University of Cagliari

***PhD in Molecular and Translational Medicine***

Nutritional and Metabolic Sciences

*Cycle XXXIII*

# *Developmental Neurotoxicity testing of chemical mixtures in zebrafish embryos*

SCIENTIFIC SECTOR OF AFFERENCE

CHIM/10


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| **PhD student:** | ***Dr. Alessandro Atzei*** |
| **PhD coordinator:** | ***Prof. Sebastiano Banni*** |
| **Supervisor:** | ***Prof. Alberto Angioni*** |
| **Co-supervisor:** | ***Dr. Harm Heusinkveld*** |


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**Alla mia famiglia, inesauribile fonte d'amore**

**e indispensabile certezza della mia vita.**

**A Kasia, che con amore e tenacia mi ha supportato**

**lungo questo percorso.**


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# Abbreviations

ACN: acetonitrile

ADME: absorption, distribution, metabolism, and excretion

ADHD: attention-deficit hyperactivity disorder

AOP: adverse outcome pathway

AOPs: advanced oxidation processes

ASD: autism spectrum disorder

BBB: biological barrier systems

BMC: benchmark concentration

BMD: benchmark dose

BMDL: benchmark dose lower

BMDU: benchmark dose upper

CAG: cumulative assessment group

CBZ: carbamazepine

CI: confidence interval

CiD: circumferential descending interneuron

CMG: common mechanism group

CNS: central nervous system

CoPA: commissural primary ascending

CRA: critical risk assessment

DA: dopamine

DEB: dynamic energy budget

DMSO: dimethyl sulfoxide

DNT: developmental neurotoxicity

DoLA: dorsal longitudinal ascending

DSW: Dutch standard water

EFSA: European Food Safety Authority

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

fHRs: higher basilar fetal heart rates

FLX: fluoxetine

GABA: gamma-aminobutyric acid

GAD: generalized anxiety disorder

GC-MS/MS: gas chromatography tandem mass spectrometry

GECI: genetic encoded calcium indicators

GMS: general morphology score

HI: hazard index

hESC: human embryonic stem cells

hiPSC: human induced pluripotent stem cell

hpf: hour post-fertilization

IATA: Integrated Approach to Testing and Assessment

LC-MS/MS: liquid chromatography tandem mass spectrometry

LOD: limit of detection

LOQ: limit of quantification

JRC: Joint Research Centre

KEs: key events

MBT: mid-blastula transition

MDD: Major depressive disorder

MeOH: methanol

MIE: molecular initiating event

MOA: mode of action

NE: norepinephrine

NHIS: National Health Interview Survey

NRC: National Research Council

NOAELs: no observed adverse effect levels

NOEC: no observed effect concentration

OCD: obsessive-compulsive disorder

ODV: O-desmethylvenlafaxine

OECD: Organization for Economic Co-operation and Development

OPs: organophosphates insecticides

PMDD: bulimia nervosa and premenstrual dysphoric disorder

PODI: point of departure index

PPP: plant protection product

QSAR: Quantitative Structure-Activity Relationship

RIVM: Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment)

RfPI: reference point index

RPF: relative potency factor

RPFL: relative potency factor lower

RPFU: relative potency factor upper

SERT: transporter enzyme for serotonin

SNRIs: serotonin and norepinephrine reuptake inhibitors

SSRI: selective serotonin reuptake inhibitors

TTC: threshold of toxicological concern

TD: toxicodynamic

TEF: toxic equivalency factor

TK: toxicokinetic

YSL: yolk syncytial layer

WHO: World Health Organization

VeLD: ventral longitudinal descending interneurons

VNX: Venlafaxine

VSD: ventricular septal defects

ZFE: zebrafish embryo

3Rs: replacement, reduction, refinement

5-HT: 5-hydroxytryptamine

5-HTT: transporter enzyme for serotonin

# Abstract

Developmental neurotoxicity (DNT) is an understudied problem. Every day, people are exposed to complex mixtures of several chemical substances via food intake, inhalation and dermal contact. Nevertheless, risk assessment is performed on single compounds only under the assumption that the individual exposure levels (below no observed adverse effect levels, NOAELs) are predictive of the mixture effect. In the EuroMix project, a method has been developed to evaluate the effects of mixtures of substances, even at or below NOAELs. This method follows the strategy proposed by the European Food Safety Authority (EFSA), and further implements the Adverse Outcome Pathway (AOP) concept as a basis. Currently, assessment of the DNT potential of compounds is performed in costly and time-consuming *in vivo* rodent studies involving a large number of animals studied over more than one generation. Therefore, from a 3Rs (Replacement, Reduction, Refinement) perspective an alternative approach is needed. The zebrafish (*Danio rerio*) embryo (ZFE) provides an interesting and potentially useful model to study DNT as neurodevelopment occurs fast with a large resemblance to the higher vertebrate including the human system. Also, from a legal perspective, experimental work with zebrafish embryos within 120 hours post-fertilization, is not considered an animal experiment. Combined with the ease of culture and the high reproduction rate this renders the ZFE a suitable model for high throughput DNT testing *in vitro*. One of the suitable readouts for DNT testing is neurobehavior since it provides integrated information on the functionality/status of the full nervous system of the embryo. Within 120 hpf the embryo develops from a fertilized egg to a fully functional embryo responsive to environmental stimuli such as light and sound (vibration). The present Ph.D. study investigated the potential human health risk caused by the simultaneous exposure of chemical substances and the need to include the mixtures in the risk assessment. To obtain a real-life picture of

environmental pollution by chemical mixtures, an UHPLC-MS/MS-MRM method was developed and validated for screeining pesticide residues on raw and processed tomatoes. Then, the attention was focused on the potential use of the zebrafish model for assessing the chemical mixtures effects in DNT. Recognised that pharmaceuticals display a well-known MOA and are known to cause DNT, their use as model compounds instead of pesticides was preferred. Therefore, the combined effect of three psychoactive pharmaceuticals of concern, Carbamazepine (CBZ), Fluoxetine (FLX), Venlafaxine (VNX) and their main metabolites, Carbamazepine 10,11 -epoxide (CBZ 10,11E), Norfluoxetine (norFLX), and Desvenlafaxine (desVNX), was studied using the zebrafish embryos as a study model. At first, single-compound concentration-effect relationships were assessed as input for dose-response modelling following the benchmark approach leading to a classification of compounds based on potency. Subsequently, a binary mixture was composed based on the relative potency of the individual compounds and tested for their effect on neurological development. To support the assessment of developmental neurotoxicity, the gene expression of three specific DNT markers was investigated.

# 1. Introduction

## 1.1. Chemical mixtures risk assessment: an overview of the current EU legislation across different sectors

Although humans and the environment are on a daily basis incessantly exposed to a multitude of substances via different routes of exposure, risk assessment of chemical substances is mainly based on exposure to individual chemicals, mostly considering only a single source (**Kienzler et al., 2014, 2016**; **Bopp et al., 2019**). Urged by the Council (2009), the European Commission (EC) published a communication (**EC, 2012**) on the concerns about the combined effect of chemicals and suggested a harmonized system which aimed for a better understanding and assessment of the risk for human health. Human exposure to individual chemicals may come from multiple sources and via multiple pathways and routes (called aggregate exposure) or to multiple chemicals via single or multiple routes (called combined or cumulative exposure) (**Figure 1**). To avoid terminology confusion, below are reported key definitions for the chemical mixture risk assessment (**Table 1**). To assess a realistic risk assessment, both aggregated and cumulative exposure need to be considered. In general, two main types of mixtures can be discriminated: intentional and coincidental. Intentional mixtures refer to combinations which are deliberately manufactured such as pesticide formulations or laundry detergent which may also include the addition of by-products released during the work processes (e.g., smelting and drink water disinfection) (also called generated mixture). Therefore, an intentional mixture is likely to be known and characterized, at least partly. Coincidental mixtures offer the hardest challenge for the risk assessment due to their unintentional combination coupled with a largely unknown composition. They are easily detectable at the environmental level, such as in surface water, drinking water and air. At the risk assessment level, intentional mixtures could be studied by applying a predictive approach, whereas the coincidental combinations could be addressed by a retrospective approach, feasible only when the adverse effect is detected. Regardless to the type of mixture, the matter gets

complicated when considering the environmental fate of each mixture component. Indeed, their composition may change over time. Such a complex scenario is also reflected at the regulatory level where, the risk assessment requirements for chemical mixtures show regulatory differences depending on type of combination, field of application (e.g., biocides, medicinal products for human use, veterinary medicinal products, cosmetics, etc.) and also between geographic regions. In general, chemical mixtures are only assessed and regulated in the case of intentional combinations (i.e., industrial chemicals, plant protection products and biocides) based on knowledge of their individual components, or by toxicity tests on the final product mixture. Contrary, the assessment of unintentional mixtures (i.e., environmental pollutants, contaminants and by-products), is predominantly not required. However, it includes limit values for some of the known individual constituents (i.e., MRLs for pesticide residues assessment in food) aimed to ensure that humans, animals and the environment are exposed to individual substances at concentrations below their threshold of concern. Nevertheless, the presence of individual safety limits does not protect human health and the environment from the potential negative effects of their combined exposure. Additionally, the assessment of the limit values does not take into account the aggregate exposure but it is strictly related to its sectorial use, therefore it might result in a non-adequate limit estimation. Together with the current pieces of EU legislation, the Member State (MS) authorities, agencies and international organizations such as EFSA, OECD, JRC, US EPA, FDA and WHO/FAO provided several guidance documents on how to apply risk assessment of mixtures. The successive section reports the main the guidance documents cited above.

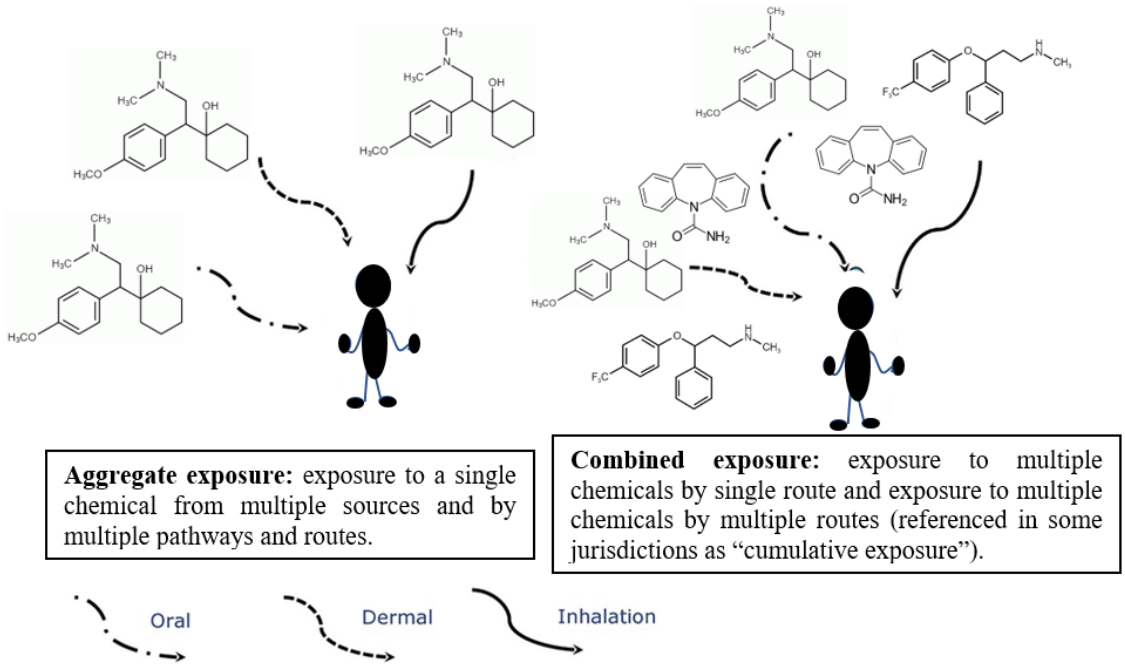


**Figure 1** Graphical comparison between aggregate and combined exposure.

**Table 1** Key definitions in the chemical mixtures risk assessment.

| Terminology | Definition | Example |
|---|---|---|
| Route | the way a chemical come in the organism | dermal exposure, oral exposure or inhalation |
| Pathway | the medium with which the chemicals are taken up | food, drinking water or air |
| Source | the places of release of chemicals | waste water treatment plant effluents or industrial emissions |

## 1.2. Different approaches and mathematical models for the assessment of combined exposure to multiple chemicals according to European Commission

According to the EU Joint Research Centre (**Kienzler et al., 2014, 2016**), the hazard of a chemical mixture can be addressed by applying two different approaches: the whole-mixture approach and the components-based approach (**Figure 2**). The whole-mixture approach provides the advantage to assess the risk based on the data of the studied mixture as well as by

the analysis of data belonging to a mixture of similar composition in terms of components and proportions. Moreover, it allows observations on each unknown material of the mixture and for potential interactions among mixture components. However, it lacks information regarding the chemicals responsible for the mixture effects besides it does not provide any information on the toxicity of the individual mixture components. In addition, the results are only applicable to mixtures that do not significantly change in their composition. For these reasons, it is not suggested as a default approach (**SCHER, SCCS, SCENIHR, 2012**). The component-based approach is based on the assumption that the components of the mixture are known as well as their corresponding modes of action (MOA). Whether the MOA is not fully understood, the assessment can be performed based on the information of other chemical groups showing similar or identical MOA. Moreover, in the worst case in which the MOA is unknown, the component based-approach can be carried out by grouping chemicals sharing a common toxicological effect. Depending whether the components of the mixture display the same mode of action or act independently, several mathematical models can be used in order to predict the combined effect of the mixtures. The toxicology of the chemical mixture can follow three principles of interaction: mixture with simple similar mode-of-action (MOA), simple dissimilar MOA or interaction between substances in a mixture (i.e., synergism or antagonism) (**Bliss, 1939**; **Macacu and Guillot, 2020**). To address the combined effect of mixtures of substances with similar MOA, dose or concentration addition (DA) is applied. In case of substances with dissimilar MOA, response addition (RA) is applied and, lastly, infra-and supra-additivity models are applied in case of interactions between mixture components (synergism or antagonism) (**Cassee et al., 1998**). The term interaction involves all kinds of joint action (at the biological target site) that deviate from both dose or response addition. In contrast, both DA and RA assume that substances do not influence each other's toxicity via interaction at the biological target site (**EFSA, 2013b**; **Bopp et al., 2015**). The DA model provides an estimation

of effect and cumulative risk of the mixture from the sum of doses/concentrations, adjusted for the difference in potency between mixture components. This assumption is based on the pharmacological concept that receptor occupancy is proportional to the concentration of the ligand and its affinity for the receptor. Thus, by summing the doses of the mixture's components, previously scaled of their potencies, it is possible derive the magnitude of the biological response. Hazard Index (HI), Relative Potency Factors (RPFs), the reference point index (RfPI, or PODI for Point of departure index), or the toxic equivalency factor (TEF) are the most common approaches used for applying the DA model. Concerning the RA model, the mixture effect is calculated as the sum responses of the individual components using the statistical concept of independent random events. Biological interaction might occur either at the toxicokinetic and toxicodynamic level. At a toxicokinetic level it describes potential changes during the processes of uptake, distribution, metabolism and excretion such as chemicals modifying the absorption or active transport of another, while, the toxicodynamic level refers to the chemical effects on biological targets like receptors, cellular target or organ. Chemical interactions may result in less (antagonistic, inhibitive, masking) or more (synergistic, potentiating) potent effects than would be expected based on either DA or RA. However, biological interactions are rarely detected at relevant exposure levels in ecological (**Cedergreen, 2014**) and even less in human hazard assessment (**Hernandez et al., 2017**). Moreover, whether interactions are observed, the deviations from DA predictions are relatively small. For these reasons, additive models provide a good solution for the chemical mixtures risk assessment. Specifically, DA model is used as a default assumption for risk assessment of chemical mixtures with similar and dissimilar MOA, providing that they produce a common adverse outcome on the same organ/system (**EFSA, 2013b**; **Kienhuis et al., 2015**; **Staal et al., 2018**; **Zoupa et al., 2020**).

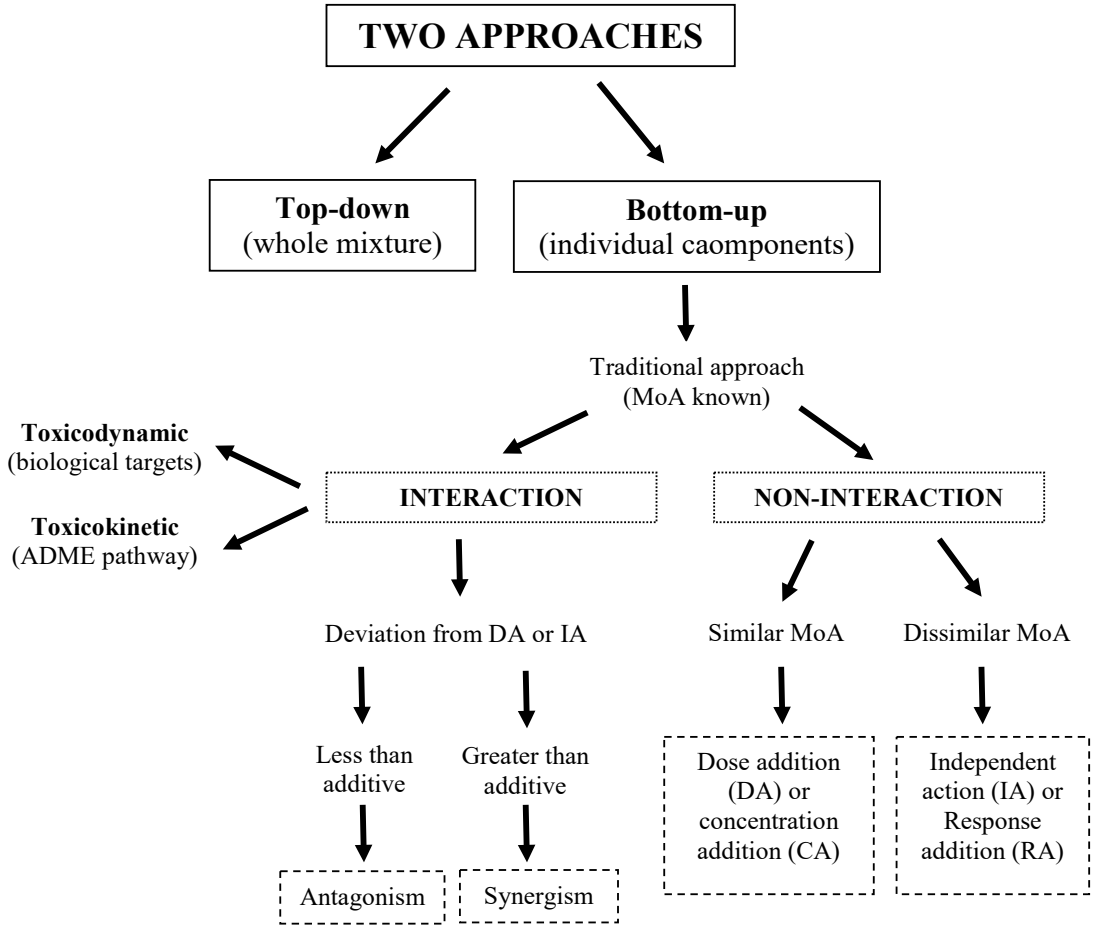


**Figure 2** Approaches used for the risk assessment of chemical mixture.

### 1.3. Relevant guidance documents across different geographic regions

An important theme commonly addressed in the guidance documents is the grouping of chemicals and the basic principles (or ground rules for their selection) underlying their selection. Chemical grouping is mostly influenced by exposure and toxicity data. While grouping based on exposure data is related to the potential co-occurrence of the components, the grouping based on toxicity is linked to toxicity similarity, or the potential for interactions, between components. Moreover, in some cases, chemical grouping can be influenced by other factors such as regulatory requirement and problem formulation. In EFSA guidance (**EFSA, 2013b**), co-exposure is not explicitly discussed as a factor for grouping in risk assessment

whereas toxicity data play a key role. Indeed, EFSA's Panel on Plant Protection Products and their Residues (PPR) proposed to only group chemicals linked to a defined endpoint, in so-called cumulative assessment groups (CAGs), using the CA model as a default to predict combined effects. CAGs are based on identifying chemicals that affect the same organ or physiological system and exhibit similar toxicological properties (adverse outcome) in that organ or system. EFSA's PPR panel applied this methodology to define groups of pesticides which are toxic to the thyroid and central nervous systems. A recent EFSA guidance document (**EFSA, 2019b**), enables a flexible approach to the grouping of chemicals, depending on problem formulation. Particularly, the potential groups can be based on several commonalities between mixture components such as regulatory sector, source, functional group(s), chemical class, breakdown products or common target organ(s). However, as more hazard data comes available, the grouping can be refined by applying mechanistic criteria such as toxicokinetic and the Adverse Outcome Pathways (AOPs). Differently than EFSA's grouping approach, US EPA **(2002b, 2016a)** grouping was based first on co-exposure, then on toxicological similarity. Exposure data are considered an important criterion of selection given that not all chemicals, pathway of exposure or uses contribute to the risk, therefore, their assessment is not required. After the exposure-based assessment, US EPA guidance recommends to group chemicals into common mechanism group (CMGs) based on similarities in chemical structure, common toxic effect and a similar sequence of key biochemical events following the initial chemical interaction. To define a CMG, sufficient evidence for a common toxicological profile (based on detailed knowledge on exposure and toxicity) need to be available. Recent communications for assessing the risks of combined exposure to multiple chemicals have been published also by the World Health Organization (WHO) in cooperation with the Food and Agriculture Organization (FAO) (**WHO, 2019**) and by the Organization for Economic Co-operation and Development (OECD) (**OECD, 2018**). Within the EuroMix project (see below), WHO/FAO

document refers specifically about the dietary risk assessment of chemical mixtures. Compared to the CAG concept proposed by EFSA, it results a more pragmatic approach which requires, for instance, the development of a database with a simple list of parameters to systematically evaluate potential mixtures existing in the real exposure scenario. Moreover, to correctly investigate dietary exposures and co-exposures, the database of single food consumption for different countries and corresponding food concentration data must be compatible. Then, a probabilistic approach is recommended to estimate dietary exposure to multiple chemicals based on individual food consumption and concentration data. Furthermore, the dual use of compounds and outdated persistent pesticides need to be considered, in order to better estimate the total dietary exposure. The OECD, through guidance document No. 296 (**OECD, 2018**) aimed to bring an overview of the technical aspect of the several approaches and methodologies currently ongoing. Special attention is paid to the application of novel *in vitro* models, considered a key point in the implementation of the 3R principle (reduce, replace, refine). OECD strongly supports the cooperation between different countries and organizations, intended to harmonize the use of tools and methodologies as well as regulatory conclusions and therefore avoiding the overlap of information. With some differences mainly related to the aim of the communication, the leading organizations share a common approach for the assessment of combined exposure to multiple chemicals. Overall, a component-based approach, where feasible, seems to be accepted as the most appropriate for a great number of mixture risk assessment. However, regardless of the approach used for risk assessment (whole mixture or component based), a tiered approach is recommended. This allows to conduct a stepwise assessment, characterized by deterministic and probabilistic approaches for both exposure and hazard assessment. For exposure, deterministic approaches may be applied at lower tiers, based on limit values such as MRLs (i.e., for pesticides) in order to provide the worst-case exposure for the mixture. Then, deterministic exposure assessments may be further

refined by applying i.e., TK models to estimate internal exposure levels. At the higher tiers, probabilistic approaches result more accurate by using distributions of the concentrations of mixture components in different food and the distributions of their consumption in the population. Similarly, at lower tiers, the hazard assessment may be performed by assuming that all components in the mixture have the same potency as the most toxic mixture component (worst-case hazard). Even if not based on the common effect, safety value such as an acceptable daily intake (ADI) can be used for the conservative risk estimation. Deterministic hazard assessment may be improved at higher tiers considering a common toxic effect and different potency among mixture components. For this purpose, the use of the common adverse effect (No Observed Adverse Effect Levels (NOAELs) or Benchmark Doses (BMDs)) for each mixture component is recommend. However, probabilistic hazard estimation can be achieved by using dose-response models and TK modelling. The tiered approach is recommended also to saving sources, indeed, whether sufficient protection is estimated at lower tiers, further analyses can be interrupted avoiding unnecessary investigations. To conclude, a common opinion among the leading organizations is that the dose addition (DA) represents the most suitable and conservative model for the chemical mixtures risk assessment.

