00e-15), allowing to observe relevant interactions between DEPs and therefore design networks. For this treatment the DEPs were linked to 8 different KEGG terms: citrate cycle (TCA cycle), starch and sucrose metabolism, pyruvate metabolism, glycolysis / gluconeogenesis, carbon metabolism,

metabolic pathways, arginine and proline metabolism, protein processing in endoplasmic reticulum. These KEGG terms had strength values varying from 0.65 (metabolic pathways) to 1.4 (TCA cycle). For 100µ/L TRA, the network had a significant PPI enrichment p-value (1.45E-06). For this treatment the DEPs were linked to 2 different KEGG terms: carbon metabolism and metabolic pathways. These KEEGs had strength values of 1.24 and 0.72 respectively. Regarding the active metabolite OTRA, exposure to 0.1µ/L of this compound resulted in a network which had a significant PPI enrichment p-value (1.36e-08). For this treatment the DEPs were linked to 4 different KEGGs: glycolysis / gluconeogenesis, metabolic pathways, arginine and proline metabolism and oxidative phosphorylation. These KEGG terms had strength values varying from 0.76 (metabolic pathways) to 1.31 (arginine and proline metabolism).

For OTRA 100µg/l exposure, the obtained network had a significant PPI enrichment p-value (3.22e-11). For this treatment the DEPs were linked to 10 different KEGGs: pentose phosphate pathway, arginine and proline metabolism, glycolysis / gluconeogenesis, fructose and mannose metabolism, oxidative phosphorylation, starch and sucrose metabolism, metabolic pathways, carbon metabolism, biosynthesis of amino acids and protein processing in the endoplasmic reticulum. These KEGG terms had strength values varying from 0.65 (metabolic pathways) to 1.45 (pentose phosphate pathway). The KEGGs terms per treatment as well as, the count in network, strength, false discovery rate values and involved proteins are summarized in the Appendix A5. (Table S6). Overall, the KEGGs affected per compound (parental/metabolite) and test concentration are presented in a heatmap (Table 3); the strength values are also shown, to better elucidate the most prominent changes.

Table 3. Suggested KEGG terms, and respective strength values, based on DEP proteins of each TRA and OTRA treatment when compared to the control group.

	TRA		OTRA	
	TRA01vsCtr	TRA100vsCtr	OTRA01vsCtr	OTRA100vsCtr
dre00030 - Pentose phosphate pathway				1.45
dre00330 - Arginine and proline metabolism	1.1		1.31	1.28
dre00010 - Glycolysis / Gluconeogenesis	1.26		1.24	1.31
dre00020 - Citrate cycle (TCA cycle)	1.4			
dre00051 - Fructose and mannose metabolism				1.44
dre00190 - Oxidative phosphorylation			1.07	1.05
dre00500 - Starch and sucrose metabolism	1.35			1.4

dre00620 - Pyruvate metabolism	1.29			
dre01100 - Metabolic pathways	0.65	0.72	0.76	0.65
dre01200 - Carbon metabolism	1.02	1.24		1.07
dre01230 - Biosynthesis of amino acids				1.16
dre04141 - Protein processing in endoplasmic reticulum	0.84			0.9

Metabolic pathways were the only KEGG suggested to be affected by all treatments. This KEGG was also the one which strength values were lower. Arginine and proline metabolism, and glycolysis/gluconeogenesis were impacted by all treatments, apart from TRA100. These pathways, however, had similar strength values for each treatment. For carbon metabolism only the OTRA01 treatment was not suggested to impact this KEGG. However, strength values show that the TRA100 treatment showed a strength value 21% and 16% higher than TRA01 and OTRA100, respectively. Evaluating the KEGG terms by compound, for TRA, eight KEGG terms were suggested at least for one treatment. Pyruvate metabolism was the only one to be exclusively impacted by TRA exposure. On the other hand, for OTRA, 10 different KEGG terms were suggested at least for one of the treatments. Effects on the pentose phosphate pathway, fructose and mannose metabolism, oxidative phosphorylation, and biosynthesis of amino acids were suggested to be affected by this active metabolite. Regarding concentration, TRA01 and OTRA100 were the ones with the more suggested KEGGs. Evaluating strength values for concentration, it is important to notice that strength values from KEGGs suggested in the two concentrations of the same compound are remarkably similar. The highest difference was found for carbon metabolism for TRA (21%).

To give a better perspective of the arrangement of the obtained networks, a k-means cluster analysis was performed to identify suitable protein clusters associated to the above mentioned KEGG terms. For the comparison between TRA01 and the control, two different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 14). Cluster 1 (red) comprises 62 different proteins and was the biggest selected cluster. This group of significant proteins was associated with five KEGG terms described before: glycolysis/gluconeogenesis; TCA cycle; starch and sucrose metabolism; pyruvate metabolism and carbon metabolism. Cluster 2 (green), the smallest of the two clusters, grouped 37 different proteins. This group of significant proteins was associated with the remaining three KEGG: arginine and proline metabolism; metabolic pathways and protein processing in endoplasmic reticulum. For the comparison between TRA100 and the control, a single cluster was found, comprising the two KEGG terms previously described to be affected within this treatment (Figure 15): metabolic pathways and carbon metabolism. This is indicative that the affected

pathways are related with the totality of the network and not with parts of it. For the comparison between OTRA01 and the control, two different clusters were found, each one related with proteins associated to different KEGG terms (Figure 16). Cluster 1 (red) was, by a small margin, the biggest cluster found, comprising 34 different proteins. This group of proteins was associated with two KEGGs: oxidative phosphorylation and metabolic pathways. Cluster two (green) comprised 31 different proteins. This group of proteins was linked to two KEGG: arginine and proline metabolism and metabolic pathways. It is important to notice that metabolic pathways are suggested to be associated with both cluster 1 and 2. Cluster 3 (blue), the smallest of the three, grouped 18 different proteins. This group of proteins was associated with the remaining KEGG: glycolysis/gluconeogenesis. Finally, for the comparison between OTRA100 and the control, two different clusters were selected, each one related with proteins associated with different KEGG terms (Figure 17). Cluster 1 (red) comprises 46 different proteins and is the biggest of the two clusters. This group of significant proteins was associated with nine of the ten suggested KEGG terms for this condition: pentose phosphate pathway; fructose and mannose metabolism; starch and sucrose metabolism; arginine and proline metabolism; glycolysis/gluconeogenesis; biosynthesis of amino acids; oxidative phosphorylation; carbon metabolism; metabolic pathways. Cluster 2 (green) grouped 42 different proteins and was the smallest cluster found. This group was associated to the remaining suggested KEGG: protein processing in endoplasmic reticulum.

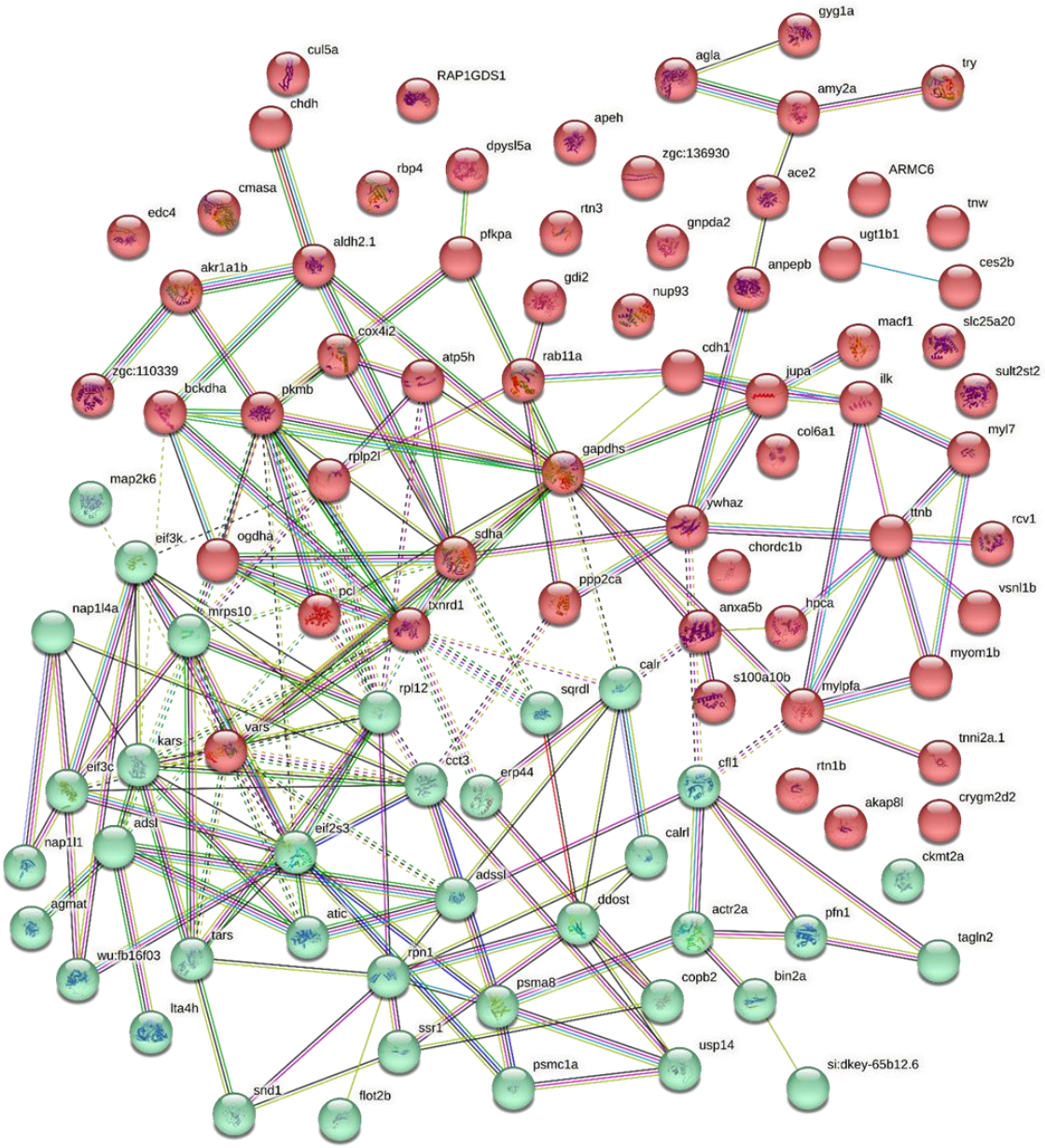

Figure 14. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L tramadol (TRA01). The red cluster was associated with KEGG terms: glycolysis/gluconeogenesis, TCA cycle, starch and sucrose metabolism, pyruvate metabolism, and carbon metabolism. The green cluster was associated to the remaining three KEGG terms: arginine and proline metabolism, metabolic pathways, and protein processing in endoplasmic reticulum.

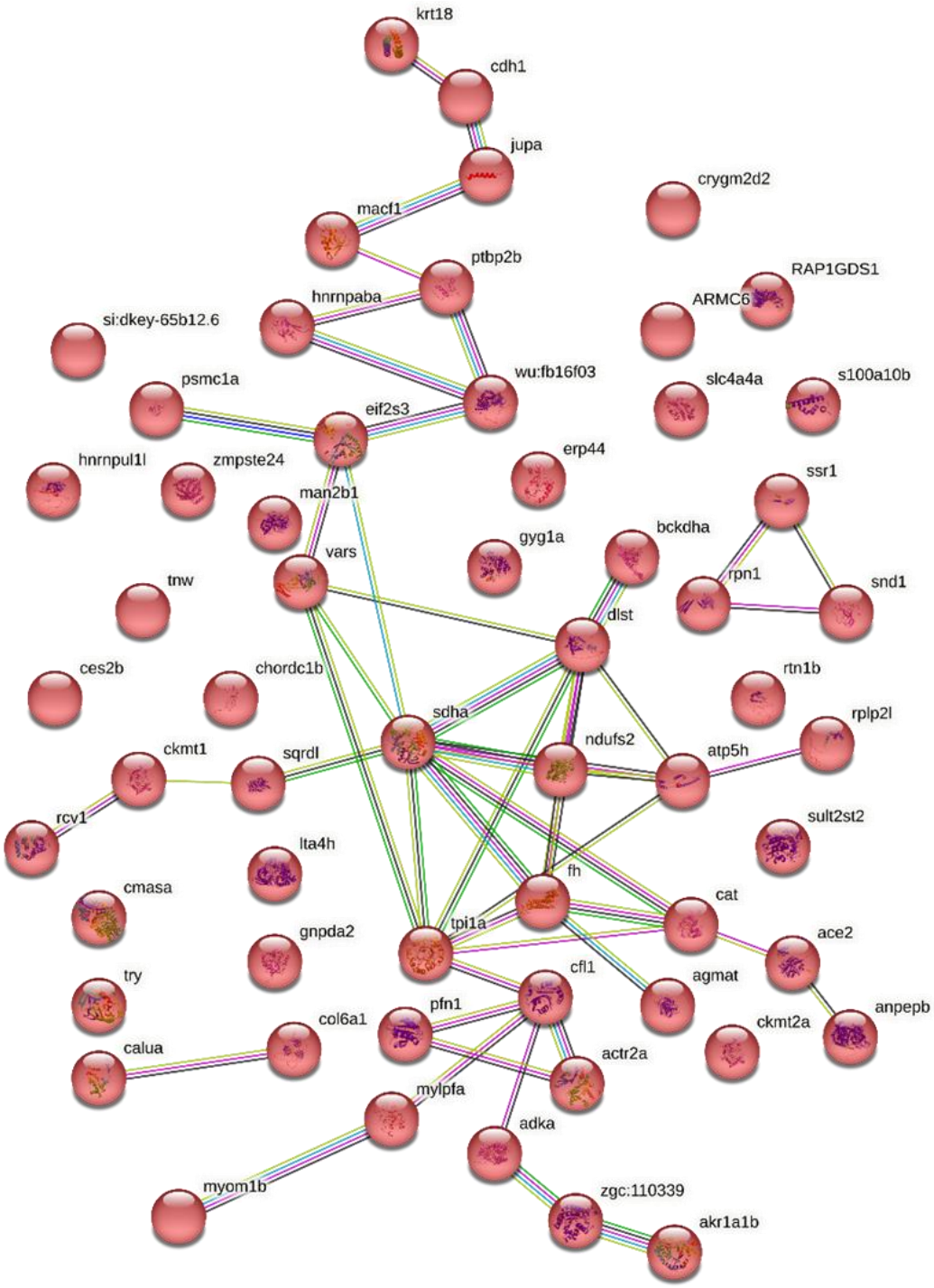

Figure 15. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L tramadol (TRA100). The red cluster was associated with KEGG terms: carbon metabolism and metabolic pathways.

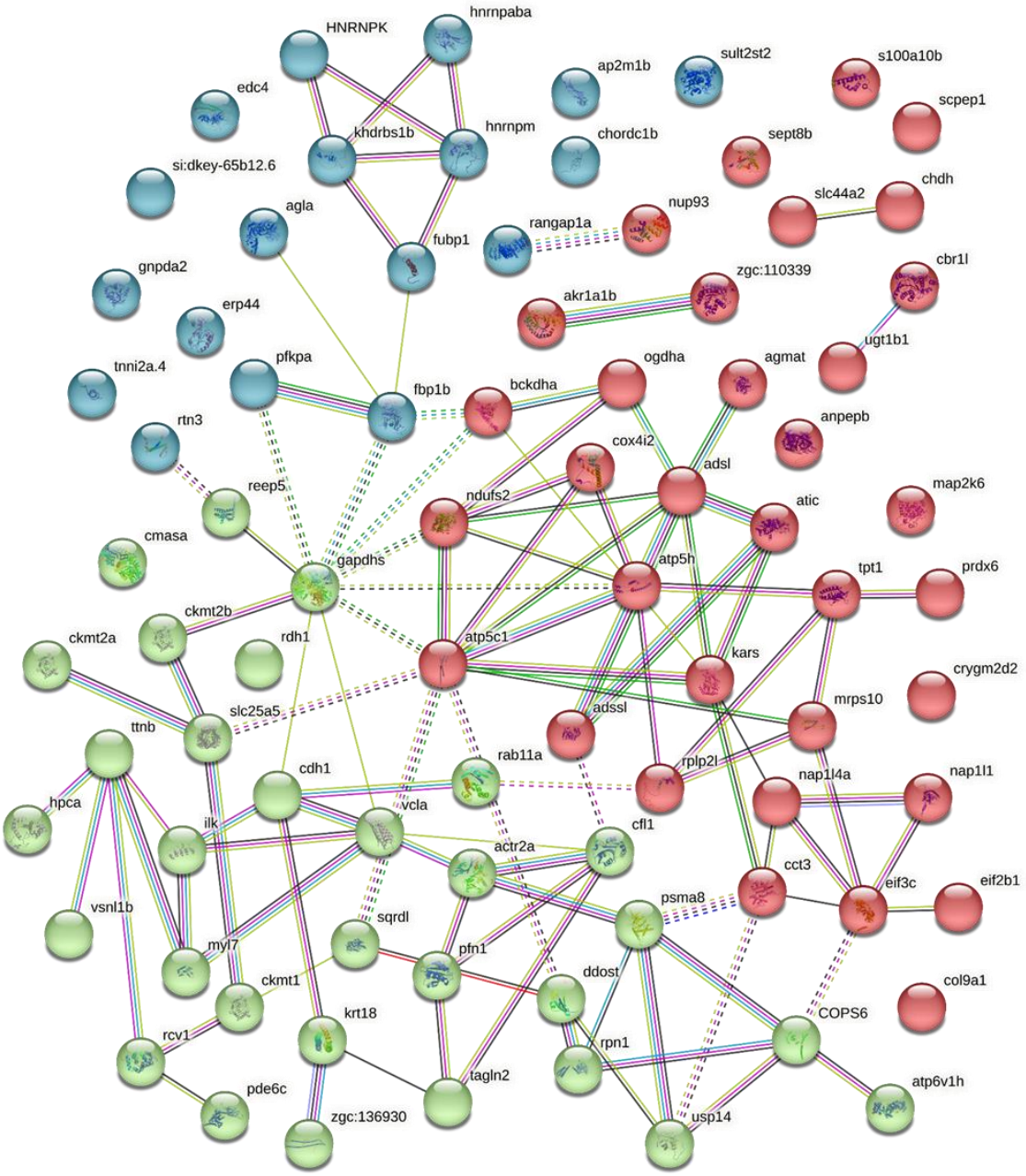

Figure 16. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 0.1µg/L o-desmethyltramadol (OTRA01). The red cluster was associated with KEGG terms: oxidative phosphorylation and metabolic pathways. The green cluster was associated to two KEGG terms: arginine and proline metabolism and metabolic pathways. The blue cluster 3 was associated to the remaining KEGG term: glycolysis/gluconeogenesis.

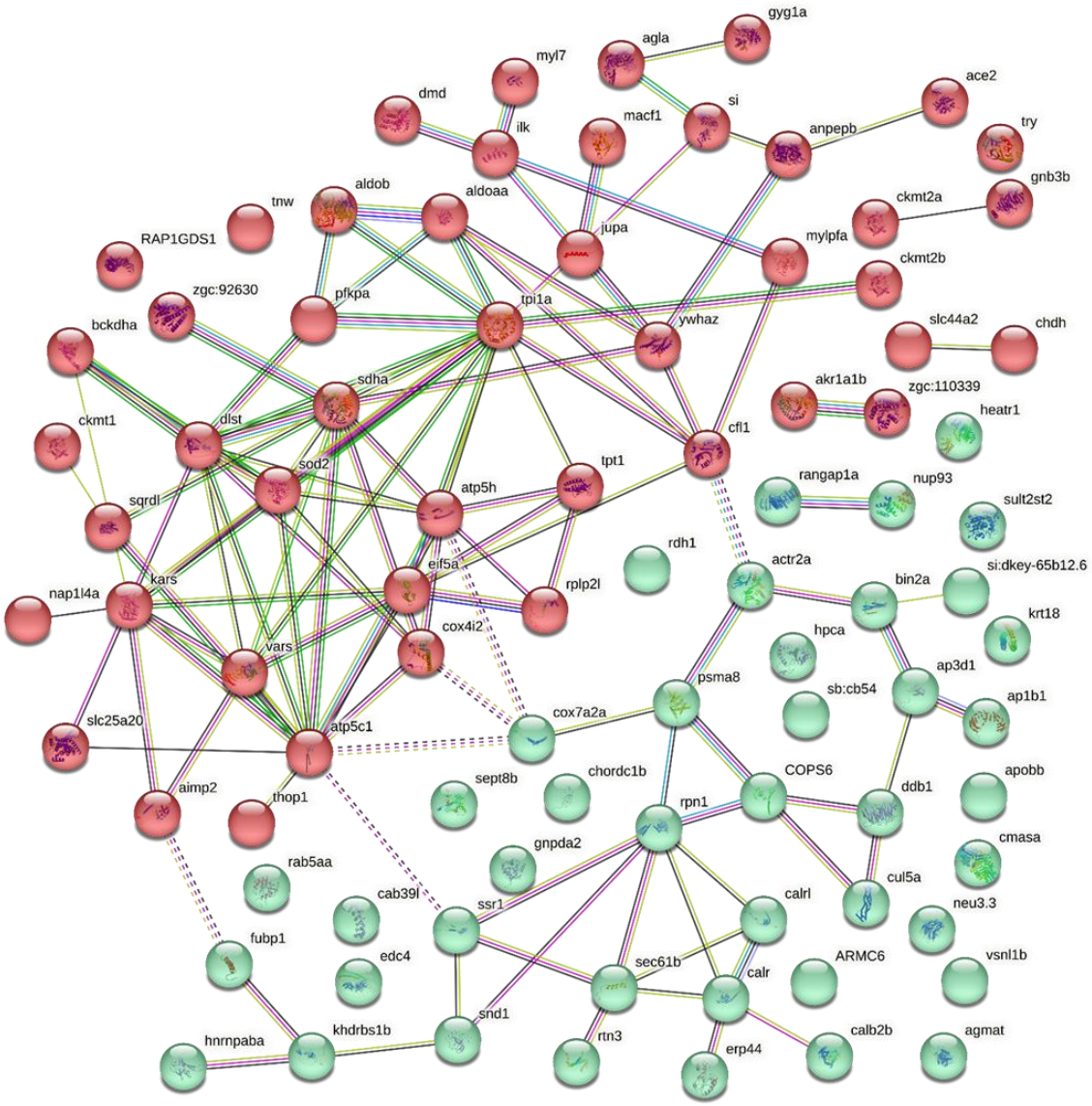

Figure 17. K-means cluster analysis of differentially expressed proteins (DEPs) as elicited by exposure to 100µg/L o-desmethyltramadol (OTRA100). The red cluster was associated with KEGG terms: pentose phosphate pathway; fructose and mannose metabolism; starch and sucrose metabolism; arginine and proline metabolism; glycolysis/gluconeogenesis; biosynthesis of amino acids; oxidative phosphorylation; carbon metabolism; metabolic pathways. The green cluster was associated with the remaining KEGG term: protein processing endoplasmic reticulum.

5.4 Discussion

Molecular biology approaches, in which proteomic studies are included, are a promising and useful tool to evaluate the toxic potential of a xenobiotic to living organisms (Ronsein et al., 2015). The great evolution of quantitative proteomic methodologies seen in the last years allows for a complex and accurate systematic study of changes in the expression of high numbers of proteins and the identification if such changes may result from exposure to a certain xenobiotic (Ronsein et al., 2015). Among these new

methodologies is shotgun proteomics and the use of the FASP method. The FASP methodology became widely used for proteomic studies, since it abolished the need of detergents use, it can be applied to a variety of sample types, and the correct application of the method results on a high quality of peptides release (Wisniewski, 2019).

5.4.1. Carbamazepine and its main metabolite

FASP methodology allowed us to globally quantify more than 3000 different proteins. Moreover, more than 2000 proteins were identified for each treatment, in a range between 67.9 and 76.3% of the total of the quantified proteins. Approximately half of the quantified proteins were common to all treatments, while the percentage of proteins quantified exclusively in one treatment was generally about 20% or lower. Therefore, the applied methodology showed suitable for this study and allowed to obtain an extensive set of data to better understand possible mechanisms underlying the toxicity of CBZ and Cbzep to zebrafish larvae.

Differentially expressed proteins

From the results of our study, CBZ and CBZep exposure altered the expression of 130 proteins in relation to the control, representing approximately 4% of the total identified/quantified proteins. Up to date, this is the first proteomics study evaluating the effects CBZ and CBZep exposure on early larval stages of zebrafish. In the available literature, only four other proteomic studies about CBZ effects were found, both using different model organisms (Yan et al., 2018; Wang et al., 2019; Navon et al., 2021; Dumas et al., 2022). Hepatic proteome of chinese rare minnows (*Gobiocypris rarus*), chronically exposed to 1, 10 and 100µg/L of CBZ, revealed that this anticonvulsant induced a sex-specific response of proteins expression. Moreover, these proteins were linked to different cellular and physiological processes such as apoptosis, cell differentiation, cell proliferation, and the respiratory chain (Yan et al., 2018). More recently, shotgun proteomics was also performed to evaluate exposure effects to 5 and 10µg/L of CBZ in the ascidian *Herdmania momus*. Authors could identify 199 proteins, 24 of which were differentially expressed between treatments (Navon et al., 2021). The differences in methodology (gel-based vs shotgun proteomics) or test models (teleost fish vs ascidian), prevent sound comparisons of the results and confirmation of trends of expression or affected pathways. Nevertheless, both studies identified differentially expressed proteins at concentrations similar to the ones tested in our study, suggesting

that CBZ exposure can induce significant alterations in protein expression in a range of aquatic species, even at low concentrations. The pattern of protein expression indicates a clear trend for over-expression for CBZ and CBZep exposure. This pattern is observable for all treatments, despite some differences in the magnitude of response. This induction of protein expression is in accordance with data already described in the literature, for different test models. CBZ exposure was reported to promote the expression of proteins related with membrane permeability in different bacterial genera at concentrations as low as 0.05mg/L (Wang et al., 2019). A more recent study conducted in the mussel *Mytilus galloprovincialis*, described that CBZ exposure was responsible to differently modulate 198 proteins in male mussels. Moreover, translation, RNA transcription, protein synthesis, transport, and catabolism processes were induced by CBZ exposure and related to the upregulation of differentially expressed proteins (Dumas et al., 2022). The most accepted mode of action of CBZ is linked to inhibition of sodium channel firing, treating seizure activity (Tolou-Ghamarie et al., 2013; Rogawski et al., 2016). However, the action of this anticonvulsant in fish species is still, mostly unknown (Ribbenstedt et al., 2022). Our data provide further information on protein expression perturbations that can be useful to better understand the mode of action these compounds in fish species, even at low concentrations.

When analysing the number of DEPs of all treatments, 95 DEPs were identified for 0.1µg/L of CBZ; 36 for 100µg/L of CBZ; 9 for 0.1µg/L of CBZep; and 32 for 100µg/L CBZep. These DEPs were linked to 18 KEGGs. The number of suggested KEGGs by treatment were: 10 for 0.1µg/L of CBZ; 4 for 100µg/L of CBZ; 7 for 0.1µg/L of CBZep; and 8 for 100µg/L of CBZep. From the obtained DEPs, protein transport protein SEC23 (sec23b), was common to all tested treatments. Therefore, this protein can be a potential biomarker of exposure for CBZ and CBZep. Moreover, CBZep treatments were grouped together in the analytical dendrogram. For CBZep, the number of affected pathways was almost similar, suggesting that the proteins impacted by the lowest concentration may have a higher importance in several physiological processes. For CBZ, the dendrogram separated both treatments. More DEPs and number of suggested KEGGs were found for CBZ01 than for the highest concentration. Moreover, the magnitude of response was considerably higher for CBZ01. This indicates a clear non-monotonic response for CBZ exposure, at least at the proteomic level. Several studies demonstrated that pharmaceutical compounds, tend to elicit non-monotonic responses in non-target species (Rodrigues et al., 2014, 2015; Rodrigues et al., 2020). Metabolomic studies also reported non-monotonic responses for both zebrafish and *Daphnia magna*, although for zebrafish monotonic responses were also described (Kovacevic et al., 2016; Ribbenstedt et al., 2022). Also, for *Daphnia magna*, a study reported the occurrence of non-monotonic

responses for population growth and behavioural effects, after exposure to different pharmaceuticals where CBZ was included (Rivetti et al., 2016). A more recent study reported that CBZ exposure caused non-monotonic responses for locomotor activity of the freshwater planarian, *Schmidtea mediterranea* (Ofoegbu et al., 2019). Altogether the results indicate a clear need to address lower concentrations in risk assessment methodologies, even more because the treatment with the most affected pathways corresponded to an environmentally relevant concentration.

Functional analysis

Functional analysis revealed that CBZ and CBZep exposure impacted a series of pathways of great importance for an organism survival. Results from the STRING software, suggested that 18 different KEGGs were affected, from the initial differentially expressed proteins associated to each tested treatment. Carbon metabolism is among of the most basic and important functions of the organism. It englobes a vast series of enzymatic steps in which sugars are transformed into metabolic precursors. These precursors are the basis for the creation of all cell biomass (Noor et al., 2010). This pathway was impacted by the lowest concentration of both compounds. To the best of our knowledge no other data about the impact of CBZ or CBZep on this pathway is available in the literature. In humans, disruption of one-carbon metabolism was associated to epilepsy (Brister et al., 2022). The parental compound CBZ is a drug used to treat epilepsy. The results of Brister at al. (2022) suggest that this drug may influence the normal functioning of the carbon metabolism pathway. Moreover, central carbon metabolism comprises several pathways associated with cellular energy and respiration (Sudarsan et al., 2014). Results from our analysis suggested that the DEPs identified were associated with several KEGGs linked to cellular energy metabolism and respiration.