### 1.4. Current scientific tools for the risk assessment of chemical mixtures

The number of potential combinations of contaminants in the environment is almost uncountable. Moreover, the synthesis, manufacture and use of chemicals worldwide registered a significant increase during the last decades, making the risk assessment more problematic. Within the risk assessment of chemical mixtures, knowledge on mode-of-action (MOA) and kinetics represent a crucial point for understanding the toxicological effects of compounds (**Heusinkveld et al., 2020**). However, the existence of more than one MOA per compound contributes to the scarcity of such information. Thus, the risk assessment of potential chemical

mixtures based mostly on common phenomenological effects among mixture components (EFSA Strategy, CAG 2) (**Nielsen et al., 2012**). To fill this lack of data, the development of alternatives to *in vivo* testing appears a necessity. For instance, the EU-funded Horizon 2020 project "EuroMix" provides a web-based toolbox (Monte Carlo Risk Assessment (MCRA)) in which the use of *in silico* and *in vitro* tools is verified *in vivo* for three adverse outcomes such as liver steatosis, adverse effects on reproduction due to endocrine disruption and, skeletal malformation/cleft palate. EuroMix findings seem to confirm the dose-addition as a default assumption for cumulative risk assessment, either *in vitro* or *in vivo* studies, for feminization and craniofacial malformation. On the other hand, more investigation needed to confirm *in vitro* liver steatosis results. Within this EU project, the zebrafish embryo has been employed as a model for the risk assessment of chemical binary mixtures known for their developmental toxicity and to induce cleft palate/craniofacial malformations **(M. Zoupa., et al., 2020)**. The following section brings an overview of the scientific tools available for the risk assessment of chemical mixtures divided in two level-analysis. The first describes the main techniques used, whereas the second report the approaches for data arrangement and interpretation.

### 1.4.1. Techniques for chemical risk assessment

#### 1.4.1.1. *In vitro* methods

*In vitro* models, such as cell lines, represent a widely used alternative to *in vivo* testing. Differently than *in vivo* approach, they are designed to respond to specific effects (biological response) under standard laboratory conditions, whereas *in vivo* studies might be influenced by non-chemical stressor leading to a more complex assessment of the chemical effect. Furthermore, they allow the contemporary analysis of several substances for different effects and different combinations (high throughput). They are also called mechanistic assays since

the biological endpoints typically investigated are activation of receptors and/or specific pathways, triggering of cellular repair mechanisms, etc. Indeed, *in vitro* findings can be used for a better understanding the MOA/s of a compound or combination of compounds, resulting helpful for prioritizing compounds for hazard assessment (**Caldwell et al., 2014**). Regarding their application in the assessment of chemical mixtures, two approaches are mostly followed: top-down and bottom up. The top-down provides the final toxic effect of the whole mixture, therefore, is gaining popularity in the environmental risk assessment where the larger part of compounds causing the overall effect are unknown. The bottom-up approach is based on the estimation of the single compound toxicity by applying a wide range of *in vitro* assays such as mitochondrial toxicity, cell viability or nuclear receptor assays. Once the single compound toxicity is well assessed, it can be used to predict mixture effects. Regardless of the approach, the main goal remains how to translate *in vitro* outcomes to adverse *in vivo* effects (**Adeleye et al., 2015**). Since the *in vitro* models lack of key aspects of a whole organism such as uptake, distribution and metabolism, a direct translation is not feasible. To date, a better estimation of the adverse *in vivo* effects is achievable by combining the *in vitro* findings to toxicokinetic models.

#### 1.4.1.2. Toxicokinetic and toxicodynamic modelling

Toxicokinetic (TK) and toxicodynamic (TD) models provide an important support for a better mechanistic understating but mostly focus on the potential interactions between mixture components. TK describe the substance fate within an organism (ADME: absorption, distribution, metabolism and excretion) whereas TD describe the interactions with biological targets and the potential harmful effects on health. In the mixture assessment, the TK model is mostly used to determine internal exposure concentrations, favouring *in vitro* to *in vivo* extrapolations (IVIVE). In addition, both models are applied to assess whether simultaneous

or sequential exposure to different mixture components reach the same target, understand metabolite generation or predicting interactions among mixture components on TK and TD level. An example of interaction at TK level occurs when one chemical in a mixture affects the ADME of other components of the mixture while an example of interaction at TD level occur when one chemical damages repair or homeostasis mechanisms. Although the literature reports examples of mixture component interactions (**Tan et al., 2011**), most of them addressed high exposure levels, whereas chemical mixtures are more likely to occur at lower exposure levels (environmental).

#### 1.4.1.3. Quantitative Structure-Activity Relationships (QSARs)

One of the most issues in the risk assessment of chemicals, both individually and in combination, is the lack of information on the properties and activities of substances. Quantitative Structure-Activity Relationship (QSAR) are mathematical models that can be used to fill substance data gaps based on their chemical structure alone, without performing *in vitro* or *in vivo* tests. They represent a predictive approach widespread in the field of chemical risk assessment due to their variety of applications. Indeed, QSARs can be used for predicting information on single compounds such as physicochemical properties and toxicological effects, for predicting directly or stepwise the combined effects and interactions of chemicals in a mixture, for assessing whether chemicals will act in a similar or dissimilar way to perform their grouping, for modelling exposure concentrations and, additionally, for calculating internal exposure concentrations by modelling internal distribution and metabolism (**Altenburger et al., 2003**). The main endpoints currently addressed through QSAR models are endocrine activity in cell-based transactivation assays, mortality, nephrotoxicity, acute (eco)toxicity, mutagenicity, genotoxicity, cancer alerts, skin sensitization, biodegradation and (chemical) behavior. Nevertheless**,** direct prediction of mixture toxicity by QSARs is rather rare. Firstly,

most QSAR models predict an $EC_{50}$ for the mixture instead of taking into account low dose effects at concentrations below the NOEC (**Kim and Kim, 2015**). Thus, these predictions can lead to overestimations of toxicity (at low concentrations the specific effects of some compounds may not be triggered yet) and therefore not being relevant for environmental exposures. Secondly, most current QSAR investigate binary mixtures and only a few are focus on multi-component mixtures. Lastly, most QSAR models focus on acute rather than chronic toxicity. To sum up, although QSAR models display a broad spectrum of applications along with several limitations, they can provide valuable input for assessing the toxicity of mixtures, particularly in a view to set up a harmonized set-strategy of analyses.

### 1.4.2. Approaches for data arrangement and interpretation

#### 1.4.2.1. Adverse Outcome Pathways (AOPs)

The Adverse Outcome Pathway (AOP) describes a sequential chain of causally linked events, starting from the molecular initiating event (MIE; at macromolecular level), via intermediate key events (KEs, at cellular or organ level) to the *in vivo* outcome of interest (adverse outcome, AO; at organism and population level). As reported in the previous sections, MOA data are pivotal for several reasons, such as determined whether mixture components follow a similar or dissimilar MOA, enable an adequately chemical grouping and prioritize the risk characterization. AOPs methodology follows OECD Guidance (**OECD, 2013**), supporting the use of MOA as a basis for understanding an adverse health or ecotoxicological effect. Their use depends on the possibility to gain insight into potential interactions together with a clear *in vivo* relevance of the considered mechanisms. However, grouping chemicals based on their MOA needs to consider that depending on the dose ranges applied, chemicals might produce a different effect by following a different AOP (**Borgert et al., 2004**).

#### 1.4.2.2. Omics

Omics techniques provide a high sensitivity tool aimed at the collective characterization and quantification of gene transcripts (transcriptomics, also called gene expression profiling) and of a pool of biological molecules such as proteins (proteomic), lipids (lipidomic) and of small molecule metabolites (metabolomic). Due to their high sensitivity, they are suitable to investigate effects at low concentration levels which meet the need to assess environmental mixture effects. By omics analyses, can be achieved a more detailed overview of toxicity pathways of single compounds by identifying the key molecular events and the sequence of complex events caused by the substance. Moreover, they are used for the identification of robust biomarkers for mixture prediction models in both human and environmental risk assessment. **Dardenne et al. (2008)** reported the effects of single compounds and binary mixtures on 14 stress gene promoters by applying both DA and RA models to describe the mixture responses. The findings showed that in many cases, both models resulted suitable for predicting mixture effects based on the individual substance responses and the differences between models was rather small. However, the application of DA and RA models aimed to data quantification is limited due to the low number of tested concentration (**Altenburger et al., 2012).** An important consideration to keep in mind when performing omics analyses, is that the effects observed at omics level need to be carefully interpreted because molecular responses do not necessarily match to an adverse outcome at the physiological level (**Beyer et al., 2014**).

#### 1.4.2.3. Read-across

Read-across is a predictive methodology that led to estimate an endpoint, test information or various proprieties (i.e., physic-chemical properties, environmental fate, human health effects and ecotoxicity) of a target chemical by the available knowledge on the same endpoint of one

or more similar chemicals (also called source chemical(s)) (**OECD, 2014**). This can be performed with a limited set of substances (analogue approach) or within a large group of substances (category approach). Structural similarity (i.e., common functional groups, common chemical class, or common precursor or breakdown products), together with proprieties and activities similarity (i.e., common molecular initiating events (MIEs) or key events (KEs)) are the most parameters used for the prediction. The latter can be both qualitative and quantitative. The first address the absence or presence of a certain property or activity whereas the second predicts a value for a certain property or endpoint such as dose-response relationship and effect concentrations (i.e., CED, NOAEL, LOAEL) (**OECD, 2014**). In the hazard assessment of chemical mixtures, read-across can follow two main applications: for untested constituents of a mixture in a component-based approach and for similar mixtures in a whole mixture approach. However, since read-across is limited to substances with adequate information about the composition and MOA (source chemical(s)), the second approach is not feasible.

#### 1.4.2.4. Threshold of Toxicological Concern (TTC)

The Threshold of Toxicological Concern (TTC) is a methodology based on a background of toxicological data, showing empirically that, given a specific endpoint, there is a threshold below which toxicity does not occur or is unlikely. Together with QSAR predictions and read-across, could be used to fill the toxicity data gaps. It is mainly applied to assess potential human health concerns of substances whit known chemical structure and estimated exposure, but for which is reporting a lack of relevant toxicity data. Therefore, in order to correctly apply the TTC approach, the classification of the substances based on their chemical structure is essential. EFSA's Scientific Committee (2012) defined the Cramer classification (**Cramer et al., 1978**) as conservative and protective of human health although a revision was required. Regarding the assessment of chemical mixtures, the Scientific Committees recommend its

application at a screening level for comparing first estimates of mixture exposure to the TTC (**SCHER, SCCS, SCENIHR, 2012**), avoiding the assessment of chemical mixtures containing substances with unknown chemical structures. Although the TTC applications largely refer to the area of human health, the development of an environmental assessment area (ecoTTC) is reported in the literature (**Belanger et al., 2015**).

#### 1.4.2.5. Integrated Approach to Testing and Assessment (IATA)

The large volume of data generated by the pool of methodologies currently available for the chemical mixture assessment, require to develop strategies aimed to evaluate and interpret them in a harmonized approach. The Integrated Approach to Testing and Assessment (IATA) provide a method designed to integrate existing knowledge coming from different information sources such as biochemical and cellular assays and computational predictive methods for testing prioritizing or elaborate assessment conclusion (**OECD 2016, 2017**). Moreover, in order to save resources, IATA deductions could lead to the refinement, reduction, and/or replacement of selected conventional tests i.e., when sufficient information is available. Nowadays, the development of IATA approach is strictly related to the improvement and availability of AOPs (**Patlewicz et al., 2014**). Indeed, testing and assessment strategy can be built up by addressing MIEs and KEs in an AOP.

## 1.5. A potential case of chemical mixtures pollution: pesticides treatment on open-field tomato plants

Pesticides are among the most common environmental pollutants (**Özkara et al., 2016**). Given their widely application to preserve the harvest by the action of weeds, insects, fungus and rodents (**Pareja et al., 2011**), agriculture is the largest consumer. Pesticides residues may

persist on the foods destined for human consumption and therefore they can lead to a major food safety risk. Although they are designed to combine efficiency with the minimal risk to human health, pesticides exposure has been related to different diseases such as asthma, diabetes, leukemia, Parkinson's disease, autism and cancer (**Ventura et al., 2015**; **Arrebola et al., 2015**; **Hernández et al., 2011**; **Moisan et al., 2015**). Tomato (*Solanum Lycopersicum* L.) is one of the most important horticultural crops in the world belonging to the Solanaceae family and which includes potatoes, peppers, and eggplants (**Knapp et al., 2016**). Open-field tomato plants can be attacked by several pests such as late blight (*Phytophthora infestans*), tomato russet mite (*Aculops lycopersici*), and a particularly harmful and highly destructive tomato moth (*Tuta absoluta*). In general, the list of authorized pesticides together with regulations, requirements and allowable limits is country-depending on food production, consumption and social concepts (**Jonghwa et al., 2018**). In Italy, according to the National Action Plan (PAN) for the sustainable use of plant protection products (PPPs), 159 pesticides have been authorized for the pest treatment on tomato. Among them, fungicides (68), insecticides (54), nematocides (13), acaricides (12), herbicide (16), plant growth regulators (4), snail killers (2), and pheromones (2). Although pesticide treatments have been conducted in good agricultural practice (GAP), there is a real possibility that these compounds may release detectable residues even if below the MRLs. Although chemical residues should not pose a risk to human health below these individual safety limits, their potential combinations are currently of concern. Moreover, given the multitude of possible chemical combinations spread in the environment, the assessment of their adverse effects is problematic. Therefore, in support of the prioritisation of the chemical mixtures assessment, preliminary multiresidue monitoring by LC-MS/MS or GC-MS/MS methods on real-life samples is required.

## 1.6. Developmental neurotoxicity (DNT): A toxicological endpoint for the risk assessment of chemical mixtures

The overall approach proposed by EFSA for grouping chemicals in CAGs and therefore assess the adverse effects of their mixtures has been applied within the EU-funded Horizon 2020 project "EuroMix". The latter provides a web-based toolbox (Monte Carlo Risk Assessment (MCRA)) in which the use of *in silico* and *in vitro* tools is verified *in vivo* for three adverse outcomes such as liver steatosis, adverse effects on reproduction due to endocrine disruption and, skeletal malformation/cleft palate. EuroMix findings seem to confirm the dose-addition as a default assumption for cumulative risk assessment, either *in vitro* or *in vivo* studies, for feminization and craniofacial malformation. On the other hand, more investigation needed to confirm *in vitro* liver steatosis results. The EFSA strategy can be applied for several toxicological endpoints representing matter of concern for human health, such as the neurotoxicity during early developmental stages. The subsequent sections report the main consequences resulting by neurodevelopmental perturbations together with an overview of the testing strategy used to prevent the spread of neurological disease and disability in children worldwide.

### 1.6.1. Developmental neurotoxicity (DNT)

Within the toxicology field, developmental neurotoxicity (DNT) represents one of the most complex and understudied issues. The development of the central nervous system (CNS) is an extended process that involves many different events at the molecular, cellular and tissue levels, such as cell proliferation and differentiation of a variety of progenitor cell types, cell migration, apoptosis, neuronal tube formation, brain segmentation, etc. (**Smirnova et al., 2014**; **Hessel et al., 2018**). Due to the still immature blood/brain-barrier (BBB), increased absorption

versus low body weight and reduced ability to detoxify exogenous chemicals, the developing CNS is more susceptible to damage caused by toxic agents than the adult CNS, and adverse effects can be more severe and less reversible than those in adults (**Rice and Barone Jr, 2000**; **Smirnova et al., 2014**). At the prenatal stage, the placenta is not an obstacle for many external agents (i.e., industrial chemicals), that therefore may reach the fetus via transfer from the maternal to the fetal circulation (**Needham et al., 2011**). Moreover, human breastmilk offers an additional route by which chemicals can reach the new-born (**Needham et al., 2011**). Neurodevelopment occurs within defined and controlled timeframes generating variable windows of vulnerability to xenobiotic exposure. Epidemiological studies suggest that perturbation of neurodevelopment by genetic or environmental factors like a chemical exposure, might cause several neurodevelopmental disorders (NDDs) such as autism spectrum disorder, intellectual disability (also known as mental retardation), attention-deficit hyperactivity disorder (ADHD), schizophrenia, cerebral palsy and impairments in vision and hearing (**Grandjean and Landrigan, 2006, 2014**; **Thapar et al., 2017**). Due to the rising of synthesis, production and daily use of chemicals worldwide, strong evidences highlight the chemicals contribution to neurodevelopmental toxicity spread (**Grandjean and Landrigan, 2006**; **Grandjean, 2013**). However, the number of chemicals with sufficient information on their developmental neurotoxicity is low in fact, DNT data for most chemicals, including environmental pollutants, industrial chemicals, drugs, pesticides, consumer products and food additives is missing (**Meyers et al., 2018**). To date, only a small number of chemicals are considered developmental neurotoxicants: methylmercury, lead, arsenic, PCBs, toluene, ethanol, manganese, fluoride, chlorpyrifos, DDT, tetrachloroethylene and the polybrominated diphenyl ethers (**EPA, 2019**). Clearly, they do not cover the entire scenario of chemicals having neurotoxic potential in early developmental stages. Hundreds of chemicals are known or

suspected to be neurotoxic to adults due to the CNS injury reported during occupational exposures, poisoning incidents, or suicide attempts.

### 1.6.2. Epidemiology of Neurodevelopmental disorders

Neurodevelopmental disorders (NDDs) affect 10-15% of all births (**Bloom et al., 2009**) with ADHD and learning disabilities having the highest prevalence rate. However, the rates reported worldwide for these and other neurodevelopmental disabilities are quite wide, supporting the difficulties in assessing the real prevalence scenario (**Kieling et al., 2011**; **Wakefield, 2010**). For instance, while US studies reported a rate of roughly 10% for ADHD and learning disabilities (**EPA, 2019**), European studies referred about 5% prevalence (**Reale and Bonati, 2018**; **Longobardi et al., 2019**; **Bachmann et al., 2017**) during childhood. Still, epidemiologic studies indicate an increasing of NDDs incidence worldwide during the last two decades (**Chiarotti and Veronesi, 2020**; **Štuhec et al., 2015**; **Perez-Crespo et al., 2019**). Brain damage caused by developmental neurotoxicity are often untreatable and commonly permanent. Additionally, diagnosis is difficult, frequently occurs at an advanced age and with treatment requiring a combination of professional therapy and pharmaceuticals. Children affected by neurodevelopmental disorders can face up difficulties with language and speech, motor skills, behaviour, memory, learning, or other neurological functions. Loss of cognitive skills (expressed as loss of IQ points) reduces academic outcomes resulting in long-term impairment of the well-being and productivity of entire societies (**Gould, 2009**). Only in the European Union, exposure to methylmercury and lead (recognized as developmental neurotoxic compounds globally) has been estimated to cause a loss of IQ points that might correspond to a financial damage ranged from €10 to €20 billion per year, indeed (**Bellanger et al., 2013**; **Pichery et al., 2011**). Furthermore, although in some cases an antisocial behaviour can result predominant, in a worse scenario, neurodevelopmental disorders can lead to criminal

behaviour, violence and substance abuse. The prevalence of developmental disorders cannot be exclusively explained by genetic factors, although the latter seem to be involved in about 30-40% of the total DNT cases (**National Research Council, 2000**). These conditions are mostly associated with a combination of genetic, biological, psychosocial and environmental risk factors. Maternal use of alcohol, tobacco, or illicit drugs during pregnancy, tough socioeconomic status, preterm birth, low birthweight, and prenatal or childhood exposure to certain contaminants are widely documented as potential environmental risks factors that may affect the CNS development (**Linnet et al., 2003**; **Weiss and Bellinger, 2006**; **Banerjee et al., 2007**). Taking into account all, the application of classic *in vivo* tests is strongly at odds with the urgency of DNT assessment due to a large number of animals involved, costs and a weak predicts towards human health. In contrast, a battery of alternative to *in vitro* tests such as cell lines and zebrafish embryos supported by mechanistic models (i.e., AOPs, QSARs) seems to better suit this need.

### 1.6.3. Testing strategies for DNT

Neurotoxic potential has been shown for a large number of chemicals currently found in the environment. However, neurotoxicity (including DNT) is hardly tested due to the lack of reliable methods. On the other hand, animal testing is still a mandatory requirement within the regulatory framework for chemical safety assessment (**Scialli and Guikema, 2012**). Nevertheless, due to the complex nature of the human brain, the predictivity of these animal tests for human health effects is limited (**NRC, 2007**; **Baker et al., 2018**), especially for developmental neurotoxicity (DNT). Indeed, although many developmental processes are conserved across mammals (**Workman et al., 2013**), the human brain shows unique abilities in terms of specific cognitive, social skills and behavioural complexity. Thus, it should not be surprising that many human neurodevelopmental disorders are not well studied in rodents

(**Bakken et al., 2016**). Despite the increasing concern regarding the chemical effects on the developing CNS, the DNT assessment is only triggered by evidence of developmental toxicity involving the nervous system, or neurotoxicity or endocrine disruption in systemic toxicity studies in adult rodents. This, may partially explain the deficit of DNT data. The current DNT test are based on the *in vivo* test guidelines (OECD TG 426 and US EPA 712-C-98-239) which are costly, time consuming, and unsuitable for testing a large number of chemicals. However, in a view to cover the entire biological process of the CNS development, a one-in-one replacement of complex *in vivo* tests with relatively easy *in vitro* tests is not practicable. Therefore, a development of an integrated test strategy characterized by a combination of alternative to *in vivo* tests based on DNT mechanistic data, is needed (**Bal-Price et al., 2015**; **Fritsche et al., 2018**; **Hessel et al., 2018**; **Piersma et al., 2018**). DNT test strategy requires knowledge of the main developmental brain processes together with their perturbation in order to smartly select the assays that better describe the key events from exposure to adverse outcome. A full achievement of this purpose is tricky at the present time. Indeed, crucial DNT pathways are still lacking together with the low number of compounds known for damaging the developing CNS. In addition, the human brain development is currently only partly understood. However, the current state of knowledge allows to develop an animal-free mechanism-based testing strategy for DNT assessing. Depending on the biological level at which the DNT evaluation aim to, *in vitro* models offer a wide test variety letting to investigate structural (i.e., morphology, differentiation and synaptogenesis), functional (i.e., measuring the function of the neurons and their network) or more complex (i.e., behaviour) endpoints. Cell lines and whole organisms represent suitable models for these purposes. Among the first, primary neuronal cells, neuronal cell lines, embryonic stem cells (ESC) and human induced pluripotent stem cells (hiPSC) are the most used. Primary neuronal cells can be isolated either from brain regions of rodent embryos or pups either from human aborted foetuses or from brain

surgery resections. *In vitro,* rodent primary neuronal cells can establish functional neuronal circuits of several cell types (**Honegger et al., 1979**) or enriched cultures of neurons or astrocyte. However, they require new *in vivo* embryos or pups for each culture (**Schmidt et al., 2017**) in addition to a lack of interspecies data extrapolation, that make their application limited. Human cell lines offer potential applications for both functional **(de Groot et al., 2016**) and morphological studies (**Radio and Mundy, 2008**) but strictly influenced by their low availability and high variability due to different genetic backgrounds. Regarding the embryonic stem cells (ESC), they are pluripotent cells widely used for measuring a large number of endpoints such as cell differentiation, proliferation, apoptosis, migration, neurite outgrowth (**Radio and Mundy, 2008**) and neuronal network formation (**Kapucu et al., 2012**; **Kiiski et al., 2013**), due to their ability of *in vitro* differentiation into any cell types of all three germ layers (endoderm, ectoderm and mesoderm). For instance, the human embryonic stem cells (hESCs) can differentiate into neuro epithelial precursors (NEP) allowing measurement of the early effects of compounds on the cells that are forming the neural tube (**Shinde et al., 2015**). The human induced pluripotent stem cell (hiPSC) are somatic cells reprogrammed into pluripotent stem cells (i.e., by retrovirus or virus action) (**Takahashi et al., 2007**) aimed to produce mature cell populations such as neural stem cells, various types of neurons (i.e., dopaminergic, cholinergic) oligodendrocyte and astrocytes (**Hu et al., 2010**; **Swistowski et al., 2010**; **Wang et al., 2013**). Cell-based assays are becoming popular also as 3D models because they seem to better mimic the *in vivo* cellular microenvironment and cellular interactions during neural development and therefore may be more physiologically relevant for DNT testing. In addition to cell lines assays, entire organisms like zebrafish embryos (*Danio rerio*), C. elegans and Xenopus laevis have been increasingly used as models for (developmental) neurotoxicity (**Leung et al., 2008**; **Peterson et al., 2008**; **McGrath and Li, 2008**; **Helmcke et al., 2010**; **Avila et al., 2012**; **de Esch et al., 2012a**; **Nishimura et al., 2015**; **Roper and Tanguay, 2018**;

**Sedensky and Morgan, 2018**). The main reason underlying this growth is based on the fact that many of the basic molecular developmental and functional aspects of the nervous system are conserved across mammalian and non-mammalian alternative species. These organisms find application for behavioural outcomes by chemical disruption. Overall, they display a well-characterized nervous system where the main neurotransmitter signalling pathways like cholinergic, glutamatergic, dopaminergic and serotonergic pathways are highly conserved (**Avila et al., 2012**; **Sullivan and Levin, 2016**). Therefore, alteration in specific behavioural endpoints (i.e., locomotor activity, chemotaxis, feeding) can be attributed to specific neuronal circuits (**Helmcke et al., 2010**). Behavioural screening assays can be used as a filter for prioritizing compounds for further DNT testing. To support behavioural findings, zebrafish and C. elegans offer transgenic fluorescent strains leading to study, by neuroimaging techniques, the effect of chemicals on specific neuronal subpopulations or morphology in a living organism (**Helmcke et al., 2010**; **Nishimura et al., 2015**). However, due to the relative simplicity of their neural system compared to the human system, the human relevance of their behavioural outcomes for DNT is under debate. The extensive body of data resulting from the *in vitro* assays brings new notions about mechanisms of action relevant for human neurodevelopment. However, whether considered individually, they cannot fully reflect the complexity of human brain development and its functionality. Consequently, also the hazard assessment may result incomplete. Based on this assumption, test batteries containing combinations of complementary assay aimed to entirely mimic the human brain development are necessary. The European Centre for the Validation of Alternative Methods (EURL-ECVAM) elaborated guidelines for the validation of alternative tests (**Hartung et al., 2004**), highlighting the concepts of biological domain, chemical domain, technical performance, and sensitivity/specificity as selection criteria for the assessment of individual and combined alternative assays (**Hartung et al., 2004**). The wealth of knowledge resulting from the joint

action of complementary alternative tests, will be available to *in silico* models for a more accurate prediction of chemical toxicity. Finally, within a test strategy framework, the zebrafish can play a key role because it represents an excellent whole-organism in *vitro* model which provides the missing link between cell models and the complexity of the in vivo brain.

### 1.7. Zebrafish (Danio rerio): an introduction

Zebrafish *(Danio rerio)* is a tropical freshwater fish, inhabitant of rivers of Himalayan region of South Asia especially India, Nepal, Bhutan, Pakistan, Bangladesh, and Myanmar (**Rahman, 1989**; **Barman, 1991**; **Talwar and Jhingran, 1991**; **Menon, 1999**; **Bhat, 2003**). It is a bony fish (teleost) that belongs to the family Cyprinidae under the class Actinopterygii (ray-finned fishes). The body of the zebrafish (**Figure 3**) is covered with five blue, horizontal, pigmented, uniform, stripes, which resemble zebra's stripes. The differences between fish genders are notable. The female has a silver stripes instead of gold and whitish, larger belly. In contrast, the male has gold stripes between the blue stripes and it is torpedo-shaped. The adult zebrafish can reach up to 4-5 cm with some length variations in the wild ones (**Arunachalam et al., 2013**). Its lifetime is different in captivity (two to five years) and in the wild (one year) (**Spence et al., 2008**; **Vishwanath, 2010**). About 28.5 °C temperature and an average pH of 8.0 are almost universally cited as ideal physical parameters in zebrafish culture **(Schaefer and Ryan, 2006**; **Spence at al., 2006**; **McClure et al., 2006**). The zebrafish is crucial and worldwide used vertebrate model organism in the scientific research, particularly in pre-clinical drug development (**Van Wijk et al., 2017**). It was first used as a biological model by George Streisinger (University of Oregon) in the 1970s. Furthermore, it is well-known for its regenerative capability, as well as has been modified by scientists to produce many transgenic strains (**White et al., 2008**). The use of zebrafish as a model organism got impetus from the 1990s when it was used to develop two large genetic mutants, one by Nobel Prize winner

Christiane Nusslein-Volhard in Tubingen, Germany, and the other by Wolfgang Driever and Mark Fishman in Boston, USA (**Khan and Alhewairini, 2018**).

**Figure 3** Zebrafish [Link: https://www.zebrafishfilm.org/].

### 1.7.1. Zebrafish development – from fertilization to hatching

The embryonic development of the zebrafish (*Danio rerio*) is characterized by seven broad periods: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching (**Kimmel et al., 1995**). A female zebrafish can produce as many as several hundreds of eggs per spawning, each of about 0.7 mm in diameter. The fertilization of a newly oocyte triggers the development of the zebrafish embryo in which a single cell, called blastomere, develops through cytoplasmic movements from the yolk to the animal pole (**Figure 4**). The development continues through the cleavage phase where the blastomere keeps to divide synchronously up to the 128-cell stage. This stage may take between 40 minutes and 2 hours. Subsequently, the embryo enters the blastula stage which is characterized by three different processes: desynchronization of the cell cycles in mid-blastula transition (MBT), the yolk syncytial layer (YSL) forms, and epiboly begins (**Kane and Kimmel, 1993**; **Kimmel et al., 1995**). During epiboly the cells form a blastoderm surrounding the yolk (**Kimmel et al., 1995**; **Rohde and Heisenberg, 2007**). The embryo goes through gastrulation during which the three germ layers, ectoderm, endoderm and mesoderm, are formed (**Kane, 1998**; **Rohde and Heisenberg, 2007**).

Whereas blastulation occurs between 2 and 5 hours after fertilization, gastrulation occurs between 5- and 10-hours following fertilization. At the end of the gastrulation phase, the tail bud is formed and the segmentation period starts. At this stage, the somite, which will mainly develop into skeletal muscle cells, develop (**Kimmel, 1988**). In addition, neurulation and early brain development take place during this stage of development. Furthermore, the tail bud elongates and even some first movements appear. The early signs of the eye, consisting of an optic cup and lens vesicle, become visible and the optic vesicle containing two otoliths is formed. At approximately 24 hours post fertilization (hpf) the basic body plan is developed. A heartbeat will be present and circulation will start shortly after. Then, pigmentation starts to show and the pectoral fins will develop. Hatching occurs between 48 and 72 hours after fertilization (**Kimmel et al., 1995**). This, however, has been shown to largely depend on the thickness of the chorion as well as the muscular activity of the embryo. Depending on these factors, hatching of some embryo may be delayed. Once it is hatched, the larvae measures about 3mm in length. In a period of about 3 days, the larval undergoes morphogenesis which is characterized by the development of various anatomical structures. By the late larval stage (about 7 days after fertilization) the organism is capable of swimming, moving its jaws and even feeding on various food material.

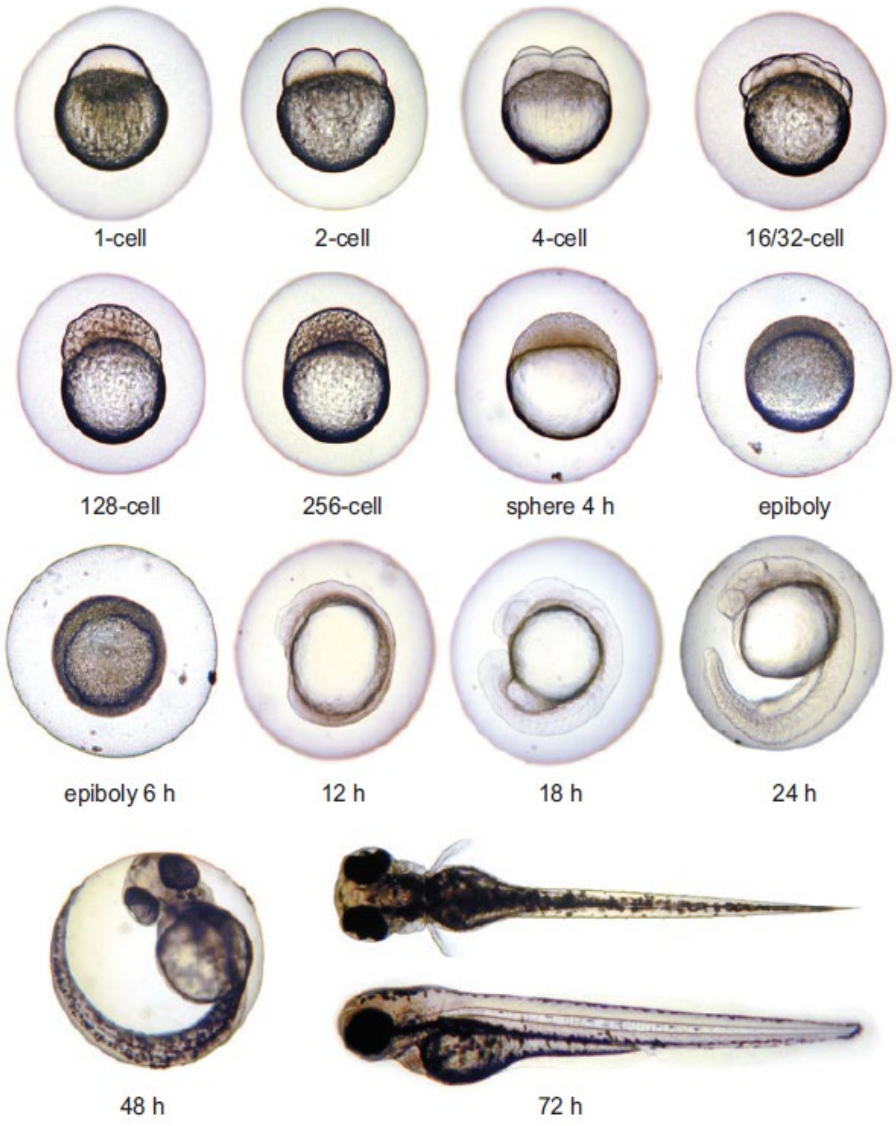


**Figure 4** Zebrafish embryo development from the 1-cell stage to hatching at 72 hpf.

### 1.7.2. Zebrafish as a model for DNT testing

To date, the zebrafish embryo is a well described model in human and environmental toxicology, considered as an alternative and efficient model for speeding up chemical hazard assessment (**Scholz et al., 2008**; **Zoupa and Machera, 2017**). In line with the 3Rs (Replacement, Reduction, Refinement) perspective, the zebrafish embryo is not considered as

an animal experimental according to the current European animal directive (2010/63/EU) up to day 5 post fertilization (dpf). Indeed, at these developmental stages, zebrafish embryos are likely to experience less or no pain, suffering, distress or lasting harm. In addition, zebrafish are oviparous, i.e., fertilization and development occur outside the mother, and therefore no euthanasia of parental animals is necessary to obtain embryos. Zebrafish present several advantages that meet the high need to develop alternative approaches to assess developmental neurotoxicity. Their small size allows for the use of multi-well plates in experimental work leading to a medium- to high-throughput, making the experiments conducted in zebrafish less expensive and time-consuming than those conducted in rodents (**Crofton et al., 2012**). They undergo external fertilization that allows to follow real-time the morphology of embryonic development due to the transparency of the eggs and embryos. Moreover, the zebrafish are easier and less expensive to house and care for than common rodent models. They show a high fecundity rate compared to rodent; roughly 200–300 embryos versus only 5–10 offspring produced by zebrafish and rodents for each mating event, respectively. The most remarkable benefit is that the zebrafish is relatively complex *vertebrate* species with a high degree of genetic, morphological and physiological homology with humans (**Kalueff et al., 2014**). Interestingly, human genetic diseases can be studied through the application of zebrafish (**Howe et al., 2013**) because its genome is fully sequenced (http://www.ensembl.org/Danio_rerio/Info/Index) and its express homologs for >70% of human genes (**Howe et al., 2013**). Compared with the *in vitro* cultured cells, zebrafish experiments lead to a stronger correlation with *in vivo* mechanism and therefore they display an important translational value. Indeed, despite being a non-mammalian animal, fundamental mechanisms of neurodevelopment are highly conserved between zebrafish, humans and other vertebrate models (**Howe et al., 2013**). Summing up, the zebrafish provides a practical and complex *in vitro* whole-organism model that is right between cells and higher vertebrates.

### 1.7.3. The zebrafish brain development

The development of zebrafish CNS occurs within 5 dpf. Specifically, neurogenesis starts around 6 h post fertilization (hpf) and at the end of gastrulation (9–10 hpf) the neural tube is formed. While the first body movements appear at 17 hpf, crucial parts of the brain such as the forebrain, diencephalon and telencephalon, midbrain, hindbrain and spinal cord are formed at 24 hpf together with the first neuron connections by axons (**de Esch et al., 2012a**; **Schmidt et al., 2017**). After 96 hpf, the nervous system is almost entirely developed, and all catecholaminergic neuron clusters, glial cell subtypes, oligodendrocytes, Schwann cells and astrocytes can be recognized (**Nishimura et al., 2015**; **Legradi et al., 2015**). Zebrafish and mammals share similar mechanisms in the early developmental stages and they express a similar range of neurochemical phenotypes, including GABA (**Higashijima et al., 2004**), glutamate (**Higashijima et al., 2004**), serotonin (**McLean and Fetcho, 2004**), dopamine (**McLean and Fetcho, 2004**), norepinephrine (**McLean and Fetcho, 2004**), glycine (**Higashijima et al., 2004**) and acetylcholine (**Panula et al., 2010**). Additionally, the development and function of biological barrier systems (BBB) in the developing CNS shows a biological similarity between the zebrafish and other vertebrates. The BBB is one of the most effective barrier systems in vertebrates and its key role in the protection of the brain against neurotoxic insults is well accepted. Finally, it displays all of the classical sensory organs such as vision, olfaction, taste, touch, balance, and hearing. Given the positive features of zebrafish described above, the effects of different chemicals on brain development can be assessed by different neurotoxicity endpoints. Among them, gene expression patterns, neural morphogenesis and neurobehavioral profiling are the most studied in the last decade (**Truong et al., 2014**; **Chueh et al., 2016**).

### 1.7.4. The development of the zebrafish motor behavior

The hindbrain and spinal cord are two key areas in the development and control of locomotion in a wide variety of vertebrates, including zebrafish (**Butler and Hodos, 1996**; **Grillner et al., 1997**; **Stein et al., 1997**; **Bass and Beker, 1997**). In the latter, spontaneous contractions, touch responses and swimming are the three sequential behaviours that appear starting at 17 hpf. Before this time, they do not show any movement. At 27 hpf, the first episodes of swimming appear in response to touch in a clear forward movement by at least one body length. However, a spontaneous swimming begins just prior to the feeding stage at 5 days, when the air bladder and sensory systems are functional. The appearance of these sequential motor behaviours is related to changes in cellular mechanisms that generate locomotion. Indeed, whereas spontaneous contractions are essentially due to electrical inputs via gap junctions rather than chemical synapses (**Saint-Amant and Drapeau, 2000**), during the touch response, for the first time, glutamatergic transmission plays a role in the developing locomotor network (**Saint-Amant and Drapeau, 2001**). The key step toward spontaneous swimming occurs in the transition from a bland and discontinuous swimming pattern (3-4 dpf) to a sustained swimming (4-5 dpf). This enhancement of locomotion may be related to the late appearance of common neuromodulators such as the biogenic amines (**Stein et al., 1997**). Serotonin (5-HT) is an important biogenic amine that have been detected in segregated populations of serotonergic neurons placed in the hindbrain and within the ventral spinal cord as early as day 1. Since 5-HT is known to promote differentiation, growth and synaptogenesis (**Lauder, 1993**), as well as being directly involved in down-regulating gap junctions in other developing networks (**Rorig and Sutor, 1996**), its early appearance in the zebrafish may simplify the transition from a network mediated by gap junction to a locomotor neuronal network driven by chemical and

electrical synaptic signals. However, although the development of the serotonergic system in the embryo already starts between 24-48 hpf, the serotonergic modulation only starts at 96 hpf when serotonergic axons have completely innervated the length of the spinal cord, from head to tail (**Airhart et al., 2007**). Thus, from this stage on there is serotonergic modulation and functional serotonin plays a role in the swimming activities modulation (**Airhart et al., 2007**; **Tufi et al., 2016**).

### 1.7.5. Behavior endpoint: modulation of locomotor activity by light adaptation and light-dark transition test

In all species, correct development of the nervous system leads to a measurable integrated outcome: behaviour. Also, zebrafish display a wide range of complex behaviours including social, anxiety, learning and memory that can be used for modelling neurodevelopment disorders. (**Friedrich et al., 2012**; **Norton, 2013**; **Levitas-Djerbi et al., 2017**). Moreover, it is well known that zebrafish larvae are sensitive to a variety of stimulus modalities such as touch, olfaction, audition, vestibular inputs, heat, and vision (**Orger et al., 2017**). The behaviours commonly investigated are thigmotaxis, startle response, optokinetic response, habituation, prey capture, sleep/awake behaviour and locomotor behaviour. Among them, the endpoint of locomotion or swimming activity is gaining popularity over the years. In mammals, two systems drive the diurnal control of locomotor activity: an endogenous circadian clock and a "masking response" to high intensity of illumination (**Mrosovsky and Hattar, 2003**; **Panda et al., 2003**). The masking response refers to changes in behaviour normally controlled by the circadian clock due to sudden changes in illumination (**Lin and Jesuthasan, 2017**). Also, zebrafish larvae show rhythmic locomotor activity guided by a circadian clock (**Cahill et al., 1998**; **Prober et al., 2006**) and a locomotor activity strongly modulated by unexpected lighting changes (**Burgess et al., 2007**; **MacPhail et al., 2009**). Particularly, zebrafish larvae display

two specific activity patterns depending on the extent of light and dark periods. Indeed, higher levels of activity occur in extended light than in extended dark whereas alternating light and dark periods produces the opposite pattern of activity (**Burgess et al., 2007**; **MacPhail et al., 2009**). While the first pattern may represent habituation to a constant condition of light or dark (**Burgess et al., 2007**; **MacPhail et al., 2009**), the rising of locomotor activity during the transition from light to dark may be attributed to the increased stress/anxiety level in zebrafish larvae (**Irons et al., 2010**). Thus, assessment of the alternating light-dark pattern in zebrafish larvae may offer a powerful tool for screening a large-scale of chemicals in toxicology and pharmacology. Here, the distance travelled by zebrafish larvae in both conditions is evaluated for understanding the neurobehavioral effects following a period of acclimatization. Indeed, the locomotor activity depends on the integrity of brain function, nervous system development, and visual pathways, (**Bilotta et al.**, **2002**). Therefore, behaviour is a sensitive endpoint for DNT assessment. Several studies observed changes in zebrafish behaviour following exposure to neuroactive drugs (i.e., d-amphetamine, cocaine, ethanol, methylmercury and chlorpyrifos) by the application of the light-dark locomotion test (**Irons et al., 2010**; **De Esch et al., 2012b**; **Ramcharitar et al., 2013**; **Asmonaite et al., 2016**; **Colon-Cruz et al., 2018**; **Mora-Zamorano et al., 2016**; **Richendrfer et al., 2012**; **Schmitt et al., 2019**). To date, due to the variations in the light-dark locomotion protocols, the comparison of data obtained across different laboratory seems problematic. The main differences are in the stage of zebrafish larvae, number of wells in multi-well plates, the length of experiments as well as the length of light and dark conditions. Consequently, inter-laboratories study plans aimed to assess the added value of the light-dark transition zebrafish test by the analysis of a consistent number of potential DNT compounds, is needed. Based on the outcomes obtained, this test can be added in the guidance lines as a tool for DNT assessment. Nevertheless, data from literature prove

that the light-dark locomotion test can be successfully used for performing large-scale high-throughput screening of thousands of neuroactive compounds.