Amino sugar and amino nucleotide sugar metabolism, as well as the galactose metabolism pathway, were other KEGGs suggested to be impacted by the lowest concentrations of both CBZ and/or CBZep. Amino sugars are present in a large number of biomacromolecules and secondary metabolism products. In living organisms, amino sugars are formed by the action of specific aminotransferases or amidotransferases (Skarbek and Milewska, 2016). On their turn nucleotide sugars are monosaccharides on their active form and act as glycosyl donors in glycosylation reactions (Betenbaugh et al., 2007). They also function as building blocks of the carbohydrates byosynthesis (Mikkola, 2020). On the other hand, galactose is an essential carbohydrate for the metabolism of the cell, contributing to the production and storage of energy in several

tissues. It is also a precursor for glycosylation (Conte et al., 2021). Herein, the galactose metabolism pathway was impacted by the CBZep01 treatment. A previous metabolomics and proteogenomic study in marine mussels revealed that CBZ exposure could influence their carbohydrate metabolism (Dumas et al., 2022), in which amino nucleotides are expected to be involved. As far as we know, our study is the first suggesting an impact of CBZ exposure on these pathways in teleost fish.

Glycolysis is the process in which glucose is degraded into lactate, generating energy for the cell (Noguchi et al., 2013). Also, gluconeogenesis is an extremely important pathway, since it is the process by which glucose is generated (Noguchi et al., 2013). In our study, glycolysis/gluconeogenesis were impacted by CBZ01 and CBZep01. It is important to notice that glycolysis is strongly related with the central carbon metabolism, and both KEGGs were impacted by the same treatments. In humans, the glycolysis pathway was described to be increased during epilepsy seizures (Yang et al., 2013). Previously, a transcriptomic study performed in salmon, reported that the glycolysis/gluconeogenesis pathway was altered by CBZ exposure (Hampel et al., 2010). Moreover, the KO (KEGG orthology) numbers were mostly associated with fold change values higher than 1, related with overexpression trends (Hampel et al., 2010). Another process linked to energy production, also affected by this anticonvulsant was oxidative phosphorylation. This KEGG was impacted by the highest concentration of both compounds. Oxidative phosphorylation is the metabolic pathway in which nutrients are oxidised by different types of enzymes, in the cell. This oxidation results in the generation and posterior release of chemical energy, that is consumed in the production of adenosine triphosphate (ATP) (Schmidt-Rohr, 2020). In mammals, CBZ exposure was already described to affect the normal oxidative phosphorylation pathway in mitochondria. The drug was reported to decrease complex IV of the electron complex chain (Cikankova et al., 2020). Also, in children, the chronic treatment with CBZ was found to affect oxidative phosphorylation, namely by a significant decrease of ATP production (Berger et al., 2010). No data on the effects of CBZ on this pathway could be found in the literature for fish species. Given the high conservation of genes and pathways between zebrafish and humans, our results suggest that this compound may also interfere with enzymatic complexes and ATP production in zebrafish larvae. Further studies will help to better understand the mechanisms underlying these interferences.

Fructose and mannose metabolism was impacted at the lowest CBZep concentration. These are monosaccharides which have several roles in living organisms. Fructose is responsible, among other functions, by signalling the insulin biosynthetic pathway, inducing triglycerides levels in plasma and regulating hepatic glucose homeostasis (Lê and Tappy, 2006). In turn, mannose is linked to a wide range of metabolic

transformations and has a stimulant action on the putative beta cell glucoreceptor (Lieu et al., 2021). Transcriptomic analysis carried out on adult salmon (*Salmo salar*) exposed to CBZ was reported to induce the fructose and mannose metabolism KEGG (Hampel et al., 2010). In this study, 5 different KO numbers were associated with this KEGG. Our study is also the first to report an alteration on the expression of proteins suggested to be linked to this pathway.

Starch and sucrose metabolism were also impacted by exposure to 0.1µg/L CBZep. Starches are one of the main energy sources for mammals. Their metabolism is catalysed by endogenous amylases, which convert starches into maltotriose, maltose, dextrins and glucose (Aller et al., 2011). Sucrose metabolism is important on several biological functions. Its importance is associated to the degradation of sucrose into different several sugars (i.e., glucose and fructose) that are essential for energy metabolism (Wong et al., 2016). Though no other study could be found about the impact of CBZ exposure on this metabolic pathway in zebrafish, other compounds were described to induce the starch and sucrose metabolic pathway. Among them are the antidepressant fluoxetine and the antibacterial and fungicide triclosan (Mishra et al., 2017; Fu et al., 2020).

Purines have a wide range of crucial functions in the cell, the most important of which is the formation of nucleic acids precursors. Purines also modulate energy metabolism and signal transduction (Maiuolo et al., 2016). Our functional analysis suggests that exposure to CBZ01 and CBZep100 affected the purine metabolism pathway. This result was in line with previous studies focused on metabolomic analysis, which reported purine metabolism as one of the pathways affected by CBZ exposure (Dumas et al., 2022 Ribbenstedt et al., 2022). This outcome may be associated to the role of purine metabolism in uric acid levels. Purines are involved in the production of uric acid in humans (Maiuolo et al., 2016). Moreover, CBZ was reported to decrease uric acid levels in human patients (Albert et al., 2015). Exposure of zebrafish larvae to CBZep01 impacted the biosynthesis of amino acids. This is a process using nitrogen in which several biochemical pathways assemble amino acids from their initial precursors (Deferrari et al., 2010). In the aquatic invertebrate *Daphnia magna*, metabolic responses to CBZ exposure were mostly associated to the depletion of several amino acids (Kovacevic et al., 2016), suggesting this may also be a possible outcome in zebrafish larvae. The metabolism of alanine, aspartate and glutamate were an example of interference of this anticonvulsant in the normal amino acids synthesis; altered metabolism was observed for the CBZep100 treatment. Alanine is a component of peptidoglycans, an effector of the leucine responsive regulation function, and inhibitor of glutamine synthetase (Reitzer, 2004). Aspartate is a precursor for several proteins and

also a nitrogen donor for purines and arginine synthesis (Reitzer, 2004). Glutamate is a precursor of different amino acids, its degradation has a critical role in the survival in acidic environments and changes in glutamate concentration have been shown to correlate with changes in osmolarity (Reitzer, 2004). No studies were found for implications of CBZep exposure on protein expression of fish species, or even mammal models. However, the parental compound was already described to interfere with some of these amino acids. CBZ consumption in humans was reported to elevate the levels of gamma glutamyl transferase, which is an enzyme included in glutamate metabolism (Hadzagic-Catibusic et al., 2017). Also, for daphnia magna, the alanine level was reported to be a potential sub-lethal bioindicator of CBZ exposure (Kovacevic et al., 2016). Other pathways related to the metabolism of amino acids were suggested to be impacted by the CBZ01 treatment, such as glycine, serine and threonine, valine, leucine and isoleucine. Serine is derived from 3-phospho-D-glycerate, an intermediate product of glycolysis, and produces several important metabolites, such as NADPH, glutathione and S-adenosylmethionine (Wu et al., 2020). Glycine is derived from serine and acts primarily as a precursor to proteins and as a neurotransmitter (Wang et al., 2013). Threonine is and essential amino acid synthesised from aspartic acid. It serves mainly as a substrate for protein synthesis, but can also be metabolised to glycine, acetyl CoA and pyruvate, which have an important role in different biological functions (Tang et al., 2021). For daphnia magna, serine and glycine were reported to be potential biomarkers of CBZ exposure (Kovacevic et al., 2016). The authors correlated these amino acids to possible alterations on energy metabolism. Our results, show that CBZ exposure also impacted other pathways involved on the energy metabolism of zebrafish larvae, such as the glycolysis/gluconeogenesis and oxidative phosphorylation. The metabolic pathway of glycine, serine and threonine was previously reported to be affected in different kidney diseases (Ribbenstedt et al., 2022). Interestingly, CBZ was suggested to induce renal toxicity in rats (Erdem Guzel et al., 2021). Valine, leucine and isoleucine are considered essential amino acids and are recognized as the branched-chain amino acids (BCAAs). The catabolism of these amino acids occurs in muscle and yields NADH and FADH2, which are used for ATP generation (Holecek, 2018). Antiepileptic drugs like CBZ cause a deficiency of biotin in human patients. This biotin deficiency interferes with a series of carboxylases essential for BCAAs metabolism (Rathman et al., 2003). Moreover, BCAAs have been linked to seizures in humans who are treated with antiepileptic drugs (Ong et al., 2021). To our knowledge, this is the first study describing an impact of CBZ on these amino acids. The results are similar to those elicited by valproic acid on different models, including aquatic species. Valproic acid, which has a

similar mode of action, was reported to impact the valine, leucine and isoleucine metabolism pathway (Andersen et al., 1994; Kovacevic et al., 2016).

Nucleocytoplasmic transport was another complex cellular pathway that was impacted by the lowest concentration of CBZ and the highest of CBZep. Nucleocytoplasmic transport has great importance in eukaryotic cells, since it is responsible for matter exchange between the nucleus and cytoplasm (Peters, 2006). The matter transported in this process comprises different biogenesis intermediates, mature RNA and ribonucleoprotein complexes (Sloan et al., 2016). This pathway thus affects a wide range of aspects related with health status of living organisms (Peters, 2006). Also, nucleocytoplasmic transport is known to be linked with cellular autophagy in humans (Tsai et al., 2012; Lagnner et al., 2017). This association between nucleocytoplasmic transport and autophagy is extremely important in the scope of our results since CBZ is recognized to induce autophagy to produce therapeutic effects (Puls et al., 2013). This suggests that CBZ impact on nucleocytoplasmic transport may occur via action on autophagy. RNA polymerase, also known as PolII, is an enzyme related with RNA synthesis that is formed by twelve different subunits (Rpb1 to Rpb12). This enzyme is responsible for different RNA functions like DNA repair, nuclear pre-mRNA splicing, nuclear export of mRNAs, initiation of translation and cytoplasmatic decay of mRNAs. Moreover, it was reported to be fundamental for the normal development of vertebrate species (Maeta et al., 2020). The RNA polymerase pathway was impacted at the highest CBZep concentration. In the literature no data linking CBZep exposure to RNA polymerase could be found. Existing data refer that CBZ can accelerate zebrafish embryonic development and disturb embryonic and larval behaviour (Qiang et al., 2016). Since RNA polymerase was found to have a critical role in vertebrates' development, the described disturbances linked to our results, can suggest that this pathway may be a possible biomarker of exposure to CBZ and CBZep.

Fatty Acids are a type of biomolecules that have different functions in different biological process, through protein interactions. Their degradation occurs through oxidation reactions that are the main source of energy for several fish species. This physiological process occurs in mitochondria and peroxisomes through the action of different enzymes (Tocher, 2010). From our results, fatty acid degradation was one of the KEGGs suggested to be impacted by CBZ01 exposure. In a previous study, CBZ was described to affect the concentrations of fatty acids in humans (Yuen et al., 2008). The authors reported that human patients who had consumed CBZ, had lower levels of docosahexaenoic acid, long-chain Omega-3 fatty acids and Omega-3 Index. The decreased level of Omega-3 Index has great importance, since it represents a risk factor for coronary heart problems, which can be lethal for humans (Yuen et al., 2008). This

gains even more relevance since cardiac muscle contraction was another suggested KEEG affected by CBZ and CBZep exposure. Moreover, CBZ was described to cause cardiotoxicity in humans, with several cardiac side effects such as sinus tachycardia, sinus and nodal bradycardia, atrioventricular block, premature ventricular contractions, and ventricular tachycardia (Koutsampasopoulos et al., 2014). From the above-mentioned data, it is possible that fatty acids degradation and cardiac muscle contraction in zebrafish may also be affected by CBZ and CBZep exposure, and even correlated. Finally, porphyrins are the essential pigments of living organisms because they comprise chlorophyll and heme, and essential co-factor involved in many biological processes. In eukaryotes, porphyrins are synthesized by a by eight different enzymes. Porphyrias are a group of disorders, resulting from defects in the normal porphyrin metabolism. These disorders produce several symptoms such as skin damage, pain, generalized weakness, delirium and seizures (Bonkovsky et al., 2013). The KEGG associated to porphyrin metabolism was suggested to be impacted by the highest concentration of both CBZ and CBZep. This is not surprising, since there are data in literature that link CBZ exposure to direct effects on this pathway. This anticonvulsant drug was reported to increase porphyrin production in rodent model and humans (Yeung Laiwah et al., 1983; Maan et al., 2021).

5.4.2 Venlafaxine and its main metabolite

Over 2000 different proteins were quantified for all treatments. Moreover, for each treatment, between 67.9 and 76.3% of the total proteins identified were quantified. Comparing different treatments, approximately more than half of the quantified proteins were common to all treatments (~56%), while the percentage of proteins quantified exclusively in one treatment was lower than 20% (maximum of 19.2%). Therefore, the applied methodology showed suitable for this study and allowed to obtain an extensive set of data to recognize possible mechanisms underlying the toxicity of VEN and ODV to zebrafish early stages of larval development.

Differentially expressed proteins

Up to date, this is the first study to evaluate VEN and ODV exposure on zebrafish first larval stages, with the FASP methodology. A study where proteomic analysis was conducted to evaluate the effects of wastewater effluents containing PCPs on wild fish, showed that between 32 and 43 proteins were significantly different in PCP impacted sites, in relation to control groups (Simmons et al., 2017). Interestingly, VEN was one

the 15 pharmaceuticals quantified in the test water (Simmons et al., 2017b). However, because the authors tested complex mixtures no direct comparisons with the present results is possible.

Herein, the global pattern of protein expression does not indicate a clear trend distinguishing VEN and ODV exposure. This may be related with the mode of action of these compounds. Venlafaxine is an antidepressant of the SNRI (serotonin and norepinephrine reuptake inhibitor) class. Also, ODV as an active metabolite has a therapeutical effect similar to its parental compound. This means that VEN and ODV act by inhibiting synaptic receptors of serotonin and norepinephrine reuptake, increasing the availability of both monoamines in the synaptic cleft (Mennigen et al., 2011). However, in mammal models, the activation of a series of pathways, was reported to be involved in the therapeutic effect of VEN (Kumar et al., 2010; Krass et al., 2011; Sossin et al, 2021). The combination of this differential action, may also be applied to zebrafish larvae, explaining the different patterns of proteins expression. Concerning a treatment-by-treatment analysis, in relation to the control group, overexpression was prominent for ODV01, while underexpression was being associated with overexpression responses and VEN0.1 eliciting underexpression, in contrast to the responses caused by ODV. The results also suggest that concentration is a major factor driving the effects of VEN on protein expression, with opposite responses obtained for high and low exposure levels. This also appears to be consistent with the initial study of Chapter 3, in which the strongest and significant differences in gene expression were found for the intermediate test concentration of 400ng/L VEN (Rodrigues et al., 2020). Also, the treatments with more affected KEGGs were 0.1µg/L of VEN and 100µg/L of ODV. This is an interesting result, not only for the number of affected KEGGs, but also for their diversity in terms of impacted pathways.

Functional analysis

Functional analysis revealed that VEN and ODV exposure is associated with a series of alterations in pathways of great importance for an organism survival. As previously referred, carbon metabolism is one of the most basic functions of a living organism. It comprises a vast series of enzymatic steps to transform sugars into metabolic precursors. Precursors resulting from these enzymatic steps, are the basis for the creation of the entire cell biomass (Noor et al., 2010). The carbon metabolism is also related with other energetic processes, such as, the citric acid cycle and the pentose phosphate pathway which is related with nucleic acids processes (Sudarsan et al., 2014). Alterations of carbon metabolism were suggested to occur upon exposure to VEN01 or ODV100

treatments. As far as we know, no studies directly reported the impact of VEN or ODV on this pathway. Indirectly, a recent study in zebrafish, studied the effect of VEN exposure on anaerobic glycolysis, which is included in the central carbon metabolism (Mehdi et al., 2019). However, the results did not support the initial hypothesis that VEN would alter the fish glycolysis. Carbon metabolism has a great heterogeneity and dynamics, and metabolites included in this pathway continuously change their dynamic according to the developmental stage, tissue, cell types, oxygen, or nutrients availability. Also, the differential metabolic rates of central carbon metabolism are related with several negative outcomes in mammal models, such as diseases like leukaemia (Zhang et al., 2020). Therefore, alterations in the dynamics of this KEGG could result in different negative outcomes in zebrafish larvae.

Another KEGG linked to energy production, that was also suggested to be impacted by all treatments except VEN100, was oxidative phosphorylation. Oxidative phosphorylation is the metabolic pathway responsible for the oxidation of nutrients in the cell by different types of enzymes. The main outcome of this pathway is the creation and posterior release of chemical energy to the production of adenosine triphosphate (ATP) (Schmidt-Rohr, 2020). Some studies, including proteomic ones, indicate oxidative phosphorylation as a role player in the depression pathway in human and animal models (Carboni et al., 2015; Allen et al., 2018). In fact, a recent study conducted in the rat brain, reported that venlafaxine administration had interference with some oxidative phosphorylation complexes (Glombik et al., 2021). These results combined with our findings, suggest a strong connection between, VEN exposure and oxidative phosphorylation, in both mammals and fish species.

Exposure to VEN and ODV was suggested to impact KEGGs involved in amino acids biosynthesis and metabolism. The arginine biosynthesis is an example of that. This pathway was affected by VEN01 and ODV100 treatments. In higher vertebrates, arginine is synthesized from citrulline in the urea cycle. This synthesis occurs by the action of arginosuccinate synthetase and arginosuccinate lyase enzymes, in a highly energy consuming process (Morris, 2004). This amino acid has an important role in ammonia removal from the organism, immune function, cell division, wound healing, and hormone release (Morris, 2004). Moreover, it is one of the main precursors of nitric oxide synthesis, which has a critical role in the regulation of blood pressure (Kibe et al., 2014). In fish, arginine also plays an essential role in growth performance and general health. Also, its synthesis from citrulline occurs at lower rates than in mammals (Hoseini et al., 2019). Therefore, arginine is an essential amino acid with limited natural availability in fish, and a disruption in its biosynthesis can lead to a decrease of the animal's health status. In the literature there are some examples of the interaction of VEN and arginine in mammal

models. Alterations in the concentration of arginine and its related products in the serum and plasma have been linked to depression (Fan et al., 2021). Increased levels of arginine were responsible to decrease the antidepressant action of VEN in mice placed under stress conditions (Kumar et al., 2010). The authors suggested that nitric oxide modulation, of which arginine is a precursor, might be involved in the therapeutic effects of VEN. The same type of effects was also reported for VEN and other antidepressants in another study (Krass et al., 2011). These reports, combined with our results, show that arginine and VEN mode of action have a close association, and can produce differences in the normal health status of the exposed animals. However, our functional analysis did not reveal KEGGs linked to nitric oxide. Also, there are no other reports concerning the effects of arginine combined with VEN exposure to zebrafish to directly compare our results. Besides arginine biosynthesis, other pathways involving amino acids were suggested to be altered. Alanine, aspartate, and glutamate metabolism was an example of that. The difference from arginine biosynthesis, is that the metabolism of the other three amino acids was only suggested for ODV100. Alanine is a component of peptidoglycans, an effector of the leucine responsive regulation function and inhibitor of glutamine synthetase (Reitzer, 2004). Aspartate is one of the precursors for asparagine, methionine, isoleucine, lysine, pyrimidines, threonine, NAD (Nicotinamide adenine dinucleotide), and pantothenate. It is also a nitrogen donor for purines and arginine synthesis, as well as, essential for transferring metabolites for C4 species of the NAD-malic enzyme (Reitzer, 2004). Glutamate is a precursor of arginine, proline, glutamine, and polyamines. Glutamate degradation has a critical role in the survival in acidic environments, and changes in glutamate concentration are directly correlated with changes in osmolarity (Reitzer, 2004). The metabolism of these three amino acids was previously described to be affected by VEN exposure. In mice, metabolomics analysis indicated that VEN exposure altered the metabolism of alanine, aspartate and glutamate, in relation to the control groups (Bai et al., 2017; Hu et al., 2018). For aquatic species, a 24h exposure of mussels to VEN, caused significant up-modulation (in males) and down-modulation (in females) of aspartylphenylalanine, a metabolite belonging to the alanine, aspartate, and glutamate metabolism (Ramirez et al., 2022). In the present study, the KEGG for alanine, aspartate and glutamate metabolism was not suggested to be altered by VEN exposure, contrarily to the ODV effect. There are no previous reports about the effects of ODV exposure on the amino acids metabolism. From the available knowledge, its mechanisms of action are expected to be similar to those elicited by VEN. It is possible that the effects of VEN upon amino acids metabolism are due to the transformation of the parental compound into ODV. However, more testing would be needed to confirm this hypothesis.

Nucleocytoplasmic transport was another complex cellular KEGG that was suggested for both compounds at the lowest tested concentration. This transport mechanism has an important role in eukaryotic cells, since it is the pathway through which the exchange of matter between the nucleus and cytoplasm occurs (Peters, 2006). The matter transported by this process englobes several biogenesis intermediates, mature RNA, and ribonucleoprotein complexes, like messenger and small nuclear RNPs; transfer and micro RNAs, and ribosomal subunits which are transported in their mature form or even as precursors (Sloan et al., 2016). Its importance is even more patent once this pathway affects a wide range of aspects related to health and disease (Peters, 2006). Up to date, no studies related VEN or ODV exposure to implications on nucleocytoplasmic transport pathway. However, omics analysis in the rat cortex revealed that, two other antidepressant compounds (imipramine and citalopram) induced overexpression of genes and proteins involved in the nucleocytoplasmic transport pathway (Palotas et al., 2004). As previously mentioned, nucleocytoplasmic transport pathway involves a series of effectors and types of matter that are transported in this process. Therefore, a most focused study about this pathway should be conducted to better understand how VEN and ODV interfere in the normal function of this important cellular process. The ribosome KEGG was suggested to be altered by ODV100 exposure. Ribosomes consist in macromolecular machines, that are ubiquitous within all cells, which main function is protein synthesis or mRNA translation. Ribosomes act on the linking of different amino acids in a specific order determined by the codon's mRNA molecules to origin polypeptide chains (de la Cruz et al., 2015). Effects of antidepressant exposure on the ribosome pathway are expected. Evidence indicates that dysregulation of pathways linked to protein synthesis is strongly related to depression disorders (Sossin et al., 2021). Moreover, the activation of mRNA translation, initiation, or elongation pathways is vital to relief depression symptoms in humans (Sossin et al., 2021). Also, sertraline, an antidepressant with action somewhat similar to VEN, was found to inhibit translation initiation in mammals (Lin et al., 2010). For ODV, or even for VEN, this study is the first one to directly link exposure of these compounds to alterations in the ribosome pathway. From the above-mentioned studies, disruption of ribosome pathway is not surprising. However, the way this pathway was affected cannot be fully explained by our work. From our results, 60S ribosomal protein L3 (rpl3), was one of the DEPs. This protein is evolutionarily conserved and has an important role on the formation of the peptidyltransferase centre (Meskauskas and Dinman, 2008; Garcia-Gomez et al., 2014). Due to its importance, and being differently expressed, rpl3 could be a potential candidate for future studies regarding ODV exposure and ribosome pathway differential response.

5.4.3 Tramadol and its main metabolite

Approximately 2000 different proteins were quantified for all treatments. Moreover, for each treatment over 1200 proteins were identified, in a range between 65.1 and 80.7% of the total quantified proteins. Comparing different treatments, more than half of the quantified proteins (57%) were common to all treatments, while the percentage of proteins quantified exclusively for one treatment was always lower than 20%. Therefore, the applied methodology showed suitable for this study and allowed to obtain an extensive set of data to better understand possible mechanism underlying the toxicity of TRA and OTRA to zebrafish larvae.

Differentially expressed proteins

From the results of protein expression obtained in our study, it is clear that TRA and OTRA altered the expression of 163 proteins. This means that approximately 8% of the quantified proteins were impacted by these compounds. As far as we know, this is the first study to evaluate TRA and OTRA exposure on zebrafish larvae using this methodology. A proteomic study was already published in 2012, but it focused on the zebrafish brain and used a gel-based approach identifying 30 differential proteins (Zhuo et al., 2012). While the sample preparation method used in our work (FASP) allows to achieve adequate amounts of proteins with confidence when compared to other methods (Araújo et al.,2021), also the use of a poll of organisms (due to their small size) in our study rather a single organ, may have interfered with the overall number of identified / quantified proteins.

The expression pattern obtained for the DEPs indicated a clear tendency for underexpression following TRA or OTRA exposure. This result is in accordance with previous findings in the literature. In a study conducted in mice exposed to TRA, proteomic analysis found 31 differential expressed proteins, in which 22 of them had their expression decreased, while 9 were increased, comparing to the control (Jiang et al., 2021). The decrease in expression found for certain proteins may be related with the TRA mode of action. One of the characteristics of TRA is its action as a SNRI (serotonin and norepinephrine reuptake inhibitor). This means that TRA acts by inhibiting synaptic receptors of serotonin and norepinephrine increasing the availability of this monoamine (Vazzana et al., 2015). Also, an inhibitory action of TRA over other types of receptors, such as the muscarinic ones, was previously reported (Shiga et al., 2002). Some of the obtained DEPs were common to all tested treatments. Examples of this are the cluster of Actin-related protein 2 (actr2a); Cluster of Zgc:153353 (agmat); Cluster of

Aminopeptidase (anpebb); ATP synthase subunit d, mitochondrial (atp5pd); Cluster of ATP-dependent 6-phosphofructokinase (pfkma); 60S acidic ribosomal protein P2 (rplp2l), among others. Therefore, these proteins can be considered potential biomarkers of exposure. In terms of affected pathways, the results showed that TRA100 was separated from the other treatments, probably due to its lower number of DEPs and impacted KEGGs. Interestingly, the treatments with stronger impact on protein expression were TRA01 and OTRA100. Differences on TRA and OTRA modes of action may possibly explain these patterns of response. Tramadol acts more as an SNRI, while OTRA has a higher affinity to opioid receptors. Such differences could reflect a differential sensitivity of those receptors to external action and, therefore, different magnitudes of response.

Functional analysis

The functional analysis indicated that TRA and OTRA exposure were linked to pathways of vital importance for homeostasis. As previously mentioned, carbon metabolism is one of the most basics aspects of life. It uses a wide range of enzymatic steps to transform sugars into metabolic precursors. These precursors are after used to create all the entire cell biomass (Noor et al., 2010). The central carbon metabolism comprises a series of important pathways for living organisms, including glycolysis and pentose phosphate pathways, as well as the citric acid cycle (Sudarsan et al., 2014). Exposure to TRA and OTRA impacted the carbon metabolism pathway in all treatments apart from the lowest concentration of OTRA. This is important since carbon metabolism is such an important process in living organisms. Impacts on its function can originate a cascade of disruption of several pathways, including the glycolysis and pentose phosphate pathways, as well as the citric acid cycle. In fact, all these pathways were found to be impacted in this study. Glycolysis is the process in which glucose is degraded into lactate (Noguchi et al., 2013), an important pathway for living organisms. Also, gluconeogenesis is an extremely important pathway, since it is the process by which glucose is generated (Noguchi et al., 2013). Glycolysis/gluconeogenesis was impacted by all treatments except TRA100. Exposure to TRA in mice was reported to interfere with the levels of glucose. At high doses, a decrease of intracellular fructose-2,6-bisphosphonate led to inhibition of the glycolysis pathway (Xia et al., 2020). Also, for mice, chronical TRA exposure significantly reduced glucose levels, compared to controls (Abazid et al., 2022). The pentose phosphate pathway is one of the most important metabolic pathways for living organisms, parallel to glycolysis. This pathway is responsible for the formation of NADPH, different pentoses, and ribose 5-phosphate, a known precursor of nucleotides synthesis (Alfarouk et al., 2020). This pathway was impacted by OTRA100 meaning that the metabolite may

have a role in the pathway function. In the study performed in mice, chronic exposure to TRA was found to stimulate the pentose phosphate pathway. At high doses, the decrease in intracellular fructose- 2,6-bisphosphonate led to inhibition of the glycolysis pathway, increasing the accumulation of fructose-6-phosphate. This accumulation was beyond the stimulation of the pentose phosphate pathway and the subsequent production of ribose and NAPDH (Xia et al., 2020).

The citrate cycle (TCA cycle) is a crucial series of reaction sequences in living organisms. It is responsible for supplying most energetic needs of complex organisms. The molecules resulting from these reactions can be used as building blocks in many important processes, such as the synthesis of fatty acids, cholesterol, steroids, purines and pyrimidines for DNA synthesis, and amino acids for building proteins (Cleveland and Morris, 2015). The TCA cycle was impacted by the lowest concentration of TRA. Opioid receptors in the brain are G protein-coupled receptors with a key regulatory role in the glutamate and glutamine dynamics. Glutamate may be synthesised from ketoglutarate, which is a Krebs cycle intermediate (Barbosa et al., 2021). It is possible that TRA exposure, can interfere with the TCA cycle via ketoglutarate, through its action on opioid receptors. In mammals, the action of TRA on opioid receptors is thought to occur mainly via OTRA. However, in the present work no alterations were found after exposure for 168hpf to OTRA. Apart from these three pathways, other processes linked to energy production and mitochondria were also affected by TRA or OTRA exposure. Oxidative phosphorylation was one of these pathways. It was impacted by both OTRA treatments. Oxidative phosphorylation is the metabolic pathway in which nutrients are oxidized by different enzymes. The result of this pathway is the release of chemical energy to adenosine triphosphate (ATP) production (Schmidt-Rohr, 2020). A 3-week exposure of adult rats to 50mg/kg TRA was reported to cause a deregulation of the oxidative phosphorylation pathway (Ezi et al., 2021). No data could, however, be found for the effects of OTRA on oxidative phosphorylation. Differences obtained for the effects of TRA and OTRA in the present work, compared to mammals, may be due to differences in sensitivity between species, life stage assessed and exposure levels.