### 1.8. Selected neuroactive compounds – pharmacological profile and clinical use

For the experiments in zebrafish a set of neuroactive compounds was selected. Indeed, established that pharmaceuticals exhibit a well-known MOA and are recognized to have a role in DNT, their use as model compounds as an alternative of pesticides was preferred.

#### 1.8.1. Carbamazepine

5H-Dibenzo[b, f]azepine-5-carboxamide (**Figure 5**), better known as Carbamazepine (CBZ), is an antiepileptic drug widely prescribed for the treatment of epilepsy, neuropathic pain and for acute manic and mixed episodes of bipolar I disorders **(Gierbolini J. et al., 2016)**. In 1962, it was first marketed for the treatment of trigeminal neuralgia for which it is currently regarded as a first-line treatment (**Harkin and Hopkinson, 2010**). However, CBZ gained success in the treatment of epilepsy due to its potentiality in suppressing seizures and its acceptable safety profile (**Zhou et al. 2011**). Epilepsy is a chronic neurological disorder affecting nearly 1–2% of the worldwide population, related to depression, anxiety and increased mortality rate (**Schmidt and Sillanpaa, 2012**). More than 65 million people worldwide have epilepsy, and around 2.4 million new cases of epilepsy are diagnosed annually (**World Health Organization, 2016**). CBZ is almost completely absorbed from the gastrointestinal tract with a peak concentration occurring between 4-10 hours since the drug assumption (**Sillanpää, 1981**). It has a high bioavailability rate (ranging from 70 to 98 % according to urinary recovery) with the plasma protein binding on the average between 60 and 70%. CBZ is generally metabolized by epoxidation to carbamazepine 10-11 epoxide (CBZ 10-11E), the major CBZ

metabolite (**Sillanpää, 1981**). CBZ 10-11E has been demonstrated to be equipotent in terms of anticonvulsant property compared to its parent compound. CBZ excretion mostly occurs in metabolized form (at least 14 metabolites have been recognized), and only 1-2% of the drug is excreted unchanged into urine (**Sillanpää, 1981**). CBZ is a well-known voltage-gate sodium channel blocker. By blocking neuronal sodium channels in the inactivated state, it stops the neuronal sodium current required to depolarize the nerve, therefore inhibiting the repetitive neuronal process that occurs during a seizure (**Fent et al., 2006**; **Harkin and Hopkinson, 2010**). Moreover, CBZ is a gamma-aminobutyric acid (GABA) agonist, a key inhibitory neurotransmitter in both developing and mature mammalian CNS. GABA receptor activation by CBZ allows the entry of chloride into the cell, avoiding the generation of an action potential.

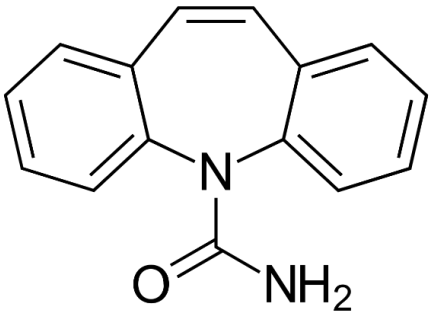


**Figure 5** Carbamazepine.

### 1.8.2. Fluoxetine

Fluoxetine (FLX, tradename Prozac) is a racemic mixture of R and S isomers (**Figure 6**), belonging to the group of selective serotonin reuptake inhibitors (SSRI). It was discovered in 1972 and approved for the commercialization by the Food and Drug Administration (FDA) in 1987. To date, fluoxetine is commonly prescribed for the treatment of major depressive disorder, panic disorder, obsessive-compulsive disorder (OCD), bulimia nervosa and premenstrual dysphoric disorder (PMDD) (**Dulawa et al., 2004**). In humans, following a single

oral dose of 40 mg, a peak plasma of FLX concentrations ranging from 15 to 55 ng/mL is observed after 6 to 8 hours. Over the concentration range from 200 to 1000 ng/mL, roughly 94.5% of FLX is bound in vitro to human serum proteins. FLX is extensively metabolized in the liver to norfluoxetine (norFLX) and a number of other unknown metabolites. The only identified active metabolite, norfluoxetine, is formed by demethylation of fluoxetine (**Hiemke and Härtter, 2000**). In animal models, S-norfluoxetine is a potent and selective inhibitor of serotonin uptake and has activity essentially equivalent to R- or S-fluoxetine (**Barclay et al., 2011**). Liver metabolism appears the primary route of elimination in order to inactive the kidney metabolites. Although a large discrepancy of the excretion rates is found in the literature, FLX seems to be excreted by urine in a range percentage of 5-30 % as unchanged parent compound (**Brooks et al., 2003a**; **Jjemba, 2006**; **Moffat et al., 2005**; **Fong and Molnar, 2008**). It acts as a potent selective inhibitor of the transporter enzyme for serotonin reuptake (SERT, 5-HTT) at the presynaptic membrane, allowing an increase of serotonin levels at postsynaptic receptor sites (**Costagliola et al., 2008**). FLX has either no effect or a small effect on the neuronal reuptake of norepinephrine and dopamine. In addition, it does not bind to cholinergic, histaminergic, or $\alpha$-adrenergic receptors.

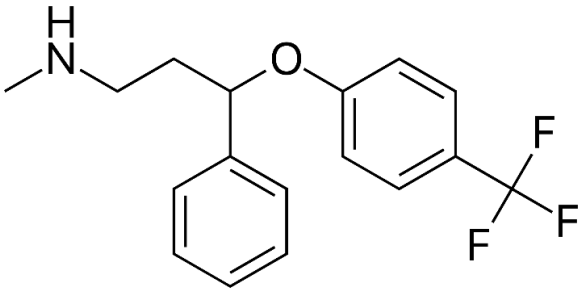


**Figure 6** Fluoxetine.

### 1.8.3. Venlafaxine

Venlafaxine (VNX, tradename Effexor) (**Figure 7**), is an antidepressant medication belonging to the drug class of serotonin (5-HT) and norepinephrine (NE) reuptake inhibitors (SNRIs) (Ilyas, S. et al., 2012; Horst, W. D. et al., 1998). It is prescribed to treat major depressive disorder (MDD), generalized anxiety disorder (GAD), panic disorder, and social phobia (Bymaster, F.P.et al., 2001). In the mid-1990s, SSNRIs were introduced on the market and, over the last decade, venlafaxine became one of the most frequently prescribed antidepressant drugs due to its faster therapeutic onset and less side effects in patients (**Sansone and Sansone, 2014**). 92% of a single VNX dose is absorbed in the gastrointestinal tract, with a distribution volume of roughly 7.5 L/kg. VNX binding to human plasma is about 27% at concentrations ranging from 2.5 to 2215 ng/mL. VNX is metabolized in the liver to its major active metabolite, O-desmethylvenlafaxine (ODV), by CYP2D6 isoenzyme (**Thase ME et al., 2009**) whereas other hepatic enzymes (CYP3A4, CYP2C19, and CYP2C9) metabolize venlafaxine and ODV to minor and less active metabolites. VNX and ODV are both potent inhibitors of neuronal serotonin and norepinephrine reuptake and weak inhibitors of dopamine reuptake (**Roseboom and Kalin, 2000**). The main route of excretion of venlafaxine and its metabolites is the renal elimination in which clearance rate O-desmethylvenlafaxine (10 h half-life) is slower than that of venlafaxine (4 h half-life) (**Thase ME et al., 2009**). VNX modulates the levels of the brain neurotransmitters serotonin and norepinephrine by blocking their reuptake transporter on presynaptic neurons, thereby increasing synaptic availability of these monoamines (**Schafer, 1999**). Although VNX is a double reuptake inhibitor, it has a stronger effect on 5-HT reuptake compared to NE (**Roseboom and Kalin, 2000**). Moreover, it shows a weak effect on dopamine (DA) reuptake although the underlying mechanism may be linked to the modulation in NE levels, since noradrenergic neurons modulate mesolimbic and mesocortical DA systems (**Muneoka et al., 2009**; **Liprando et al., 2004**). Given this low modulation on dopamine

transmission, VNX is also referred to as a serotonin-norepinephrine-dopamine reuptake inhibitor (SNDRI).

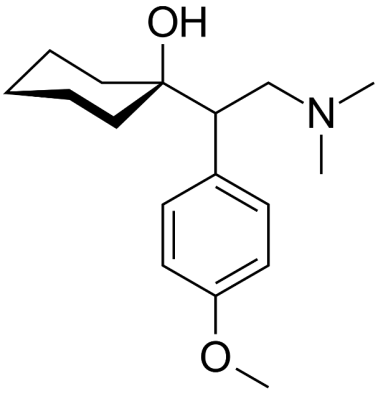


**Figure 7** Venlafaxine.

### 1.9. Why do the selected neuroactive compounds may present concern for DNT?

Neuroactive compounds are specifically designed to cause a biological or physiological effect in humans or animals by affecting the central nervous system and its components (**Fent et al., 2006**). For instance, they are sufficiently lipophilic for crossing cellular membranes in addition to be relatively persistent to metabolic inactivation before having the desired therapeutic effect. The exposure of these compounds to the developing CNS may represent a serious risk to human health because damages occurring during this stage are mostly irreversible and they may lead to short and long-term neurobehavioral effects. Pharmacological treatments with the studied compounds are recurrent during pregnancy. Indeed, about 0.5–2.5% of pregnant women are exposed to antiepileptic drugs (AEDs) for epilepsy and other various conditions (i.e. pain syndromes, psychiatric disorders and chronic migraine) (**Holmes et al., 2001**) while roughly 10-16 % of the pregnant women are at risk to present a major depressive disorder (**Austin et al., 2007**; **Yonkers et al., 2009**) which is mostly treated with serotonin reuptake inhibitors (SRIs) (**Taouk et al., 2018**). Moreover, all the targets compounds are capable to cross the placenta barrier in addition to being transported to the infant through breast feeding (**Ewing et**

**al., 2015; Rampono et al., 2009**) increasing therefore the risk of negative neurodevelopmental outcomes. Among the latter, potential impairments of language and psychomotor development at childhood and school age as well as neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) are the most investigated DNT endpoints (**Harrington et al., 2013, 2014**; **Boukhris et al., 2016**; **Man et al., 2017, 2018**). Despite that, in order to avoid maternal and fetal risks associated with the illness itself and considered the overall safety of these pharmaceuticals (**Einarson et al., 2004**; **Viguera et al., 2007**), pregnant women commonly continue the treatments as demonstrated by the high rate of prescriptions worldwide (**Calisto and Esteves, 2009**). This may explain an additional human health concern linked to the spread of these pharmaceuticals (and their transformation products) in the environment. Indeed, like most of the drugs, they end up into the environment by human excretion (**Williams, 2008**; **Cunningham et al., 2006**) or by inadequate disposal of unused or expired human medications. Consequently, they are usually detected at different environmental matrices such as wastewaters, surface, ground and drinking waters, soils, sediment and even animal tissues (**Rao et al., 2014**; **Expósito et al., 2018**; **Mole and Brooks, 2019**; **Metcalfe et al., 2010**). Although at environmental level they are detected at concentrations much lower than the doses therapeutically required, they might alter crucial processes in sensitive non-targets, including children and pregnant women. This reflection gained attention when it was reported the capacity of some pharmaceuticals to interfere with ecosystem at very low concentrations (**Halling-Sørensen et al., 1998**). In addition, given the presence in the environment of different pharmaceutical contaminants (**Aus der Beek et al., 2016**), the low concentrations of single compounds represent an unreliable safety for human health. Indeed, by the sum of low doses, they may form therapeutically active mixtures. Finally, the DNT assessment also needs to include the risk from the major metabolites of neuroactive compounds which are habitually detected in the environment (**Metcalfe et al., 2010**;

**Lajeunesse et al., 2013**; **Gurke et al., 2015**; **Alygizakis et al., 2016**). They may strongly contribute to the final therapeutic effect because sometimes they are detected at concentrations higher than the parent compounds and, some of them, are equipotent to the parent compound.

# 2. Material and Methods

## 2.1. Analytical assessment of pesticides multiresidue

### 2.1.1. Pest treatment, samples collection and processing

Pest treatments followed an integrated production strategy which included the supervision of specialized technicians about the use of authorized pesticides. For instance, attention was paid on pesticides with the shortest pre-harvest interval, lowest toxicity and the minimum environmental persistence. The collection of the tomato samples was carried out in an area of 558 ha located in the province of Oristano and Middle Campidano (Sardinia, Italy), in August 2019. A total of 159 fields, ranging from 10 to 1 Ha in size, with seven tomato cultivars (Taylor 28.9%, Creso 19.5%, Dask 21.4%, Docet 19.5%, Datterino 1.3%, Rapidus 1.9%, and undefined cultivar 7.5%) were selected. Raw tomato samples were manually collected in 15 kg boxes, considering plant density and spacing. Then, 1 kg of samples per ha was pooled and transported to the laboratory. Ten samples for each typology of purée, triple concentrated paste, fine pulp and diced (from now on called processed tomatoes), were obtained from 5 batches of processing and analysed during the working month.

### 2.1.2. Chemicals and reagents

All the tested compounds used for qualitative and quantitative analysis were certified analytical standards (≥99.5% purity) from Dr Ehrenstorfer (Lab service Analitica, Milan, Italy). Acetonitrile (ACN) and methanol (MeOH) were LC/MS grade solvents (Sigma Aldrich, Milan, Italy). Formic acid was reagent grade (>95%, Honeywell, Sigma Aldrich), ammonium formate solution 5 M (0.315 g $mL^{-1}$) (G1946-85021, Agilent Technologies). QuEChERS reagents were: Part No.: 5982-6650, 4 g MgSO, 4.1 g NaCl, 1 g trisodium citrate dihydrate, 0.5 g disodium hydrogen citrate sesquihydrate (EN Method 15662, Agilent Technologies, Milan, Italy); Part No.: 5982-5056, 150 mg PSA, 900 mg $MgSO_4$ (EN Method, fruit and vegetable,

Agilent Technologies, Milan, Italy). MilliQ water with a conductivity less than 18.2 MΩ was obtained from an integrated Millipore purification system (MilliQ integral, Merck, Milan, Italy). The stock solution of pesticide standards (~1000 mg $L^{-1}$) was prepared by weighing about 10 mg of pesticide in a 10 mL volumetric flask, filling up to volume with ACN. Mixed multiresidue pesticide intermediate solution was prepared at 5 mg $L^{-1}$ in ACN. The working solutions were prepared daily by diluting the stock solutions with eluent mixture (MeOH + $H_2O$ at 0.1% formic acid and 0.5 mM ammonium formate).

### 2.1.3. Sample preparation

The samples of raw tomato were prepared on the same day of collection by chopping and homogenized with a semi-industrial blender. Processed tomatoes were directly blended in a 600 W stainless steel food blender (Girmi, Rimini, Italy). In general, 10 g of homogenized sample was weighed in a 50 mL test tube and, thereby agitated in the vortex (Reax Top, Heidolph, Germany) with 10 mL of ACN for 1 min. Then, 6.5 g of QuEChERS salt (Part No.: 5982-6650) was added and, thus agitated for 2 min in the vortex and an additional 15 min in a rotatory shaker. The sample was further centrifuged for 5 min at 4000 RPMs and 10 ˚C (Centrifuge 5810 R, Eppendorf AG 22331 Hamburg). 6 mL of the supernatant was recovered and transferred to a 15 mL test tube containing 1 g of the second QuEChERS salt (Part No.: 5982-5056, Agilent, Milan, Italy). The same procedure of extraction was also repeated for the 15 mL test tube. Finally, the resulted organic solution was filtered at 0.45 µm (PTFE, Thermo Scientific, Waltham, MA, USA) and transferred to a 1.8 mL vial for LC-MS/MS analysis.

### 2.1.4. UHPLC-MS/MS analysis

A UHPLC Agilent 1290 Infinity II LC coupled with an Agilent 6470 Triple Quad LC-MS/MS mass detector was used. The chromatography column was a ZORBAX Eclipse Plus C18 (2.1

× 150 mm, 1–8 µm). A binary gradient composed of water (A) and methanol (B) solutions with the addition of 5 mM ammonium formate and 0.1% formic acid was set as follows: T = 0 A 95%, T = 50 sec A 95%, T = 3.50 min A 60%, T = 17 min A 2% and 10 extra min of post-run at 95% of A solution. The total duration of the run was 27 min while the flow was 0.3 mL/min with 2 µL of sample volume injected. Both mass detector gas and sheath-gas were set at 350 °C whereas gas flow and sheath-gas flow were 10 L $min^{-1}$ and 12 L $min^{-1}$, respectively. Additional settings were: gas nebulizer at 30 psi, ion capillary at 4000 V. MassHunter ChemStation was the software used.

### 2.1.5. Method validation

The analytical method was validated according to SANTE Guidelines assessing linearity, selectivity, precision, method detection limits (LOD), method quantification limits (LOQ), accuracy in terms of recovery, uncertainty, and matrix effect (**SANTE/12682, 2019**). Since no blank certified control sample has been available on the market, a control field (i.e., row tomatoes) was set up on a reliable farm. Each typology of processed tomatoes were also used as a control matrix. A total of six blank control samples for each of the five matrices (i.e., raw tomatoes and four typologies of processed tomatoes) were spiked with the mixed multiresidue standard at 5xLOQ and analysed in one day for repeatability (RSDr, intraday $n$ = 30). In comparison, reproducibility ($RSD_{wR}$) was calculated by the analysis of two samples for each matrix in six separate days ($n$ = 60). Each sample belonged to an independent experiment. Recovery assays were carried out fortifying blank control at LOQ and 10xLOQ with the mixed multiresidue pesticide standard and, left standing for 30 min. Three replicates samples of each concentration were analyzed for each matrix (n = 30), as reported above. Recovery results were analyzed using matrix control standard calibration curves. The instrumental sequence was conducted according to SANTE indications. The matrix effect was evaluated by comparing the

analytical responses of the active ingredients in ACN + 0.1% formic acid with those prepared with blank control matrix extracts. Linearity was assessed by analyzing five standard calibration curves performed in triplicate, both in solvent and blank control matrix extracts. It was admitted as acceptable when the coefficient of determination was above 0.990. Selectivity was assessed comparing extracts from control matrices with those spiked at the LOQ value. The absence of peaks at the retention times of the studied compounds was a criterion for confirmation method selectivity. The expanded measurement uncertainty (U), a quantitative parameter of the reliability of the analytical method, was calculated by multiplying the combined uncertainty ($u'$) by a coverage factor k = 2, to accomplish a level of confidence of 95%, using the following Equations:

$$u' = u'(bias)^2 + u'(precision)^2; \quad (1)$$

$$U = k \times u' \quad (2)$$

The instrument LOD and LOQ were calculated as three and ten times the signal to noise ratio (S/N) (**Regulation(EC) NO 396, 2005**; **Shrivastava et al., 2011**).

### 2.1.5. Industrial processing

Tomato samples were mechanically collected from the field in 350 kg bins and brought to the industrial plant within three hours. Then, the tomatoes were left to stand shortly before being subjected to washing and visual selection to reject any tomatoes that are immature, over-ripe, rotten, or damaged. The selected tomatoes were subjected to a blanching step to facilitate skin removal in the subsequent peeling stage. After that, tomatoes followed two different production lines. The first led to purée, triple concentrated paste, fine pulp, and the second to diced tomatoes. Steps of refilling, acidic adjustment, and concentration (only for triple concentrated

paste), packing and sealing, followed by pasteurization and cooling were carried out before storing and marketing.

### 2.2. DNT assessment

#### 2.2.1. Chemicals

The test compounds carbamazepine (CBZ, CAS 298-46-4; cat. no. C4024), carbamazepine 10,11-epoxide (CBZ10,11E, CAS 36507-30-9; cat. no. C4206), fluoxetine hydrochloride (FLX, CAS 54910-89-3; cat. no. F132), norfluoxetine hydrochloride (norFLX, CAS 57226-68-3; cat. no. F133), phenytoin (PHT, CAS 57-41-0, cat. no. P1290000), venlafaxine hydrochloride (VNX, CAS 93413-69-5; cat. no. V7264) and desvenlafaxine hydrochloride (desVNX, CAS 300827-87-6; cat. no. D2069) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). VNX, FLX and their metabolites were diluted directly in embryo medium (see below). CBZ, its metabolites and PHT were dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and further diluted in embryo medium, with a final concentration of 0.1% DMSO.

#### 2.2.2. Maintenance of fish and egg spawning

Experiments with zebrafish (*Danio rerio*) were performed at two locations, *i.e.* at the zebrafish facilities of National Institute for Public Health and the Environment (RIVM), Bilthoven, and Free University Amsterdam (VU), both using a breed of AB-line zebrafish obtained from the European Zebrafish Resource Center (Karlsruhe, Germany); in addition, a breeding line of fish which were originally obtained as commercial wild-type import (Ruinemans Aquarium BV, Monfoort, The Netherlands) was used at RIVM to replicate some of the single compound experiments. Fish were kept and bred under similar standard conditions in both facilities, using

7.5 L ZebTec tanks (Tecniplast S.p.A, Buguggiate, Italy), and with a photoperiod of 14/10h light/dark (gradual on and off turning), temperature maintained at 27.5 ± 1 °C, pH at 7.5 ± 0.5, and conductivity at 500 ± 100 µS. Fish were fed twice a day with SDS 100, 200, 400 or small granules (Special Diet Services, Essex, UK) depending on the age of the fish, and supplemented with *Artemia salina* (three times per day in-house cultured live artemia for larvae and young juveniles; defrosted artemia obtained from Ruto Frozen Fish Food Zevenhuizen, The Netherlands, once daily for adults). To obtain embryos for experiments, females were separated from males four days prior to spawning and fed a artemia three times/day. The afternoon before spawning, two females and two males were reunited as breeding units in breeding tanks, and spawning initiated by next morning light.

### 2.2.3. Zebrafish Embryotoxicity Test (ZFET)

The Zebrafish Embryotoxicity Test (**Hermsen et al., 2011**) was applied to determine the embryotoxicity potency of the test compounds in the fish. Spawned eggs were collected with a sieve and rinsed thoroughly with embryo medium (demineralized water supplemented with 100 mg/l $NaHCO_3$, 20 mg/l $KHCO_3$, 200 mg/l $CaCl_2 \cdot 2H2O$, and 180 mg/l $MgSO_4 \cdot 7H2O$) and quality was checked under a microscope. Batches with less than 10% coagulated eggs and limited egg deformations were pooled. Eggs at 4-32-cell stage were selected transferred within 2.5 hours post fertilization (hpf) to a 6 well-plate (10 eggs per well) containing 5 mL test medium with a dilution range of each test compound in embryo medium, including maximum dissolution as highest concentrations (**Table 2**), each with appropriate solvent controls (0.1% DMSO for CBZ, its metabolites and PHT, plain embryo medium for FLX, VNX and their metabolites) after that immediately moved to a 24- well plate (1 egg and 2 mL per well). The 24-well plates were kept in an incubator at 26.5±1°C with a light/dark cycle of 14/10h. After 3 days post-fertilization (dpf), development and teratological effects of the embryos were

evaluated under a light microscope as described previously (**Hermsen et al., 2011**). In brief, development was scored using a integrative semi-quantitative scoring system (General Morphology Score, GMS) for specific developmental endpoints, including detachment of tail, formation of somite, development of eyes, movement, heartbeat, blood circulation, pigmentation of head- body, pigmentation of tail, pectoral fin, protruding mouth, and hatching. In addition, teratological effects were scored as present or absent as a total teratology score, considering pericardial edema, yolk sac edema, eye edema, malformation of the head, absence/malformation of sacculi/otoliths, malformation of tail, malformation of heart, modified chorda structure, scoliosis, rachischisis, and yolk deformation.

### 2.2.4. Light dark transition test

Exposure to single compounds was performed starting within the first 2.5hpf and terminating at 5 dpf for evaluation of the swimming activity. The fertilized eggs were firstly exposed in a 6 well-plate (20 eggs per concentration and solvent control) containing 5 mL of test medium and kept in an incubator at 27.5±0.5°C up to 5 dpf. Before performing the behaviour test, embryos were moved along with 300 µL of test medium to a 96 well-plate (1 embryo per well) for a total of twelve (*n=12*) embryos per concentration and solvent control. At 120hpf after acclimatization for 30' light free swimming activity was recorded in the ZebraBox (Viewpoint, France) during three repeated triggers of light-dark transitions in 10' periods. Sensitivity was set at 20 whereas thresholds were 10 (Burst) and 1 (Freezing). Locomotor activity was evaluated as the duration of movement, using the Zebralab Quantization software (Viewpoint, France), which gives "time in activity" as output. Occasionally occurring embryos with observable morphological aberrations were excluded from behaviour testing to avoid obvious non-neurological causes for observed effects on locomotor activity (**Selderslaghs et al., 2010**). For the first set of experiments, behaviour was tested in a dose-response set-up at

concentrations below visually observable embryotoxicity in the ZFET or at the dissolution limit in case were no embryotoxicity was observed. Half-logarithmic dilutions were applied as shown in **Table 3** with *n=12* replicates per concentration, and exposure from <2.5 - 120hpf. These experiments were replicated at the two locations VU and RIVM. Environmentally relevant concentrations were tested and repeated for the parent compounds at concentrations ranges reported in **Table 3** including a concentration of effect. Sensitive window of exposure and (ir)reversibility of effects were studied using a single effective concentration for each compound (nominal concentrations 200-10-300 µM for respectively CBZ, FLX, and VNX), with limited exposure windows (<2.5-96hpf, 96-120hpf), as compared to full period of exposure (<2.5-120hpf) and appropriate solvent controls.

### 2.2.5. Mixture design

Zebrafish embryos were exposed to the binary mixtures of carbamazepine-fluoxetine (CBZ-FLX), carbamazepine-venlafaxine (CBZ-VNX) and venlafaxine-fluoxetine (VNX-FLX) and combined to DSW solvent control groups. For each mixture, the concentrations of the second compound B were expressed as equivalents of the first (reference) compound A, thus adjusting for the difference in potency using a relative potency factor (RPF) (**Staal et al., 2018**). CBZ was chosen as reference compound in its combinations while VNX was the reference compound of the VNX-FLX mixture. The RPFs were calculated using a dedicated function in the PROAST software (see below) and/or by comparing the $BMC_{50}$ of the two compounds (**Table 4**). The resulting RPFs enabled to design an equipotent dose-range of the mixtures aiming to cover the intermediate part of the single dose-response curve of the reference compound. In addition to the 1:1 ratio of equipotency, the excess ratios 1:3 and 3:1 were investigated, to account for potency/sensitivity variations between experiments (**Table 5**). Exposure to mixtures was performed as described for the single-compound analyses.

Exposures to individual compounds were always repeated together with the mixture as reference for the mixture dose response. Behavioural tracking was performed at 120hpf as described above.

### 2.2.6. RNA isolation and quantitative real-time PCR

Gene expression of specific markers related to DNT, derived from a previous study in fathead minnows (**Thomas et al., 2012**), was measured at $BMC_{50}$ values, i.e. the benchmark dose where 50% of the motor activity of described above was inhibited (see below), derived from a behaviour experiment with matched conditions, and based on pooled data from the three dark blocks. The analysis provided the following $BMD_{50}$ values: 115 µM CBZ, 6 µM FLX and 107 µM VNX. Six replicate pools per condition, each containing 10-12 embryos, were exposed during 0-120 hpf, then euthanized in liquid nitrogen and stored at -80ºC. For RNA isolation, the RNeasy Mini kit (QIAGEN, Venlo, the Netherlands) was used according to the manufacturer's protocol. Briefly, frozen embryos were pulverized using a tissue homogenizer (Omni TH) in a 2 ml Eppendorf tube, lysed in Qiazol and chloroform, and centrifuged. The aqueous phase was removed, mixed with EtOH (70%) and RNA was extracted using the dedicated RNeasy column. The concentration of RNA was measured on the NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific, Waltham MA, USA) as A260/A280 and A260/A280 ratios, and RNA integrity was assessed on the Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using the RNA 6000 Nano Chip kit (Agilent). Samples with a NanoDrop score ≥1.8-2.0 and a RNA Integrity Number (RIN) between 7 and 10 were considered of sufficient quality for further qPCR analysis. The samples of isolated RNA were stored at -80°C. For qPCR, RNA was transcribed to complementary DNA (cDNA) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific, Landsmeer, the Netherlands) according to manufacturer's protocol. The target genes were

amplified during qPCR with the Applied Biosystems 7500 fast real-Time PCR system with software v2.0.6. Expression of the following genes was investigated: *gabra6a* (Genbank: NM_200731.1), *grin1a* (Genbank: NM_001076714.2), *dlg4* (Genbank: NM_214728.1) (Applied Biosystems). As negative control the following housekeeping genes were used, *gapdh* (Genbank: NM_001115114.1), *actb1* (Genbank: NM_131031.1) and *hprt1* (Genbank: NM_212986.1). All targets were obtained as standard assays (Applied Biosystems).

### 2.2.7. Statistical analysis

#### 2.2.7.1. ZFET - single compounds

Morphology and teratology score obtained from ZFET as well data from behaviour testing were used to perform a benchmark dose-response analysis through R statistical software package v3.6.0-4.0.0 (also available as web application: https://proastweb.rivm.nl/ and as integrated part in the EuroMix toolbox: https://mcra-test.rivm.nl/EuroMix/WebApp/#/) with work package PROAST v67.0-69.0 (https://www.rivm.nl/en/proast/ (**Staal et al., 2018**). The dose-response analysis enables the estimation of a benchmark concentration (BMC) at a defined critical effect size (CES). A $BMC_5$ (BMC at CES=5%) was derived for the ZFET, and $BMC_5$ and a $BMC_{50}$ for behaviour testing. The estimated BMC is reported along with its lower (BMCL) and upper (BMCU) bound at its 90% confidence interval.

#### 2.2.7.2. Light dark transition test - single compounds

All the dose-response analyses were repeated with exponential and Hill models. The data of the different exposure windows are presented as mean±SD time (s) in activity per 10' measurement of the first dark block only. Second and third dark blocks did not provide additional information (see supplementary data), whereas the light blocks did not show any

statistical differences whether compared to the control (see supplementary data). Significance was calculated with a two sided ANOVA for repeated measures using SPSS Statistics (IBM, 24.0.0.1) ($P<0.05$). Activity throughout a test session was the dependent variable, whereas light condition (light or dark) or exposure to compounds was the independent variable.

#### 2.2.7.3. Gene expression – single compounds

The fold change was determined using the efficiency-corrected delta comparative quantification method and students t-test (unpaired, two tailed) were performed to determine significance ($P<0.05$).

#### 2.2.7.4. Mixtures and light dark transition test

The PROAST software was also used to calculate relative potency factor (RPF), combining the data of the refence and second compound dose-response analysis. The evaluation of the dose-addition was performed both in a visual and quantitative way. The first way was applied by visual comparison of the dose-response curve fitted to both the single-compound responses and the mixture responses, after expressing all concentrations in equivalent units of the reference compound after transformation using the RPF. Dose addition is likely when all data (mixture and single-compound) are described by the fitted curve. The visual assessment was supported by a quantitative evaluation, that consisted in a comparison between the RPFs-CIs calculated with and without mixture data (**Table 6**). When the dose-addition holds, an overlap of the RPFs-CIs is expected, which can be quantified by dividing the RPFL (relative potency factor lower) of the higher interval to the RPFU (relative potency factor upper) of the lower interval. Ratios greater than 1 indicate a relatively large deviation from dose addition, while a ratio smaller than 1 means that there is no evidence of deviation from dose addition.

**Table 2** Test compounds and exposure dose-ranges in ZFET.

| Compound | Code | CAS | Dose-range (μM) |
|---|---|---|---|
| Carbamazepine | CBZ | 298-46-4 | 0, 5, 25, 50, 250 |
| Carbamazepine 10,11-epoxide | CBZ10,11E | 36507-30-9 | 0, 0.003, 0.03, 0.35, 3.5, 35 |
| Fluoxetine | FLX | 54910-89-3 | 0, 0.0089, 0.089, 0.89, 8.9, 89.9 |
| Norfluoxetine | norFLX | 57226-68-3 | 0, 0.006, 0.06, 0.6, 6, 60 |
| Phenytoin | PHT | 57-41-0 | 0,3,10,30,100,300 |
| Venlafaxine | VNX | 93413-69-5 | 0, 0.03, 0.3, 3.18, 31.86, 318.61 |
| Desvenlafaxine | desVNX | 300827-87-6 | 0, 0.03, 0.33, 3.33, 33.35, 333.51 |

**Table 3** Exposure dose-range of the single compound dose-response analysis at both experimental and environmental levels.

| Compound | Tested dose-ranges (μM) | |
|---|---|---|
| | Experiments environmental levels | Dose response experiments |
| Carbamazepine | 0, 0.0032, 0.010, 0.032, 0.1,100 | 0, 0.3, 1, 3, 10, 30, 100, 200 |
| Carbamazepine 10,11 -epoxide | - | 0, 0.03, 0.1, 0.3, 1, 3, 10, 30 |
| Fluoxetine | 0.00001, 0.000032, 0.0001, 0.00032,1 | 0, 0.03, 0.1, 0.3, 1, 3, 10 |
| Norfluoxetine | - | 0 , 0.03, 0.1, 0.3, 1, 3, 10 |
| Phenytoin | - | 0, 0.3, 1, 3, 10, 30, 100, 300 |
| Venlafaxine | 0, 0.00032, 0.001, 0.0032, 0.01,100 | 0, 0.3, 1, 3, 10, 30, 100, 300 |
| Desvenlafaxine | - | 0, 0.3, 1, 3, 10, 30, 100, 300 |

**Table 4** RPFs of the three compound combinations used for designing the mixture experiments.

| Reference compound | Second compound | RPF* |
|---|---|---|
| Carbamazepine | Fluoxetine | 50.50 |
| Carbamazepine | Venlafaxine | 12.65 |
| Venlafaxine | Fluoxetine | 10.26 |

**Table 5** Design of the mixture carbamazepine-venlafaxine.

| Ratio | Final concentration (μM CBZ equivalents) | CBZ concentration (μM) | VNX concentration (μM) |
|---|---|---|---|
| | | 0.00 | 0.00 |
| 1:0 | 0.00 | 0-10-30-100-150-200 | 0.00 |
| 0:1 | 0.00 | 0.00 | 0-0.3-1-3-10-30-100 |
| 1:3 | 3.00 | 0.75 | 0.18 |
| 3:1 | 3.00 | 2.25 | 0.06 |
| 1:1 | 3.00 | 1.50 | 0.12 |
| 1:3 | 10.00 | 2.50 | 0.59 |
| 3:1 | 10.00 | 7.50 | 0.20 |
| 1:1 | 10.00 | 5.00 | 0.40 |
| 1:3 | 33.00 | 8.25 | 1.96 |
| 3:1 | 33.00 | 24.75 | 0.65 |
| 1:1* | 33.00 | 16.50 | 1.30 |
| 1:3 | 100.00 | 25.00 | 5.93 |
| 3:1 | 100.00 | 75.00 | 1.98 |
| 1:1 | 100.00 | 50.00 | 3.95 |
| 1:3 | 200.00 | 50 | 11.86 |
| 3:1 | 200.00 | 150 | 3.95 |
| 1:1 | 200.00 | 100 | 7.91 |

As an example, the final concentration of 33 μM carbamazepine equivalents in a 1:1 mixture composed of 33*0.5 = 16.50 μM carbamazepine and 33*0.5/12.65 = 1.30 μM venlafaxine, where RPF = 12.65-VNX. The dose-range mixture aimed to cover the intermediate part of the single dose–response curve of the reference.

**Table 6** Quantitative evaluation of the dose-addition: effect of mixtures on RPF of single compounds.

| Reference compound | Second compound | RPF | | | | |
|---|---|---|---|---|---|---|
| | | single compounds | | compounds and mix | | |
| | | Lowest | Highest | Lowest | Highest | Ratio of overlap[a] |
| Carbamazepine | Fluoxetine | 51.7 | 63 | 47.5 | 61 | 0.85 |
| Carbamazepine | Venlafaxine | 4.26 | 5.91 | 5.11 | 9.3 | 0.86 |
| Venlafaxine | Fluoxetine | 9.67 | 13.1 | 5.25 | 12.9 | 0.75 |

[a]ratio of overlap was obtained by dividing the RPFL (relative potency factor lower) of the higher interval to the RPFU (relative potency factor upper) of the lower interval. It indicates overlap of RPF confidence intervals without and with mixture. Ratio < 1 supports the hypothesis that the mixture effect can be predicted by dose-addition.

# 3. Results

### 3.1. Analytical assessment

#### 3.1.1. Validation Method

The proposed LC-MS/MS-MRM method allowed the analysis of 116 pesticides, 85 of which authorized on tomatoes (**Table 7**). Any difference was detected comparing calibration curves prepared in pure solvents and blank matrix. Therefore, multistandard calibration curves were prepared at five points with minimum and maximum values at LOQ and 100xLOQ in blank control matrix extracts showing correlation coefficients ($r^2$) ranging from 0.9959 to 1.0000 and RSD% max 8.65%. Linearity was above the condition set for method validation (**Table 8**). No interfering peaks were detected in the chromatographic range of interest, and no further clean-up was necessary, showing a reasonable specificity of the method (**Figure 8**). Accuracy ranged from 76.6 to 115.3% at LOQ level, and from 75.5 to 109.5% at 10xLOQ (**Table 8**). Moreover, minimum and maximum coefficient of variability ranged from 0.1 to 19.6% (**Table 8**). The average value of all recoveries was 94.6% ± 0.09%. Repeatability (RSDr; n = 30) and within laboratory reproducibility (RSDwR; n = 60) showed good results below 19.1%. Maximum and minimum RSD% were 18.3% and 3.4% in RSDr, and 19.4% and 5.4% in RSDwR (**Table 8**), with an average value of 11.11% ± 33.6%. According to average recoveries and RSDwR, expanded uncertainty (U) for all pesticides was below 50% of the default values for both spiking levels. The instrument limits of quantification (LOQs) and of determination (LODs), calculated as 10-fold and 3-fold the signal-to-noise ratio, were far below the MRLs set by the European Community (**Table 8**), with LOQ values ranging from 2.35 μg $kg^{-1}$ for benthiavalicarb to 6.49 μg $kg^{-1}$ for allethrin.

### 3.1.2. Raw and processed tomatoes

The analysis of raw tomatoes allowed the identification of 46 pesticides among the 116 searched with the validated MRM method. The cultivar Dask and Creso were the most polluted, accounting for 36 residues. A total of 1390 residues have been found spread in all samples. Azoxystrobin (141 times), dimethomorph (106), and chlorantraniliprole (102) were the most frequent in raw tomatoes. These pesticides showed the highest levels of residues, followed by fenarimol (97), spinosyn A (83), and emamectin benzoate (72) (**Table 9**). In 83.2% of the analysis, the residues were below the LOQ of the method and were not quantifiable. Multiresidue pollution has been registered in many samples, with a maximum number of 22 pesticide residues found in a sample of the cultivar Creso. 35% of the samples showed pesticide residues ≤ 5,31%, between five and 10,22% between 10 and 15, while only 12% showed more than 16 pesticide residues. The analysis of the processed products showed no residues detectable above the LOD of the method (**Table 9**).

**Table 7** Active ingredients, types, and LC-MS/MS-MRM m/z ions used for qualitative and quantitative analysis.

| Nr | Pesticide | Type of pesticide¥ | $t_R$ (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|---|
| 1 | Cyromazine* | IGR | 2.86 | 167 | 68<br>85 |
| 2 | Methamidophos | I | 2.89 | 142 | 94<br>125 |
| 3 | Acephate | I | 3.47 | 184 | 125<br>143 |
| 4 | Formetanate* | I/A | 3.63 | 222 | 65<br>165 |
| 5 | Pymetrozine* | I | 3.84 | 218 | 79<br>105 |
| 6 | Omethoate | I | 3.84 | 214 | 109<br>125 |
| 7 | Propamocarb* | F | 3.87 | 189 | 102<br>74 |
| 8 | Oxamyl* | N | 4.45 | 237 | 72<br>90 |
| 9 | Methomyl* | I | 4.77 | 163 | 88<br>106 |
| 10 | Flonicamid* | I | 4.81 | 230 | 174<br>203 |
| 11 | Thiamethoxam* | I | 4.90 | 292 | 181<br>211 |
| 12 | Carbendazim | F | 5.00 | 192 | 132<br>160 |
| 13 | Monocrotophos | I | 5.06 | 224 | 127<br>193 |
| 14 | Chlordimeform | A | 5.15 | 197 | 117<br>152 |
| 15 | Cypermethrin* | I | 5.52 | 433 | 89<br>133 |
| 16 | Imidacloprid* | I | 5.55 | 256 | 128<br>175 |
| 17 | Methiocarb* | I | 5.77 | 242 | 170<br>185 |
| 18 | Dimethoate* | I | 6.04 | 230 | 79<br>125 |
| 19 | Acetamiprid* | I | 6.05 | 223 | 56<br>126 |
| 20 | Cymoxanil* | F | 6.51 | 199 | 110<br>128 |
| 21 | Thiacloprid* | I | 6.64 | 253 | 90<br>126 |
| 22 | Atrazine-desethyl | H | 6.68 | 188 | 68<br>146 |
| 23 | Aldicarb | I | 7.30 | 213 | 89<br>116 |
| 24 | Pirimicarb* | I | 8.05 | 239 | 72<br>182 |
| 25 | Dichlorvos | I | 8.27 | 221 | 109<br>127 |
| 26 | Thiophanate-methyl* | F | 8.40 | 343 | 93<br>151 |

**Table 7 Cont.**

| Nr | Pesticide | Type of pesticide¥ | $t_R$ (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|---|
| 27 | Metribuzin* | H | 8.51 | 215 | 84<br>187 |
| 28 | Carbofuran | I | 8.58 | 222 | 123<br>165 |
| 29 | Carbaryl | I | 9.06 | 202 | 127<br>145 |
| 30 | Imazalil* | F | 9.54 | 297 | 159<br>201 |
| 31 | Fosthiazate* | N | 9.60 | 284 | 61<br>227 |
| 32 | Disulfoton-Sulfoxide | I | 9.73 | 291 | 157<br>185 |
| 33 | Flutriafol* | F | 10.49 | 302 | 95 |
| 34 | Metalaxyl* | F | 10.59 | 280 | 160<br>220 |
| 35 | Methidathion | I | 10.71 | 303 | 145 |
| 36 | Azinphos-methyl | I | 10.95 | 318 | 132<br>261 |
| 37 | Chlorantraniliprole* | I | 11.00 | 483 | 285<br>453 |
| 38 | Pyrimethanil* | F | 11.01 | 200 | 82<br>107 |
| 39 | Azoxystrobin* | F | 11.44 | 404 | 226<br>329 |
| 40 | Diethofencarb | F | 11.45 | 268 | 124<br>226 |
| 41 | Propanil | H | 11.61 | 218 | 127<br>162 |
| 42 | Fenamidone* | F | 11.68 | 312 | 92<br>236 |
| 43 | Diclobutrazol | F | 11.69 | 328 | 70<br>159 |
| 44 | Boscalid* | F | 11.86 | 343 | 272<br>307 |
| 45 | Dimethomorph* | F | 12.13 | 388 | 165<br>301 |
| 46 | Mandipropamid* | F | 12.16 | 412 | 328<br>356 |
| 47 | Benthiavalicarb* | F | 12.24 | 340 | 72<br>180 |
| 48 | Molinate | H | 12.33 | 188 | 83<br>126 |
| 49 | Chloroxuron | H | 12.45 | 291 | 125<br>164 |
| 50 | Myclobutanil* | F | 12.48 | 289 | 70<br>125 |
| 51 | Bifenazate* | A | 12.49 | 301 | 170<br>198 |
| 52 | Cyproconazole* 1 | F | 12.58 | 292 | 70<br>125 |
| 53 | Triadimenol* | F | 12.64 | 296 | 70<br>99 |