Another affected pathway was that of the pyruvate metabolism. This pathway was impacted by exposure to TRA01. Pyruvate is an important molecule for several physiological processes of eukaryotic metabolism. Pyruvate is the final product of glycolysis and has an important function in the transport into mitochondria as a crucial input for the citric acid cycle carbon flux. In mitochondria, pyruvate boosts ATP production by oxidative phosphorylation and different pathways linked to the citric acid cycle (Gray et al., 2014). An impact of TRA on pyruvate metabolism was previously reported in the literature. A study conducted in human cell lines showed that exposure

to 600µM of TRA increased the expression of the ALDOC (Aldolase, Fructose-Bisphosphate C) gene, which in turn increases ATP but impairs the conversion of pyruvate to acetyl CoA, due to an increase in PDK (Pyruvate dehydrogenase kinase) expression. Also, authors reported an inhibition of NDUFS1 (NADH:Ubiquinone Oxidoreductase Core Subunit S1) expression. The increase in PDK expression coupled with the decrease in NDUFS1 expression results in the accumulation of pyruvate (Faria et al., 2016). Summing up, it appears that TRA and OTRA expression can influence vital functions in the zebrafish metabolism, like ATP production, cellular respiration, and energy metabolism. In part, alterations in these functions are somehow expected, since there are some evidences arising from omics data, including proteomics, that opioids like morphine, influence the energy metabolism in brain (Antolak et al., 2017). Nevertheless, there are several proteins involved in the cellular energy metabolism making difficult to explain in detail the mechanisms of toxicity. Moreover, studies are mainly conducted in mammal models at concentrations higher than the ones we tested.

Fructose and mannose metabolism was impacted at the highest OTRA concentration. Fructose and mannose are monosaccharides with great importance in living organisms. Fructose, among others, is responsible for signaling the insulin biosynthetic pathway, increasing plasma triglycerides and altering hepatic glucose homeostasis (Lê and Tappy, 2006). In its turn, mannose is involved in several metabolic transformations and stimulates the putative beta cell glucoreceptor (Lieu et al., 2021). Recently, a study conducted in mice exposed to 20 and 50mg/Kg of TRA, reported differences in the animals' metabolites. For the highest concentration, differentially changed metabolites, in relation to control, were associated to sphingolipid, fructose, and mannose metabolism (Xia et al., 2020). Our results showed that only OTRA100 elicited changes in these pathways. In contrast, starch and sucrose metabolism were impacted by both opioids, TRA01 and OTRA100. Starches are among the most important energy source for mammals. They are metabolised by the action of amylases secreted in the organism, which convert it to maltotriose, maltose, dextrins, and glucose (Aller et al., 2011). Sucrose metabolism is important on several biological functions, although not in its own. Its importance is associated to the degradation of sucrose into different several sugars (i.e. glucose and fructose) that are essential for energy metabolism (Wong et al., 2016). Up to date there is no information about a direct link between TRA exposure and effects on starch and sucrose metabolism in fish or even in mammals. However, the interaction between opioids and sugars like starch and sucrose was recently studied and a strong interaction between them was reported (Jewett et al., 2022). Opioid antagonists, like naloxone, reduced imbibition of sucrose in mammals (Jewett et al., 2022). Also, sucrose intake was found to lower µ-opioid availability in the porcine brain (Winterdahl et al.,

2019). It is possible that the action of TRA or OTRA on opioid receptors may also interfere with sugar metabolism in zebrafish larvae.

Exposure of zebrafish larvae to OTRA also impacted amino acids biosynthesis. This functional process was affected by the highest OTRA concentration. The biosynthesis of amino acids is the result of several biochemical pathways with the function of assembly of amino acids from initial precursors. The biosynthesis of amino acids is distinct from other molecules, due to the use of nitrogen in the process (Deferrari et al., 2010). Recent investigation has shown that chronic TRA exposure resulted in changes on proteomic and metabolomic expression in a mammal model (Jiang et al., 2021). The authors found differentially expressed metabolites related to the biosynthesis of amino acids, among the changed pathways. In the present study, a clear example of alterations in the biosynthesis of amino acids elicited by opioid exposure was related to the arginine and proline metabolism. This pathway was affected by exposure to all treatments, except TRA100. This is the main pathway for the biosynthesis of the amino acids arginine and proline from glutamate, but also ornithine and citrulline, (Wu et al., 2009). In the study of Jiang et al. (2021) the metabolite creatinine was altered on animals exposed to TRA, in relation to the control. The alterations found were related to the arginine and proline metabolism, as well as glycine, serine and threonine metabolism (Jiang et al., 2021).

The endoplasmic reticulum is the main cellular organelle responsible for the correct processing of nascent proteins and any disturbance of this organelle function can cause stress in organisms (Placido et al., 2014). Strong evidence suggests that opioid exposure at early stages of development can lead to several neurobehavioral defects later in the organism life span. Nonetheless, the underlying neurobiological mechanisms related to these effects are yet to be elucidated (Tsai et al., 2019). Tramadol is not an exception, and it was already reported to induce oxidative and endoplasmic reticulum stress in the brain of different test models, including zebrafish (Zhuo et al., 2012; Awadalla and Salah-Eldin, 2016; Faria et al., 2016). In the present work, the data showed that TRA01 and OTRA100 affected the KEGG related to protein processing in the endoplasmic reticulum.

5.5 Conclusion

Omics approaches, namely proteomics, are a promising and continuously improving tool for environmental toxicology studies. These methodologies allow researchers to have a more concise image of the pathways affected by exposure to environmental contaminants, in a wide range of living organisms, even in non-target species for these compounds or their metabolites. Regarding the pharmaceutical products investigated in

the present study, the existing literature is mainly focused on mammalian species and chronic exposure periods. Also, metabolites are somehow neglected in this kind of analysis. This study is one of the first focusing on exposure of zebrafish first larval stages to CBZ, VEN, TRA or their main metabolites using shotgun proteomics analysis. The results obtained, allowed the identification of 130 proteins differentially expressed after 168h of exposure to CBZ and CBZep. The expression of these proteins followed a trend of overexpression, which was in line with data available in literature. These 130 proteins affected 18 different KEGGs with different physiological functions on zebrafish larvae, such as energy metabolism, amino acid synthesis and metabolism, fatty acids degradation and cardiac function. Results also pointed that 0.1µg/L of CBZ and 100µg/l of CBZep, were the treatments with more affected pathways, which is worrying, since 0.1µg/L is a concentration of CBZ found in environmental samples. These results also highlight the non-monotonic response elicited of CBZ that is typically found for different pharmaceutical compounds in non-target species, as well as possible differences in fish sensibility and between the mode of action of the parental compound and the metabolite.
For the VEN block of chemicals, the shotgun analysis allowed the overall identification of 42 DEPs in VEN and ODV treatment groups. The expression of these proteins did not follow a particular trend, which can be explained by the mode of action of these compounds and supports previous data on the non-monotonic responses described in the literature exhibited for different mammal or fish models. These 42 proteins were related with 7 different suggested KEGGs, involved in different functions in the organism, such as energy metabolism, amino acid synthesis and metabolism, and protein synthesis. The results pointed that 0.1µg/L of VEN and 100µg/l of ODV were the treatments with more altered pathways.
The shotgun analysis carried out for the TRA block, has shown that TRA and OTRA exposure affected 162 proteins in zebrafish larvae, which were mainly underexpressed in relation to control groups. This is in accordance with the expected mode of action of these compounds found for mammal models. These significantly different expressed proteins affected 12 different KEGGs with different functions on the organism, such as energy metabolism, cellular respiration, mitochondria and amino acid and protein synthesis. Results also indicated that 0.1µg of TRA and 100µg/l of OTRA were the treatments affecting most of the altered pathways identified and highlighted differences in the modes of action of the parental compound and the metabolite.
To sum up, the results presented herein indicate that exposure to CBZ, VEN, TRA or their main metabolites (CBZep, ODV, OTRA) can disrupt basal protein expression of zebrafish larvae and important pathways in fish species. They also suggest that the metabolites may be somewhat less potent than the respective parental compounds to

disrupt protein expression. Nevertheless, ultimately, because they disrupt so important cellular pathways, they can compromise vital physiological functions of exposed animals, even at low, environmentally relevant, concentrations. The evidence highlights the crescent need to intensify the monitoring of these compounds in the aquatic environment and include them in risk assessment analysis.

5.6 References

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Chapter 6: Integrative discussion: Adverse Outcome Pathways (AOPs)

Chapter 6: Integrative discussion: Adverse Outcome Pathways (AOPs)

6.1 General introduction

The Adverse Outcome Pathways (AOPs) framework was initially proposed to address the need to conciliate new tools in predictive testing with existing knowledge at the molecular level, creating new approaches to ecological risk assessment (Ankley *et al*., 2010). An AOP is a structured representation of linked biological events to the outcomes they will lead. The interaction between a particular contaminant and a biomolecule will trigger an initial molecular event(s) - MIE(s), which will reflect into a series of key events at higher biological organisation levels, ultimately resulting in an adverse outcome relevant to ecological risk assessment (Ankley *et al*., 2010; Kramer *et al*., 2011). An AOP encompasses a wide range of events occurring through different levels of biological organisation, of which the information can be obtained from a wide range of testing methodologies (Ankley *et al*., 2010). This workflow and the different relationships it addresses, makes it a trustworthy tool to use in predictive approaches in the fields of toxicology, environmental toxicology and risk assessment (Ankley *et al*., 2010; Kramer *et al*., 2011). The data presented in Chapters 4 and 5 of this thesis represent a significant contribution to the AOPs under development for these compounds (https://aopwiki.org; ID: 91; AOP: 214; 233; 234). In this chapter, we combined those data with the data available in the existing literature (some of them presented in the discussion of results of those chapters) and wrapped up the information to propose new AOP drafts for the exposure of zebrafish embryos and early larvae to carbamazepine, venlafaxine, tramadol, or their respective transformation products, providing an integrated discussion of the results presented herein.

6.2. Draft Adverse Outcome Pathways

6.2.1 Carbamazepine

In mammals, the mode of action of carbamazepine is still under discussion. One of the focuses in carbamazepine action is the neuronal acetylcholine receptor subunit alpha-4 (CHRNA4). Mutations in CHRNA4 have been researched for their involvement in genetically transmissible forms of epilepsy. This gene encodes the alpha4 subunit of nicotinic acetylcholine receptors (ACHA4), and the alteration of this ligand-gated ion channel was the first one linked with seizures (Bertrand, 2002). Also, CHRNA4 has been reported to be connected to autosomal dominant nocturnal frontal lobe epilepsy (Neng et al., 2020). Carbamazepine was reported to inhibit neuronal nicotinic receptors, including the alpha 4 subunit (CHRNA4) (Picard et al., 1999). Also, carbamazepine was

described as effective in treating frontal lobe epilepsy (Picard et al., 1999). Some of the carbamazepine's therapeutical effects are linked to the blockage of voltage-gated sodium channels (Tolou-Ghamarie et al., 2013). The sodium channel (SCN) family of genes generates and propagates the action potential in neurons (Tolou-Ghamarie et al., 2013). Carbamazepine acts by decreasing the firing of SCN in case of a seizure. Sodium channels are formed by three different subunits: one alpha and two beta subunits. The alpha subunit acts as a voltage sensor and forms the channel's pore, while the beta subunits regulate the function of SCN (Meisler and Kearney, 2005). Different genes encode the different subunits: SCN($_{1–10}$)A genes encode the subunit alpha, and the beta subunits are encoded by SCN($_{1–4}$)B genes (Lin et al., 2021). Action on the neuronal acetylcholine receptor subunit alpha-4 (CHRNA4) and the sodium channel family of genes (SCN), were taken as MIEs for the drafting of our AOP (Figure 1) (https://aopwiki.org/; ID: 91).

As visible in this draft, no linkages to adverse outcomes could be found at the cellular level. On the other hand, different organ responses were presented. Both MIEs are implicated in a direct action on neurotransmission. Data from the literature shows that genetic variations in CHRNA4 can attenuate the synaptic and extrasynaptic transmission of GABA and cause abnormal glutamate release during slow-wave sleep (Zhu et al., 2008). More recently, Breckel et al. (2015) showed that polymorphisms in this gene could affect the dopaminergic and cholinergic systems, impacting cognitive functions, as well as altering the effect of nicotine on distractor interference. Besides CHRNA4, the SCN gene family is linked to different neurological dysfunctions. Among these are developmental and epileptic encephalopathy, a group of complex disorders with early onset (during the first year of the patients' life) of hard-to-control seizures, movement disturbances, intellectual disability and elevated risk of sudden death (Meisler et al., 2021). Exacerbation epilepsies related to mutations in SCN1A and SCN8A genes, occurring through action of the GABAergic system were also described (Muligan et al., 2019; Yu et al., 2020). In the present work, it was possible to determine differences in the expression profile of different genes related to monoaminergic systems, such as the serotonergic, the noradrenergic and the dopaminergic. Also, the functional analysis resulting from shotgun proteomics indicated that some pathways linked with the metabolism of some amino acids, which have a role in neurotransmission (i.e., glycine, glutamate), were affected by carbamazepine and its main metabolite (Chapter 5). Effects on neurotransmission can ultimately lead to several dysfunctions of the impacted organism. Therefore, carbamazepine may jeopardise the homeostasis of non-target fish, with adverse outcomes resulting from exposure to this anticonvulsant and its transformation products. A good example is the potential to interfere with the organism's

energy budget and consequent fitness. Shotgun proteomic analysis performed in the scope of our work showed that CBZ and CBZep, impacted different metabolic pathways linked to energy metabolism and respiration (i.e., glycolysis; oxidative phosphorylation). However, it's not possible to infer how these metabolic pathways are connected to CHRNA4 and SCN.

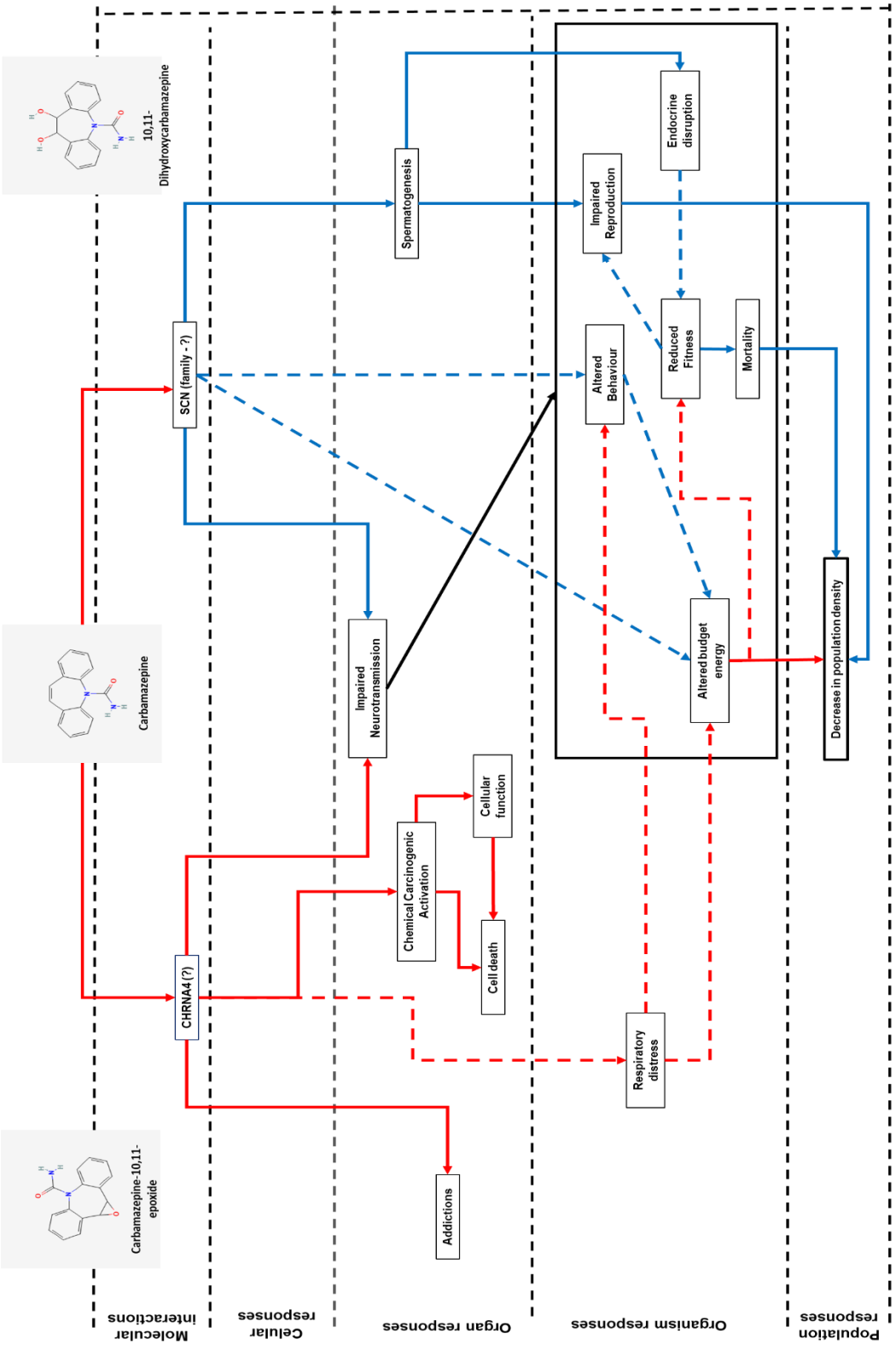

Figure 1. Draft adverse outcome pathway (AOP) for carbamazepine and its main metabolites. Solid arrows represent confirmed direct effects, while intermittent refer to indirect effects still to be confirmed. Red arrows are associated to the action on the neuronal acetylcholine receptor subunit alpha-4 (CHRNA4), blue arrows are associated to the action on the sodium channel gene family (SCN).

From our draft, CHRNA4 is directly linked to other organ responses, such as addictions and carcinogenic chemical activation. As previously mentioned, CHRNA4 encodes the nicotinic acetylcholine receptor a4 subunit and is considered a focal gene candidate for nicotine dependence in mammals (Han et al., 2011). Neuronal nicotinic acetylcholine

receptors like CHRNA4 are ligand-gated ion channels that are expressed in cellular membranes, including cancer cells (Zhao, 2016). From the proposed draft, SCN genes are directly involved with spermatogenesis and reproduction. The literature shows that sodium channels are directly involved with sperm production and maturation in humans. Pinto and colleagues (2009) demonstrated that SCN_{1-10} genes were all expressed in functional mature spermatozoa. Disruption of spermatogenesis can cause serious endocrine disruption and a deficit of reproductive function in males. Ultimately, this disruption can lead to decreased fitness, threatening population maintenance.

6.2.2 Venlafaxine

In mammals, venlafaxine has distinct grades of reuptake inhibition depending on the ingested concentration. At lower doses, it acts as an SSRI, inhibiting mainly the reuptake of serotonin, while at higher doses, it inhibits the reuptake of both serotonin and norepinephrine (Redrobe et al., 1998). This is related to a higher affinity of venlafaxine to the serotonin transporter than the norepinephrine one (Montgomery, 2008). The calculated inhibitory constants (KIs) of venlafaxine were 82nM for serotonin and 2480nM for norepinephrine (Bymaster et al., 2001). Although lower than the affinity to serotonin and norepinephrine transporters, venlafaxine also has an affinity (KI=7647nM) to the dopamine transporter and, at high doses, may affect the reuptake of dopamine (Bymaster et al., 2001; Sansone and Sansone, 2014). Based on the information available, the MIEs proposed for the draft AOP of venlafaxine were the serotonin (SLC6A4 or SERTA), norepinephrine (SLC6A2 or NET) and dopamine (SLC6A3 or DAT) transporters (Figure 2) (https://aopwiki.org; AOP: 214). At the cellular level, the adenosine 3',5'-cyclic monophosphate (cAMP) signalling pathway has a major role in the effects elicited by venlafaxine. It is directly linked to all monoamines resulting from the identified MIEs and leads to several responses from the organism. The cAMP molecule is a major messenger involved in the regulation of the organism's metabolism and cellular biological functions. Its functions include, among others, the regulation of neurotransmitter synthesis, membrane protein activity and transcription factors; as well as involvement in the synaptic transmission in ganglia (Metz and Ziff et al., 1991; Patterson et al., 2001; Guseva et al., 2014; Yan et al., 2016).

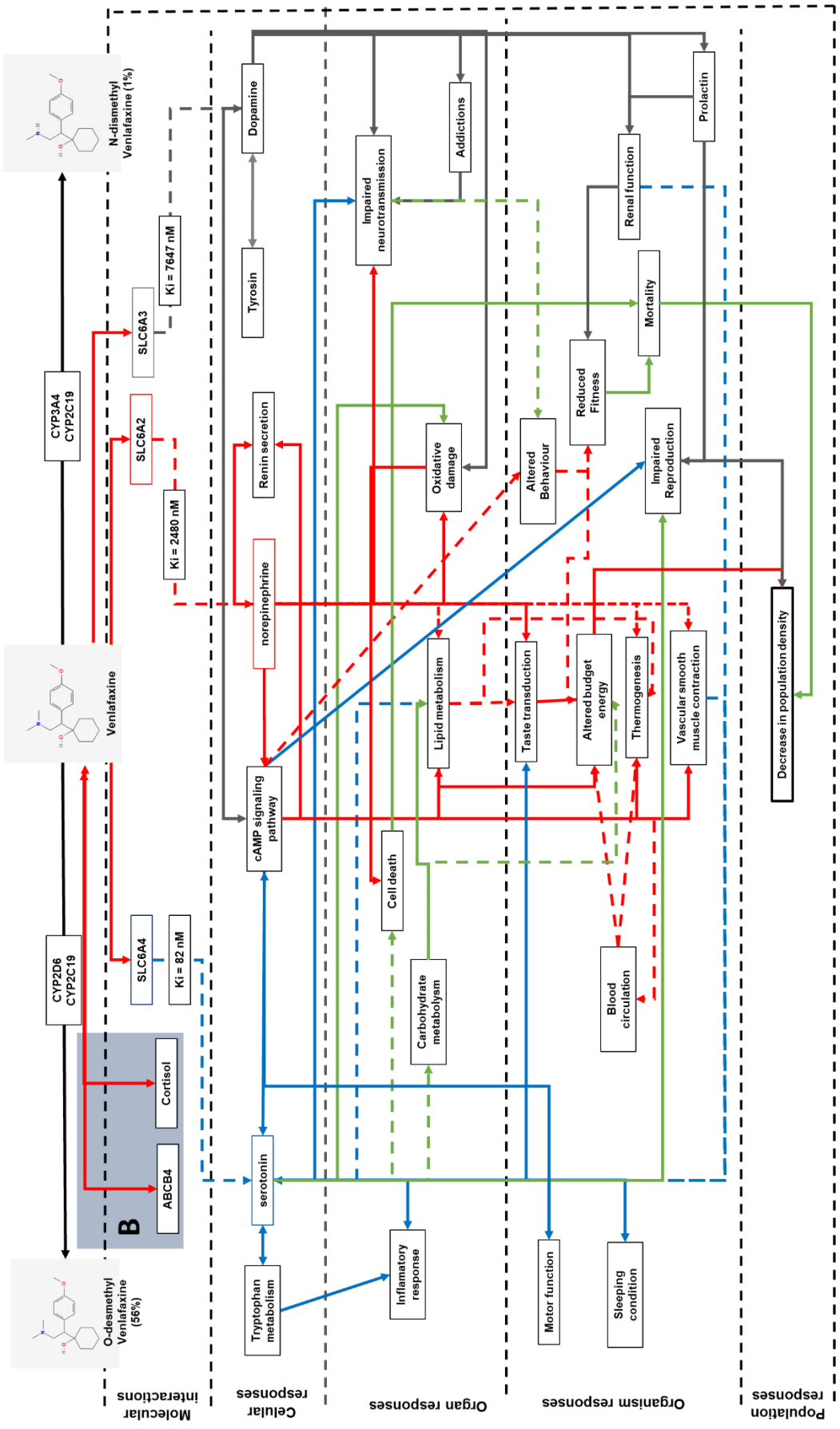

Figure 2. Draft adverse outcome pathway (AOP) for venlafaxine exposure. Solid arrows represent direct confirmed effects, while intermittent ones refer to indirect effects still to be confirmed. Red arrows are associated to norepinephrine action, blue for serotonin action, grey for dopamine and green for the compound itself.

Neurotransmission is also affected by all monoamines represented in our draft AOP. Serotonin, norepinephrine and dopamine are monoamine neurotransmitters in the vertebrate central nervous system. These monoamines are extremely important to the organism's physiology and play a crucial role in the modulation of several brain functions, such as learning, cognitive control, addictive behaviours, sensory transduction, motor function and sleep condition (Kreke and Dietrich, 2008; Zhang et al., 2010; Panlilio et al., 2012; Palacios, 2016). From the results discussed in the previous chapters, it was possible to observe that the expression of dopamine transporters and receptors and the monoamine oxidase was significantly different in organisms exposed to venlafaxine and its metabolites compared to the control. This indicates that all monoaminergic systems can be impacted upon exposure, therefore affecting the neurotransmission processes occurring via these systems. The impact on neurotransmission may be reflected in several physiological functions. This was the case of the increasing number of embryonic anomalies and the significant decrease in sensorimotor responses found for VEN and ODV. Such alterations can interfere with the organism's survival. Also, the proteomics analysis suggested that some metabolic pathways are affected by exposure to VEN and ODV. These were mainly related with energy and amino acid metabolism, which can impact vital biological processes. The results reported herein are supported by a recent literature review, which compiled all ecotoxicological effects caused by different antidepressants (venlafaxine included) on aquatic species (Salahinejad et al., 2021).

Besides the monoamine transporters, two other MIEs were considered: *abcb4* and *cortisol*. Those two MIEs are connected to the mode of action of venlafaxine in the organism. Nevertheless, no calculated inhibitory constants are available for them since venlafaxine is not an inhibitor of any of them. However, their disruption can lead to adverse effects on the exposed organism (Figure 3). In the zebrafish, *abcb4* plays the role of the functional p-glycoprotein. Furthermore, it shows the same functions as ABCB1, i.e., the functional p-glycoprotein in mammals (Fisher et al., 2013), which includes the efflux of unmodified compounds from the cells (phase 0). By itself, studies in mammals have shown that venlafaxine is a substrate of the functional p-glycoprotein (Karlsson et al., 2010). The *Abcb4* multi-xenobiotic transporter is indirectly linked to lipid metabolism, acting at the atherogenesis, affecting the lipids in macrophages (Pennings et al., 2007). Gene a*bcb4* was one of the genes whose expression was significantly changed in zebrafish larvae exposed to VEN, ODV and NDV. Cortisol is a steroid hormone which acts as a regulator of a wide range of vital processes in the organism, such as metabolism and immune response (Thau et al., 2021). Cortisol and catecholamines, mainly epinephrine and norepinephrine, are involved in the regulation of various metabolic responses (Dickerson and Kemeny, 2004). In zebrafish, venlafaxine was

described to regulate the organism's cortisol levels by upregulation of the *cAMP-pCREB* pathway. This led to an enhancement in the activity of zebrafish larvae in light/dark assays, mainly during the dark periods (Tang et al., 2019). Moreover, from the sensorimotor assay performed in this work, exposure to VEN and ODV resulted in decreased sensorimotor responses. Alterations in the behavioural phenotype may imply an alteration of feeding and escape responses that, ultimately, can reduce the survival rate and the population fitness.

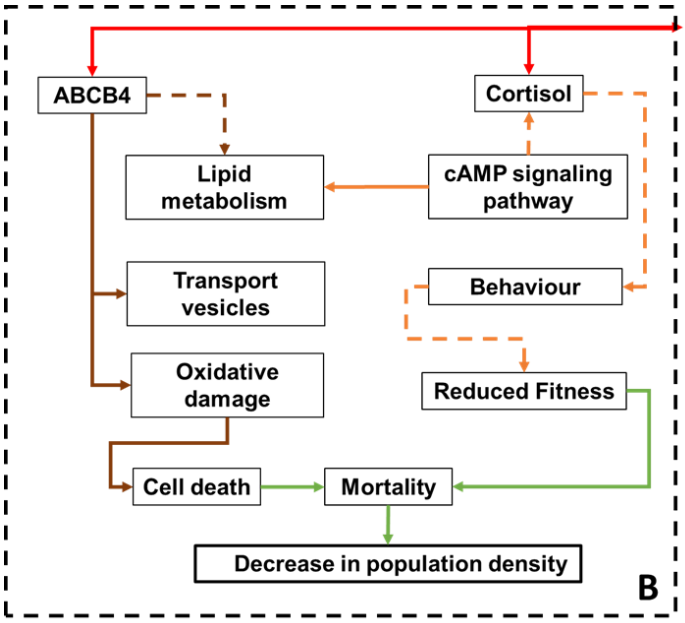

Figure 3. Schematic representation of a draft adverse outcome pathway (AOP) for abcb4 and cortisol

6.2.3 Tramadol

In mammals, tramadol is considered an opioid analgesic and an SNRI (Vazzana et al., 2015). Tramadol acts in different receptors of serotonergic, noradrenergic and opioid systems. This compound is a strong serotonin and noradrenaline reuptake inhibitor and a serotonin-releasing agent. (Vazzana et al., 2015). Besides the action on monoaminergic systems, it is also known that this opioid analgesic is an agonist of the μ-opioid receptor and, at a lower level, is an agonist of the δ- and κ-opioid receptors. An antagonistic effect was also reported upon M1 and M3 muscarinic acetylcholine receptors (Vazzana et al., 2015). Tramadol was also described to act on other receptors, such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) or the neurokinin 1 receptor (NK1), among others (Minami et al., 2011; Myiano et al., 2015). Although there is evidence of tramadol action on these receptors, the drug's

affinity to them is pretty low, and the mode of action is still not fully known (Minami et al., 2011; Myiano et al., 2015). Based on this, for tramadol and its active metabolite OTRA the serotonin (SLC6A4 or SERTA), norepinephrine (SLC6A2 or NET) transporters, μ-, δ-, and κ-opioid receptors (OPRM1; OPRD1; OPRK1) and muscarinic cholinergic receptors M1 and M3 (CHRM1; CHRM3) were considered as MIEs of the draft AOP (Figure 4) (https://aopwiki.org; AOPs: 214; 233; 234).