**Table 7 Cont.**

| Nr | Pesticide | Type of pesticide¥ | $t_R$ (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|---|
| 54 | Iprovalicarb* | F | 12.70 | 321 | 119<br>186 |
| 55 | Fenhexamid* | F | 12.74 | 302 | 55<br>97 |
| 56 | Azinphos-ethyl | I | 12.76 | 346 | 132<br>55 |
| 57 | Tetraconazole* | F | 12.77 | 372 | 159<br>108 |
| 58 | Cyproconazole* 2 | F | 12.78 | 292 | 70<br>125 |
| 59 | Mepanipyrim* | F | 12.80 | 224 | 77<br>106 |
| 60 | Spirotetramat* | I | 12.80 | 374 | 270<br>302 |
| 61 | Flufenacet* | H | 12.81 | 224 | 152<br>194 |
| 62 | Ethoprop* | I/N | 12.91 | 243 | 97<br>131 |
| 63 | Bupirimate* | F | 12.93 | 317 | 166 |
| 64 | Cyazofamid* | F | 12.97 | 325 | 108<br>217 |
| 65 | Flusilazole | F | 13.24 | 316 | 165<br>247 |
| 66 | Cyprodinil* | F | 13.27 | 226 | 65<br>77 |
| 67 | Fenamiphos* | I | 13.27 | 304 | 217<br>234 |
| 68 | Iprodione* | F | 13.41 | 330 | 245 |
| 69 | Aclonifen* | H | 13.51 | 265 | 182<br>218 |
| 70 | Penconazole* | F | 13.65 | 284 | 70<br>159 |
| 71 | Tebuconazole* | F | 13.76 | 308 | 70<br>125 |
| 72 | Napropamide* | H | 13.82 | 272 | 58<br>171 |
| 73 | Benalaxyl* | F | 13.96 | 326 | 91<br>148 |
| 74 | Spinosyn *A | I | 14.14 | 732 | 98<br>142 |
| 75 | Zoxamide* | F | 14.17 | 336 | 159<br>187 |
| 76 | Pyraclostrobin* | F | 14.18 | 388 | 163<br>194 |
| 77 | Cyflufenamid | F | 14.24 | 413 | 241<br>295 |
| 78 | Bitertanol | F | 14.27 | 338 | 70<br>100 |
| 79 | Clofentezine* | A | 14.28 | 303 | 102<br>138 |
| 80 | Phosalone | I/A | 14.29 | 368 | 111<br>182 |
| 81 | Metrafenone* | F | 14.46 | 409 | 209<br>227 |

**Table 7 Cont.**

| Nr | Pesticide | Type of pesticide¥ | $t_R$ (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|---|
| 82 | Difenconazole* | F | 14.58 | 406 | 251<br>337 |
| 83 | Chlorpyrifos-methyl* | I | 14.63 | 322 | 125<br>290 |
| 84 | Ametoctradin* | F | 14.69 | 276 | 186<br>191 |
| 85 | Spinosyn* D | I | 14.71 | 746 | 98<br>142 |
| 86 | Indoxacarb* | I | 14.73 | 528 | 150<br>203 |
| 87 | Cycloate | H | 14.78 | 216 | 83<br>154 |
| 88 | Hexaflumuron | IGR | 14.81 | 461 | 141<br>158 |
| 89 | Trifloxystrobin* | F | 14.82 | 409 | 150<br>186 |
| 90 | Quizalofop-ethyl* | H | 15.32 | 373 | 255<br>271 |
| 91 | Cycloxydim* | H | 15.37 | 326 | 101<br>180 |
| 92 | Buprofezin* | I | 15.54 | 306 | 106<br>201 |
| 93 | Tebufenpyrad* | I/A | 15.55 | 334 | 117<br>145 |
| 94 | Emamectin Benzoate* | I | 15.59 | 887 | 126<br>158 |
| 95 | Propaquizafop* | H | 15.61 | 444 | 299<br>327 |
| 96 | Metaflumizone* | I | 15.63 | 507 | 116<br>178 |
| 97 | Oxadiazon* | H | 15.73 | 362 | 220 |
| 98 | Allethrin | I | 15.77 | 303 | 103<br>135 |
| 99 | Piperonyl butoxide* | S | 15.83 | 356 | 119<br>177 |
| 100 | Chlorpyriphos* | I | 16.03 | 350 | 97<br>198 |
| 101 | Hexythiazox* | I/A | 16.07 | 353 | 168<br>228 |
| 102 | Pyriproxyfen* | I | 16.12 | 322 | 96<br>185 |
| 103 | Pendimethalin* | H | 16.13 | 282 | 194<br>212 |
| 104 | Flufenoxuron | I | 16.21 | 489 | 141<br>158 |
| 105 | Propargite | A | 16.35 | 368 | 81<br>231 |
| 106 | Lufenuron* | I | 16.53 | 510 | 141<br>158 |
| 107 | Etoxazole* | A | 16.54 | 160 | 113<br>141 |
| 108 | Fenpyroximate(E)* | A | 16.66 | 422 | 107<br>366 |

**Table 7 Cont.**

| Nr | Pesticide | Type of pesticide¥ | $t_R$ (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|---|
| 109 | Deltamethrin* | I/A | 16.85 | 523 | 181<br>281 |
| 110 | Acrinathrin* | I/A | 16.92 | 559 | 181<br>208 |
| 111 | Pyridaben* | I/A | 17.09 | 365 | 147<br>309 |
| 112 | Tau Fluvalinate* | I/A | 17.62 | 503 | 181<br>208 |
| 113 | Fenarimol | F | 17.86 | 331 | 238<br>313 |
| 114 | Etofenprox* | I | 17.88 | 394 | 107<br>177 |
| 115 | Bifenthrin | I | 17.91 | 442 | 166<br>181 |
| 116 | Famoxadone* | F | 18.84 | 392 | 93 |

*pesticide authorized on tomatoes
¥A: acaricide; F: fungicide; H: herbicide; I/A: acaricide/insecticide; I: insecticide; S: synergist a.i. IGR: insect growth regulator; I/N: insecticide/nematocide.
[Corrias F., **Atzei A.,** et al., 2020]

**Table 8** Linearities, curves, LODs and LOQs and method validation parameters for the analysis of 116 target pesticides in tomato in LC-MS/MS.

| Pesticide | Linearity | Linear regression equation | $R^2$ ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | $RSD_r$ (5xLOQ) | $RSD_{wR}$ (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g $kg^{-1}$) | | | (mg $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Cyromazine | LOQ – 411 | y=2741924x + 8425 | 0.9988 ± 0.07 | 0.60 | 1.37 | 4.11 | 115.3 ± 4.9 | 108.8 ± 1.7 | 11.2 | 12.4 | 29.4 |
| Methamidophos | LOQ – 410 | y=4887931x + 21107 | 0.9989 ± 0.06 | 0.01* | 1.37 | 4.10 | 105.4 ± 14.3 | 93.1 ± 10.8 | 9.0 | 12.9 | 38.3 |
| Acephate | LOQ – 435 | y=375881x – 144 | 1.0000 ± 0.10 | 0.01* | 1.45 | 4.35 | 96.5 ± 13.3 | 90.6 ± 19.4 | 12.0 | 17.1 | 39.8 |
| Formetanate | LOQ – 413 | y=14913520x – 5868 | 1.0000 ± 0.01 | 0.30 | 1.38 | 4.13 | 84.5 ± 1.9 | 84.4 ± 9.3 | 8.6 | 16.9 | 33.6 |
| Pymetrozine | LOQ – 473 | y=696973x + 6250 | 0.9959 ± 0.08 | 0.50 | 1.58 | 4.73 | 101.3 ± 6.7 | 98.2 ± 5.8 | 13.9 | 14.0 | 16.5 |
| Omethoate | LOQ – 621 | y=32351x + 372 | 0.9987 ± 0.07 | 0.01* | 2.07 | 6.21 | 87.4 ± 7.5 | 85.1 ± 5.9 | 7.5 | 15.1 | 31.8 |
| Propamocarb | LOQ – 417 | y=43247201x – 8829 | 0.9998 ± 0.18 | 4.00 | 1.39 | 4.17 | 99.6 ± 6.7 | 98.5 ± 2.7 | 13.5 | 12.9 | 14.3 |
| Oxamyl | LOQ – 401 | y=5159083x -5579 | 1.0000 ± 0.01 | 0.01 | 1.34 | 4.01 | 94.8 ± 11.1 | 80.9 ± 0.3 | 5.3 | 8.1 | 38.6 |
| Methomyl | LOQ – 410 | y=5917725x + 7746 | 0.9997 ± 0.14 | 0.01 | 1.37 | 4.10 | 111.8 ± 10.4 | 107.2 ± 2.3 | 9.6 | 10.3 | 31.0 |
| Flonicamid | LOQ – 394 | y=784761x + 1544 | 0.9998 ± 0.13 | 0.50 | 1.31 | 3.94 | 95.5 ± 9.6 | 91.0 ± 12.9 | 15.4 | 11.4 | 29.4 |
| Thiamethoxam | LOQ – 524 | y=11189678x + 71281 | 0.9986 ± 0.08 | 0.20 | 1.75 | 5.24 | 107.8 ± 8.3 | 104.5 ± 2.5 | 7.4 | 9.8 | 22.6 |
| Carbendazim | LOQ – 400 | y=5329930x + 3082 | 1.0000 ± 0.01 | 0.30* | 1.33 | 4.00 | 76.6 ± 1.7 | 83.7 ± 4.1 | 8.1 | 12.8 | 43.2 |
| Monocrotophos | LOQ – 414 | y=17644763x + 44731 | 0.9993 ± 0.11 | 0.01* | 1.38 | 4.14 | 96.4 ± 11.1 | 79.8 ± 7.4 | 6.5 | 10.7 | 41.8 |
| Chlordimeform | LOQ – 396 | y=853728x - 1979 | 0.9998 ± 0.01 | - | 1.32 | 3.96 | 85.6 ± 7.4 | 75.5 ± 0.7 | 14.2 | 11.4 | 43.1 |
| Cypermethrin | LOQ – 518 | y=5132597x + 20901 | 0.9979 ± 0.12 | 0.50 | 1.73 | 5.18 | 86.6 ± 4.1 | 86.3 ± 7.4 | 12.6 | 11.8 | 29.7 |
| Imidacloprid | LOQ – 321 | y=5358564x + 20834 | 0.9990 ± 0.20 | 0.50 | 1.07 | 3.21 | 107.4 ± 13.7 | 107.0 ± 1.2 | 4.2 | 6.7 | 32.1 |
| Methiocarb | LOQ - 387 | y=31899495x + 73553 | 0.9994 ± 0.16 | 0.20 | 1.29 | 3.87 | 76.9 ± 1.9 | 80.0 ± 5.2 | 5.6 | 9.0 | 44.7 |
| Dimethoate | LOQ – 407 | y=9144917x + 45650 | 0.9984 ± 0.08 | 0.02 | 1.36 | 4.07 | 105.3 ± 5.0 | 96.7 ± 2.7 | 12.3 | 8.4 | 17.7 |
| Acetamiprid | LOQ – 398 | y=13742437x + 39503 | 0.9992 ± 0.79 | 0.50 | 1.33 | 3.98 | 98.1 ± 8.7 | 88.1 ± 1.8 | 6.1 | 10.7 | 26.7 |

**Table 8** Cont.

| Pesticide | Linearity | Linear regression equation | R2 ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | RSDr (5xLOQ) | RSDwR (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g kg$^{-1}$) | | | (mg kg$^{-1}$) | (g kg$^{-1}$) | (g kg$^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Cymoxanil | LOQ – 435 | y=7081921x + 5097 | 0.9998 ± 0.00 | 0.40 | 1.45 | 4.35 | 99.1 ± 5.8 | 88.6 ± 5.8 | 11.4 | 14.9 | 24.1 |
| Thiacloprid | LOQ – 439 | y=4714164x + 21318 | 0.9988 ± 0.55 | 0.50 | 1.46 | 4.39 | 111.8 ± 5.7 | 106.6 ± 1.2 | 11.2 | 12.8 | 24.4 |
| Atrazine-desethyl | LOQ – 430 | y=8730484x + 17192 | 0.9996 ± 0.03 | - | 1.43 | 4.30 | 102.0 ± 5.2 | 93.5 ± 3.8 | 8.4 | 11.5 | 18.0 |
| Aldicarb | LOQ – 458 | y=22774x - 3 | 0.9989 ± 0.04 | 0.02* | 1.53 | 4.58 | 99.8 ± 0.2 | 99.9 ± 0.2 | 10.0 | 8.1 | 10.6 |
| Pirimicarb | LOQ – 410 | y=30848679x + 25212 | 0.9994 ± 0.02 | 0.50 | 1.37 | 4.10 | 109.8 ± 9.6 | 108.2 ± 1.8 | 9.5 | 7.8 | 27.7 |
| Dichlorvos | LOQ – 410 | y=450147x + 175 | 1.0000 ± 0.01 | 0.01* | 1.37 | 4.10 | 87.2 ± 4.3 | 81.7 ± 2.0 | 8.7 | 17.1 | 32.6 |
| Thiophanate-methyl | LOQ – 406 | y=17268518x - 53036 | 0.9997 ± 0.32 | 1.00 | 1.35 | 4.06 | 93.1 ± 10.0 | 80.2 ± 4.6 | 18.1 | 7.9 | 38.4 |
| Metribuzin | LOQ – 427 | y=3761325x + 17279 | 0.9983 ± 0.74 | 0.10 | 1.42 | 4.27 | 98.9 ± 10.7 | 108.4 ± 2.4 | 5.6 | 12.4 | 26.3 |
| Carbofuran | LOQ – 416 | y=27252840x + 33458 | 0.9996 ± 0.01 | 0.002* | 1.39 | 4.16 | 96.3 ± 2.9 | 94.9 ± 1.5 | 8.5 | 7.7 | 10.7 |
| Carbaryl | LOQ – 410 | y=14046644x + 7098 | 0.9998 ± 0.22 | 0.01* | 1.37 | 4.10 | 110.4 ± 5.0 | 100.2 ± 2.1 | 8.4 | 11.5 | 22.9 |
| Imazalil | LOQ – 431 | y=972258x + 947 | 0.9999 ± 0.21 | 0.50 | 1.44 | 4.31 | 78.8 ± 8.2 | 77.5 ± 2.2 | 13.0 | 16.7 | 46.0 |
| Fosthiazate | LOQ – 397 | y=720338x + 1656 | 0.9997 ± 0.12 | 0.02 | 1.32 | 3.97 | 97.2 ± 2.1 | 98.7 ± 0.1 | 7.5 | 10.6 | 6.4 |
| Disulfoton-Sulfoxide | LOQ – 471 | y=12702349x + 19964 | 0.9998 ± 0.32 | - | 1.57 | 4.71 | 110.8 ± 7.0 | 107.6 ± 1.2 | 4.6 | 16.4 | 24.7 |
| Flutriafol | LOQ – 470 | y=3625x + 183 | 0.9986 ± 0.04 | 0.80 | 1.57 | 4.70 | 106.9 ± 8.1 | 104.9 ± 1.0 | 15.2 | 12.4 | 21.0 |
| Metalaxyl | LOQ – 390 | y=4371x + 183 | 0.9986 ± 0.04 | 0.20 | 1.30 | 3.90 | 85.6 ± 6.9 | 84.3 ± 13.1 | 9.5 | 10.2 | 36.7 |
| Methidathion | LOQ – 424 | y=396329x – 101 | 0.9994 ± 0.14 | 0.02* | 1.41 | 4.24 | 93.6 ± 11.4 | 92.9 ± 2.9 | 10.2 | 11.1 | 26.2 |
| Azinphos-methyl | LOQ – 464 | y=398216x + 390 | 0.9998 ± 0.58 | 0.05* | 1.55 | 4.64 | 98.2 ± 9.9 | 93.6 ± 2.3 | 8.5 | 19.4 | 22.5 |
| Chlorantraniliprole | LOQ - 399 | y=585240x + 2 | 0.9997 ± 0.01 | 0.60 | 1.33 | 3.99 | 92.1 ± 5.8 | 89.6 ± 4.5 | 7.4 | 15.7 | 22.2 |
| Pyrimethanil | LOQ – 395 | y=1208059x – 4250 | 0.9991 ± 0.05 | 1.00 | 1.32 | 3.95 | 109.7 ± 5.5 | 105.4 ± 0.3 | 7.4 | 15.7 | 20.9 |

**Table 8** Cont.

| Pesticide | Linearity | Linear regression equation | R2 ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | RSDr (5xLOQ) | RSDwR (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g kg$^{-1}$) | | | (mg kg$^{-1}$) | (g kg$^{-1}$) | (g kg$^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Azoxystrobin | LOQ – 431 | y=37027632 + 20546 | 0.9999 ± 0.31 | 3.00 | 1.44 | 4.31 | 103.4 ± 5.5 | 99.7 ± 4.2 | 6.7 | 19.8 | 14.5 |
| Diethofencarb | LOQ – 385 | y=17914008x +75801 | 0.9990 ± 1.21 | 0.70* | 1.28 | 3.85 | 97.0 ± 14.2 | 83.9 ± 14.6 | 6.4 | 15.5 | 43.4 |
| Propanil | LOQ – 392 | y=1172102x – 4083 | 0.9972 ± 0.09 | 0.01* | 1.31 | 3.92 | 90.9 ± 5.1 | 91.0 ± 5.8 | 12.5 | 13.0 | 22.0 |
| Fenamidone | LOQ – 390 | y=19532597x + 43718 | 0.9995 ± 1.06 | 1.00 | 1.30 | 3.90 | 96.5 ± 9.5 | 103.2 ± 1.3 | 9.4 | 14.5 | 21.5 |
| Diclobutrazol | LOQ – 391 | y=145538x + 476 | 0.9993 ± 0.22 | - | 1.30 | 3.91 | 101.5 ± 0.7 | 102.0 ± 0.2 | 8.7 | 10.3 | 3.8 |
| Boscalid | LOQ – 408 | y=4252887x + 877 | 1.0000 ± 0.30 | 3.00 | 1.36 | 4.08 | 104.5 ± 8.6 | 105.9 ± 0.3 | 14.1 | 15.5 | 20.6 |
| Dimethomorph | LOQ - 394 | y=14639124x + 39105 | 0.9980 ± 0.16 | 1.00 | 1.31 | 3.94 | 93.8 ± 17.4 | 97.9 ± 2.5 | 8.9 | 8.7 | 35.5 |
| Mandipropamid | LOQ – 455 | y=5148550x - 20963 | 0.9983 ± 5.08 | 3.00 | 1.52 | 4.55 | 85.6 ± 11.9 | 82.1 ± 8.4 | 5.6 | 14.8 | 40.6 |
| Benthiavalicarb | LOQ – 235 | y=4160577x + 5366 | 0.9994 ± 0.97 | 0.30 | 0.78 | 2.35 | 111.4 ± 5.8 | 106.8 ± 0.9 | 9.7 | 11.5 | 24.0 |
| Molinate | LOQ – 621 | y=47567x – 140 | 0.9999 ± 0.93 | 0.01* | 2.07 | 6.21 | 92.2 ± 3.1 | 85.9 ± 4.6 | 8.1 | 15.4 | 24.5 |
| Chloroxuron | LOQ – 425 | y=12252052x – 45885 | 0.9984 ± 0.19 | 0.01* | 1.42 | 4.25 | 105.7 ± 5.7 | 102.3 ± 0.4 | 4.5 | 10.5 | 15.7 |
| Myclobutanil | LOQ – 411 | y=123049x - 122 | 0.9997 ± 0.07 | 0.30 | 1.37 | 4.11 | 83.1 ± 6.1 | 91.8 ± 0.7 | 13.4 | 13.7 | 32.5 |
| Bifenazate | LOQ – 399 | y=1008299x + 76836 | 0.9961 ± 1.07 | 0.50 | 1.33 | 3.99 | 87.0 ± 14.1 | 90.8 ± 19.6 | 5.6 | 15.5 | 44.4 |
| Cyproconazole 1 | LOQ – 410 | y=14106016x + 110236 | 0.9985 ± 4.29 | 0.05 | 1.37 | 4.10 | 109.2 ± 7.2 | 105.7 ± 1.1 | 10.8 | 18.6 | 22.6 |
| Triadimenol | LOQ – 495 | y=3828694x + 27482 | 0.9970 ± 5.79 | 1.00 | 1.65 | 4.95 | 84.9 ± 13.3 | 96.4 ± 8.1 | 8.4 | 15.7 | 38.8 |
| Iprovalicarb | LOQ – 408 | y=4408560x + 40826 | 0.9967 ± 0.22 | 0.70 | 1.36 | 4.08 | 90.6 ± 14.0 | 102.9 ± 0.4 | 12.4 | 13.5 | 33.7 |
| Fenhexamid | LOQ – 436 | y=3699942x + 18108 | 0.9991 ±1.57 | 2.00 | 1.45 | 4.36 | 102.8 ± 5.9 | 82.3 ± 3.6 | 3.4 | 18.5 | 36.8 |
| Azinphos-ethyl | LOQ – 391 | y=367433x + 492 | 0.9997 ± 0.39 | 0.02* | 1.30 | 3.91 | 79.1 ± 9.5 | 95.7 ± 0.5 | 7.6 | 15.4 | 43.2 |
| Tetraconazole | LOQ – 516 | y=9182219x – 30061 | 0.9993 ± 0.09 | 0.10 | 1.72 | 5.16 | 106.8 ± 7.4 | 109.5 ± 0.9 | 12.1 | 15.3 | 22.4 |
| Cyproconazole 2 | LOQ – 410 | y=5446075x - 17111 | 0.9994 ± 5.95 | 0.05 | 1.37 | 4.10 | 105.3 ± 7.5 | 102.7 ± 1.1 | 7.7 | 13.5 | 18.3 |