Tramadol is considered an analgesic prodrug, which means that the parental compound has a lower potency in terms of analgesic action than its active metabolite (Miotto et al., 2017). Therefore, the reported adverse outcomes are due to the action of both the parental compound and the active metabolite (OTRA). As previously mentioned, tramadol acts as an SNRI, and thus the proposed draft for TRA and OTRA is similar to that of VEN and its metabolites in what concerns the MIEs SLC6A4 and SLC6A2. Therefore, the cAMP signalling pathway and impaired neurotransmission are the main focus for adverse outcomes directly linked to these MIEs. These outcomes elicit various responses from the organism. These include i) regulation of neurotransmitter synthesis, membrane protein activity and transcription factors, ii) involvement in synaptic transmission in ganglia, iii) involvement in learning, cognitive control, addictive behaviours, sensory transduction, motor function and sleep condition (Metz et al., 1991; Patterson et al., 1996; Kreke and Dietrich, 2008; Zhang et al., 2010; Panlilio et al, 2012; Guseva et al., 2014; Palacios, 2016; Yan et al., 2016). From the results discussed in the previous chapters, it was possible to observe that the serotonin transporter expression differed significantly in organisms exposed to tramadol and its metabolites compared to controls. This indicates that the serotonergic system may be impaired, with implications for the neurotransmission processes. This impact on neurotransmission may disturb several physiological functions and reflect at higher levels of organisation. This was the case with the increased rates of embryonic anomalies and the significant decrease in sensorimotor responses found for TRA and OTRA, which can interfere with the organism's survival.

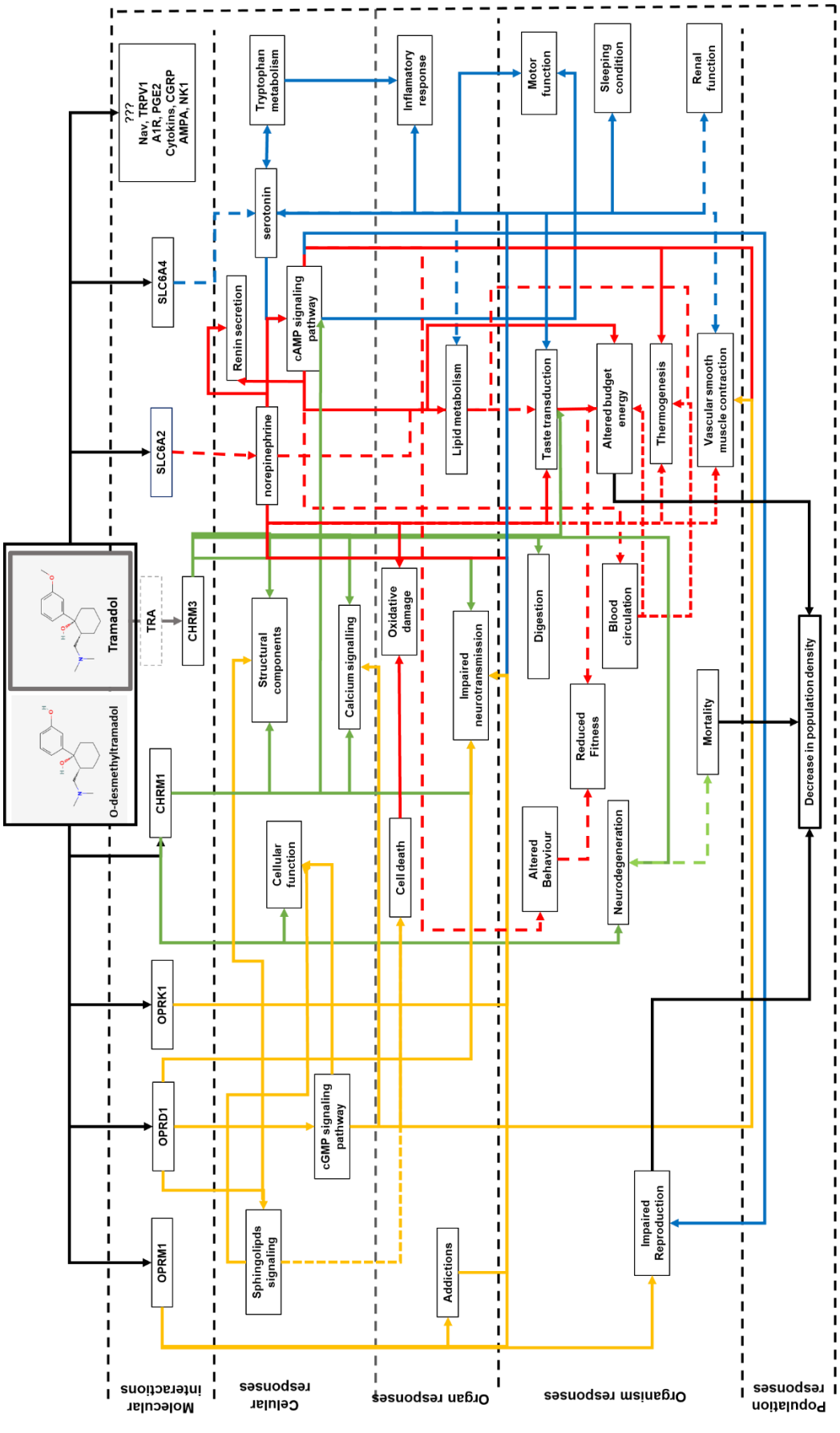

Figure 4. Draft adverse outcome pathway (AOP) for tramadol, o- and n-desmethyltramadol exposure. Solid arrows represent direct confirmed effects, while intermittent arrows refer to indirect effects still to be confirmed. Red arrows are associated to norepinephrine action, blue arrows represent the serotonin action, yellow arrows indicated the opioid receptors and green arrows represent the muscarinic receptors.

For the μ-, δ-, and κ-opioid receptors (OPRM1; OPRD1; OPRK1), several adverse outcomes are predicted in the draft of Figure 4. As in the case of monoamines, all these receptors are involved in neurotransmission, reinforcing that exposure to tramadol and its metabolites should have a major influence on this process. The OPRD1 receptor is directly involved with sphingolipids (André et al., 2008). Sphingolipids have recognised functions on the structure of cellular membranes, lipoproteins, and skin, among others (Merrill, 2008). The gene expression analysis (Chapter 4) showed that *pparg* was significantly impacted by exposure to TRA and its metabolites. This gene is reported to be involved in the regulation of lipid metabolism in fish (Wafer et al., 2017). Also, results from the shotgun proteomics (Chapter 5) showed that TRA exposure impacts the TCA cycle, which among others, is responsible for the synthesis of lipids (Cleveland and Morris, 2015). These factors reinforce the implications of TRA and its metabolites on lipid metabolism and its potential impact on several biological processes.

Tramadol can cause several adverse outcomes via its action on the muscarinic cholinergic receptors M1 and M3 (CHRM1; CHRM3), as indicated by the draft AOP (Figure 4). In addition, CHRM3 should be an MIE exclusive for the parental compound since the metabolite OTRA was previously shown to have little to no effect on this muscarinic receptor in Xenopus oocytes (Makamura et al., 2005). Like the other MIEs, neurotransmission is also an aspect that these receptors can influence. Furthermore, the results of the shotgun proteomics (Chapter 5) showed that TRA and OTRA exposure impacted several pathways associated with vital biological processes. Examples are the pathways for carbon metabolism, oxidative phosphorylation and pyruvate metabolism. These pathways are linked to ATP production, energy metabolism and cellular respiration. All of these biological processes interfere with normal homeostasis and, ultimately, can decrease the organism's chances of survival.

6.3 Conclusion

Overall, these new draft AOPs bring crucial information at various levels. From the viewpoint of human health, they contribute with information for a better understanding of the action of these pharmaceuticals and their side effects. From an environmental perspective, they bring information and insight into potential hazardous effects on a key trophic level of freshwater ecosystems, useful for monitoring and regulatory decisions. The following steps will be to improve these new drafts, e.g., with further network analysis, the linking of proteomics and genomics data, and protein-ligand analysis, as a function of the concentration levels, to obtain a more refined tool for environmental risk assessment.

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Chapter 7: Conclusions and future investigation

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This study clearly demonstrates that pharmaceutical products, not only the parental compounds but also some of their metabolites, have negative impact on zebrafish embryos and early larval stages. This impact was translated on increases of developmental anomalies, reduced sensorimotor responses, different patterns of gene expression, and significant differences in the proteome of exposed animals, impacting different pathways that are critical for the organism's survival. The main conclusions of the investigation developed for this thesis are presented in the following of the text.

The literature review presented in chapter 2 allowed to produce a concentration heatmap to easily identify the contaminants found at higher levels in various types of water samples and plan future research. The wealth of information generated also made clear that toxicological information was lacking more for pharmaceutical products than pesticides.

In chapter 3, fluoxetine elicited higher mortality than norfluoxetine. Both compounds increased the embryonic malformation rates in zebrafish. Pigmentation anomalies were the most frequently found. Opposing trend were found for gene expression patterns elicited by norfluoxetine and fluoxetine. Exposure to venlafaxine led to an increase in the rate of malformations (depigmentation and spinal deformities mainly), impaired the sensorimotor reflexes and altered the expression of genes belonging to the serotonergic, noradrenergic and dopaminergic pathways, as well as nuclear receptors related to lipid and drug metabolism. Exposure to a cocktail of levels of norfluoxetine and venlafaxine found in environmental samples caused different interaction effects, depending on the level of biological organisation and the signalling pathways possibly affected (e.g., serotonergic, noradrenergic and/or dopaminergic). In particular, increased mortality and embryo malformations were found relative to the control and single exposures, as well as antagonism and inverse agonism at the molecular level. These works indicated embryo pigmentation and the expression of several genes (e.g., *adra2b*, *5-ht2c*, *drd1b*, *vmat2* as promising biomarkers of fish exposure to selective monoamine reuptake inhibitors.

In chapter 4, in the presence of different concentrations of VEN, CBZ and TRA, as well as their metabolites (ODV, NDV, CBZep, diCBZ, OTRA and NTRA) mortality was not affected. On the other hand, exposure to the parental compounds or one of their respective metabolites (ODV, CBZep, and OTRA) lead to an increase in the rate of gross anomalies during embryonic development. Of the tested compounds, CBZ, CBZep, ODV and TRA were those eliciting the highest malformation rates, compared to the control. The most affected endpoint was the deformation of the yolk sac, often with small edemas.

All tested compounds significantly decreased larvae responses on a sensorimotor assay. Some of the significant differences registered for some anomalies and sensorimotor responses were found at environmentally relevant concentrations. This happened not only for parental compounds but also for metabolites. More importantly, exposure to the metabolite ODV was responsible for a higher increase of anomalies, than its parental compound. Among the gene evaluated, *abcc1*, *abcc2*, *abcg2a*, *nrf2*, *pparg* and *raraa* showed expression models common to the three drug blocks, suggesting that exposure to the tested pharmaceuticals may increase cellular efflux, antioxidant defences and adipogenesis in zebrafish. Additionally, for the CBZ and VEN blocks some genes showed the same pattern of expression for the parental compound and the corresponding metabolites; *drd2b*, *5-ht2c*, *5-ht1a*, *abcg2a* for the CBZ block, and *rxrbb*, *rxraa*, *abcb4* for the VEN block. These are promising candidates as biomarkers of exposure.

In chapter 5, the shotgun proteomics identified 130 proteins differentially expressed after 168h of exposure to CBZ and CBZep, which affected 18 KEGGs with different physiological functions on zebrafish larvae, such as energy metabolism, amino acid synthesis and metabolism, fatty acids degradation and cardiac function. From these proteins, sec23b is a potential biomarker of exposure for these compounds For VEN and ODV, 42 DEPs were found, which affected seven KEGGs, involved in pathways related to energy metabolism, amino acid synthesis and metabolism, and protein synthesis. For TRA and OTRA, 162 DEPs were found, which were associated to 12 KEGGs related to energy metabolism, cellular respiration, mitochondria and amino acid and protein synthesis. Several DEPs were found to be potential biomarkers of exposure (ie., actr2aa; agmat; anpebb; atp5pd; pfkma; rplp2l) for these compounds. For all drugs, the most affected treatments were the lowest test concentration of the parental compound (0.1µg/L) and the highest (100µg/L) of the respective metabolite, indicating their potentially lower potency.

Finally, in chapter 6, the integration of all data generated allowed to contribute for the AOPs of these drugs (https://aopwiki.org; ID: 91; AOP: 214; 233; 234) by generating new drafts and bringing additional knowledge relevant from both the human and environmental health perspectives.

Although this work presents a great effort to better understand the effects of metabolites on aquatic species, it only scratches the surface of what can be done in this field of knowledge. Existing data regarding the effects of drug metabolites is still sparse, and the potential number of harmful transformation products in the environment is substantially higher than the number of transformation products studied in detail in this work, and even the number of compounds whose concentrations were determined in the literature review that was performed in the first chapters. It is important to notice that

some obtained data, were not possible to include in appropriate time. To understand if the embryos chorion has a protective effect against xenobiotics, molecular analysis using qPCR technique for the same set of genes was performed for embryos exposed to the tested compounds and concentrations until 56hpf. Analysis to understand if embryos are already impacted at this stage, as well as compare the expression patterns between 56 and 168hpf, is still ongoing. Also, the AOP drafts need further to be analysed and detailed. To better elucidate some of the networks, determination of some biochemical biomarkers in the exposed animals will add relevant information at another important level of biological organisation. In the short period of time, these two points should be the focus of future work.

From this work itself, some questions arise for potential long-term investigation. Firstly, only single exposures of the compounds were performed for most of the transformation products investigated herein. In the natural environment, aquatic species are threatened by a mixture of different compounds in water. Therefore, it would be important to test complex mixtures of different metabolites and parental compounds and address their toxicity and potential relationships that can occur between them (e.g., antagonism, synergism, reverse antagonism). Also, it would be important to address possible delayed effects of the exposure during the embryonic phase on the swimming and reproductive behaviours, as well as to establish behavioural tests to assess preference or avoidance for contaminated medium. Moreover, the same methodology performed in this work and above-mentioned in the future perspectives, should be carried out for other groups of compounds as the case of the pesticides, which were initially thought to be included in the present research.

In conclusion, non-target organisms like zebrafish may already suffer adverse effects at different levels of biological organization due to the presence of pharmaceutical metabolites in ecosystems. However, future developments in this area are needed for a better understanding of the effects of these compounds on these organisms and provide useful knowledge and assessment tools to be applied in new, more efficient water treatments, monitoring campaigns, risk assessment calculations and even to establish new legislation defining regulatory levels of metabolites in natural and effluent waters.

Appendix A1. Occurrence of pharmaceutical and pesticide transformation products in freshwater: update on environmental levels, toxicological information and future challenges

Table S1. Pesticides transformation products detected and quantified in real water samples and detection methods used.

Parental compound	Byproduct	Concentration (ng/L)	Water compartment	Detection method	Reference
Triazine herbicide					
Atrazine	Desethylatrazine	620	groundwater	GC-EPD, HPLC-MS-MS and HPLC-UV	Fava et al., (2010)
		81	storm flow	LC-MS/MS	McKnight (2015)
		46	base flow		
		100	groundwater		
		540	groundwater	GC-MS/MS	Kolpin et al., (2004)
		850	river water	LC-MS	Verstraeten et al., (2002)
		860	well collector		
		850	groundwater		
	desiosopropylatrazine	32	storm flow	LC-MS/MS	McKnight (2015)
		100	groundwater		
		620	groundwater	GC-MS/MS	Kolpin et al., (2004)
		200	wetland	SPE-HPLC	Papadopoulos et al., (2007)
		80	groundwater	GC-EPD, HPLC-MS-MS and HPLC-UV	Fava et al., (2010)
	Hydroxyatrazine	11600	river water	LC-MS	Verstraeten et al., (2002)
		730	well collector		
		170	groundwater	GC-MS/MS	Kolpin et al., (2004)
	desethylhidroxyatrazine	9 × 10^4	groundwater	GC-MS/MS	Kolpin et al., (2004)
	desiosopropyl-hydroxyatrazine	120	river water	LC-MS	Verstraeten et al., (2002)
		450	well collector		
		3840	wetland	SPE-HPLC	Papadopoulos et al., (2007)
	didealkylatrazine	260	river water	LC-MS	Verstraeten et al., (2002)
		250	well collector		
		2680	groundwater	GC-MS/MS	Kolpin et al., (2004)

	deethyldeisopropylatrazine	890	groundwater	unknown	Steele and Cania (2005)
		314	drinking water	UHPLC-ESI-MS/MS	Wang et al., (2020a)
Therbuthylazine	desethyl-terbuthylazine	5210	wetland	SPE-HPLC	Papadopoulos et al., (2007)
		80	groundwater	GC-EPD, HPLC-MS-MS and HPLC-UV	Fava et al., (2010)
		120	groundwater		
	hydroxy-therbuthylazine	73	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
		1.2401 × 10^5	wetland	SPE-HPLC	Papadopoulos et al., (2007)
		2900	river water	unknown	Velisek et al., (2014)
	desethyl-hydroxy-terbuthylazine	2680	wetland	SPE-HPLC	Papadopoulos et al., (2007)
Cyanizine	deethylcyanizide acid	2190	groundwater	GC-MS/MS	Kolpin et al., (2004)
		460	well collector	LC-MS	Verstraeten et al., (2002)
		380	treated water		
	cyanizine acid	440	groundwater	GC-MS/MS	Kolpin et al., (2004)
		130	river water	LC-MS	Verstraeten et al., (2002)
		300	well collector		
		140	treatd water		
	cyanizine amide	110	groundwater	GC-MS/MS	Kolpin et al., (2004)
		430	river water	LC-MS	Verstraeten et al., (2002)
		1450	well collector		
		250	treatd water		
Metamitron	desamino-metamitron	11	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Hexazinone	demethyl hexazinone B	12.8	surface water	LC-MS/MS	Mahler et al., (2021)
Simazine	hidroxysimazine	250	surface water	LC-MS/MS	Mahler et al., (2021)
Chloroacetanilides herbicides					
Metolachlor	metolachlor ESA	2920	groundwater	GC-MS/MS	Kolpin et al., (2004)
		620	river water	LC-MS	Verstraeten et al., (2002)
		510	well collector		
		87	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	metolachlor OXA	3600	groundwater	GC-MS/MS	Kolpin et al., (2004)
		450	river water	LC-MS	Verstraeten et al., (2002)

		240	well collector		
		121	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	metolachlor metabolite CGA357704	61	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	metolachlor metabolite NOA413173	290	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	deschlorometolachlor	453	groundwater	UPLC-MS/MS	Amalric et al., (2013)
	2-hidroxy-metolachlor	68	groundwater	UPLC-MS/MS	Amalric et al., (2013)
	metolachlor morpholimnone	950	groundwater	UPLC-MS/MS	Amalric et al., (2013)
Acetochlor	acetochlor ESA	21	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
		490	river water	LC-MS	Verstraeten et al., (2002)
		550	well collector		
		1560	groundwater	GC-MS/MS	Kolpin et al., (2004)
	acetochlor OXA	1040	river water	LC-MS	Verstraeten et al., (2002)
		1310	well collector		
		2090	groundwater	GC-MS/MS	Kolpin et al., (2004)
	acetochlor sulfinylacetic acid	2607	surface water	LC-MS/MS	Mahler et al., (2021)
	hydroxyacetochlor	451	surface water	LC-MS/MS	Mahler et al., (2021)
Metazachlor	metazachlor ESA	620	river water	LC-MS	Verstraeten et al., (2002)
		510	well collector		
		72	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	metazachlor OXA	293	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
		450	river water	LC-MS	Verstraeten et al., (2002)
		420	well collector		
Alachlor	alachlor ESA	5690	groundwater	GC-MS/MS	Kolpin et al., (2004)
		350	river water	LC-MS	Verstraeten et al., (2002)
		340	well collector		
		250	groundwater		
	alachlor OXA	4170	groundwater	GC-MS/MS	Kolpin et al., (2004)
		280	river water	LC-MS	Verstraeten et al., (2002)
		390	well collector		
		1170	bank filtered water		

Terbumeton	terbumeton-deethyl	63.9	river water	UHPLC-LC-MS/MS	Calvo et al., (2021)
Organochlorine herbicides/insecticides					
Endosulfan	endosulfan sulfate	18	surface water	SPE-GC/MS	Planas et al., (2006)
		38	stream water	SPE-GC-MS	Laabs et al., (2002)
		18	river water		
		4	surface water		
DDT	p,p'-DDT	744	river water	GC-ECD	Veljanoska-Sarafiloska et al., (2013)
		60	lake water		
		50	river water	LC-ESI-MS	Wan et al., (2005)
	o,p'-DDT	100	river water	LC-ESI-MS	Wan et al., (2005)
	p,p'-DDE	25	river water	GC-ECD	Veljanoska-Sarafiloska et al., (2013)
		900	river water	LC-ESI-MS	Wan et al., (2005)
	o,p'-DDE	800	river water	LC-ESI-MS	Wan et al., (2005)
	p,p'-DDD	13	river water	GC-ECD	Veljanoska-Sarafiloska et al., (2013)
		650	river water	LC-ESI-MS	Wan et al., (2005)
	o,p'-DDD	870	river water	LC-ESI-MS	Wan et al., (2005)
	p-p'-DDA	1749	river water	LC-ESI-MS	Wan et al., (2005)
	p,p'-DDH	28	river water	LC-ESI-MS	Wan et al., (2005)
	p,p'-DBP	166	river water	LC-ESI-MS	Wan et al., (2005)
	P,P'-DHU	10	river water	LC-ESI-MS	Wan et al., (2005)
Chlorothalonil	chlorothalonil metabolite R417888	55	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	4-Hydroxychlorothalonil	39117	surface water	LC-MS/MS	Mahler et al., (2021)
Dimethachlor	dimethachlor metabolite CGA369873	39	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Pentachloronitrobenzene	pentachloroaniline	4	surface water	SPE-GC/MS	Planas et al., (2006)
Carbamete pesticides					
Carbofuran	carbofuran-3-hydroxy	93	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
		1.546 × 10^6	surface water	HPLC-GC/MS	Otieno et., (2010)
	3-ketocarbofuran	8.94 × 10^5	surface water	HPLC-GC/MS	Otieno et., (2010)
Triallate	2,3,3-trichloro-prop-2-en-sulfonic acid	63	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)

Aldicarb	aldicarb sulfoxide	2 × 10^5	groundwater	LC-MS	Soriano et al., (2001)
		52	river water	GC/NPD HPLC	Boquene and Franco (2005)
	aldicarb sulfone	83	river water	GC/NPD HPLC	Boquene and Franco (2005)
		2.5x10^5	groundwater	LC-MS	Soriano et al., (2001)
		60	drinking water	LC-MS/MS	Sjerps et al., (2019)
Organophosphate pesticides					
Dichlorvos	2-2 dichloroacetic acid	49	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Parathion	4-nitrophenol	96	storm flow	LC-MS/MS	McKnight (2015)
	paraoxon-ethyl	200	drinking water	LC-MS/MS	Sjerps et al., (2019)
Glyphosate	aminomethylphosphonic acid	6.6x10^4	surface water	GC-NPD	Struger el al., (2008)
		3600	surface water	GC/MS-LC/MS	Battaglin et al., (2005)
		1210	drinking water	LC-MS/MS	Sjerps et al., (2019)
Benzonitrile herbicide					
Dichlobenil	dichlorobenzamide	79	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
		190	storm flow	LC-MS/MS	McKnight (2015)
		1700	base flow		
		2100	groundwater		
		3.11 × 10^6	groundwater	SPE-GC-MS	Porazzi el al., (2005)
		14	groundwater	SPE–HPLC–MS/MS	Huntscha et al., (2012)
	2,6-dichlorobenzonitrile	<5.1x10^4	groundwater	SPE-GC-MS	Porazzi el al., (2005)
	2,6-dichlorobenzoic acid	4.9x10^4	groundwater	SPE-GC-MS	Porazzi el al., (2005)
	2,6-dichlorophenol	64	base flow	LC-MS/MS	McKnight (2015)
Phenylpyrrole insecticides					
Fiprunil	fiprunil sulfone	36	municipal wastewater	GPC/GC-MS	Weston and Ludy (2014)
		18	surface water	LC-MS/MS	Mahler et al., (2021)
	fiprunil sulfide	14.8	municipal wastewater	GPC/GC-MS	Weston and Ludy (2014)
		10.6	surface water	LC-MS/MS	Mahler et al., (2021)
	fiprunil desulfinyl	11.5	municipal wastewater	GPC/GC-MS	Weston and Ludy (2014)

		10.6	surface water	LC-MS/MS	Mahler et al., (2021)
	fiprunil amide	84	surface water	LC-MS/MS	Mahler et al., (2021)
Potox-inhibitor herbicide					
Flumioxazin	3,4,5,6-tetrahydrophthalic acid	49	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Clorophenoxy herbicide					
Dichlorprop	2-(4-chlorophenoxy)propanoic acid	309	storm flow	LC-MS/MS	McKnight (2015)
		690	base flow		
		110	groundwater		
Phenoxy herbicide					
2-methyl-4-chlorophenoxyacetic acid	4-chlor-2-methylphenol	85	storm flow	LC-MS/MS	McKnight (2015)
		100	groundwater		
Chloroacetamide herbicide					
Dimethenamid	dimethenamid ESA	36	surface water	LC/MS/MS	Vargo (2003)
	dimethenamid OXA	25	surface water	LC/MS/MS	Vargo (2003)
	P-dimethenamid metabolite 27	140	groundwater	UPLC-MS/MS	Kowal et al., (2013)
Pyridazinone pesticides					
Chloridazon	chloridazon-methyl-desphenyl	930	drinking water	LC-MS/M	Sjerps et al., (2019)
		679	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	desphenylchloridazon	1.3x10^4	groundwater	SPE-LC-MS/MS-UPLC	Loos et al., (2010)
		3500	drinking water	LC-MS/M	Sjerps et al., (2019)
Phthalimide fungicide					
Captan	1R,6S-6-Carbamoylcyclohex-3-ene-1	64	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
	1,2,3,6-Tetrahydrophthalimide	39	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Urea pesticides					
Diuron	3-4-dichloroaniline	1496	surface water	SPE-GC/MS	Planas et al., (2006)

		3800	groundwater	GC/MS	Batista et al., (2002)
	dcpmu	359	surface water	LC-MS/MS	Mahler et al., (2021)
Strobirulin fungicides					
Trifloxystrobin	trifloxystrobin metabolite NOA 413161	45	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Dimoxystrobin	dimoxystrobin metabolite 505-M08	70	groundwater	UPLC-MS/MS	Kowal et al., (2013)
Acylalanine fungicides					
Metaxyl	metaxyl M-CGA108906	275	groundwater	UPLC-MS/MS	Kowal et al., (2013)
	metaxyl M-CGA62826	50	groundwater	UPLC-MS/MS	Kowal et al., (2013)
Oxyacetanilide herbicides					
Flufenacet	flufenacet M2	90	groundwater	UPLC-MS/MS	Kowal et al., (2013)
	flufenacet OA	40	groundwater	UPLC-MS/MS	Kowal et al., (2013)
Quinoline herbicides					
Quinmerac	BH-518-2	90	groundwater	UPLC-MS/MS	Kowal et al., (2013)
Phosphorothioate insecticides					
Demeton	demeton-s-methyl	1	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Triazole fungicides					
Paclobutrazol	pacobutrazol CGA149907	20	ground and surface water	LC-MS/MS	Reemtsma et al., (2013)
Systemic fungicides					
Benomyl	carbendazin	30	drinking water	LC-MS/MS	Sjerps et al., (2019)
Dichlofuanid	n,n-dimethylsulfamide	270	drinking water	LC-MS/MS	Sjerps et al., (2019)
Triazinone herbicides					
Metribuzin	desamino-metribuzin	3881	surface water	LC-MS/MS	Mahler et al., (2021)
Isoxazole herbicides					
Isoxaflutole	Diketonitrile isoxaflutole	2134	surface water	LC-MS/MS	Mahler et al., (2021)

	Isoxaflutole acid RPA	928	surface water	LC-MS/MS	Mahler et al., (2021)
Isoxazole herbicides					
Imidacloprid	desnitro-imidacloprid	167	drinking water	UHPLC-ESI-MS/MS	Wan et al., (2020b)
		4.5	surface water		
	imidacloprid urea	4.5	drinking water	UHPLC-ESI-MS/MS	Wan et al., (2020b)
		21.2	surface water		

Table S2. Pharmaceutical transformation products detected and quantified in real water samples and detections methods used.

Parental compound	Byproduct	Concentration (ng/L)	Water compartment	Detection method	Reference
Antidepressants and other psychotropic drugs					
Venlafaxine	o-desmethylvenlafaxine	50	WWTP upstream	UPLC/TOQ-MS	Archer et al., (2017)
		174.9	WWTP downstream		
		84	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
		10x10^4	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		1600	WWTP trickling filter		
		345.9	STP influent	SPE-LC-MS	Santos et al., (2010)
		330	STP efluent		
		68.7	river water		
		1600	WWTP efluent	SPE-MCX	Metcalfe et al., (2010)
		2600	untreated wastewater		
		1637	treated wastewater		
		828	WWTP influent	GC-MS	Rua-Gomez et al., (2012)
		3302	untreated wastewater		
		865	WWTP influent	UHPLC-MS/MS	Paíga et al., (2019)
		2014	WWTP influent		
	n-desmethylvenlafaxine	3.9	groundwater	SPE–HPLC–MS/MS	Huntscha et al., (2012)
		259	untreated wastewater	SPE-MCX	Metcalfe et al., (2010)
		217	treated wastewater		
	n,n-Didesmethylvenlafaxine	103.5	WWTP influent	HPLC-MS/MS	Garcia-Galan et al., (2020)
	n,o-didesmethylvenlafaxine	150	WWTP influent	LC-MS/MS	Schlüsener et al., (2015)
		140	WWTP efluent		

Fluoxetine	norfluoxetine	63	influent	PLE-SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2011)
		13	efluent		
		2.8	surface water		
		0.77	drinking water	SPE-LC-MS/MS	Santos et al., (2010)
		9.3	STP influent	HF-LPME-HPLC-MS	
		2.4	STP efluent		
		1.3	river water	SPE-LC-MS/MS	
		51.2	WWTP influent	UHPLC	Paiga et al., (2016)
		45.2	WWTP influent		
		38.5	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		30	effluent		
Sertraline	desmethylsertraline	5	STP influent	SPE-LC-MS/MS	Lajeunesse et al., (2008)
		4.7	STP efluent		
		4.5	river water		
Citalopram	desmethylcitalopram	200	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		140	WWTP trickling filter		
		80	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		72.5	effluent		
		133	untreated wastewater	SPE-MCX	Metcalfe et al., (2010)
		111	treated wastewater		
		364	WTTP effluent	UHPLC-MS/MS	Paíga et al., (2016)
Bupropion	hydroxybupropion	2600	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		4300	WWTP trickling filter		
		150	unknown	LC/Q-TOF-MS	Ferrer and Thurman (2012)
	hydrobupropion	5700	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		3500	WWTP trickling filter		
Amitriptyline	nortriptyline	185.8	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		53.8	WTTP effluent		
		4.5	STP inffluent	SPE-LC-MS/MS	Lajeunesse et al., (2008)
		3.8	STP effluent		
		0.73	river water		
	10-hidroxy-amitriptyline	64	WWTP effluent	UPLC-MS/MS	Batt el al., (2008)
Verapamil	norverapamil	71	WWTP effluent	UPLC-MS/MS	Batt el al., (2008)
Modafinil	modafinil acid	560	wastewater	LC-MS/MS	Oliveira et al., (2015)
		320	WWTP influent		
		120	WWTP efluent		
Zolpiden		340	wastewater	LC-MS/MS	Oliveira et al., (2015)

	zolpiden phenyl-4-carboxilic acid	370	WWTP influent		
		390	WWTP efluent		
Caffeine	paraxantine	110	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
Methylphenidate and Ethylphenidate	ritalinic acid	1.8	groundwater	SPE–HPLC–MS/MS	Huntscha et al., (2012)
		450	wastewater	LC-MS/MS	Oliveira et al., (2015)
		310	WWTP influent		
		130	WWTP efluent		
Ketamine	norketamine	330	WWTP influent	SPE-LC-MS/MS	Lin et al., (2014)
Levatiracetam	Levatiracetam acid	240	raw wastewater	SPE-LC-MS/MS	Zhang et al., (2020)
		75.3	WWTP efluent		
		86.2	surface water		
Anticonvulsants					
Carbamazepine	carbamazepine-10,11-epoxide	398.8	WWTP upstream	UPLC/TOQ-MS	Archer et al., (2017)
		752.2	WWTP downstream		
		105	river water	UPLC-QqLIT	Gros et al., (2012)
		1.024x10^4	WWTP influent		
		7480	WWTP efluent		
		44.4	river water	UHPLC	Paiga et al., (2016)
		4.7	WWTP influent		
		88	WWTP efluent		
		63.4	WWTP efluent		
		1.6208x10^4	municipal wastewater	UPLC-QqLIT MS/MS	Petrovic et al., (2014)
		128	drinking water		
		500	STP influent	SPE-GC-MS	Santos et al, (2010)
		300	STP efluent		
		1667	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
		33.7	groundwater	SPE–HPLC–MS/MS	Huntscha et al., (2012)
	licarbazepine	22.7	WWTP upstream	UPLC/TOQ-MS	Archer et al., (2017)
		56.9	WWTP downstream		
		255	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
		1900	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		490	WWTP trickling filter		

		4.8	surface water	SPE-LC.MS/MS	Zhang et al., (2020)
	oxcarbazepine	480	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		23	WWTP trickling filter		
	10,11-dihydroxicarbamazepine	250	WWTP activate sludge	LC/Q-TOF-MS and LC-TOF-MS	Writer et al., (2013)
		180	WWTP trickling filter		
		4000	WWTP influent	LC-MS/MS	Bahlmann et al., (2014)
		3400	WWTP efluent		
		80	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
		4	surface water	SPE-LC.MS/MS	Zhang et al., (2020)
	2-hidroxycarbamazepine	52	river water	UPLC-QqLIT	Gros et al., (2012)
		928	WWTP influent		
		646	WWTP efluent		
		1.5939x10^4	municipal wastewater	UPLC-QqLIT MS/MS	Petrovic et al., (2014)
		160	drinking water		
		61.7	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
		48	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
	3-hidroxycarbamazepine	39.9	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Diazepam	temazepam	10.6	WWTP inffluent	LC-MS/MS	Wu et al., (2015)
		1.3	river water		
		254.7	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		249.5	WTTP effluent		
	nordiazepam	2.4	surface water	GC-MS	Togola and Budzinski (2008)
		8.3	STP effluent		
		63.9	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		16	WTTP effluent		
	oxazepam	250	STP effluent	GC-MS/MS	Herberer (2002)
		40	surface water	UHPLC/MS	Hass et al., (2012)
		155.1	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		65.3	WTTP effluent		
	3-hidroxydiazepam	860	WTTP inffluent	LC/MS/MS	Hummel et al., (2006)
		630	WTTP effluent		

	desmethyl-diazepam	12.9	groundwater	SPE-LC-MS/MS	Lopez-Serna et al., (2013)
		2.85	WWTP influent	HPLC-MS/MS	Garcia-Galan et al., (2020)
Primidone	phenylethylmalonamide	540	groundwater	UHPLC/MS	Hass et al., (2012)
		420	WWTP efluent	UHPLC/MS	Hass et al., (2012)
Lamotrigine	lamotrigine-2-n-glucoronide	17	groundwater	SPE-LC/Q-TOF-M	Ferrer and Thurman (2010)
Opioids					
Tramadol	n-desmethyltramadol	16	WWTP upstream	UPLC/TOQ-MS	Archer et al., (2017)
		74	WWTP downstream		
		7192.8	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		792.5	WTTP effluent		
		181	surface water		
		209	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		341	effluent		
		208	raw wastewater	LC-MS/MS	Krizman-Matasic *et al.*, (2018)
		249	secondary effluent		
	o-desmethyltramadol	978	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		671	effluent		
		331	WWTP influent	GC-MS	Rua-Gomez et al., (2012)
		345	WWTP influent		
		207.6	WWTP upstream	UPLC/TOQ-MS	Archer et al., (2017)
		577.3	WWTP downstream		
		671	raw wastewater	LC-MS/MS	Krizman-Matasic *et al.*, (2018)
		890	secondary effluent		
Codeine	norcodeine	120	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		84.5	effluent		
		161.3	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		72.1	WTTP effluent		
	dihydrocodeine	155	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		232	effluent		
		1030.6	WTTP inffluent	SPE-LC-MS/MS	

		568.6	WTTP effluent		Baker and Kasprzyk-Hordern (2013)
Morphine	normorphine	86	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		300	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		47.1	WTTP effluent		
		26	river water	LC-MS/MS	Krizman-Matasic et al., (2018)
Methadone	EDDP	106	crude wastewater	UPLC-MS/MS	Petrie et al., (2016)
		87.5	effluent		
		342.2	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		162.3	WTTP effluent		
		8.2	groundwater	SPE-LC-ESI-MS/MS	Jurado et al., (2012)
	EMDP	5.6	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		3.3	WTTP effluent		
Propoxyphene	Norpropoxyphen	612.4	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		301.3	WTTP effluent		
Buprenorphine	norbuprenorphine	18.9	WTTP inffluent	SPE-LC-MS/MS	Baker and Kasprzyk-Hordern (2013)
		77.3	WTTP effluent		
Fentanil	norfentanil	770	wastewater	LC-MS/MS	Oliveira et al., (2015)
		300	WWTP influent		
		240	WWTP efluent		
Nonsteroidal anti-inflammatory drugs (NSAID)					
Ibuprofen	hydroxy ibuprofen	317	river water	UHPLC	Paiga et al., (2016)
		198	WWTP influent		
		349	WTTP effluent		
		334	WWTP influent		
		780	WTTP effluent		
		990	STP inffluent	SPE-GC-MS	Santos et al., (2010)
		50	STP effluent		
		68.7	WWTP impacted river	UPLC-MS/MS	Huerta et al., (2016)

		2.2909x10^4	WWTP influent	UHPLC-MS/MS	Paíga et al., (2019)
		577	WWTP influent		
	carboxy ibuprofen	1.20365x10^5	WWTP inffluent	UHPLC	Paiga et al., (2016)
		430	STP effluent	SPE-GC-MS	Santos et al., (2010)
		680	river water		
Acetylsalicylic acid	salicylic acid	2178.2	STP effluent	SPE-GC-MS/MS	Verenitch et al., (2006)
		371.5	river water		
		286.7	lake water		
		1.27x10^4	STP inffluent	SPE-GC-MS	Lee et al., (2005)
		320	STP effluent		
		620	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Diclofenac	3-hydroxy diclofenac	300	river water	LC-MS/MS	Scheurell et al., (2009)
	4-hydroxy diclofenac	48.2	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
		147	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
	5-hydroxy diclofenac	300	river water	LC-MS/MS	Scheurell et al., (2009)
		856.5	WWTP influent	HPLC-MS/MS	Garcia-Galan et al., (2020)
	Diclofenac amide	502	wastewater	LC-MS/MS	Gomez-Canela et al., (2021)
N-phenylanthranilic acid	Acridone	28	WTTP inffluent	UPLC-QqLIT	Gros et al., (2012)
		26	WTTP effluent		
		17.5	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
		8.2	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Acridone	Acridin	16.8	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
		15.8	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Analgesics and antipyretics					
Acetaminophen/Paracetamol	acetaminophen glucoronide	3570	river water	HPLC-DAD	Santos et al., (2013)
		360	river water		
		4.32*10^5	WWTP effluent	SPE-LC-MS/MS and HPLC-DAD	Sunkara and Wells (2010)
	acetaminophen sulfate	3.3x10^4	WWTP inffluent	SPE-LC-MS/MS and HPLC-DAD	Sunkara and Wells (2010)
		4.19x10^5	WWTP effluent		
	p-Aminophenol	1630	river water	HPLC-DAD	Santos et al., (2013)
		1125	river water		

		7000	WWTP inffluent	SPE-LC-MS/MS and HPLC-DAD	Sunkara and Wells (2010)
Metamizole	4-acetylamino-antipyrine	1200	WWTP efluent	LC-ESI-QTOF-MS	Herrera-Lopez et al., (2014)
		362	groundwater	LC-QTRAP-MS	Teijon et al., (2010)
		4400	wastewater	LC-MS/MS	Gomez-Canela et al., (2021)
Aminophenazone	4-formylaminoantipyrine	660	WWTP efluent	LC-ESI-QTOF-MS	Herrera-Lopez et al., (2014)
		275	groundwater	LC-QTRAP-MS	Teijon et al., (2010)
Lipid lowering drugs					
Clofibrate	clofibric acid	2593	STP inffluent	SPE-HPLC-MS	Santos et al., (2010)
		487	STP effluent		
		651	WWTP influent	HPLC-ESI-MS/MS	Roberts and Thomas (2006)
		44	WTTP effluent		
		164	surface water	UPLC–ESI/MS/MS	Baker and Kasprzyk-Hordern (2008)
Atorvastatin	o-hydroxy atorvastatine	<0,50	drinking water	SPE-LC-MS/MS	Benotti et al., (2009)
	p-hydroxy atorvastatine	<0,50	drinking water	SPE-LC-MS/MS	Benotti et al., (2009)
Sinvastatin	hidroxy sinvastatin acid	10	WWTP influent	SPE-LC-ESI-MS/MS	Vanderford and Snyder (2006)
Proton pump inhibitors					
Omeprazole	4-hydroxy omeprazole sulfide	0.028	surface water	LC-MS/MS	Garcia-Lor et al., (2014)
		0.29	WWTP effluent		
	5-hydroxy omeprazole	0.004	surface water	LC-MS/MS	Garcia-Lor et al., (2014)
		0.25	WWTP effluent		
	4-hydroxy omeprazole	60	surface water	SPE-LC-MS/MS	Boleda et al., (2013)
β-blockers					
Atenolol	metropolol acid	74	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
		298	WWTP influent	LC-HRMS	Rubirola et al., (2014)
		2500	WWTP efluent		
	atenolol-desisopropyl	6.4	groundwater	SPE–HPLC–MS/MS	Huntscha et al., (2012)
Propanolol	4-hidroxy-propanolol	21.4	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Cough Supressants					

Dextromethorphan	desmethyl-dextrophan	10	environmental samples	LC/Q-TOF-MS	Ferrer and Thurman (2012)
Diuretic/antihyperthensives					
Chlorothiazide	hydrochlorothiazide	311	WWTP impacted river	UPLC-MS/MS	Huerta et al., (2016)
Enalaprilat	Enalapril	385	wastewater	LC-MS/MS	Gomez-Canela et al., (2021)
		12.5	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
H2 (histamine 2) blockers					
Ranitidine	ranitidine N-oxide	196	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
Antibiotics and antibacterial drugs					
Sulfamethazole	desamino-sulphametazole	6	groundwater	HPLC-MS/MS	Reh et al., (2013)
	4-nitrosulphametazole	4.1	groundwater		
	n-acetyl sulfamethoxazole	5.5	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
		73.8	river water	TFC-LC-MS/M	Lopez-Serna et al., (2012)
Sulphadiazine	n-acetyl-sulphadiazine	37.2	groundwater	SPE-LC-ESI-MS/MS	Lopez-Serna et al., (2013)
Sulphamerazine	n-acetyl-sulphamerazine	18	groundwater	SPE-LC-ESI-MS/MS	Garcia-Galan et al., (2011)
Sulphamethazine	n-acetyl-sulphamethazine	57	groundwater	SPE-LC-ESI-MS/MS	Garcia-Galan et al., (2011)
Erythromicine	anydroerythromicine	300	groundwater	SPE-LC-MS	Focazio et al., (2008)
Sulfapyridine	n-acetyl-sulfapyridine	6	groundwater	SPE-LC-ESI-MS/MS	Garcia-Galan et al., (2011)
Anthelmintic drugs					
Triclabendazole	triclabendazole sulfone	9	surface water	SPE-LC/MS	Charuaud et., (2019)
	triclabendazole sulfoxide	8	surface water	SPE-LC/MS	Charuaud et., (2019)
Calcium channel blocker					
Nifepidine	dehydronifedipine	14.7	raw wastewater	SPE-LC-MS/MS	Zhang et al., (2020)
		16.8	WWTP efluent		
		25.5	surface water		
		19	groundwater	SPE-LC-MS	Focazio et al., (2008)

Diltiazem	desmethyldiltiazem	110	WWTP efluent	UPLC-MS/MS	Batt el al., (2008)
Estrogens					
Estrone	4-hidroxyestrone	14	WWTP influent	SPE-GC–NCI–MS	Xiao et al., (2001)
		27	WWTP efluent	SPE–HPLC–MS/MS	Gentili et al., (2002)
	16α-hidroxyestrone	71.7	WWTP influent	SPE-GC-MS	Jiang et al., (2005)
Estradiol	2-hidroxyestradiol	15	WWTP influent	SPE–HPLC–MS/MS	Gentili et al., (2002)
Antidiabetics					
Metformin	Guanylurea	400	WWTP influent	SPE-LC-ESI-MS/MS	Vanderford and Snyder (2006)
		1860	WWTP efluent		
		4502	river water	HILIC-QTOF-MS	Tisler and Zwiener (2018)
	Methylbiguanide	122	wastewater	HILIC-QTOF-MS	Tisler and Zwiener (2018)
		31	river water		
Antivirals					
Oseltamivir	oseltamivir carboxylate	42.7	WWTP influent	SPE-LC-MS/MS	Prasse et al., (2010)
		17.3	WWTP efluent		
Abacavir	carboxy-abacavir	960	WWTP influent	LC-MS/MS	Funke et al., (2016)
		1320	WWTP efluent		
		92	surface water		
	descyclopropyl-abacavir	80	WWTP influent	LC-MS/MS	Funke et al., (2016)
Emitricitabine	carboxy-emitricitabine	250	WWTP influent	LC-MS/MS	Funke et al., (2016)
		750	WWTP efluent		
		280	surface water		
		80	drinking water		
	emitricitabine-s-oxide	240	WWTP influent	LC-MS/MS	Funke et al., (2016)
		130	WWTP efluent		
Acyclovir	carboxy-acyclovir	3420	WWTP influent	LC-MS/MS	Funke et al., (2016)
		6690	WWTP efluent		
		750	surface water		
		41	drinking water		

Lamivudine	carboxy-lamivudine	84	WWTP influent	LC-MS/MS	Funke et al., (2016)
		530	WWTP efluent		
		230	surface water		
		84	drinking water		
Antiplatelet drugs					
Clopidrogel	clopidrogel acid	190	WWTP efluent	LC-MS/MS	Oliveira et al., (2015)
		790	wastewater		
Angiotensin receptor blocker					
Valsartan	valsartan acid	3000	wwtp treatment	LC-MS/MS	Hermes et al., (2018)
		100	WWTP influent		
		5000	WWTP efluent		
		>1000	surface water		
Anti-gout drug					
Allopurinol	Oxypirunol	>3000	wwtp treatment	LC-MS/MS	Hermes et al., (2018)
		300	drinking water	SPE-LC-MS/MS	Funk et al., (2015)
		380	groundwater		
		21700	wastewater		
		22600	surface water		
Steroids					
Testosterone	Nandrolone	5.4	raw wastewater	SPE-LC-MS/MS	Zhang et al., (2020)
		4.3	WWTP efluent		
Expectorant					
Bromhexine	Ambroxol	1047	raw wastewater	SPE-LC-MS/MS	Zhang et al., (2020)
		4.9	WWTP efluent		
		7.8	surface water		

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Appendix A2. Differential molecular responses of zebrafish larvae to fluoxetine and norfluoxetine

Table S1. Genebank accession numbers, name, function and detailed primer information for the studied target and reference genes.

Gene	Accession number	Function	Primers Sequence (5'→3')	Final Concentration (nM)	Amplicon Length (bp)	Efficiency (%)
5-ht1aa	NM_001123321.1	Serotonin receptor	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	300	80	125
5-ht2c	NM_001129893.1	Serotonin receptor	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	1000	89	126.4
abcb4	JQ014001	ABC transporter	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	ABC transporter	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	ABC transporter	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	ABC transporter	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
adra2a	NM_207637.2	Norepinephrine receptor	F: AGCGTTTTGTGACTGCTGTG R: TAATGGGATTGAGGGAGCTG	300	86	114
adra2b	NM_207638.1	Norepinephrine receptor	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	1000	80	119.7
adra2c	NM_207639.1	Norepinephrine receptor	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	1000	80	133.8
ahr2	NM_001007789.2	Aryl hydrocarbon nuclear receptor	F: TTCTGTTGCCGATTCAGATG R: CTTGTTTTGCCCATGGAGAT	300	96	113.8
Cat	NM_130912.1	Antioxidant enzyme	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113
Cu/Zn sod	Y12236	Antioxidant enzyme	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110
cyp1a1	NM_131879.1	Phase I biotransformation enzyme	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102
cyp3a65	NM_001037438.1	Phase I biotransformation enzyme	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91
Dat	NM_131755.1	Dopamine transporter	F: ACGTCAATTCTCTTTGGAGT R: TCCTCGATATCATCACTGAA	150	86	97
drd1b	NM_001135976.2	Dopamine receptor	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	600	98	117.2
drd2b	NM_197936.1	Dopamine receptor	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	300	96	110.7
Gstπ	NM_131734	Phase II biotransformation enzyme	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119
Mao	NM_212827.2	Monoamine oxidase	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	300	151	105
Net	XM_689046.5	Norepinephrine transporter	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	300	92	117
Pparα	NM_001161333.1	Peroxisome proliferator activated nuclear receptor	F: CATCTTGCCTTGCAGACATT R: CACGCTCACTTTTCATTTCAC	600	81	88.3
Pparβ	AF342937.1	Peroxisome proliferator activated nuclear receptor	F: GCGTAAGCTAGTCGCAGGTC R: TGCACCAGAGAGTCCATGTC	600	204	81.6

pparγ	DQ839547.1	Peroxisome proliferator activated nuclear receptor	F: GGTTTCATTACGGCGTTCAC F: TGGTTCACGTCACTGGAGAA	600	250	87
pxr	DQ069792.1	Pregnane X nuclear receptor	F: CTTTTTCAGACGTGCGATGA R: TTGGCACTGTCTTCTGTTGC	300	94	112.7
raraa	NM_131406.2	Retinoic acid nuclear receptor	F: GTAGTGGAGTGTGGATGTGAA R: GTGCTGATGTCTGATGGATGA	300	118	108.7
rarab	NM_131399.1	Retinoic acid nuclear receptor	F: ATGGATTACTACCACCAGAAC R: TCTCCACAGAGTGATTCGAGC	300	115	109.4
rarga	NM_131339.1	Retinoic acid nuclear receptor	F: CCCGCCAACTGTACGATGTCA R: GGGTCCAGTCCAGCATAGAAA	300	79	117.6
rxraa	NM_001161551.1	Retinoid X receptor	F: ATTCAATGGCATCTCCTG R: GCGGCTTAATATCCTCTG	600	99	101.8
rxrab	NM_131153.1	Retinoid X receptor	F: CGCCGCATCAAATCACATAAAC R: TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
rxrbb	NM_131238.1	Retinoid X receptor	F: TCACAACTTGGGCGTGGAGGC R: CGCATCTTGCAGACCAGCTCAG	300	105	100.7
rxrga	NM_131217.2	Retinoid X receptor	F: ATCTCAGTTCTTCGTTGCAGGTAG R: CGTTGATGATGGATGGGTGATGG	300	105	99.6
rxrgb	NM_001002345.1	Retinoid X receptor	F: CGCGGAATGGATACTCACG R: GCTGATGACGGACGGATGAC	300	114	97.7
serta / slc6a4a	NM_001039972.1	Serotonin transporter	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	300	73	100
vmat2	NM_001256225.2	Vesicular monoamine transporter	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	150	231	133
actb1	NM_131031.1	Reference gene	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5
ef1	NM_131263.1	Reference gene	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
rpl8	NM_200713.1	Reference gene	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

Protocol A1. Identification of amplicons procedure

To evaluate if the identity of the sequences was correct, qPCR (polymerase chain reaction) was performed in a Biometra thermocycler. qPCR reactions were performed with the following components, volumes, and concentrations, in a 20µL volume per reaction: 2µL MgCl2 (2.5mM), 4µL of 5x buffer, 1µL of forward primer and 1µL of reverse primer (1µM), 0.4 µL of DNTP's (0.2 mM), 0.1µL of TaqPolimerase (Promega) (2.5U), 9.5µL water, and 2µL of cDNA template (obtained from polls of ~40 zebrafish embryos at 80hpf stage). The reaction was performed with the following protocol: 2 minutes of denaturation at 94ºC; 40 cycles with a denaturation period of 30 seconds, followed by 30 seconds of annealing or hybridization at 51ºC, 54ºC, 55 ºC (54ºC for all genes except for vmat (51ºC), 5-ht2c and drd1b (55ºC)) and a final 30 second period of polymerisation at 72ºC; and 10 minutes at 72 ºC for a further elongation. Band size was assessed on a 2% agarose gel buffered with 1µLof TAE1x Gel Red, under UV light. Bands with the amplicon expected size were cut from and purified using the illustra GFX PCR DNA and Tm Gel Band Purification Kit (GE Healthcare). The fragments were inserted into the

vector pGEM (pGEM(R) -T Easy Vector Systems -Promega) and introduced into *E. coli* with NovaBlue Singles Competent Cells (Novagen). Selected colonies were developed on solid medium for 10 hours (35 g/L of LB Broth, ampicillin 0.1 mg/ml, X-gal 100mM and IPTG 0.1mM) at 37ºC. Plasmids were isolated from 5mL of culture medium with 5µL ampicillin and incubated overnight at 37ºC, with continuous stirring. DNA extraction was performed according to the Wizard Kit Plus SV Minipreps DNA Purification System (Promega), manufacturer instructions. Final products were sequenced by Stabvida (Portugal) and the sequence's identity was verified with the Alignment Basic Local Search Tool (Blast) at the National Centre for Biotechnology Information (NCBI).

Protocol A2. qPCR reaction volumes

qPCR reactions were performed with the following components and volumes per well: 10µL of PerfeCTa *SYBR Green* SuperMix (Quantabio), 4µL of water, 2µL of forward primer, 2µl of reverse primer and 2µL of cDNA in a total volume of 20µL.

Table S2. Results of the analysis of variance (ANOVA) performed for the normalised expression of each of the genes of the clusters with significant results in the MANOVA (cluster e, g and h) to investigating differences of treatments relative to the control solvent.

Gene	F(4,15)	p
Cluster e		
cyp3a65	5.309	0.007
drd2b	6.791	0.003
Dat	23.323	<0.000001
Mao	9.44	0.001
5-ht1a	2.74	0.068
Cluster g		
Net	0.792	0.548
serta	5.115	0.008
pparb	8.198	0.001
ahr2	6.305	0.003
Cluster h		
abcc2	6.512	0.003
Gst	3.654	0.029
rarab	19.45	<0.000001
raraa	2.182	0.121
rarga	24.729	<0.000001
rxrga	11.348	<0.000001
rxraa	7.282	0.002
5-ht2c	7.547	0.002
drd1b	2.568	0.081

Table S3. Nominal and exposure concentrations, or recovery%, reported for fluoxetine and norfluoxetine in previous works.

Nominal concentration (ng/L)	Real concentration (ng/L)	Recovery (%)	Sampling time	Media replacement	Quantification method	Reference
racemic-fluoxetine						
100	94.6 (46.4+48.2)	>80%	Every renewal	Daily	UPLC-ESI-MS/MS	[29]
Fluoxetine						
100	104	Not reported	24 hours	Daily	LC-MS/MS	[44]
1000	1000					
10000	9830					
100000	94300					
1000000	971000					

500 10000	440 8720	>87%	Daily	Daily	UPLC-MS/MS	[45]
(s)-fluoxetine						
100	105.4	>80%	Every renewal	Daily	UPLC-ESI-MS/MS	[29]
(r)-fluoxetine						
100	89.4	>80%	Every renewal	Daily	UPLC-ESI-MS/MS	[29]
(s)-norfluoxetine						
3500		62.5				
15000	Not reported	84.1	day 1, 2 and 3	Not reported	SPE-HPLC-FD	[46]
28000		91.1				
(r)-norfluoxetine						
3500		99.1				
15000	Not reported	102	day 1, 2 and 3	Not reported	SPE-HPLC-FD	[46]
28000		103				
racemic- norfluoxetine						
100	102 (46.4+48.2)	>80%	Every renewal	Daily	UPLC-ESI-MS/MS	[29]

The above mentioned studies were conducted under controlled laboratorial conditions. UPLC-ESI-MS/MS,ultra-performance liquid chromatography-electrospray tandem mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; SPE-HPLC-FD, Solid-phase extraction with high-performance liquid cromatography coupled with Chirobiotic V and fluorescence detection

Appendix A3. Norfluoxetine and venlafaxine in zebrafish larvae: single and combined toxicity of two pharmaceutical products relevant for risk assessment

Table S1. Accession numbers (Genbank), function and primer information for the target genes investigated in this study and the three reference genes used.