**Table 8** Cont.

| Pesticide | Linearity | Linear regression equation | R2 ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | RSDr (5xLOQ) | RSDwR (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g kg$^{-1}$) | | | (mg kg$^{-1}$) | (g kg$^{-1}$) | (g kg$^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Mepanipyrim | LOQ – 407 | y=328904x – 348 | 0.9991 ± 2.87 | 1.50 | 1.36 | 4.07 | 96.6 ± 2.1 | 100.8 ± 0.6 | 13.3 | 19.1 | 8.5 |
| Spirotetramat | LOQ – 503 | y=2820842x + 73839 | 0.9997 ± 0.67 | 2.00 | 1.68 | 5.03 | 85.6 ± 5.6 | 98.5 ± 1.7 | 6.5 | 18.3 | 29.9 |
| Flufenacet | LOQ – 414 | y=10522988x + 50386 | 0.9983 ± 8.65 | 0.05 | 1.38 | 4.14 | 89.9 ± 8.6 | 89.9 ± 0.4 | 10.5 | 14.8 | 28.4 |
| Ethoprop | LOQ – 377 | y=5056608x + 20031 | 0.9990 ± 2.42 | 0.02 | 1.26 | 3.77 | 89.5 ± 10.1 | 88.4 ± 1.2 | 8.9 | 10.4 | 29.2 |
| Bupirimate | LOQ – 396 | y=7147770x + 5118 | 0.9998 ± 0.37 | 2.00 | 1.32 | 3.96 | 91.8 ± 4.5 | 81.9 ± 10.2 | 5.4 | 18.8 | 33.1 |
| Cyazofamid | LOQ – 405 | y=54070x + 229 | 0.9992 ± 0.72 | 0.60 | 1.35 | 4.05 | 101.7 ± 1.0 | 102.7 ± 0.2 | 5.6 | 12.8 | 5.0 |
| Flusilazole | LOQ – 457 | y=9842141x + 54831 | 0.9982 ± 2.81 | 0.01* | 1.52 | 4.57 | 106.4 ± 8.2 | 104.1 ± 0.2 | 5.8 | 14.9 | 20.7 |
| Cyprodinil | LOQ – 408 | y=2997671x + 13504 | 0.9992 ± 0.05 | 1.50 | 1.36 | 4.08 | 93.9 ± 15.1 | 79.8 ± 1.9 | 4.6 | 18.7 | 45.0 |
| Fenamiphos | LOQ – 547 | y=2610314x + 116029 | 0.9991 ± 0.92 | 0.04 | 1.82 | 5.47 | 103.9 ± 7.1 | 100.7 ± 1.2 | 11.2 | 15.6 | 16.5 |
| Iprodione | LOQ – 455 | y=82937x + 89 | 0.9997 ± 0.22 | 5.00 | 1.52 | 4.55 | 94.4 ± 4.0 | 96.5 ± 4.2 | 9.9 | 15.8 | 14.0 |
| Aclonifen | LOQ – 393 | y=96057x + 89 | 0.9997 ± 0.22 | 0.01 | 1.31 | 3.93 | 97.5 ± 1.2 | 96.2 ± 0.7 | 12.4 | 12.5 | 7.0 |
| Penconazole | LOQ – 398 | y=6090424x + 15097 | 0.9992 ± 0.43 | 0.10 | 1.33 | 3.98 | 104.6 ± 6.2 | 102.4 ± 0.1 | 9.1 | 11.4 | 15.2 |
| Tebuconazole | LOQ – 401 | y=19544180x + 77610 | 0.9989 ± 0.56 | 0.90 | 1.34 | 4.01 | 102.1 ± 2.1 | 104.4 ± 1.1 | 7.1 | 10.4 | 8.4 |
| Napropamide | LOQ – 413 | y=6724980x + 62380 | 0.9964 ± 0.81 | 0.10 | 1.38 | 4.13 | 108.4 ± 6.2 | 104.8 ± 11.1 | 8.5 | 13.8 | 25.6 |
| Benalaxyl | LOQ – 481 | y=23788991x + 92142 | 0.9982 ± 0.19 | 0.50 | 1.60 | 4.81 | 100.6 ± 4.9 | 108.6 ± 1.1 | 8.9 | 9.4 | 17.4 |
| Spinosyn A | LOQ – 432 | y=1125007x – 3076 | 0.9997 ± 0.01 | 0.70 | 1.44 | 4.32 | 77.0 ± 4.1 | 76.7 ± 0.6 | 15.5 | 14.5 | 46.8 |
| Zoxamide | LOQ – 410 | y=5772043x + 12727 | 0.9994 ± 0.04 | 0.5 | 1.37 | 4.10 | 92.1 ± 9.4 | 81.5 ± 4.8 | 16.5 | 13.2 | 35.7 |
| Pyraclostrobin | LOQ – 418 | y=13354734x – 5243 | 1.0000 ± 3.84 | 0.30 | 1.39 | 4.18 | 94.5 ± 8.2 | 92.8 ± 3.9 | 5.8 | 15.7 | 21.0 |
| Cyflufenamid | LOQ – 408 | y=5945864x + 38431 | 0.9977 ± 0.65 | 0.04 | 1.36 | 4.08 | 87.5 ± 17.6 | 91.1 ± 1.1 | 17.4 | 18.5 | 40.0 |
| Bitertanol | LOQ – 406 | y=6031192x + 40609 | 0.9969 ± 0.80 | 0.01* | 1.35 | 4.06 | 105.1 ± 1.7 | 102.6 ± 0.9 | 11.2 | 14.6 | 9.8 |

**Table 8** Cont.

| Pesticide | Linearity | Linear regression equation | R2 ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | RSDr (5xLOQ) | RSDwR (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g kg$^{-1}$) | | | (mg kg$^{-1}$) | (g kg$^{-1}$) | (g kg$^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Clofentezin | LOQ – 408 | y=1910622x -533 | 0.9999 ± 0.95 | 0.30 | 1.36 | 4.08 | 83.2 ± 11.4 | 103.7 ± 3.8 | 8.9 | 8.1 | 42.8 |
| Phosalone | LOQ – 578 | y=6702437x + 16186 | 0.9995 ± 0.02 | 0.01* | 1.93 | 5.78 | 99.6 ± 3.4 | 94.2 ± 3.1 | 3.4 | 12.8 | 12.8 |
| Metrafenone | LOQ – 468 | y=9239760x + 20728 | 0.9999 ± 0.02 | 0.40 | 1.56 | 4.68 | 96.4 ± 1.9 | 101.0 ± 4.9 | 5.6 | 8.4 | 11.4 |
| Difenconazole | LOQ – 485 | y=20779327x + 20116 | 0.9999 ± 0.02 | 2.00 | 1.62 | 4.85 | 97.4 ± 7.3 | 95.8 ± 1.8 | 10.0 | 9.4 | 16.3 |
| Chlorpyrifos-methyl | LOQ – 414 | y=65820x - 225 | 0.9999 ± 0.71 | 0.50 | 1.38 | 4.14 | 91.2 ± 7.9 | 79.1 ± 2.3 | 9.1 | 11.4 | 37.5 |
| Ametoctradin | LOQ – 317 | y=8626247x + 1875 | 0.9999 ± 0.01 | 2.00 | 1.06 | 3.17 | 96.1 ± 7.1 | 92.8 ± 0.4 | 8.4 | 18.7 | 18.2 |
| Spinosyn D | LOQ – 432 | y=209477x - 615 | 0.9997 ± 0.41 | 0.70 | 1.44 | 4.32 | 92.9 ± 7.6 | 89.4 ± 4.1 | 12.7 | 5.6 | 23.8 |
| Indoxacarb | LOQ – 450 | y=1790071x - 2915 | 0.9985 ± 0.01 | 0.50 | 1.50 | 4.50 | 94.1 ± 1.5 | 82.2 ± 4.1 | 8.4 | 18.9 | 29.5 |
| Cycloate | LOQ – 502 | y=598248x – 1864 | 0.9974 ± 0.15 | - | 1.67 | 5.02 | 101.6 ± 6.7 | 101.3 ± 0.1 | 10.1 | 15.4 | 13.8 |
| Hexaflumuron | LOQ – 419 | y=633012x – 654 | 0.9977 ± 1.28 | - | 1.40 | 4.19 | 81.8 ± 10.2 | 81.7 ± 1.3 | 5.6 | 14.7 | 41.0 |
| Trifloxystrobin | LOQ – 432 | y=17415017x + 17609 | 0.9999 ± 0.27 | 0.70 | 1.44 | 4.32 | 96.7 ± 7.3 | 94.7 ± 2.0 | 7.6 | 8.4 | 17.1 |
| Quizalofop-ethyl | LOQ – 398 | y=3145820x -5888 | 0.9998 ± 0.18 | 0.40 | 1.33 | 3.98 | 100.8 ± 5.7 | 100.1 ± 1.5 | 11.3 | 5.4 | 11.8 |
| Cycloxydim | LOQ – 407 | y=137254x + 708 | 0.9986 ± 0.01 | 1.50 | 1.36 | 4.07 | 85.2 ± 7.8 | 96.3 ± 0.8 | 14.2 | 12.1 | 30.9 |
| Buprofezin | LOQ – 465 | y=20507228x + 51039 | 0.9998 ± 0.89 | 1.00 | 1.55 | 4.65 | 95.0 ± 8.0 | 93.9 ± 0.7 | 8.4 | 14.0 | 19.2 |
| Tebufenpyrad | LOQ – 401 | y=4341528x + 15573 | 0.9994 ± 1.27 | 0.80 | 1.34 | 4.01 | 83.6 ± 6.8 | 81.7 ± 1.8 | 9.7 | 9.4 | 36.7 |
| Emamectin Benzoate | LOQ – 522 | y=3632831x - 14712 | 0.9998 ± 0.25 | 0.02 | 1.74 | 5.22 | 98.8 ± 5.2 | 95.2 ± 3.1 | 12.4 | 7.4 | 13.7 |
| Propaquizafop | LOQ – 431 | y=2547342x – 6777 | 0.9971 ± 0.02 | 0.05 | 1.44 | 4.31 | 99.6 ± 8.8 | 100.0 ± 2.5 | 15.4 | 16.1 | 18.1 |
| Metaflumizone | LOQ – 410 | y=949238x – 6681 | 0.9983 ± 0.03 | 0.60 | 1.37 | 4.10 | 90.1 ± 3.8 | 75.6 ± 8.6 | 10.6 | 13.0 | 41.8 |
| Oxadiazon | LOQ – 403 | y=726169x – 4189 | 0.9983 ± 0.05 | 0.05 | 1.34 | 4.03 | 94.3 ± 4.8 | 88.1 ± 0.4 | 8.4 | 8.4 | 21.4 |
| Allethrin | LOQ – 649 | y=439397x – 5650 | 0.9963 ± 0.22 | - | 2.16 | 6.49 | 87.7 ± 4.3 | 81.2 ± 0.1 | 10.1 | 10.4 | 32.7 |

**Table 8** Cont.

| Pesticide | Linearity | Linear regression equation | R2 ± RSD% | MRL | LOD | LOQ | Apparent recovery (%, n = 15) | | RSDr (5xLOQ) | RSDwR (5xLOQ) | U* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (g kg$^{-1}$) | | | (mg kg$^{-1}$) | (g kg$^{-1}$) | (g kg$^{-1}$) | LOQ | 10 x LOQ | n = 30 | n = 60 | |
| Piperonyl butoxide | LOQ – 404 | y=31536094x + 11745 | 0.9999 ± 0.01 | - | 1.35 | 4.04 | 104.8 ± 5.5 | 100.9 ± 1.8 | 5.6 | 9.7 | 14.6 |
| Chlorpyriphos | LOQ – 395 | y=638457x - 2533 | 0.9992 ± 0.01 | 0.01 | 1.32 | 3.95 | 104.5 ± 5.1 | 109.5 ± 6.0 | 11.4 | 7.4 | 16.1 |
| Hexythiazox | LOQ – 358 | y=10457257x – 49960 | 0.9987 ± 0.01 | 0.50 | 1.19 | 3.58 | 97.9 ± 5.8 | 97.1 ± 0.5 | 14.3 | 14.1 | 12.5 |
| Pyriproxyfen | LOQ – 418 | y=6633898x – 13776 | 0.9994 ± 0.02 | 1.00 | 1.39 | 4.18 | 83.4 ± 4.8 | 77.7 ± 3.5 | 15.8 | 12.8 | 40.2 |
| Pendimethalin | LOQ – 333 | y=1172935x - 2381 | 0.9999 ± 0.01 | 0.05 | 1.11 | 3.33 | 84.2 ± 6.3 | 81.0 ± 4.6 | 14.2 | 8.4 | 36.9 |
| Flufenoxuron | LOQ – 391 | y=3953910x + 11436 | 0.9990 ± 0.48 | 0.50* | 1.30 | 3.91 | 81.9 ± 7.4 | 79.5 ± 1.5 | 9.7 | 9.5 | 40.7 |
| Propargite | LOQ – 382 | y=6439838x + 13171 | 0.9997 ± 1.64 | 0.01* | 1.27 | 3.82 | 86.8 ± 6.0 | 81.8 ± 1.2 | 14.1 | 14.5 | 33.5 |
| Lufenuron | LOQ – 437 | y=568999x + 552 | 0.9993 ± 0.09 | 0.50 | 1.46 | 4.37 | 93.5 ± 5.4 | 96.3 ± 0.6 | 7.5 | 12.3 | 15.7 |
| Etoxazole | LOQ – 516 | y=393488x + 312 | 0.9998 ± 0.02 | 0.07 | 1.72 | 5.16 | 88.7 ± 6.6 | 83.2 ± 2.7 | 18.3 | 17.2 | 31.5 |
| Fenpyroximate(E) | LOQ – 460 | y=24740316x + 67928 | 0.9987 ± 0.41 | 0.20 | 1.53 | 4.60 | 96.2 ± 8.0 | 95.2 ± 1.2 | 10.3 | 9.3 | 18.0 |
| Deltamethrin | LOQ – 345 | y=212931x + 1529 | 0.9999 ± 0.01 | 0.07 | 1.15 | 3.45 | 96.8 ± 5.7 | 79.9 ± 1.1 | 4.8 | 10.3 | 36.0 |
| Acrinathrin | LOQ - 475 | y=48200x - 207 | 0.9998 ± 0.27 | 0.10 | 1.58 | 4.75 | 89.9 ± 12.2 | 88.8 ± 10.7 | 5.4 | 8.3 | 34.6 |
| Pyridaben | LOQ – 418 | y=18219756x + 7898 | 0.9999 ± 0.07 | 0.30 | 1.39 | 4.18 | 98.6 ± 2.6 | 88.7 ± 9.2 | 8.6 | 13.4 | 23.2 |
| Tau - Fluvalinate | LOQ – 431 | y=17740157x - 22927 | 0.9999 ± 0.13 | 0.10 | 1.44 | 4.31 | 85.0 ± 11.4 | 94.2 ± 3.8 | 4.5 | 14.1 | 34.7 |
| Fenarimol | LOQ – 444 | y=167645x + 140 | 0.9999 ± 0.57 | 0.02* | 1.48 | 4.44 | 84.2 ± 19.4 | 86.3 ± 10.2 | 11.1 | 5.6 | 48.7 |
| Etofenprox | LOQ – 387 | y=5530818x + 37481 | 0.9981 ± 0.24 | 1.00 | 1.29 | 3.87 | 92.4 ± 5.6 | 93.3 ± 0.5 | 7.2 | 13.1 | 18.0 |
| Bifenthrin | LOQ – 417 | y=39808x + 128 | 0.9989 ± 0.58 | 0.30* | 1.39 | 4.17 | 80.4 ± 12.6 | 76.2 ± 1.4 | 11.1 | 8.7 | 49.2 |
| Famoxadone | LOQ - 376 | y=168731x - 107 | 1.0000 ± 0.07 | 2.00 | 1.25 | 3.76 | 91.7 ± 10.9 | 94.4 ± 7.1 | 8.7 | 9.8 | 27.6 |

*Pesticides not allowed in EU with an MRL on tomatoes. [Corrias F., **Atzei A.,** et al., 2020]

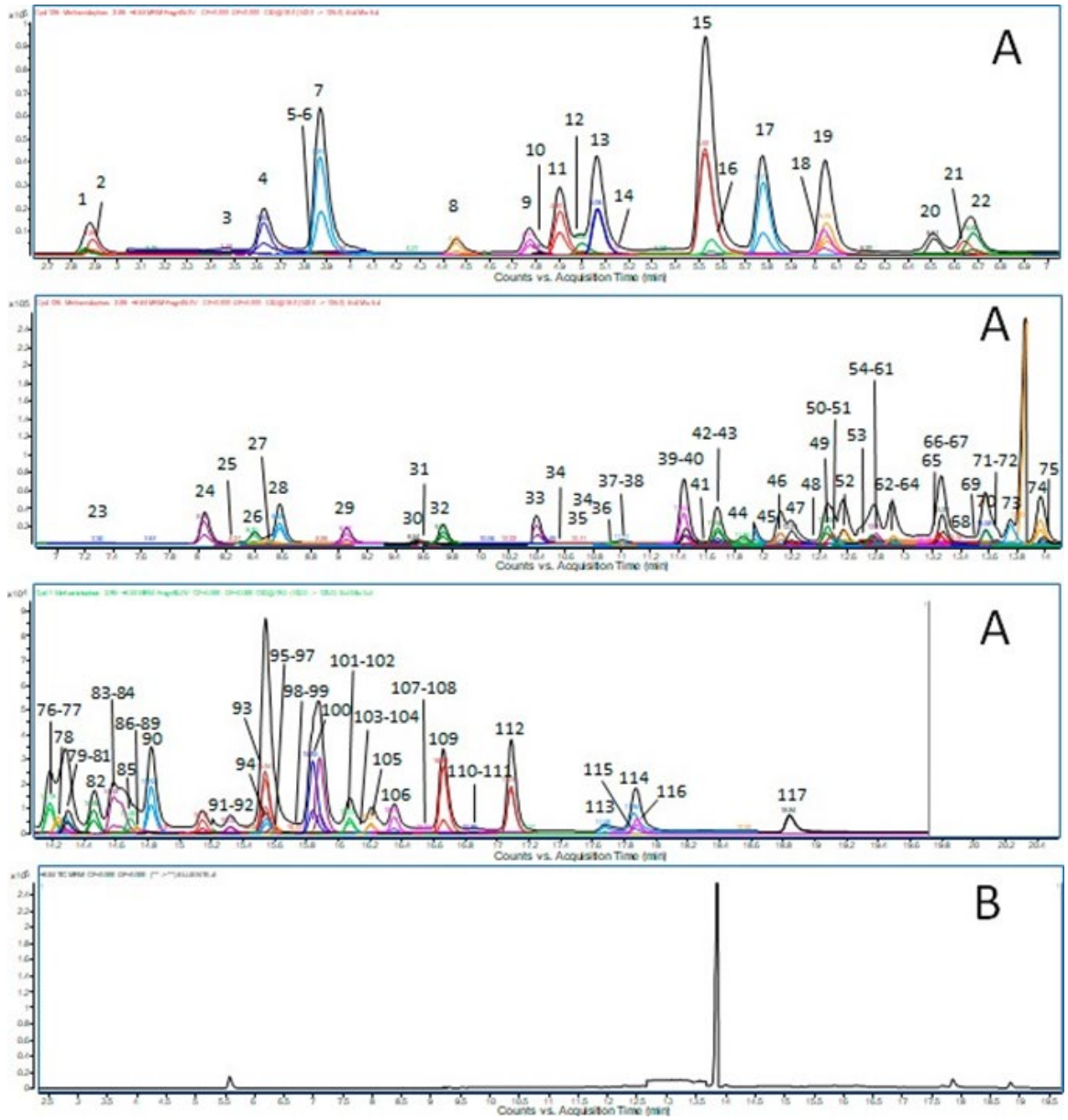


**Figure 8** MRM condensed chromatogram at LOQ value of the 116 pesticides analysed in the present study (**A**), and blank matrix (**B**). [Corrias F., **Atzei A.,** et al., 2020]

**Table 9** Pesticide residues concentration (minimum and maximum µg/kg) in raw tomatoes and processed products analyzed during the survey.

| Pesticide | Samples* | min-max (average) (g kg$^{-1}$) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | frequency | Raw Tomatoes | | | | | | | Puree | Triple Concentrate | Pulp | Diced tomatoes |
| | | Creso (31) | Dask (34) | Datterino (2) | Docet (31) | Rapidus (3) | Taylor (46) | Mixed (12) | | | | |
| Formetanate | 68 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ -20.58 (5.69) | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Propamocarb | 15 | <LOQ | <LOQ-26.87 (4.71) | <LOD | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Flonicamid | 9 | <LOQ | <LOQ-10.49 (5.73) | <LOD | <LOD | <LOD | 9.55 | <LOD | <LOD | <LOD | <LOD | <LOD |
| Carbendazim* | 2 | <LOD | <LOD | <LOD | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Imidacloprid | 25 | <LOQ | <LOQ | <LOD | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Methiocarb | 8 | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Dimethoate | 1 | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Acetamiprid | 15 | <LOQ | <LOQ | <LOD | <LOQ | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Cymoxanil | 1 | <LOD | <LOD | <LOD | <LOD | <LOD | 6.51 | <LOD | <LOD | <LOD | <LOD | <LOD |
| Thiacloprid | 4 | <LOQ | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Atrazine-desethyl* | 1 | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Metribuzin | 1 | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Carbofuran* | 5 | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Chlorantraniliprole | 102 | <LOQ-111.76 (29.37) | <LOQ-205.19 (44.45) | 29.74-40.81 (35.28) | <LOQ –50.95 (16.04) | 51.78 | <LOQ-139.75 (23.11) | <LOQ-37.10 (22.03) | <LOD | <LOD | <LOD | <LOD |
| Pyrimethanil | 13 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Azoxystrobin | 141 | 17.99-201.98 (80.80) | <LOQ-32.75 (8.27) | <LOQ | <LOQ | 26.96 | <LOQ-129.65 (22.00) | 7.67-72.16 (31.15) | <LOD | <LOD | <LOD | <LOD |
| Fenamidone | 4 | <LOD | <LOQ | <LOQ | <LOD | <LOD | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Boscalid | 15 | <LOQ -45.13 | 7.59-69.62 (38.60) | <LOD | <LOQ | <LOQ | <LOQ-442.23 (112.86) | <LOD | <LOD | <LOD | <LOD | <LOD |
| Dimethomorph | 106 | <LOQ | <LOQ-170.19 (31.45) | <LOQ | <LOQ | 7.70 | 27.42-71.13 (47.23) | <LOQ-655.78 (264.91) | <LOD | <LOD | <LOD | <LOD |
| Iprovalicarb | 11 | 5.80-10.77 (15.60) | <LOD | <LOD | 15.59 | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD |

**Table 9** Cont.

| Pesticide | Samples* frequency | min-max (average) (µg kg$^{-1}$) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Raw Tomatoes | | | | | | | Puree | Triple Concentrate | Pulp | Diced tomatoes |
| | | Creso (31) | Dask (34) | Datterino (2) | Docet (31) | Rapidus (3) | Taylor (46) | Mixed (12) | | | | |
| Tetraconazole | 49 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ-34.21 (18.72) | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Spirotetramat | 67 | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Penconazole | 4 | <LOQ | 4.22 | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Tebuconazole | 21 | <LOQ | <LOQ | <LOD | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Benalaxyl | 62 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Spinosyn A | 83 | <LOQ–70.61 (11.60) | <LOQ-216.78 (24.54) | <LOQ | <LOQ-39.65 (27.03) | <LOQ | 36.79 | <LOQ-9.83 (4.51) | <LOD | <LOD | <LOD | <LOD |
| Zoxamide | 5 | <LOQ | <LOQ | <LOD | <LOD | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Pyraclostrobin | 42 | <LOQ -128.01 (22.25) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ-22.78 (11.50) | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Clofentezine | 1 | <LOD | <LOD | <LOD | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Phosalone* | 9 | <LOQ | <LOD | <LOQ | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Difenconazole | 14 | <LOQ-54.00 (24.87) | <LOQ | <LOD | <LOD | <LOD | 20.79 | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Ametoctradin | 60 | <LOQ-134.60 (27.30) | <LOQ-606.10 (86.72) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Spinosyn D | 49 | <LOQ-97.36 (38.09) | <LOQ-352.24 (92.88) | <LOD | <LOQ- 117.68 (86.79) | <LOD | <LOQ-349.00 (70.46) | 10.32-12.62 (11.46) | <LOD | <LOD | <LOD | <LOD |
| Indoxacarb | 4 | 11.20-17.60 (14.40) | 6.25 | <LOD | <LOD | <LOD | 7.31 | <LOD | <LOD | <LOD | <LOD | <LOD |
| Trifloxystrobin | 2 | <LOD | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Quizalofop-ethyl | 2 | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Emamectin Benzoate | 72 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ-7.97 (5.58) | <LOQ-17.95 (6.92) | <LOD | <LOD | <LOD | <LOD |
| Piperonyl butoxide | 59 | <LOQ-4.62 (4.62) | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |
| Chlorpyriphos | 14 | <LOQ | <LOQ | <LOD | <LOQ | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Hexythiazox | 38 | 4.87-18.57 (13.84) | 6.18-43.72 (16.65) | 9.56 | 4.87-11.52 (7.12) | <LOD | 7.76-23.47 (12.58) | 17.89 | <LOD | <LOD | <LOD | <LOD |

**Table 9** Cont.

| Pesticide | Samples* frequency | min-max (average) (g kg$^{-1}$) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Raw Tomatoes | | | | | | | Puree | Triple Concentrate | Pulp | Diced tomatoes |
| | | Creso (31) | Dask (34) | Datterino (2) | Docet (31) | Rapidus (3) | Taylor (46) | Mixed (12) | | | | |
| Pyriproxyfen | 2 | <LOQ | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Pendimethalin | 1 | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Fenpyroximate(E) | 2 | <LOD | 12.30 | <LOD | <LOD | <LOD | <LOQ | <LOD | <LOD | <LOD | <LOD | <LOD |
| Deltamethrin | 26 | <LOQ-12.48 (8.02) | <LOQ-7.47 (4.05) | | <LOQ- 7.11 (5.57) | | <LOQ-15.41 (7.19) | 4.78 | <LOD | <LOD | <LOD | <LOD |
| Fenarimol* | 97 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOD | <LOD | <LOD | <LOD |

*Non authorized pesticided in tomatoes. [Corrias F., **Atzei A.,** et al., 2020]

### 3.2. DNT assessment

#### 3.2.1. Zebrafish Embryotoxicity Test (ZFET)

After exposing the zebrafish embryos to the test compounds up to 72 hpf only FLX and its metabolite norFLX resulted in a concentration-dependent decrease in GMS, with a $BMC_{05}$ of 31.2 (CI 7.22-57.2) μM and 32.19 (CI 14.9-59.7) μM (**Fig. 9**). Particularly, a high mortality rate of the embryos was observed when exposed to the highest tested concentrations of FLX and norFLX, 89.9 and 60 μM, respectively. No developmental delay was observed with the other compounds, and no teratogenicity was detected.