Gene	Accession number	Function	Primers Sequence (5'→3')	Final Concentration (nM)	Amplicon Length (bp)	Efficiency (%)
5-ht1aa	NM_001123321.1	Serotonin receptor	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	300	80	125
5-ht2c	NM_001129893.1	Serotonin receptor	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	1000	89	126.4
abcb4	JQ014001	ABC transporter	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	ABC transporter	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	ABC transporter	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	ABC transporter	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
adra2a	NM_207637.2	Norepinephrine receptor	F: AGCGTTTTGTGACTGCTGTG R: TAATGGGATTGAGGGAGCTG	300	86	114
adra2b	NM_207638.1	Norepinephrine receptor	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	1000	80	119.7
adra2c	NM_207639.1	Norepinephrine receptor	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	1000	80	133.8
ahr2	NM_001007789.2	Aryl hydrocarbon nuclear receptor	F:TTCTGTTGCCGATTCAGATG R:CTTGTTTTGCCCATGGAGAT	300	96	113.8
cat	NM_130912.1	Antioxidant enzyme	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113
Cu/Zn sod	Y12236	Antioxidant enzyme	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110
cyp1a1	NM_131879.1	Phase I biotransformation enzyme	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102
cyp3a65	NM_001037438.1	Phase I biotransformation enzyme	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91
Dat	NM_131755.1	Dopamine transporter	F:ACGTCAATTCTCTTTGGAGT R:TCCTCGATATCATCACTGAA	150	86	97
drd1b	NM_001135976.2	Dopamine receptor	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	600	98	117.2
drd2b	NM_197936.1	Dopamine receptor	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	300	96	110.7
Gstπ	NM_131734	Phase II biotransformation enzyme	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119

Mao	NM_212827.2	Monoamine oxidase	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	300	151	105
Net	XM_689046.5	Norepinephrine transporter	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	300	92	117
Pparα	NM_001161333.1	Peroxisome proliferator activated nuclear receptor	F:CATCTTGCCTTGCAGACATT R:CACGCTCACTTTTCATTTCAC	600	81	88.3
Pparβ	AF342937.1	Peroxisome proliferator activated nuclear receptor	F:GCGTAAGCTAGTCGCAGGTC R:TGCACCAGAGAGTCCATGTC	600	204	81.6
Pparγ	DQ839547.1	Peroxisome proliferator activated nuclear receptor	F:GGTTTCATTACGGCGTTCAC F:TGGTTCACGTCACTGGAGAA	600	250	87
Pxr	DQ069792.1	Pregnane X nuclear receptor	F: CTTTTTCAGACGTGCGATGA R:TTGGCACTGTCTTCTGTTGC	300	94	112.7
Raraa	NM_131406.2	Retinoic acid nuclear receptor	F:GTAGTGGAGTGTGGATGTGAA R:GTGCTGATGTCTGATGGATGA	300	118	108.7
Rarab	NM_131399.1	Retinoic acid nuclear receptor	F:ATGGATTACTACCACCAGAAC R:TCTCCACAGAGTGATTCGAGC	300	115	109.4
Rarga	NM_131339.1	Retinoic acid nuclear receptor	F:CCCGCCAACTGTACGATGTCA R:GGGTCCAGTCCAGCATAGAAA	300	79	117.6
Rxraa	NM_001161551.1	Retinoid X receptor	F:ATTCAATGGCATCTCCTG R:GCGGCTTAATATCCTCTG	600	99	101.8
Rxrab	NM_131153.1	Retinoid X receptor	F:CGCCGCATCAAATCACATAAAC R:TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
Rxrbb	NM_131238.1	Retinoid X receptor	F:TCACAACTTGGGCGTGGAGGC R:CGCATCTTGCAGACCAGCTCAG	300	105	100.7
Rxrga	NM_131217.2	Retinoid X receptor	F:ATCTCAGTTCTTCGTTGCAGGTAG R:CGTTGATGATGGATGGGTGATGG	300	105	99.6
Rxrgb	NM_001002345.1	Retinoid X receptor	F:CGCGGAATGGATACTCACG R:GCTGATGACGGACGGATGAC	300	114	97.7
serta / slc6a4a	NM_001039972.1	Serotonin transporter	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	300	73	100
vmat2	NM_001256225.2	Vesicular monoamine transporter	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	150	231	133
actb1	NM_131031.1	Reference gene	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5
ef1	NM_131263.1	Reference gene	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
rpl8	NM_200713.1	Reference gene	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

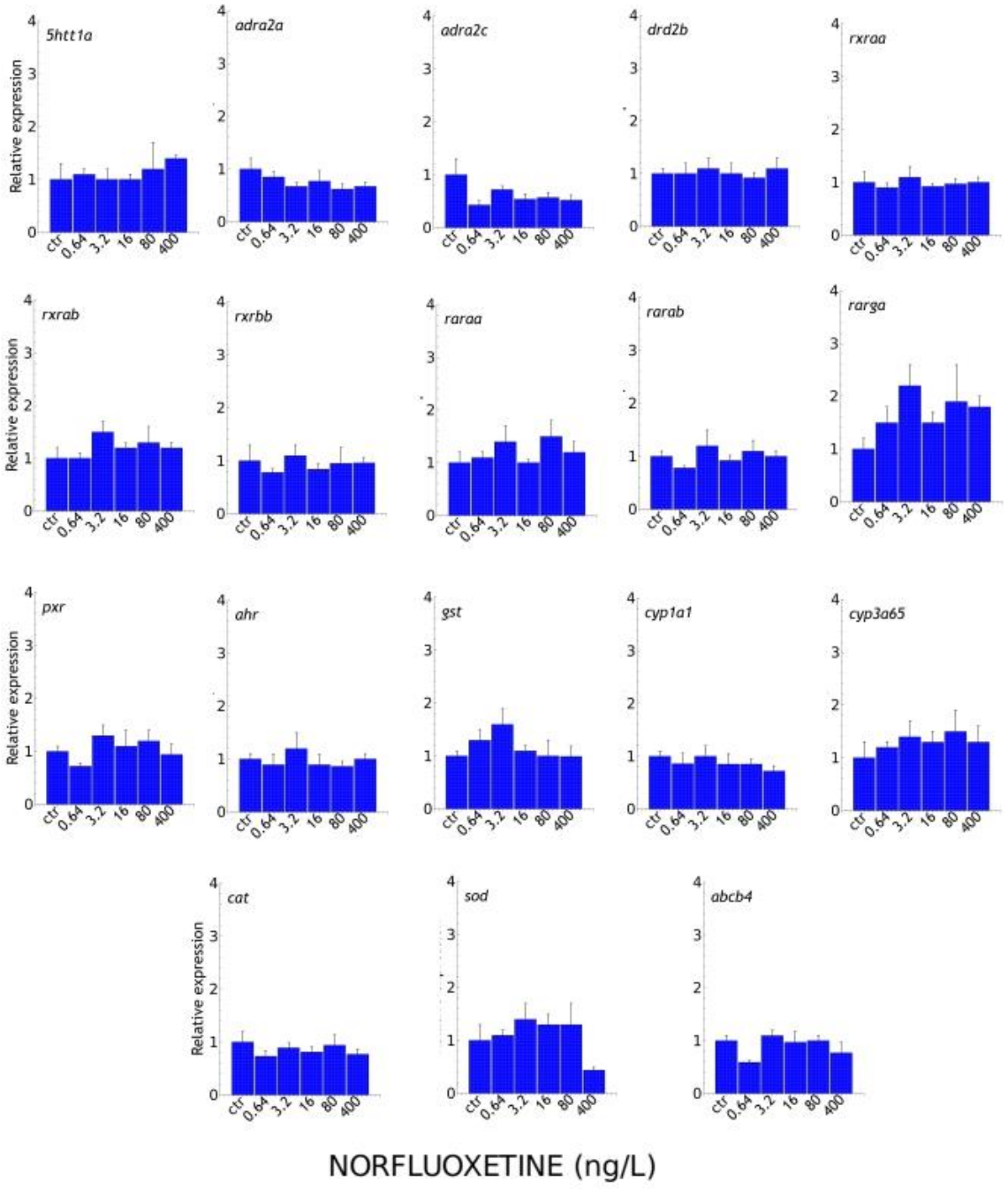

Figure S1. Gene expression levels determined in zebrafish larvae exposed to norfluoxetine for 80 hpf and in controls.

Table S2. ANOVA results for the exposure of zebrafish larvae to norfluoxetine for 80 hpf.

Gene	MS Model	df Model	MS Residual	df Residual	*F*	*p*
abcc2	1.290	5	0.919	18	1.403	0.270
abcg2a	0.736	5	1.074	18	0.685	0.641
abcb4	1.519	5	0.856	18	1.776	0.169
abcc1	1.981	5	0.727	18	2.725	0.053
Gst	1.169	5	0.953	18	1.226	0.337
Cu/Zn sod	1.796	5	0.779	18	2.305	0.087
cyp1a1	0.637	5	1.101	18	0.579	0.716
cyp3a65	0.441	5	1.155	18	0.382	0.855
Cat	0.511	5	1.136	18	0.450	0.808
Raraa	0.822	5	1.050	18	0.783	0.575
Rarab	0.683	5	1.088	18	0.628	0.681
Rarga	1.346	5	0.904	18	1.489	0.242
Rxraa	0.446	5	1.154	18	0.387	0.851
Rxrab	1.090	5	0.975	18	1.118	0.386
Rxrbb	0.376	5	1.173	18	0.321	0.894
Rxrgb	0.747	5	1.070	18	0.698	0.632
Rxrga	0.465	5	1.149	18	0.405	0.839
Ppara	1.052	5	0.985	18	1.068	0.410
Pparb	0.683	5	1.088	18	0.627	0.681
Pparg	0.742	5	1.072	18	0.693	0.635
Pxr	1.274	5	0.924	18	1.380	0.278
ahr2	0.545	5	1.126	18	0.484	0.784
5-ht2c	1.565	5	0.843	18	1.856	0.152
drd1b	1.172	5	0.952	18	1.230	0.336
drd2b	0.220	5	1.217	18	0.181	0.966
adra2b	1.207	5	0.942	18	1.281	0.315
adra2c	1.472	5	0.869	18	1.694	0.187
adra2a	0.905	5	1.026	18	0.882	0.513
Dat	1.590	5	0.836	18	1.902	0.144
Serta	0.262	5	1.205	18	0.217	0.950

Net	0.412	5	1.163	18	0.354	0.875
vmat2	1.327	5	0.909	18	1.460	0.251
Mao	1.143	5	0.960	18	1.190	0.353
5-ht1a	0.393	5	1.169	18	0.336	0.884

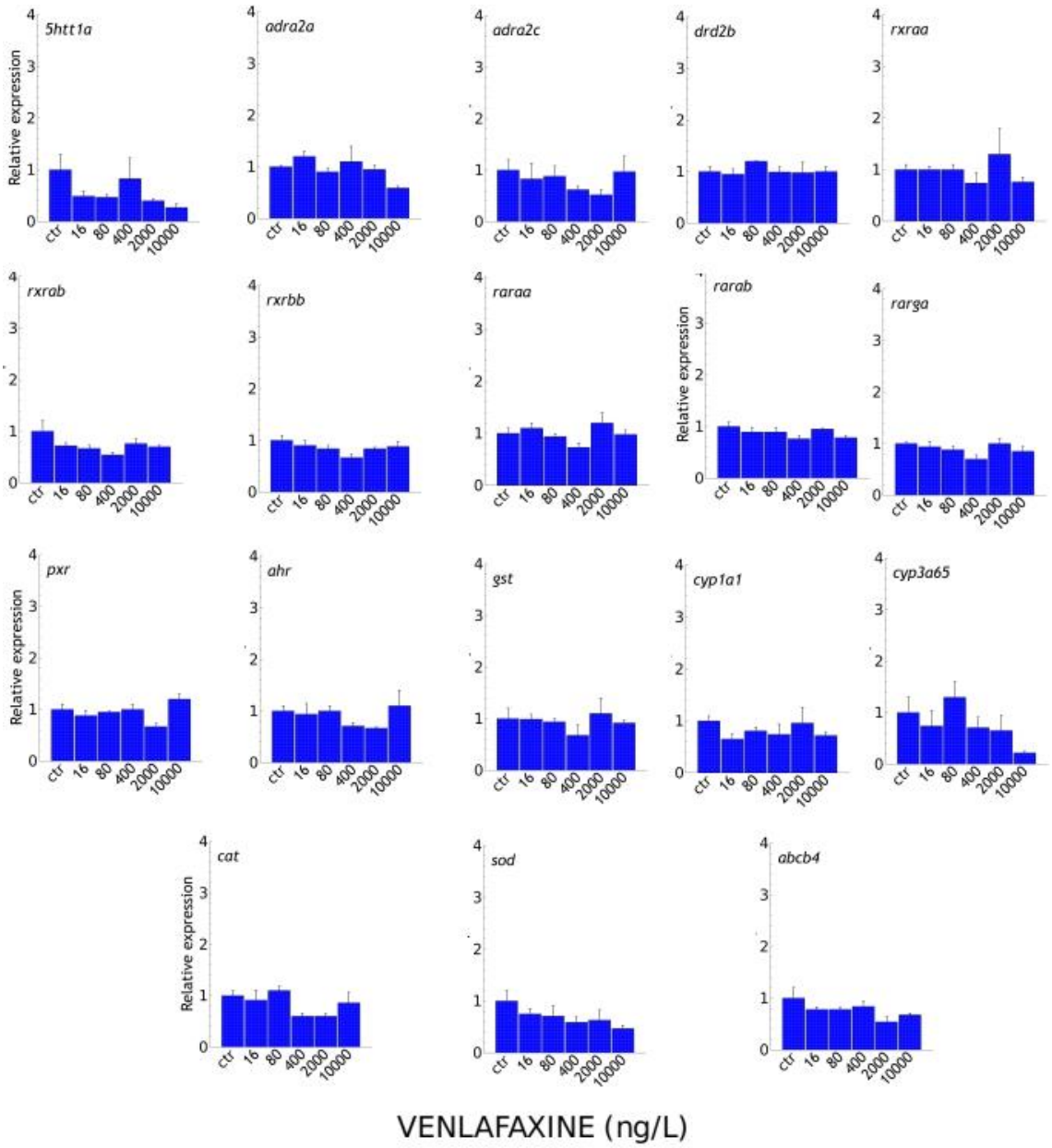

Figure S2. Gene expression levels determined in zebrafish larvae exposed to venlafaxine for 80 hpf and in controls.

Table S3. ANOVA results for the exposure of zebrafish larvae to venlafaxine for 80 hpf. Genes showing significant differences among test treatments are highlighted in bold.

Gene	MS Model	*df* Model	MS Residual	*df* Residual	*F*	*p*
abcc2	1.332	5	0.908	18	1.467	0.249
abcg2a	**2.358**	**5**	**0.623**	**18**	**3.786**	**0.016**
abcb4	1.655	5	0.818	18	2.024	0.124
abcc1	**2.460**	**5**	**0.594**	**18**	**4.140**	**0.011**
Gst	0.708	5	1.081	18	0.654	0.662
Cu/Zn sod	1.386	5	0.893	18	1.552	0.224
cyp1a1	0.797	5	1.056	18	0.754	0.594
cyp3a65	1.524	5	0.854	18	1.784	0.167
Cat	1.760	5	0.789	18	2.231	0.096
Raraa	1.368	5	0.898	18	1.523	0.232
Rarab	1.617	5	0.828	18	1.952	0.135
Rarga	1.365	5	0.898	18	1.520	0.233
Rxraa	0.822	5	1.049	18	0.784	0.575
Rxrab	1.727	5	0.798	18	2.165	0.104
Rxrbb	1.195	5	0.946	18	1.264	0.322
Rxrgb	**3.101**	**5**	**0.416**	**18**	**7.446**	**<0.001**
Rxrga	**2.090**	**5**	**0.697**	**18**	**2.998**	**0.039**
Ppara	**2.465**	**5**	**0.593**	**18**	**4.157**	**0.011**
Pparb	**2.486**	**5**	**0.587**	**18**	**4.232**	**0.010**
Pparg	**3.127**	**5**	**0.409**	**18**	**7.641**	**<0.001**
Pxr	1.988	5	0.726	18	2.740	0.052
ahr2	0.946	5	1.015	18	0.932	0.487
5-ht2c	**2.282**	**5**	**0.644**	**18**	**3.545**	**0.021**
drd1b	**2.385**	**5**	**0.615**	**18**	**3.877**	**0.015**
drd2b	0.538	5	1.128	18	0.477	0.789
adra2b	**2.902**	**5**	**0.472**	**18**	**6.155**	**0.002**
adra2c	0.878	5	1.034	18	0.849	0.533
adra2a	1.925	5	0.743	18	2.591	0.062
Dat	1.918	5	0.745	18	2.573	0.063
Serta	1.997	5	0.723	18	2.763	0.051

Net	**2.217**	**5**	**0.662**	**18**	**3.348**	**0.026**
vmat2	**2.570**	**5**	**0.564**	**18**	**4.556**	**0.007**
Mao	**2.881**	**5**	**0.478**	**18**	**6.033**	**0.002**
5-ht1a	1.616	5	0.829	18	1.949	0.136

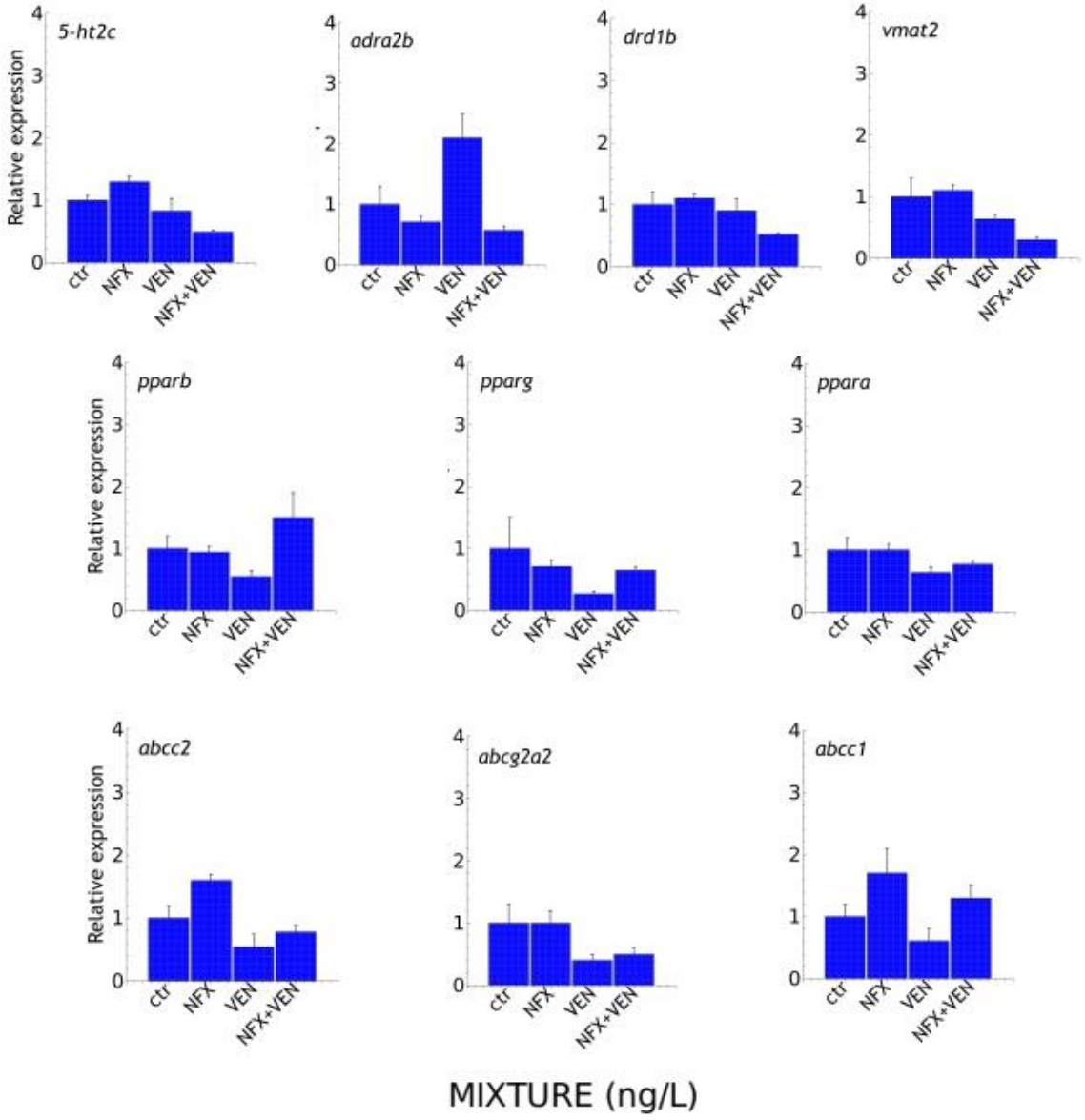

Figure S3. Gene expression levels determined in zebrafish larvae exposed to venlafaxine, norfluoxetine and mixture for 80 hpf and in controls.

Table S4. ANOVA results for the exposure of zebrafish larvae to a mixture of norfluoxetine and venlafaxine for 80 hpf. Genes showing significant differences among test treatments are highligthed in bold.

Gene	MS Model	***df*** Model	MS Residual	***df*** Residual	*F*	*p*
abcc2	**3.366**	**3**	**0.355**	**11**	**9.493**	**0.002**
abcg2a	1.751	3	0.795	11	2.201	0.145
abcc1	**2.344**	**3**	**0.633**	**11**	**3.702**	**0.046**
Ppara	1.402	3	0.890	11	1.575	0.251
Pparb	2.305	3	0.644	11	3.579	0.050
Pparg	**2.365**	**3**	**0.628**	**11**	**3.767**	**0.044**
5-ht2c	**3.113**	**3**	**0.424**	**11**	**7.348**	**0.006**
drd1b	**2.640**	**3**	**0.553**	**11**	**4.775**	**0.023**
adra2b	**3.425**	**3**	**0.339**	**11**	**10.116**	**0.002**
vmat2	**3.557**	**3**	**0.303**	**11**	**11.756**	**0.001**

Table S5. Reported data about differences from nominal to real concentrations for venlafaxine and norfluoxetine. Values for nominal and real concentrations, recovery rates, sampling time, media replacement and quantification methods are shown, if available.

Venlafaxine						
Nominal concentration (ng/L)	**Real concentration (ng/L)**	**Recovery (%)**	**Sampling time**	**Media replacement**	**Quantification method**	**Reference**
300 30000	Not reported	96	24, 96 and 144h	Daily	UHPLC-TQMS	Study conducted with zebrafish embryo Hodcovikova et al., 2019
200 1000	260 ± 8 1020 ± 14	106 to 117	2 to 7 days after exposure	Daily (40%)	SPE-QTRAP	Study conducted with immature rainbow trout Melnyk-Lamont et al., 2014
(s)-norfluoxetine						

3500		62.5				Study conducted with extract obtained from wastewater effluents Ribeiro et al., 2014
15000	Not reported	84.1	day 1, 2 and 3	Not reported	SPE-HPLC-FD	
28000		91.1				
(r)-norfluoxetine						
3500		99.1				Study conducted with extract obtained from wastewater effluents Ribeiro et al., 2014
15000	Not reported	102	day 1, 2 and 3	Not reported	SPE-HPLC-FD	
28000		103				

The above mentioned studies were conducted under controlled laboratorial conditions. UHPLC-TQMS, ultra-high-performance liquid chromatography coupled with mass spectrometry; SPE-QTRAP, Solid-phase extraction-liquid chromatrography mass spectrometry; SPE-HPLC-FD, Solid-phase extraction with high-performance liquid cromatography coupled with Chirobiotic V and fluorescence detection

Appendix A4. Carbamazepine, venlafaxine, and tramadol metabolites: toxicological effects on zebrafish embryos and larvae

Table S1. Accession numbers (Genbank), function and primer information for the target genes investigated in this study and the three reference genes used.

Gene	Accession number	Function	Primers Sequence (5'→3')	Final Concentration (nM)	Amplicon Length (bp)	Efficiency (%)
5-ht1a	NM_001123321.1	Serotonin receptor	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	300	80	125
5-ht2c	NM_001129893.1	Serotonin receptor	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	1000	89	126.4
abcb4	JQ014001	ABC transporter	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	ABC transporter	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	ABC transporter	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	ABC transporter	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
adra2b	NM_207638.1	Norepinephrine receptor	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	1000	80	119.7
adra2c	NM_207639.1	Norepinephrine receptor	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	1000	80	133.8
ahr2	NM_001007789.2	Aryl hydrocarbon nuclear receptor	F:TTCTGTTGCCGATTCAGATG R:CTTGTTTTGCCCATGGAGAT	300	96	113.8
Keap	NM_182864.2	Antioxidant enzyme	F: TGATGGACAAACCCAACTCA R: CACTGGACAGGAAACCACCT	300	n.d	117
nrf2	NM_182889.1	Antioxidant enzyme	F: TGGCCCTGAAGAATTTAACG R: CCCGGTGAGAAGCTCTGTAG	300	n.d	108
cyp3a65	NM_001037438.1	Phase I biotransformation enzyme	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91
dat	NM_131755.1	Dopamine transporter	F:ACGTCAATTCTCTTTGGAGT R:TCCTCGATATCATCACTGAA	150	86	97
drd1b	NM_001135976.2	Dopamine receptor	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	600	98	117.2
drd2b	NM_197936.1	Dopamine receptor	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	300	96	110.7
gstpi	NM_131734	Phase II biotransformation enzyme	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119
mao	NM_212827.2	Monoamine oxidase	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	300	151	105

net	XM_689046.5	Norepinephrine transporter	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	300	92	117
pparα	NM_001161333.1	Peroxisome proliferator activated nuclear receptor	F:CATCTTGCCTTGCAGACATT R:CACGCTCACTTTTCATTTCAC	600	81	88.3
pparβ	AF342937.1	Peroxisome proliferator activated nuclear receptor	F:GCGTAAGCTAGTCGCAGGTC R:TGCACCAGAGAGTCCATGTC	600	204	81.6
pparγ	DQ839547.1	Peroxisome proliferator activated nuclear receptor	F:GGTTTCATTACGGCGTTCAC F:TGGTTCACGTCACTGGAGAA	600	250	87
pxr	DQ069792.1	Pregnane X nuclear receptor	F: CTTTTTCAGACGTGCGATGA R:TTGGCACTGTCTTCTGTTGC	300	94	112.7
raraa	NM_131406.2	Retinoic acid nuclear receptor	F:GTAGTGGAGTGTGGATGTGAA R:GTGCTGATGTCTGATGGATGA	300	118	108.7
rarab	NM_131399.1	Retinoic acid nuclear receptor	F:ATGGATTACTACCACCAGAAC R:TCTCCACAGAGTGATTCGAGC	300	115	109.4
rarga	NM_131339.1	Retinoic acid nuclear receptor	F:CCCGCCAACTGTACGATGTCA R:GGGTCCAGTCCAGCATAGAAA	300	79	117.6
rxraa	NM_001161551.1	Retinoid X receptor	F:ATTCAATGGCATCTCCTG R:GCGGCTTAATATCCTCTG	600	99	101.8
rxrab	NM_131153.1	Retinoid X receptor	F:CGCCGCATCAAATCACATAAAC R:TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
rxrbb	NM_131238.1	Retinoid X receptor	F:TCACAACTTGGGCGTGGAGGC R:CGCATCTTGCAGACCAGCTCAG	300	105	100.7
rxrga	NM_131217.2	Retinoid X receptor	F:ATCTCAGTTCTTCGTTGCAGGTAG R:CGTTGATGATGGATGGGTGATGG	300	105	99.6
rxrgb	NM_001002345.1	Retinoid X receptor	F:CGCGGAATGGATACTCACG R:GCTGATGACGGACGGATGAC	300	114	97.7
sert / slc6a4a	NM_001039972.1	Serotonin transporter	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	300	73	100
vmat2	NM_001256225.2	Vesicular monoamine transporter	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	150	231	133
actb1	NM_131031.1	Reference gene	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5
ef1	NM_131263.1	Reference gene	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
rpl8	NM_200713.1	Reference gene	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

Table S2. Results of the multiple regression analysis done for the carbamazepine block.

Gene	*R2*	*df*	*F*	*p*
Sert	0.86	14, 45	9.45	<0.00001
abcg2a	0.86	14, 45	9.31	<0.00001
rarab	0.86	14, 45	8.81	<0.00001
rarga	0.85	14, 45	8.23	<0.00001
Mao	0.84	14, 45	7.52	<0.00001
rxrgb	0.83	14, 45	7.21	<0.00001
pparg	0.83	14, 45	7.11	<0.00001
adra2c	0.83	14, 45	7.09	<0.00001
rxrbb	0.83	14, 45	6.93	<0.00001
nrf2	0.81	14, 45	6.04	<0.00001
rxrab	0.80	14, 45	5.83	<0.00001
abcc1	0.80	14, 45	5.76	<0.00001
ppara	0.80	14, 45	5.63	<0.00001
abcc2	0.79	14, 45	5.45	<0.00001
drd2b	0.79	14, 45	5.40	<0.00001
raraa	0.79	14, 45	5.23	0.00001
Dat	0.78	14, 45	5.11	0.00001
cyp3a	0.78	14, 45	5.07	0.00001
Keap	0.78	14, 45	4.89	0.00002
vmat	0.77	14, 45	4.72	0.00003
5-ht2c	0.77	14, 45	4.62	0.00004
Gstpi	0.76	14, 45	4.49	0.00006
rxrga	0.76	14, 45	4.36	0.00008
adra2b	0.76	14, 45	4.33	0.00009
rxraa	0.76	14, 45	4.27	0.00010
ahr2	0.75	14, 45	4.18	0.00012
Net	0.75	14, 45	4.14	0.00014
5ht1a	0.74	14, 45	3.82	0.00030
Pxr	0.73	14, 45	3.56	0.00059
pparb	0.72	14, 45	3.49	0.00071
abcb4	0.71	14, 45	3.22	0.00144
drd1b	0.66	14, 45	2.43	0.01227

Table S3. Results of the multiple regression analysis done for the venlafaxine block.

Gene	*R2*	*df*	*F*	*p*
ahr2	0.94	14, 45	26.5	<0.00001
gstpi	0.92	14, 45	18.0	<0.00001
rarab	0.90	14, 45	13.9	<0.00001
raraa	0.89	14, 45	12.4	<0.00001
abcc1	0.89	14, 45	11.8	<0.00001
nrf2	0.88	14, 45	11.6	<0.00001
drd1b	0.87	14, 45	10.0	<0.00001
abcg2a	0.87	14, 45	9.72	<0.00001
abcc2	0.86	14, 45	9.00	<0.00001
pparg	0.86	14, 45	8.86	<0.00001
dat	0.85	14, 45	8.63	<0.00001
rxrab	0.85	14, 45	8.15	<0.00001
rxraa	0.84	14, 45	8.02	<0.00001
mao	0.83	14, 45	7.21	<0.00001
rxrbb	0.82	14, 45	6.82	<0.00001
abcb4	0.81	14, 45	6.21	<0.00001
5ht1a	0.81	14, 45	6.16	<0.00001
vmat	0.81	14, 45	6.15	<0.00001
net	0.81	14, 45	6.13	<0.00001
rarga	0.80	14, 45	5.85	<0.00001
rxrgb	0.80	14, 45	5.63	<0.00001
adra2c	0.80	14, 45	5.53	0.00001
pxr	0.79	14, 45	5.50	0.00001
rxrga	0.79	14, 45	5.47	0.00001
drd2b	0.79	14, 45	5.31	0.00001
sert	0.75	14, 45	4.14	0.00014
pparb	0.74	14, 45	3.93	0.00023
ppara	0.72	14, 45	3.54	0.00063
adra2b	0.70	14, 45	3.09	0.00204
keap	0.68	14, 45	2.73	0.00547
5-ht2c	0.68	14, 45	2.71	0.00572
cyp3a	0.66	14, 45	2.48	0.01083

Table S4. Results of the multiple regression analysis done for the tramadol block.

Gene	*R2*	*df*	*F*	*p*
nrf2	0.97	14, 45	51.9	<0.00001
dat	0.97	14, 45	46.2	<0.00001
keap	0.96	14, 45	43.1	<0.00001
rarga	0.96	14, 45	33.7	<0.00001
abcc1	0.95	14, 45	32.5	<0.00001
abcc2	0.95	14, 45	29.5	<0.00001
raraa	0.94	14, 45	26.1	<0.00001
rxrga	0.94	14, 45	24.5	<0.00001
pxr	0.94	14, 45	22.4	<0.00001
ahr2	0.93	14, 45	22.1	<0.00001
abcb4	0.92	14, 45	18.3	<0.00001
abcg2a	0.92	14, 45	17.5	<0.00001
pparg	0.92	14, 45	17.2	<0.00001
sert	0.92	14, 45	17.1	<0.00001
drd2b	0.92	14, 45	17.1	<0.00001
rxraa	0.91	14, 45	15.7	<0.00001
mao	0.90	14, 45	14.0	<0.00001
rxrbb	0.90	14, 45	13.6	<0.00001
cyp3a	0.89	14, 45	12.5	<0.00001
5ht1a	0.88	14, 45	11.3	<0.00001
rxrab	0.88	14, 45	11.1	<0.00001
ppara	0.88	14, 45	10.9	<0.00001
adra2c	0.87	14, 45	10.1	<0.00001
vmat	0.86	14, 45	8.92	<0.00001
pparb	0.86	14, 45	8.88	<0.00001
net	0.85	14, 45	8.63	<0.00001
gstpi	0.84	14, 45	7.87	<0.00001
rarab	0.83	14, 45	7.29	<0.00001
drd1b	0.82	14, 45	6.84	<0.00001
rxrgb	0.81	14, 45	6.30	<0.00001
5-ht2c	0.81	14, 45	6.29	<0.00001
adra2b	0.81	14, 45	6.24	<0.00001

Component	PLS summary TRA, 80,9% of sum of squares of the dependent variables was explained by the extracted components.									
	R²X	R²X(Cumul.)	Eigenvalues	R²Y	R²Y(Cumul.)	Q²	Limit	Q²(Cumul.)	Significance	Iterations
1	0.071429	0.071429	1.000000	0.432982	0.432982	0.26152	0.00	0.261516	S	2
2	0.071429	0.142857	1.000000	0.065366	0.498348	0.03014	0.00	0.283770	S	2
3	0.071429	0.214286	1.000000	0.071613	0.569961	0.05099	0.00	0.320289	S	2
4	0.071429	0.285714	1.000000	0.072672	0.642633	0.05846	0.00	0.360023	S	2
5	0.071429	0.357143	1.000000	0.064193	0.706827	0.09118	0.00	0.418377	S	2
6	0.071429	0.428571	1.000000	0.028642	0.735469	0.02991	0.00	0.435775	S	2
7	0.071429	0.500000	1.000000	0.020329	0.755797	0.04462	0.00	0.460953	S	2
8	0.071429	0.571429	1.000000	0.016152	0.771950	0.01092	0.00	0.466840	NS	2
9	0.071429	0.642857	1.000000	0.006591	0.778541	-0.01636	0.00	0.458118	NS	2
10	0.071429	0.714286	1.000000	0.009210	0.787751	-0.01524	0.00	0.449857	NS	2
11	0.071429	0.785714	1.000000	0.010125	0.797876	0.01226	0.00	0.456601	NS	2
12	0.071429	0.857143	1.000000	0.005201	0.803077	0.00491	0.00	0.459269	NS	2
13	0.071429	0.928571	1.000000	0.005466	0.808542	0.00402	0.00	0.461443	NS	2

Component	PLS summary CBZ, 61,4% of sum of squares of the dependent variables was explained by the extracted components.									
	R²X	R²X(Cumul.)	Eigenvalues	R²Y	R²Y(Cumul.)	Q²	Limit	Q²(Cumul.)	Significance	Iterations
1	0.071429	0.071429	1.000000	0.157031	0.157031	0.14842	0.00	0.148420	S	2
2	0.071429	0.142857	1.000000	0.224271	0.381302	0.12907	0.00	0.258337	S	2
3	0.071429	0.214286	1.000000	0.075717	0.457019	0.06917	0.00	0.309641	S	2
4	0.071429	0.285714	1.000000	0.030017	0.487036	0.01487	0.00	0.319908	S	2
5	0.071429	0.357143	1.000000	0.031483	0.518519	0.02029	0.00	0.333711	S	2
6	0.071429	0.428571	1.000000	0.020319	0.538838	-0.01283	0.00	0.325164	NS	2
7	0.071429	0.500000	1.000000	0.019009	0.557846	-0.00242	0.00	0.323528	NS	2
8	0.071429	0.571429	1.000000	0.012128	0.569974	-0.00436	0.00	0.320580	NS	2
9	0.071429	0.642857	1.000000	0.013581	0.583556	0.00790	0.00	0.325945	NS	2
10	0.071429	0.714286	1.000000	0.014014	0.597570	-0.00139	0.00	0.325012	NS	2
11	0.071429	0.785714	1.000000	0.008159	0.605729	-0.02054	0.00	0.311147	NS	2
12	0.071429	0.857143	1.000000	0.004528	0.610257	-0.00613	0.00	0.306923	NS	2
13	0.071429	0.928571	1.000000	0.003231	0.613488	0.00179	0.00	0.308161	NS	2

Component	PLS summary VEN, 66,7% of sum of squares of the dependent variables was explained by the extracted components.									
	R²X	R²X(Cumul.)	Eigenvalues	R²Y	R²Y(Cumul.)	Q²	Limit	Q²(Cumul.)	Significance	Iterations
1	0.071429	0.071429	1.000000	0.254482	0.254482	0.17134	0.00	0.171342	S	2
2	0.071429	0.142857	1.000000	0.144868	0.399350	0.11870	0.00	0.269707	S	2
3	0.071429	0.214286	1.000000	0.091789	0.491139	0.09082	0.00	0.336030	S	2
4	0.071429	0.285714	1.000000	0.060613	0.551752	0.03168	0.00	0.357063	S	2
5	0.071429	0.357143	1.000000	0.036987	0.588739	0.00778	0.00	0.362065	S	2
6	0.071429	0.428571	1.000000	0.021171	0.609910	-0.02304	0.00	0.347367	NS	2
7	0.071429	0.500000	1.000000	0.017400	0.627310	-0.01045	0.00	0.340550	NS	2
8	0.071429	0.571429	1.000000	0.007465	0.634776	-0.03089	0.00	0.320179	NS	2
9	0.071429	0.642857	1.000000	0.008419	0.643195	-0.02592	0.00	0.302558	NS	2
10	0.071429	0.714286	1.000000	0.007007	0.650202	-0.02506	0.00	0.285080	NS	2
11	0.071429	0.785714	1.000000	0.006083	0.656285	-0.00987	0.00	0.278022	NS	2
12	0.071429	0.857143	1.000000	0.010340	0.666625	-0.01496	0.00	0.267225	NS	2
13	0.071429	0.928571	0.999999	0.000000	0.666625	-0.09783	0.00	0.195539	NS	2

Figure S1. Results of the partial least squares analysis done to investigate gene expression responses of the three drug blocks, i.e. carbamazepine (CBZ), venlafaxine (VEN) and tramadol (TRA).

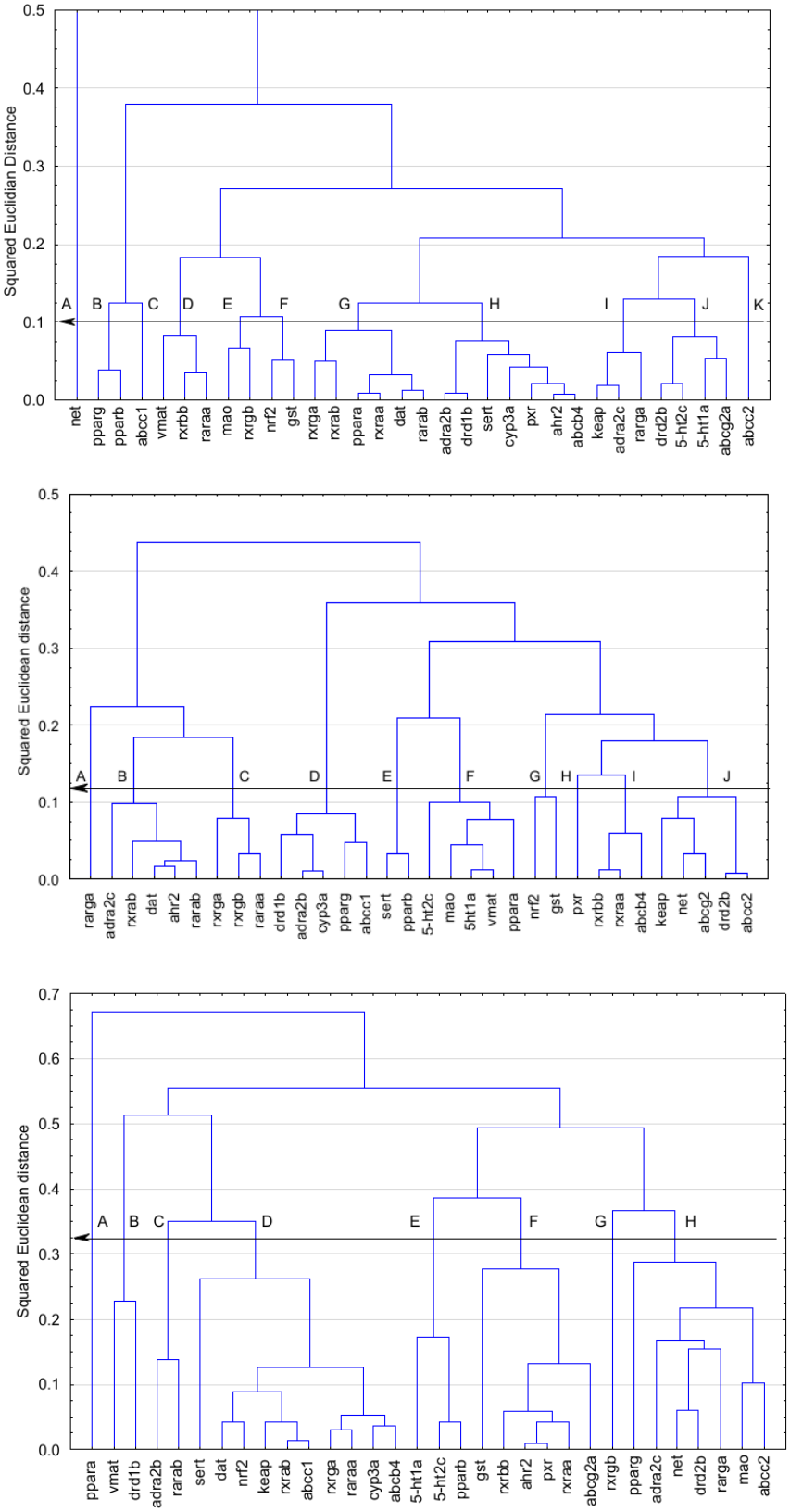

Figure S2. Groups of genes with similar response patterns identify through the Cluster Analysis done with significant Y components extracted from the Partial Least Squares Regression performed for the drug blocks carbamazepine (top), venlafaxine (middle) and tramadol (bottom). The arrows represent the cut-off value for cluster identification and the capital letters indicated the clusters.

Appendix A5. Comparison of the effects of pharmaceuticals and their metabolites on zebrafish larvae through shotgun proteomics

Table S1. Discrimination of the total significant proteins per each CBZ or CBZep treatment, comparing to control

Protein	CTR	CBZ01	CBZ100	CBZep01	CBZep100	CBZ01	CBZ100	CBZep01	CBZep100	p*sig
acp2	0	9E+06	2E+06	2387267	3870600	*				0,006267
Adka	0	2E+07	5E+06	0	0	*				0,000747
adsl	3E+07	2E+07	2E+07	3.3E+07	1.3E+08				*	0,022422
afg3l2	0	6E+06	1E+07	7044200	3070533		*			0,017665
alad	0	2E+07	6E+07	6750000	6506133		*		*	0,028824
aldh3a2b	0	2E+07	1E+07	7685933	6173600	*	*			0,017544
alyref	0	7E+07	2E+07	1.6E+07	18367000	*				0,003210
ankhd1	0	4E+06	3E+06	830333	1656167	*	*		*	0,002484
anxa1d	0	2E+07	2E+07	1.5E+07	27722667	*	*		*	0,014284
ap3m2	0	6E+06	0	0	0	*				0,000507
apo1b	9E+07	6E+08	9E+07	9.3E+07	1.11E+08	*				0,019012
arl3b	0	2E+07	5E+06	0	0	*				0,002199
bckdha	0	5E+07	3E+07	8499000	39138000	*				0,000824
bfsp2	0	1E+07	2E+07	9953000	19173667	*	*		*	0,027594
blmh	2E+05	9E+06	0	1759567	8345967		*		*	0,001187
btbd17b	0	4E+06	0	0	1480667	*			*	0,004616
cdh2	0	2E+06	1E+07	6321533	17930800				*	0,021733
cfl2	4E+06	2E+07	3E+07	6628000	24475033				*	0,022771
cirbpa	6E+07	3E+07	0	4.3E+07	0	*		*		0,011735
clic3	2E+06	9E+06	2E+06	0	0	*				0,012154
cmasa	0	2E+07	5E+06	0	3453000	*				0,003216
col14a1a	0	2E+06	4E+06	0	0	*	*			0,011795
coro1cb	1E+07	4E+07	6E+07	5.2E+07	95579667				*	0,019857
cox6c	2E+07	1E+08	2E+08	4.3E+07	85016667		*			0,023360

cox7a2a	2E+07	1E+08	2E+07	1.8E+07	18056667	*				0,019910
cplx4a	3E+06	2E+07	3E+06	2066533	4487867	*				0,019614
cpne3	3E+06	7E+06	2E+06	7188467	13869667				*	0,029153
cpvl	0	2E+07	0	5795667	5795667	*				0,005420
cryaa	0	7E+06	4E+07	6428667	35366667		*		*	0,002197
crygmx	2E+08	1E+08	4E+08	3.3E+07	2.48E+08			*		0,033358
csnk2a1	0	2E+07	0	0	0	*				0,000404
ctbp1	4E+07	9E+07	1E+08	6.9E+07	94552333		*			0,031511
cyb5r2	0	2E+07	1E+07	4408667	4408667	*				0,031406
dcps	0	9E+06	1E+07	1.3E+07	24436333		*	*	*	0,015576
ddost	0	2E+07	3E+06	0	5341333	*				0,001367
eci2	0	2E+07	0	0	0	*				0,008405
elocb	0	4E+07	0	2.9E+07	24529333	*				0,028256
emilin3a	0	7E+06	0	0	0	*				0,000129
erp44	8E+06	3E+07	4E+07	1.6E+07	18183333		*			0,022407
fga	0	2E+07	2E+07	1.1E+07	15374433	*	*		*	0,005318
Filamin B	5E+05	9E+06	1E+07	2315300	8239633	*	*		*	0,017849
fkbp1aa	1E+07	1E+08	2E+08	8.9E+07	1.27E+08	*	*		*	0,009489
gclc	0	1E+07	0	0	0	*				0,000011
gcsha	0	1E+07	5E+06	2978033	5254700	*				0,006992
gda	2E+06	7E+06	0	0	0	*				0,030761
gfap	7E+07	1E+08	1E+08	4.5E+07	90528333	*	*			0,033433
glulb	0	1E+07	5E+06	3948667	58342000				*	0,009716
gorasp2	0	8E+06	0	0	2770067	*				0,006789
guk1a	0	6E+06	675200	0	0	*				0,000087
gyg1a	0	4E+07	5E+07	1.6E+07	0		*			0,005571
hadh	1E+07	6E+07	3E+07	1.1E+07	20448333	*				0,027668
hexb	5E+06	4E+07	6E+06	7485000	0	*				0,004302

hk1	6E+07	3E+07	5E+07	2.3E+07	30965333			*		0,010361
hmgb3a	5E+07	2E+08	1E+08	3.7E+07	63508000	*				0,028872
hmgn3	0	1E+07	7E+06	0	0	*				0,030691
hmgn6	0	9E+05	1E+06	911033	5166167				*	0,006210
hmgn7	0	1E+07	2E+07	1.2E+07	15410333				*	0,012650
homer1b	0	0	3E+06	3261133	7986833		*	*	*	0,006065
igf2bp2a	0	7E+06	8E+06	3855000	14718667	*				0,027600
itgb1a	0	3E+07	1E+07	0	0	*				0,002772
khdrbs1b	2E+07	1E+08	3E+07	3.8E+07	83713333	*				0,026968
lrrc17	0	2E+07	0	5340667	6550333	*				0,026794
lrrc59	0	9E+06	2E+06	2667933	2667933	*				0,025834
mt-atp8	0	3E+07	0	0	0	*				0,000473
naa40	0	1E+07	5E+06	0	1540967	*	*			0,001364
ncalda	1E+07	3E+07	1E+07	0	0	*				0,012361
ndufb4	0	4E+07	4E+07	3E+07	23398667	*	*	*		0,009058
ndufs8a	7E+06	4E+07	4E+06	4721000	8798000	*				0,020976
nme2a	0	8E+07	2E+07	3.3E+07	39285667	*				0,013699
no code	0	4E+07	6E+07	1.5E+07	47700667					0,017569
no code	7E+07	4E+07	5E+07	2.6E+07	40104667					0,019001
nudt21	0	2E+07	0	0	0	*				0,000068
nup98	0	9E+06	7E+06	5860267	7735833	*	*		*	0,017656
nutf2l	2E+06	2E+07	2E+06	5338667	6895700	*				0,024011
oxsr1a	5E+06	1E+07	5E+07	4.4E+07	45442333		*	*	*	0,025587
papss2a	0	1E+07	0	2967600	2967600	*				0,006725
pcp4a	0	7E+06	9E+06	2096733	7097200	*	*		*	0,002030
pfdn4	0	2E+08	1E+07	1.1E+07	16368333	*				0,003971
pgam2	1E+07	1E+08	4E+07	2.4E+07	48061000	*				0,019820
picalmb	1E+06	7E+06	1E+06	0	0	*				0,004613

pin4	0	1E+07	985100	2166433	2166433	*				0,002709
polr2h	0	1E+07	0	0	6083633	*			*	0,000921
ppp2r1ba	1E+08	8E+07	7E+07	3.9E+07	7915833		*	*	*	0,019537
prps1a	3E+06	3E+07	0	5197333	5197333	*				0,004881
psat1	1E+07	9E+07	0	1.6E+07	15786000	*				0,007884
psmb6	0	3E+07	6E+06	6886667	6886667	*				0,021982
ptk7a	1E+06	0	2E+07	0	5899000		*			0,000962
pvalb7	0	3E+07	1E+07	0	0	*				0,002979
rab42a	0	0	6E+06	0	1521567		*			0,024296
rab5aa	0	8E+07	0	2E+07	30239000	*				0,007357
rad23aa	0	0	5E+07	7796100	14015100		*		*	0,000628
rhot1a	0	4E+06	0	0	0	*				0,000084
rnf20	3E+06	2E+07	7E+06	6583300	9244067	*				0,014828
rpa2	0	2E+07	0	0	0	*				0,000109
rpia	0	3E+07	0	0	2323633	*				0,000015
rpl27a	2E+07	2E+08	9E+06	0	0					0,009663
rtn1a	1E+07	9E+07	2E+07	1.1E+07	11315667	*				0,017800
s100a10a	0	4E+07	0	0	0	*				0,000000
s100s	0	8E+06	1E+06	0	0	*				0,002948
scg2b	0	2E+07	2E+07	1.5E+07	17728333	*			*	0,006259
sec23b	0	2E+07	1E+07	9197000	14859333	*	*	*	*	0,008620
serpinb1l3	0	3E+07	1E+07	8618167	10209333	*				0,020217
setd3	0	9E+06	4E+06	0	0	*				0,002441
si:ch211-113a14.11	1E+08	8E+08	2E+08	1.2E+08	2.01E+08	*				0,011904
si:ch211-222l21.1	3E+07	1E+08	1E+08	2.9E+07	14131667	*				0,016034
si:dkey-183j2.10	0	2E+07	0	0	0	*				0,000029
slc25a11	0	5E+07	1E+07	1.1E+07	7810000	*				0,025631
snap91a	0	1E+07	0	0	0	*				0,000000

snu13b	2E+07	1E+08	2E+07	0	0	*				0,005912
ssb	8E+06	3E+07	2E+07	1.7E+07	28796000	*				0,031752
ssr4	5E+06	6E+07	0	1.3E+07	12579000	*				0,007011
sub1a	7E+06	1E+08	5E+07	6E+07	42531333	*				0,025794
synpo2la	0	0	9E+06	0	2725233		*			0,002616
thbs1b	5E+06	1E+07	2E+06	4023567	7359400	*				0,023986
thoc2	0	1E+07	5E+06	2265367	0	*				0,023701
tia1l	0	2E+06	2E+07	0	3352667		*			0,006559
tpi1b	9E+07	3E+08	1E+08	9.4E+07	1.19E+08	*				0,014720
tyrp1b	1E+07	3E+07	2E+07	1.8E+07	20700333	*				0,031847
ube2ia	5E+06	4E+07	1E+07	6511000	6511000	*				0,021630
ubxn1	4E+06	2E+07	1E+07	0	8084333	*				0,032901
ufm1	0	3E+07	4E+06	0	1022800	*				0,000729
uqcrc1	4E+07	6E+07	2E+08	5.1E+07	1.24E+08		*		*	0,002470
uraha	0	2E+07	0	0	0	*				0,000458
wdr61	0	4E+07	4E+07	0	16467667	*	*			0,003973
xrn1	0	7E+06	2E+06	1467767	2769867	*				0,005360
yars1	4E+06	2E+07	3E+07	1.9E+07	44667333		*		*	0,014495
ywhabl	1E+08	3E+08	2E+08	1.1E+08	1.42E+08	*				0,012123
ywhag2	3E+07	3E+08	1E+08	8E+07	1.45E+08	*	*		*	0,001760
Zgc:100868	0	1E+06	9E+06	5493333	15222333		*		*	0,017713
zgc:122983	6E+07	2E+08	6E+07	6.9E+07	1.02E+08	*				0,022217

Table S2. Affected keggs per CBZ or CBZep treatment comparing to control, as well as the count in network, strenght, false discovery rate values and involved proteins.

Compound	CBZ					CBZep				
Comparison	CBZ0.1vsCtr (PPI enrichment p-value:0.00274)				CBZ100vsCtr (PPI enrichment p-value:0.00766)	CBZep0.1vsCtr (PPI enrichment p-value:0.0000000361)				CBZep100vsCtr (PPI enrichment p-value: 0.000286)
KEGG Pathways	dre00010 - Glycolysis / Gluconeogenesis	dre00230 - Purine metabolism	dre00260 - Glycine, serine and threonine metabolism	dre00520 - Amino sugar and nucleotide sugar metabolism	dre01100 - Metabolic pathways	dre01200 - Carbon metabolism	dre01230 - Biosynthesis of amino acids	dre03013 - RNA transport	dre00071 - Fatty acid degradation	dre00280 - Valine, leucine and isoleucine degradation	dre00190 - Oxidative phosphorylation	dre00860 - Porphyrin and chlorophyll metabolism	dre01100 - Metabolic pathways	dre04260 - Cardiac muscle contraction	dre00010 - Glycolysis / Gluconeogenesis	dre00051 - Fructose and mannose metabolism	dre00052 - Galactose metabolism	dre00500 - Starch and sucrose metabolism	dre00520 - Amino sugar and nucleotide sugar metabolism	dre01100 - Metabolic pathways	dre01200 - Carbon metabolism	dre00190 - Oxidative phosphorylation	dre00230 - Purine metabolism	dre00250 - Alanine, aspartate and glutamate metabolism	dre00860 - Porphyrin and chlorophyll metabolism	dre01100 - Metabolic pathways	dre03013 - RNA transport	dre04260 - Cardiac muscle contraction	dre03020 - RNA polymerase
Count in Network	3 of 74	7 of 157	3 of 44	3 of 60	24 of 1672	6 of 127	5 of 83	4 of 162	3 of 53	3 of 54	7 of 135	3 of 46	12 of 1672	7 of 129	4 of 74	2 of 44	2 of 33	4 of 36	5 of 60	5 of 1672	3 of 127	7 of 135	5 of 157	4 of 47	3 of 46	16 of 1672	5 of 162	5 of 129	3 of 32
Strenght	1.06	1.1	1.28	1.15	0.61	1.12	1.23	0.84	1.2	1.19	1.47	1.57	0.62	1.49	2.01	1.93	2.06	2.32	2.2	0.75	1.65	1.42	1.21	1.64	1.52	0.69	1.2	1.29	1.68
False discovery rate	0.0484	0.00017	0.0209	0.0307	5.86E-07	0.00045	0.00065	0.0487	0.029	0.029	4.05E-06	0.014	0.0025	4.05E-06	3.82E-06	0.0063	0.0043	3.71E-07	2.52E-08	0.0263	0.0014	6.69E-06	0.00055	0.00017	0.0026	6.69E-06	0.00055	0.00028	0.0011
Involved genes	*tpi1b; pgam2; aldh3a2b*	*urah; gda; guk1a; nme2a; adka; prps1a; papss2a*	*gcsha; psat1; pgam2*	*cyb5r2; hexa;cmasa*	*urah; gda; guk1a; nme2a; atp8; hexa; cmasa; cox7a2a; ndufs8a;*	*gcsha; psat1; pgam2; prps1a; tpi1b; rpia*	*psat1; pgam2; tpi1b; rpia; prps1a*	*nup98; ube2i; thoc2; alyref*	*hadh; zgc:92030; aldh3a2b*	*hadh; bckdha; aldh3a2b*	*cox6c; uqcrc1*	*alad*	*cox6c; uqcrc1; alad; gyg1a; aldh3a2b*	*cox6c; uqcrc1*	*hk1*	*hk1*	*hk1*	*hk1*	*hk1*	*hk1*	*hk1*	*uqcrc1*	*adsl*	*adsl; glulb*	*alad*	*adsl; alad; glulb; uqcrc1*	*nup98*	*uqcrc1*	*polr2h*

Table S3. Discrimination of the total significant proteins per each VEN or ODV treatment, comparing to control

Protein	CTR	VEN01	VEN100	ODV0.1	ODV100	VEN0.1	VEN100	ODV0.1	ODV100	p
adam10b	0.0E+00	0.0E+00	0.0E+00	4.5E+06	1.8E+06					0.004858
aldh1l1	5.7E+06	1.7E+06	4.5E+06	1.6E+06	4.9E+06	*		*		0.018634
atp6v1h	5.9E+06	4.2E+06	6.7E+06	5.5E+06	3.7E+06				*	0.039914
cap1	2.4E+07	1.7E+07	2.4E+07	1.7E+07	1.7E+07	*		*	*	0.012709
capn2a	5.0E+06	4.4E+06	5.0E+06	5.5E+06	3.1E+06				*	0.024736
col4a5	1.4E+07	4.3E+06	1.6E+07	0.0E+00	0.0E+00	*		*	*	0.02332
cops6	0.0E+00	0.0E+00	1.0E+07	5.9E+06	0.0E+00		*			0.000353
cpne3	4.8E+06	3.5E+06	5.0E+06	2.2E+06	0.0E+00				*	0.04346
csnk1a1	4.2E+06	0.0E+00	4.6E+06	3.2E+06	1.3E+06				*	0.030599
eif3ba	7.7E+06	1.8E+06	9.6E+06	1.6E+06	5.1E+06	*		*	*	0.03192
ela3l	7.6E+06	1.3E+07	1.5E+07	1.0E+07	1.1E+07	*	*			0.013539
fabp3	5.8E+07	1.7E+07	4.1E+07	1.3E+07	2.4E+07	*		*	*	0.004784
Fga	1.2E+07	7.2E+06	7.8E+06	3.2E+06	2.4E+06	*		*	*	0.000577
fth1b	5.1E+06	1.6E+06	5.6E+06	2.8E+06	0.0E+00				*	0.021753
fubp1	1.2E+07	3.8E+06	1.2E+07	2.2E+07	5.2E+06			*		0.025825
Gapdh	5.3E+07	2.5E+07	3.0E+07	5.8E+07	2.8E+07	*			*	0.025402
Glrx	1.4E+07	1.5E+06	1.6E+06	1.3E+07	4.9E+06	*	*		*	3.63E-05
glud1b	1.2E+07	6.9E+06	1.0E+07	7.2E+06	9.5E+06	*		*	*	0.030721
got1	2.0E+07	1.3E+07	1.5E+07	1.9E+07	1.4E+07	*			*	0.047796
gsta.1	4.5E+07	6.3E+07	3.0E+07	1.4E+08	4.7E+07			*		0.027501
Hnrnpu	7.5E+06	1.1E+07	9.9E+06	1.6E+07	7.0E+06		*	*		1.8E-05
khdrbs1b	0.0E+00	0.0E+00	0.0E+00	0.0E+00	2.6E+06				*	0.00249
l1cama	1.6E+07	1.3E+07	2.0E+07	1.7E+07	2.8E+06			*		4.46E-07

ndufs7	6.4E+06	6.5E+06	2.5E+06	0.0E+00	0.0E+00	*		*	*	0.037624
neu3.3	0.0E+00	0.0E+00	1.8E+06	2.2E+06	0.0E+00		*	*		0.002722
plxnb2a.1	0.0E+00	6.2E+05	7.6E+05	2.2E+06	0.0E+00			*		0.018688
polr2a	0.0E+00	1.8E+06	0.0E+00	6.0E+06	6.7E+05			*		0.000981
rab7a	4.8E+06	0.0E+00	2.2E+06	2.1E+07	4.5E+06			*		0.007192
rpl3	1.9E+07	8.0E+06	1.7E+07	1.3E+07	9.9E+06				*	0.041321
rplp2l	6.2E+07	7.6E+07	9.3E+07	1.2E+08	7.8E+07			*		0.030785
ruvbl2	4.7E+06	6.4E+05	5.4E+06	1.9E+06	2.8E+06	*		*	*	0.016181
Sgca	0.0E+00	0.0E+00	6.9E+05	1.1E+07	1.0E+06			*		0.048192
sh3gl2a	0.0E+00	0.0E+00	0.0E+00	2.8E+06	2.1E+06			*	*	0.002225
si:ch73-167i17.6	0.0E+00	1.5E+06	0.0E+00	8.2E+06	4.2E+06			*		0.006169
slc17a6a	0.0E+00	0.0E+00	0.0E+00	3.9E+06	2.0E+06			*	*	0.001098
slc25a3b	2.6E+07	1.9E+07	2.6E+07	2.7E+07	5.0E+07				*	0.038967
slc51a	0.0E+00	0.0E+00	0.0E+00	2.9E+06	0.0E+00			*		1.33E-11
smc3	2.2E+06	3.0E+06	6.4E+06	2.5E+06	1.5E+06				*	0.04697
sptbn1	4.0E+06	5.4E+06	4.9E+06	5.6E+06	4.7E+06			*		0.004758
Tnxba	4.5E+06	2.8E+06	6.1E+06	7.0E+06	1.8E+06				*	0.027529
Umps	6.6E+05	4.5E+06	1.2E+06	8.0E+06	3.3E+06			*		0.014759
Xpot	0.0E+00	0.0E+00	0.0E+00	2.3E+06	0.0E+00			*		2.63E-06

Table S4. Affected keggs per VEN or ODV treatment comparing to control, as well as the count in network, strenght, false discovery rate values and involved proteins.

Compound	VEN					ODV							
Comparison	VEN0.1vsCtr (PPI enrichment p-value: 0.00415)					ODV0.1vsCtr (PPI enrichment p-value: 0.0438)			ODV100vsCtr (PPI enrichment p-value:1.53e-05)				
KEGG Pathways	dre00220 - Arginine biosynthesis	dre00190- Oxidative phosphorylation	dre01200 - Carbon metabolism	dre03013 - RNA transport	dre01100 - Metabolic pathways	dre00190- Oxidative phosphorylation	dre03013 - RNA transport	dre01100 - Metabolic pathways	dre00190- Oxidative phosphorylation	dre03010 - Ribosome	dre00220 - Arginine biosynthesis	dre01200 - Carbon metabolism	dre00250 - Alanine, aspartate and glutamate metabolism
Count in Network	2 of 29	6 of 135	4 of 127	3 of 162	10 of 1672	6 of 135	4 of 162	10 of 1672	4 of 135	8 of 128	2 of 29	3 of 127	2 of 47
Strenght	1.9	1.71	1.56	1.33	0.84	1.5	1.25	0.63	1.38	1.7	1.74	1.28	1.53
False discovery rate	0.0376	8.78E-07	0.00072	0.0376	0.00013	0.0000189	0.017	0.017	0.006	2.24E-09	0.0316	0.0316	0.0476
Involved genes	*glud1b; got1*	*ndufs7*	*glud1b; got1*	*eif3ba*	*glud1b; got1; gadph*	*ndufs7*	*eif3ba; xpot*	*ndufs7; glud1b; neu3.3; upms; gsta.1*	*ndufs7; atpv61h*	*rpl3*	*got1; glud1b*	*got1; glud1b; gapdh*	*got1; glud1b*

Table S5. Discrimination of the total significant proteins per TRA or OTRA treatment, comparing to control

Protein	CTR	TRA01	TRA100	OTRA 0.1	OTRA 100	TRA 0.1	TRA100	OTRA 0.1	OTRA 100	p
ace2	6.8E+06	2.3E+06	0.0E+00	5.7E+06	1.2E+06	*	*		*	0.002616
actr2a	2.0E+07	1.3E+07	9.9E+06	9.3E+06	9.6E+06	*	*	*	*	0.008405
adka	7.8E+06	4.1E+06	0.0E+00	4.1E+06	5.1E+06		*			0.039876
adsl	1.2E+07	2.6E+06	1.0E+07	5.0E+06	1.0E+07	*		*		0.02163
adssl	2.3E+06	4.8E+05	2.0E+06	0.0E+00	1.8E+06	*		*		0.048576
agla	9.3E+06	5.2E+06	7.7E+06	5.5E+06	5.5E+06	*		*	*	0.002441
agmat	1.2E+07	5.9E+06	2.4E+06	4.5E+06	1.6E+06	*	*	*	*	0.004613
aimp2	5.6E+06	3.8E+06	8.3E+06	0.0E+00	1.3E+07				*	0.009489
akap8l	0.0E+00	7.6E+06	1.4E+06	0.0E+00	2.0E+06	*				0.000458
akr1a1b	8.2E+06	0.0E+00	0.0E+00	1.5E+06	2.6E+06	*	*	*	*	0.000109
aldh2.2	8.1E+06	0.0E+00	5.1E+06	7.6E+06	3.6E+06	*				0.014828
aldoaa	1.7E+07	1.5E+07	1.4E+07	1.8E+07	2.7E+07				*	0.044565
aldob	8.9E+06	5.9E+06	1.1E+07	1.2E+07	1.5E+07				*	0.026794
amy2a	2.5E+07	1.4E+07	1.8E+07	1.8E+07	2.0E+07	*				0.041416
anpepb	1.1E+07	7.5E+06	5.7E+06	4.9E+06	7.2E+06	*	*	*	*	0.002948
anxa5b	1.8E+07	9.3E+06	1.6E+07	2.0E+07	1.7E+07	*				0.04868
ap1b1	1.3E+07	1.3E+07	1.1E+07	1.4E+07	8.4E+06				*	0.030691

ap2m1b	3.8E+06	0.0E+00	0.0E+00	9.6E+06	1.4E+06			*		0.012123
ap3d1	5.6E+06	4.5E+06	4.1E+06	3.8E+06	1.1E+06				*	0.006559
apeh	1.4E+06	0.0E+00	1.2E+06	1.7E+06	6.0E+05	*				0.040328
apobb	3.4E+07	3.1E+07	3.5E+07	2.9E+07	2.1E+07				*	0.005571
armc6	3.0E+06	5.9E+05	0.0E+00	1.3E+06	0.0E+00	*	*		*	0.012154
atic	2.2E+07	6.2E+06	1.3E+07	0.0E+00	8.0E+06	*		*		0.017544
atp5f1c	1.4E+08	1.1E+08	1.1E+08	7.5E+07	5.9E+07		*	*	*	0.023986
atp5pd	1.1E+08	6.6E+07	6.7E+07	4.2E+07	4.1E+07	*	*	*	*	0.00203
atp6v1h	5.0E+06	4.7E+06	4.4E+06	7.1E+06	4.0E+06			*		0.021733
bckdha	5.3E+06	0.0E+00	1.8E+06	7.0E+05	0.0E+00	*	*	*	*	0.000824
bin2a	3.7E+06	0.0E+00	1.1E+06	0.0E+00	1.6E+06	*			*	0.026968
cab39l	6.6E+06	5.7E+06	6.0E+06	6.9E+06	4.6E+06				*	0.013699
calb2b	3.7E+07	3.0E+07	2.8E+07	2.6E+07	2.2E+07				*	0.036722
calr	5.8E+06	2.0E+06	5.4E+06	3.8E+06	3.0E+06	*			*	0.006992
calr3b/calrl	3.3E+07	2.2E+07	2.6E+07	3.3E+07	1.7E+07	*			*	0.020976
calua	6.2E+06	4.3E+06	0.0E+00	3.3E+06	3.4E+06		*			0.009663
cat	1.0E+07	7.2E+06	3.3E+06	7.3E+06	6.0E+06		*			0.025587
cbr1l	2.1E+07	2.0E+07	1.6E+07	1.3E+07	1.4E+07			*		0.040678

cct3	1.4E+07	7.8E+06	9.3E+06	8.0E+06	1.5E+07	*		*		0.024011
cdh1	9.8E+06	2.5E+06	3.3E+06	0.0E+00	6.5E+06	*	*	*		0.034737
ces2b	3.8E+06	0.0E+00	1.0E+07	3.9E+06	1.4E+06	*	*			1.53E-05
cfl1l	1.3E+07	0.0E+00	0.0E+00	3.4E+06	4.5E+06	*	*	*	*	0.004302
chdh	2.8E+06	0.0E+00	2.3E+06	7.7E+05	8.9E+05	*		*	*	0.011904
chordc1b	1.1E+06	0.0E+00	0.0E+00	0.0E+00	0.0E+00	*	*	*	*	3.08E-07
ckmt1	3.8E+07	3.2E+07	2.9E+07	2.7E+07	2.6E+07		*	*	*	0.009058
ckmt2a	7.6E+07	4.5E+07	4.5E+07	4.0E+07	4.0E+07	*	*	*	*	0.028824
ckmt2b	8.0E+07	5.6E+07	5.1E+07	3.1E+07	4.2E+07			*	*	0.022217
cmasa	4.2E+06	0.0E+00	1.1E+06	1.0E+06	0.0E+00	*	*	*	*	0.000747
col6a1	7.8E+06	1.2E+07	4.6E+06	9.3E+06	5.6E+06	*	*			0.012361
col9a1b	2.9E+07	1.0E+07	1.4E+07	5.5E+07	2.3E+07			*		0.049572
copb2	3.3E+06	1.1E+07	4.7E+06	6.4E+06	5.9E+06	*				0.038548
cops6	5.3E+06	6.3E+06	0.0E+00	0.0E+00	2.8E+06			*	*	0.007357
cox4i2	1.4E+07	2.7E+06	9.6E+06	6.2E+06	6.8E+06	*		*	*	0.027594
cox7a2a	1.1E+08	1.1E+08	1.1E+08	1.1E+08	7.3E+07				*	0.045793
crygm2d2	1.9E+07	0.0E+00	0.0E+00	0.0E+00	1.6E+07	*	*	*		0.01265
cul5a	3.6E+06	0.0E+00	1.7E+06	1.4E+06	0.0E+00	*			*	0.017656

ddb1	4.2E+06	2.0E+06	2.5E+06	1.7E+06	0.0E+00				*	0.031511
ddost	1.3E+07	5.1E+06	7.3E+06	4.1E+06	7.0E+06	*		*		0.033433
dlst	0.0E+00	2.8E+06	3.7E+06	1.9E+06	4.4E+06		*		*	0.042882
dmd	3.4E+06	2.3E+06	2.3E+06	2.9E+06	1.6E+06				*	0.047248
dpysl5a	1.8E+06	6.2E+06	4.4E+05	1.5E+06	9.9E+05	*				0.015576
edc4	2.6E+06	4.9E+05	1.7E+06	0.0E+00	0.0E+00	*		*	*	0.000921
eif2b1	8.5E+06	4.3E+06	7.7E+06	2.2E+06	5.4E+06			*		0.046304
eif2s3	1.0E+07	7.1E+06	7.1E+06	7.8E+06	8.2E+06	*	*			0.031847
eif3c	1.2E+07	1.7E+07	1.2E+07	7.8E+06	9.2E+06	*		*		0.004616
eif3k	8.8E+06	3.6E+06	6.5E+06	8.5E+06	6.7E+06	*				0.016034
eif5a	2.3E+06	0.0E+00	2.7E+06	0.0E+00	9.9E+06				*	0.028872
erp44	4.1E+06	0.0E+00	7.8E+05	0.0E+00	1.5E+06	*	*	*	*	0.002199
fbp1b	4.1E+07	2.7E+07	2.7E+07	1.9E+07	2.5E+07			*		0.025834
fh	1.1E+07	8.3E+06	1.6E+07	1.0E+07	8.8E+06		*			0.022422
flot2b	2.6E+07	5.8E+06	1.6E+07	1.5E+07	1.7E+07	*				0.019537
fubp1	1.9E+07	1.7E+07	9.3E+06	5.1E+06	5.7E+06			*	*	0.005912
gapdhs	2.8E+07	4.3E+07	1.7E+07	1.2E+07	2.0E+07	*		*		0.001367
gdi2	1.6E+07	2.5E+07	1.6E+07	1.5E+07	1.6E+07	*				0.00862

gnb3b	0.0E+00	1.1E+06	0.0E+00	0.0E+00	2.4E+07				*	2.92E-05
gnpda2	3.9E+06	0.0E+00	2.2E+06	0.0E+00	0.0E+00	*	*	*	*	6.84E-05
gyg1a	3.4E+07	2.3E+07	4.3E+07	3.1E+07	2.3E+07	*	*		*	0.005318
heatr1	1.5E+06	1.8E+06	2.4E+06	1.8E+06	3.8E+05				*	0.049749
hnrnpaba	8.8E+06	4.5E+06	3.1E+06	2.5E+06	1.6E+07		*	*	*	0.00542
hnrnpk	5.9E+06	1.8E+06	2.5E+06	1.3E+07	3.2E+06			*		0.006259
hnrnpm	1.0E+07	4.8E+06	1.2E+07	1.7E+06	1.2E+07			*		0.02336
hnrnpul1l	7.5E+06	5.5E+06	4.5E+06	6.6E+06	5.3E+06		*			0.046092
hpca	1.4E+07	9.8E+06	1.2E+07	1.0E+07	9.0E+06	*		*	*	0.017849
ilk	2.1E+06	0.0E+00	1.6E+06	5.1E+05	0.0E+00	*		*	*	1.07E-05
lta4h	8.6E+06	7.7E+05	0.0E+00	5.9E+06	6.5E+06		*			0.049284
jupa	1.5E+07	5.2E+06	5.0E+06	9.3E+06	7.0E+06	*	*		*	0.00247
kars1	6.0E+06	0.0E+00	2.8E+06	0.0E+00	0.0E+00	*		*	*	0.032901
khdrbs1b	2.2E+07	1.2E+07	2.0E+07	4.6E+06	3.0E+06			*	*	0.003973
krt18a.1	3.7E+07	3.1E+07	2.8E+07	2.3E+07	3.0E+07		*	*	*	0.003971
lta4h	8.6E+06	7.7E+05	0.0E+00	5.9E+06	6.5E+06	*	*			0.049284
macf1a	1.9E+06	0.0E+00	0.0E+00	1.2E+06	6.3E+05	*	*		*	0.00621
man2b1	7.2E+06	1.3E+07	1.4E+07	9.5E+06	5.8E+06		*			0.041671

map2k6	3.3E+06	6.2E+05	2.3E+06	6.3E+05	2.1E+06	*		*		0.025794
myl7	2.8E+06	0.0E+00	1.5E+06	6.6E+05	8.9E+05	*		*	*	0.023701
mylpfa	1.8E+08	1.0E+08	1.2E+08	1.4E+08	1.6E+08	*	*		*	0.003216
myom1b	6.6E+06	4.0E+06	4.7E+06	5.4E+06	5.8E+06	*	*			0.033358
nap1l1	9.2E+06	4.1E+06	8.0E+06	3.8E+06	1.1E+07	*		*		0.035313
nap1l4a	1.1E+07	0.0E+00	5.8E+06	0.0E+00	0.0E+00	*		*	*	0.002484
ndufs2	2.0E+07	2.3E+07	5.9E+07	4.2E+07	1.6E+07		*	*		0.001364
neu3	2.3E+06	0.0E+00	1.2E+06	2.5E+06	0.0E+00	*			*	0.004881
nup93	5.5E+06	4.5E+06	2.5E+06	1.4E+06	9.2E+05	*		*	*	0.009716
ogdha	8.3E+06	4.8E+06	6.2E+06	4.4E+06	5.7E+06	*		*		0.019857
pcxa	7.7E+06	4.6E+06	8.0E+06	9.5E+06	6.2E+06	*				0.039998
pde6c	8.5E+06	5.6E+06	8.2E+06	1.3E+07	6.6E+06			*		0.047041
pfkma	6.0E+06	5.0E+05	2.4E+06	1.4E+06	1.0E+06	*	*	*	*	0.030761
pfn1	3.3E+07	0.0E+00	6.8E+06	6.5E+06	2.0E+07	*	*	*		0.00176
pkmb	1.3E+07	0.0E+00	7.1E+06	9.5E+06	7.7E+06	*				0.011795
ppp2caa	6.4E+06	1.0E+06	6.3E+06	5.7E+06	5.2E+06	*				0.014495
prdx6	9.1E+06	4.9E+06	9.1E+06	1.5E+06	8.9E+06			*		0.019012
prss1	3.1E+07	1.8E+07	1.7E+07	2.9E+07	1.9E+07	*	*		*	0.000628

psma8	4.1E+07	2.8E+07	3.4E+07	3.1E+07	2.2E+07	*		*	*	0.000404
psmc1a	1.1E+07	6.2E+06	4.6E+06	1.0E+07	1.0E+07	*	*			0.021982
ptbp2b	3.2E+06	0.0E+00	8.9E+06	0.0E+00	0.0E+00		*			0.031752
rab11a	1.9E+07	1.3E+07	1.5E+07	1.0E+07	1.4E+07	*		*		0.031406
rab5aa	2.4E+07	1.4E+07	1.5E+07	2.3E+07	3.8E+06				*	0.048674
rangap1a	2.3E+06	1.2E+06	2.5E+06	4.9E+06	0.0E+00			*	*	0.000962
rap1gds1	8.6E+06	5.0E+06	6.1E+06	6.9E+06	5.8E+06	*	*		*	0.0276
rbp4	1.6E+07	3.3E+07	1.6E+07	1.1E+07	1.2E+07	*				0.043263
rcvrn2	2.7E+07	1.6E+07	1.8E+07	2.0E+07	1.7E+07	*	*	*		0.006725
rdh1	4.2E+06	1.4E+06	2.7E+06	0.0E+00	0.0E+00			*	*	0.007884
reep5	0.0E+00	0.0E+00	0.0E+00	2.4E+07	0.0E+00			*		8.39E-05
rpl12	3.4E+07	2.2E+07	3.6E+07	2.8E+07	3.2E+07	*				0.025631
rplp2l	1.6E+08	9.8E+07	1.2E+08	1.1E+08	8.5E+07	*	*	*	*	0.000473
rpn1	9.6E+06	0.0E+00	0.0E+00	0.0E+00	2.7E+06	*	*	*	*	8.65E-05
rtn1b	1.9E+07	1.3E+07	1.1E+07	1.8E+07	1.4E+07	*	*			0.019001
rtn3	1.4E+08	7.3E+07	1.1E+08	8.2E+07	7.8E+07	*		*	*	0.01982
s100a10b	2.6E+07	0.0E+00	0.0E+00	6.5E+06	1.7E+07	*	*	*		0.002197
sb:cb283	1.8E+07	1.5E+07	1.5E+07	1.8E+07	9.2E+06				*	0.010361

scpep1	0.0E+00	9.2E+05	1.5E+06	3.0E+06	0.0E+00			*		0.017665
sdha	2.4E+06	6.7E+05	4.3E+05	1.3E+06	0.0E+00	*	*		*	0.00536
sec61b	2.4E+07	2.6E+07	2.7E+07	2.1E+07	6.8E+06				*	0.006267
sept8b	1.3E+07	9.0E+06	1.3E+07	4.0E+06	3.2E+06			*	*	0.043948
si	1.2E+07	1.0E+07	1.1E+07	1.3E+07	7.1E+06				*	0.006065
Si:dkey-65b12.6	9.0E+06	6.8E+06	7.0E+06	5.3E+06	4.6E+06	*	*	*	*	0.000129
slc25a20	3.5E+06	0.0E+00	2.2E+06	3.3E+06	0.0E+00	*			*	0.039345
slc25a5	8.4E+07	8.9E+07	1.0E+08	1.2E+08	8.1E+07			*		0.020217
slc44a2	5.1E+06	2.1E+06	2.5E+06	1.1E+06	0.0E+00			*	*	0.022407
slc4a4a	4.9E+06	1.1E+07	1.6E+07	3.9E+06	4.0E+06		*			0.011735
snd1	1.0E+07	6.8E+06	6.6E+06	1.1E+07	7.4E+06	*	*		*	0.007011
sod2	2.4E+07	1.8E+07	2.0E+07	2.7E+07	1.2E+07				*	0.006789
sqor	3.0E+06	0.0E+00	0.0E+00	0.0E+00	0.0E+00	*	*	*	*	1.11E-07
ssr1	1.9E+07	8.1E+06	9.4E+06	1.2E+07	1.4E+07	*	*		*	0.001187
sult2st2	6.4E+06	1.5E+06	0.0E+00	0.0E+00	0.0E+00	*	*	*	*	0.002979
tagln2	1.6E+07	9.3E+06	1.2E+07	1.0E+07	1.2E+07	*		*		0.027668
tars1	5.4E+06	3.7E+06	4.7E+06	3.9E+06	6.4E+06	*				0.029153
thop1	1.3E+07	1.2E+07	1.0E+07	9.8E+06	8.2E+06				*	0.049523

tnn	1.3E+07	9.7E+06	9.8E+06	1.1E+07	9.7E+06	*	*		*	0.035234
tnni2a.1	8.9E+06	0.0E+00	3.8E+06	5.4E+06	1.2E+07	*				0.019614
tnni2a.4	3.9E+07	2.9E+07	4.5E+07	6.7E+07	2.4E+07			*		0.017713
tpi1a	3.0E+07	3.0E+07	4.4E+07	2.8E+07	1.5E+07		*		*	0.017569
tpt1	8.9E+07	6.0E+07	6.3E+07	4.9E+07	4.2E+07			*	*	0.01472
ttn.2	8.5E+06	7.0E+06	7.5E+06	7.2E+06	7.9E+06	*		*		0.03712
txnrd3	5.1E+06	4.6E+05	4.9E+06	7.1E+06	3.1E+06	*				0.000729
ugt1b1	4.3E+06	0.0E+00	3.1E+06	9.3E+05	3.0E+06	*		*		0.037588
rps20	2.9E+07	7.1E+06	2.7E+07	7.1E+06	3.4E+07	*		*		0.014284
usp14	1.3E+07	2.6E+06	4.9E+06	3.0E+06	6.3E+06	*		*		0.028256
vars1	6.7E+06	3.1E+06	0.0E+00	5.9E+06	2.9E+06	*	*		*	0.00321
vcla	1.0E+07	6.3E+06	7.7E+06	4.3E+06	6.6E+06			*		0.039307
vsnl1b	2.7E+07	1.7E+07	2.7E+07	1.6E+07	1.8E+07	*		*	*	0.022771
wu:fb16f03	0.0E+00	3.5E+06	2.7E+06	2.4E+06	0.0E+00	*	*			0.0178
ywhaz	3.4E+07	1.8E+07	2.4E+07	2.9E+07	1.9E+07	*			*	0.024296
Zgc:110339	9.3E+06	0.0E+00	2.5E+06	0.0E+00	3.5E+06	*	*	*	*	0.000507
Zgc:136930	1.0E+07	2.5E+06	7.9E+06	3.8E+06	1.3E+07	*		*		0.002709
zgc:92630	1.2E+07	8.9E+06	1.0E+07	1.1E+07	4.4E+06				*	0.01991

zmpste24	5.2E+06	6.8E+06	1.7E+07	1.1E+07	6.0E+06		*			0.038934

Table S6. Affected keggs per TRA or OTRA treatment comparing to control as well as, the count in network, strenght, false discovery rate values and involved proteins.

Compound	TRA									OTRA								
Comparison	TRA0.1vs Ctr (PPI enrichment p-value: 2.00e-15)							TRA100vs Ctr (PPI enrichment p-value: 1.45e-06)		OTRA0.1vs Ctr (PPI enrichment p-value: 1.36e-08)			OTRA100vs Ctr (PPI enrichment p-value: 3.22e-11)					
KEGG Pathways	dre00020 - Citrate cycle (TCA cycle)	dre00500 - Starch and sucrose metabolism	dre00620 - Pyruvate metabolism	dre00010 - Glycolysis / Gluconeogenesis	dre00330 - Arginine and proline metabolism	dre01200 - Carbon metabolism	dre04141 - Protein processing in endoplasmic reticulum	dre01100 - Metabolic pathways	dre01200 - Carbon metabolism	dre01100 - Metabolic pathways	dre00010 - Glycolysis / Gluconeogenesis	dre01100 - Metabolic pathways	dre00330 - Arginine and proline metabolism	dre00190 - Oxidative phosphorylation	dre00500 - Starch and sucrose metabolism	dre00010 - Glycolysis / Gluconeogenesis	dre01200 - Carbon metabolism	dre01100 - Metabolic pathways	dre00330 - Arginine and proline metabolism	dre00190 - Oxidative phosphorylation	dre00030 - Pentose phosphate pathway	dre00051 - Fructose and mannose metabolism	dre01230 - Biosynthesis of amino acids	dre04141 - Protein processing in endoplasmic reticulum
Count in Network	3 of 32	3 of 36	3 of 41	5 of 74	3 of 63	5 of 127	5 of 191	28 of 167	5 of 127	20 of 167	4 of 74	30 of 167	4 of 63	5 of 135	3 of 36	5 of 74	5 of 127	25 of 167	4 of 63	5 of 135	3 of 32	4 of 44	4 of 83	5 of 191
Strenght	1.4	1.35	1.29	1.26	1.1	1.02	0.84	0.65	1.24	0.72	1.24	0.76	1.31	1.07	1.4	1.31	1.07	0.65	1.28	1.05	1.45	1.44	1.16	0.9
False discovery rate	0.0344	0.038	0.0452	0.0027	0.0406	0.0211	0.0204	6.00E-09	0.0028	1.85E-07	0.0134	7.45E-13	0.0134	0.0134	0.0149	0.0015	0.0084	6.48E-08	0.0084	0.0084	0.0133	0.003	0.0133	0.0227
Involved genes	*ogdha; sdha; pcl*	*gyg1a; agla; amy2a*	*aldh2.1; pkmb; pcl*	*akr1a1b; aldh2.1; pfkpa; gapdhs; pkmb*	*agmat; ckmt2a; aldh2.1*	*pfkpa; pkmb; ogdha; pcl; sdha*	*calr; calrl; ddost; rpn1; ssr1*	*gyg1a; agla; amy2a; ugt1b1; anpepb; gnpda2; pfkpa; aldh2.1; akr1a1b; cmasa; chdh; cox4i2; bckdha; pkmb; atp5h; gapdhs; sdha; ogdha; pcl; sqrdl; agmat; adsl; atic; adssl; ddost; rpn1; ita4h; ckmt2a*	*tpi1a; fh; cat; sdha; pfkpa*	*tpi1a; fh; cat; sdha; rpn1; gyg1a; bckdha; ckmt1; sqrdl; ndufs2; atp5h; cmasa; ita4h; gnpda2; agmat; ckmt2a; anpep; adka; akr1a1b; pfkpa*	*fbp1b; pfkpa; akr1a1b; gapdhs*	*fbp1b; pfkpa; akr1a1b; gapdhs; agmat; ckmt2a; ckmt2b; ckmt1; ndufs2; cox4i2; atp5c1; atp5h; atp6v1h; gnpda2; agla; chdh; ugt1b1; cbr1l; ogdha; anpepb; cmasa; bckdha; adsl; prdx6; atic; adssl; sqrdl; ddost; rpn1; pde6c*	*agmat; ckmt2a; ckmt2b; ckmt1*	*ndufs2; cox4i2; atp5c1; atp5h; atp6v1h*	*gyg1a; agla; si*	*aldob; aldoaa; pfkpa; tpi1a; akr1a1b*	*aldob; aldoaa; pfkpa; tpi1a; sdha*	*gyg1a; agla; si; aldob; pfkpa; aldoaa; anpepb; ckmt2a; ckmt2b; chdh; akr1a1b; tpi1a; sdha; bckdha; ckmt1; sqrdl; atp5h; cox4i2; atp5c1; cox7a2a; gnpda2; rpn1; agmat; neu3.3; cmasa*	*ckmt2a; ckmt2b; ckmt1; agmat*	*sdha; atp5h; cox4i2; atp5c1; cox7a2a*	*aldob; aldoaa; pfkpa*	*aldob; aldoaa; pfkpa; tpi1a*	*aldob; aldoaa; pfkpa; tpi1a*	*ssr1; rpn1; sec61b; clar; calrl*

Autor: Pedro Miguel Carneiro Rodrigues

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