#### 3.2.2. Single compound dose-response analysis

As a first step in locomotor analysis, the locomotor activity was evaluated after exposing to the high range of laboratory concentrations (**Table 3**). Here, all test compounds except CBZ10,11E, showed a dose-dependent decrease in the dark period of embryos locomotor activity. The example output of the Quantization protocol with CBZ (**Figure 10A**) illustrates the multitude of parameters which can be analysed, including light-dark transition values, initial and peak values, change over the three measurement blocks, etc. This report further considers the analysis of the first 10' dark block only (**Figure 10B**); in this study, the second and third blocks did not provide additional information, and there were no significant effects in light blocks (**Figure 10C**; **Table 10**). Total duration of activity in that 10' section was recorded per embryo and analysed for dose responses in dark and light (**Figure 10B**). Given the absence of an effect during light with each compound, this parameter was not considered of informative use and not further reported. In this way, the independent replicate experiments of CBZ, FLX and VNX displayed a strong reproducibility also between the two different laboratories, resulting in a graphical and quantitative overlap of each replicate dose-response

curve and their respectively CIs (**Figure 11A-B-C-D-E-F**). Quantified behaviour data for FLX, PHT and VNX are reported as supplementary data in the end of the work. When summarizing the results of the single compounds dose-response analysis (**Table 11**), it appeared that FLX and VNX had a similar potency ($BMC_{05}$ ranges of 0.17-0.65 and 0.26-1.34µM, respectively), and that CBZ and PHT were about 85x less potent compared to these two compounds ($BMC_{05}$-CBZ range 51.55-82.25 µM; $BMC_{05}$-PHT 45.13µM). The metabolites were either ineffective (CBZ10,11E) or showed a lower potency than their respective parent compounds ($BMC_{05}$ around 2 and 6 µM for norFLX and desVNX, respectively). While the 5% CES is applicable for risk assessment, a 50% CES was calculated using the same data sets for more accurate relative potency analysis for design of the subsequent mixture experiments. These values (**Table 11**) revealed FLX as the most potent compound ($BMC_{50}$ average around 3µM), followed by a approximative 17x and 50x lower potency of VNX and CBZ, respectively. Exposure to the parent compounds, CBZ, FLX and VNX was repeated at environmentally relevant concentrations, to confirm an absence of effect at these levels (**Table 3**). Indeed, no effect was observed at both light and dark period (not shown).

### 3.2.3. Gene expression

To better investigate the possible developmental neurotoxicity mechanism induced by CBZ, FLX and VNX in zebrafish embryos, the gene expression of the specific DNT markers, *grin1a*, *dlg4* and *gabra6a,* was studied. The mRNA expression of all the target markers was upregulated when exposing to 115 µM CBZ ($P<0.05$), although *dlg4* was most markedly affected by the CBZ exposure, reaching a double relative quantification compared to the control group (**Figure 12**). In contrast, both exposures to 10 µM FLX and 107 µM VNX showed a significant upregulation of *gabra6a* mRNA expression only ($P<0.05$) (**Figure 12**).

### 3.2.4. Different windows exposure

Different exposure periods were investigated to assess the persistence of the pharmaceuticals on the zebrafish locomotor activity. Embryos were exposed to the effective concentrations of CBZ, FLX and VNX (200, 10 and 300 µM, respectively) during (<2.5-120, <2.5-96 and 96-120 hpf time intervals. In all conditions, the exposures induced a decreased embryo activity already at 96 hpf over the dark periods ($P<0.05$) (**Figure 13A**), although the inhibition with FLX appeared less pronounced than with the other two compounds. Removal of the pharmaceuticals at 96 hpf led to a motor activity recovery at 120hpf with CBZ and FLX, but not with VNX (**Figure 13B**). Furthermore, the acute exposure (96-120 hpf) also induced a decreased activity compared to control ($P<0.05$), and was almost as effective as the chronic treatment (<2.5-120 hpf) with all three compounds.

### 3.2.5. RPF estimation for mixture designing

For the purpose to design an accurate mixture experiments, a RPF was estimated for each binary combination of the test compounds, based on their relative potency (expressed as $BMC_{50}$). A combined dataset including the first dark periods of each compound was analysed in a single run by PROAST for CBZ-FLX and VNX-FLX mixtures whereas, in the case of CBZ-VNX mixture, a manual comparison was preferred. This analysis revealed that FLX was 50x and 10x more potent than CBZ and VNX, respectively, whereas the latter was 12x more potent than CBZ (**Table 4**).

### 3.2.6. Mixture results

The binary mixtures of CBZ-FLX, CBZ-VNX and VNX-FLX, together with the exposure to single compounds, were qualitatively and quantitatively evaluated in order to assess the dose-addition model in predicting the combined effect of the chemical mixtures. The mixtures dose-response curves (**Figure 14A-B- C**) describe the behaviour of the single compounds along with the three compound combinations in a equipotent (1:1) and near-equipotent (1:3; 3:1) ratios. The visual evaluation shows that both single compounds and mixtures responses do not deviate from the fitted curve describing the trend of the entire pool of data. In the event of deviation from dose-addition, the graphical assessment would show a shifting of the mixture response either to the right (less than dose addition) or to the left (more than dose addition). A quantitative evaluation objectively supported the graphic estimation by comparing the RPF-CI calculated with and without mixture (**Table 7**). Indeed, a ratio overlap below one, was obtained for all the studied binary mixtures, supporting that the mixture data did not affect the RPF, which is in line with dose-addition. This conclusion is true for the combination of compounds with similar (VNX-FLX) and dissimilar MOA (CBZ-FLX and CBZ-VNX).

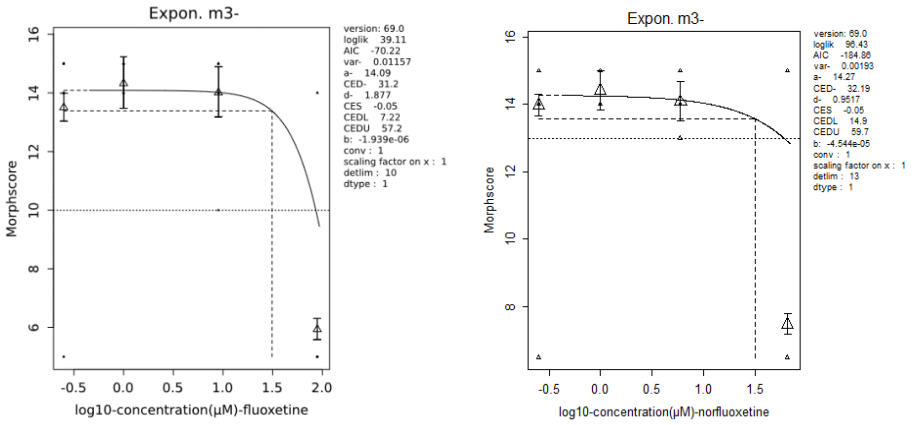


**Figure 9** Dose-response curves of general morphology score performed at 72 hpf for fluoxetine and norfluoxetine. X- axis shows compounds dose-range on log scale.

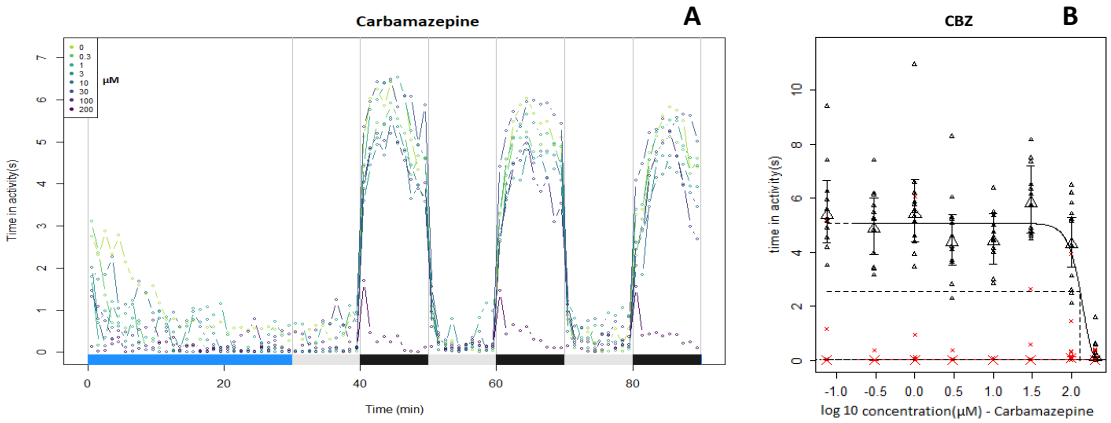


**Figure 10A** A plot overview showing the entire light-dark transition test applied for the CBZ experiment, after 0-120hpf exposure. The blue bar indicates the acclimatization period while the gray and black bars represent the light and dark period, respectively. X-axis shows the whole experiment time whereas the y-axis shows the average time (s) spent in activity within one min by the zebrafish embryos (n=12). Each dot shows the average time (s) spent in activity by 12 embryos/concentration in one minute of recording, whereas different colours represent different concentrations reported on the upper left legend.

**Figure 10B** A dose-response curve of an individual CBZ experiment. The data set belong to the first light-dark block. The red crosses/lines show the light period whereas the black triangles/lines show the dark period. X-axis shows the CBZ dose-range (µM) in log-scale whereas the y-axis shows the average time (s) spent in activity within 1 min by the zebrafish embryos (n=12). Each small symbol shows the average time (s) spent in activity by 12 embryos within one min while the large symbol represents the geometric means together with their confidence intervals (error bars).

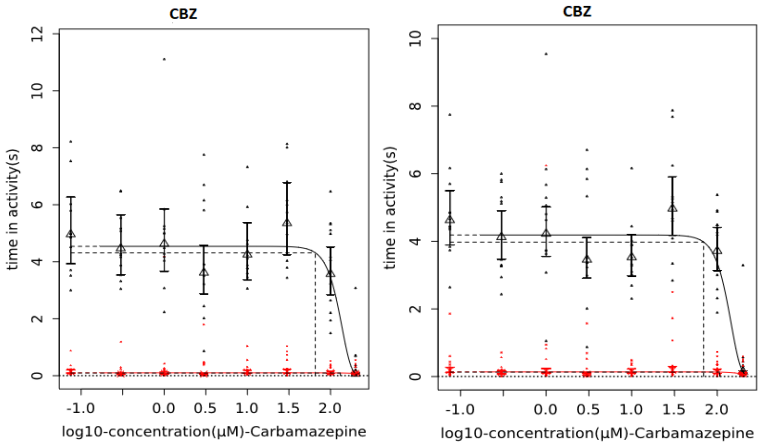


**Figure 10C** Dose-response curves of an individual CBZ experiment. The data set belong to the second and third light-dark block, respectively. This experiment (plot overview and first block reported in Fig 2A-B) was performed at the RIVM.

**Table 10** Carbamazepine CEDs at 5% effect level of each dark period of the L-D transition test.

| Block 1 | Block 2 | Block 3 |
|---|---|---|
| $CED_{05}$(CI) | $CED_{05}$(CI) | $CED_{05}$(CI) |
| 67.16 (42.7-69.1) | 65.58 (37.8-67.8) | 69.39 (47.1-71.3) |

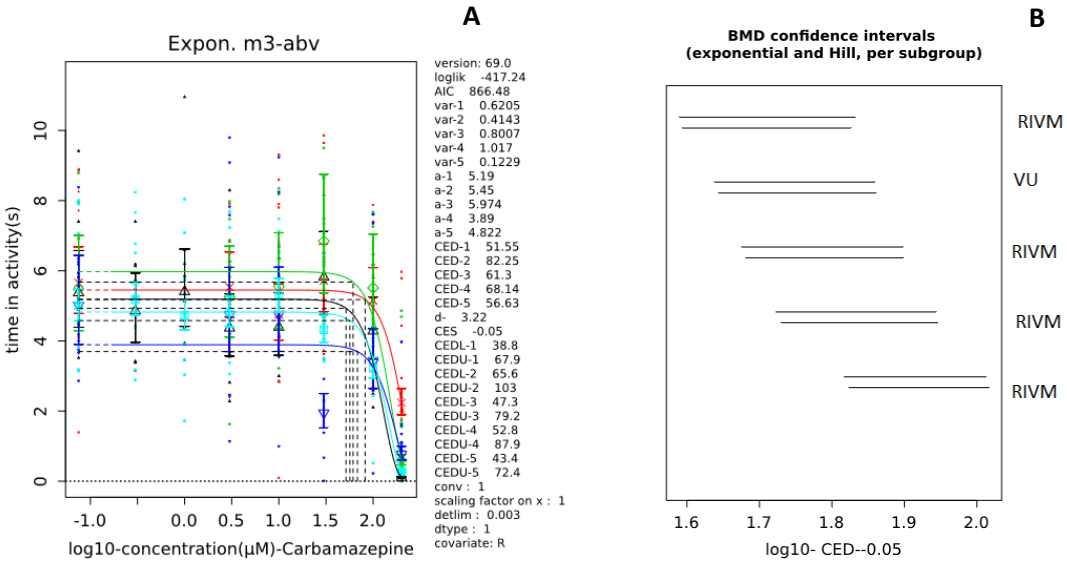


**Figure 11A** A dose-response curve plot showing five combined replicates of CBZ (dark periods only). The individual experiments were used as covariate. One replicate of each compound was performed at VU, the others at RIVM.

**Figure 11B** Distribution of BMDs over the five repeated experiments of CBZ. Each pair of lines represents the confidence interval (CI) of an independent experiment, related to the exponential (upper) and Hill (lower) model. X-axis, log10 of concentration in µM. The five experiments produce one overlapping cluster, within a combined range of 51.55 to 82.25 µM.

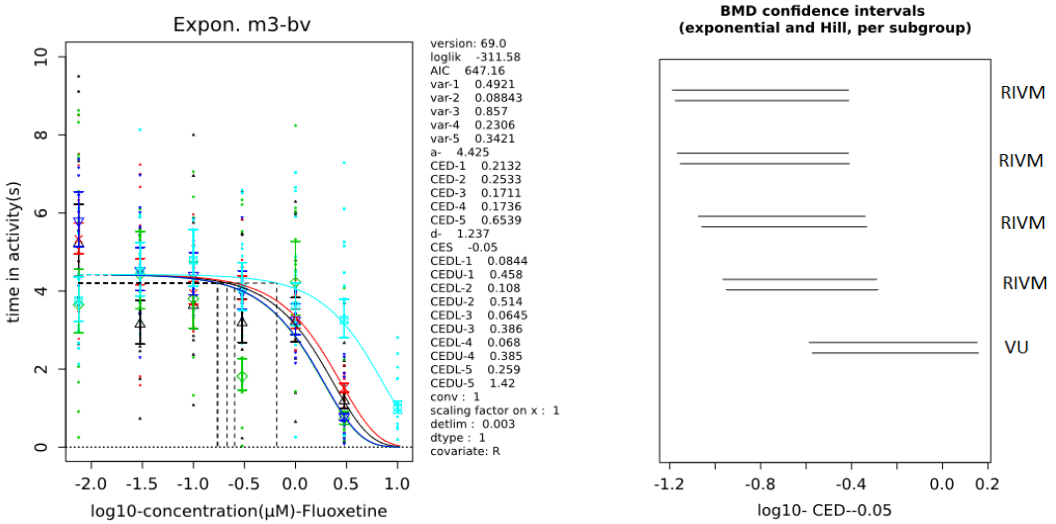


**Figure 11C** A dose-response curve plot showing five combined replicates of FLX (dark periods only). The individual experiments were used as covariate. One replicate of each compound was performed at VU, the others at RIVM.

**Figure 11D** Distribution of BMDs over the five repeated experiments of FLX**.** Each pair of lines represents the confidence interval (CI) of an independent experiment, related to the exponential (upper) and Hill (lower) model. X-axis, log10 of concentration in µM. The five experiments produce one cluster of overlapping CED-CIs, ranging from 0.17 to 0.65 µM.

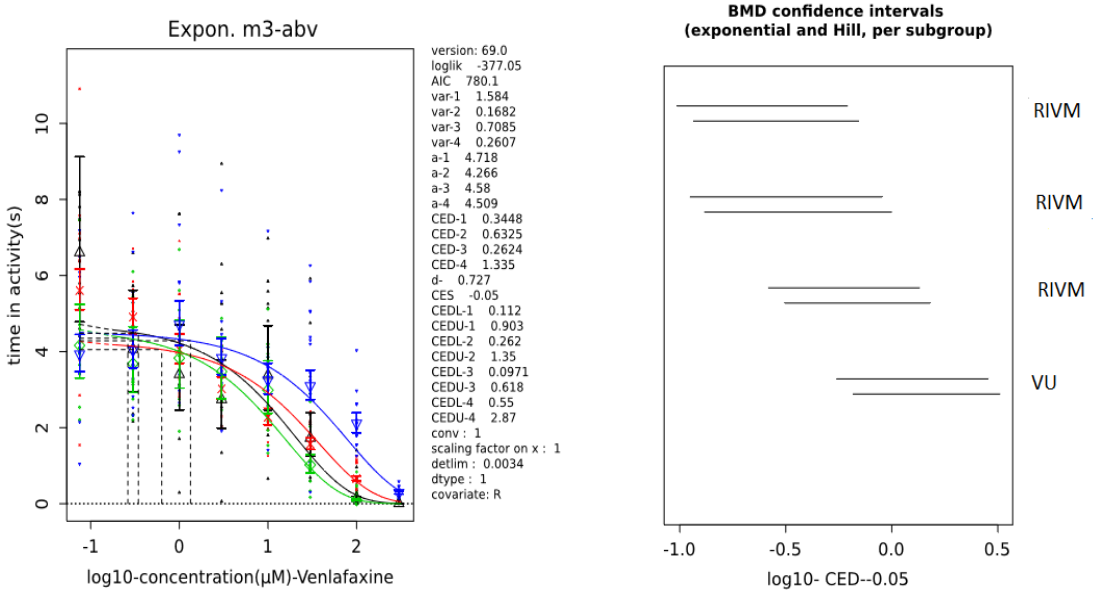


**Figure 11E** A dose-response curve plot showing four combined replicates of VNX (dark periods only). The individual experiments were used as covariate. One replicate of each compound was performed at VU, the others at RIVM. **Fig. 11F.** Distribution of BMDs over the four repeated experiments of VNX. Each pair of lines represents the confidence interval (CI) of an independent experiment, related to the exponential (upper) and Hill (lower) model. X-axis, log10 of concentration in µM. The four experiments produce one cluster of overlapping CED-CIs, ranging from 0.26 to 1.34 µM.

**Table 11** DR curves results of the single-compound analysis at experimental level, at both 5% and 50% CES.

| | $BMC^{a}_{05}$ (µM) | $BMC_{05}$-$CI^{b}$(µM) | $BMC_{05}$ (mg/L) | $BMC_{05}$-CI(mg/L) |
|---|---|---|---|---|
| | | Experimental level | | |
| Carbamazepine | 51.55-82.25 | 38.8-103 | 12-19 | 9.16-24.33 |
| Carbamazepine 10,11-epoxide | - | - | - | - |
| Fluoxetine | 0.17-0.65 | 0.06-1.42 | 0.05-0.2 | 0.02-0.44 |
| Norfluoxetine | 2.21 | 0.2-6.03 | 0.65 | 0.06-1.78 |
| Phenytoin | 45.13 | 4.14-52.5 | 11.38 | 1.04-13.24 |
| Venlafaxine | 0.26-1.34 | 0.10-2.87 | 0.07-0.37 | 0.03-0.8 |
| Desvenlafaxine | 6.06 | 0.69-26.7 | 1.6 | 0.18-7.03 |
| | $BMC^{a}_{50}$ (µM) | $BMC_{50}$-$CI^{b}$(µM) | $BMC_{50}$ (mg/L) | $BMC_{50}$-CI(mg/L) |
| | | Experimental level | | |
| Carbamazepine | 116-185.1 | 103-216 | 27.4-43.73 | 19-51.03 |
| Fluoxetine | 1.41-5.40 | 1.01-6.77 | 0.44-1.67 | 0.31-2.09 |
| Venlafaxine | 9.53-48.45 | 6.19-63.9 | 2.64-13.44 | 1.72-17.73 |

-No effect.

[a] Benchmark concentration of the single-compound analysis calculated at the 5% and 50 % effect level. The values represent the highest and the lowest $BMC_{05}$ / $BMC_{50}$ of 5 (CBZ and FLX) and 4 (VNX) independent experiments in the exponential and Hill models. The experiments on the metabolites were performed once.

[b] Confidence interval (CI) of the $BMC_5$ / $BMC_{50}$. The values represent the highest $BMCU_{05}$ / $BMCU_{50}$ and the lowest $BMCL_{05}$ / $BMCL_{50}$ of 5 (CBZ and FLX) and 4 (VNX) independent experiments in the exponential and Hill models.

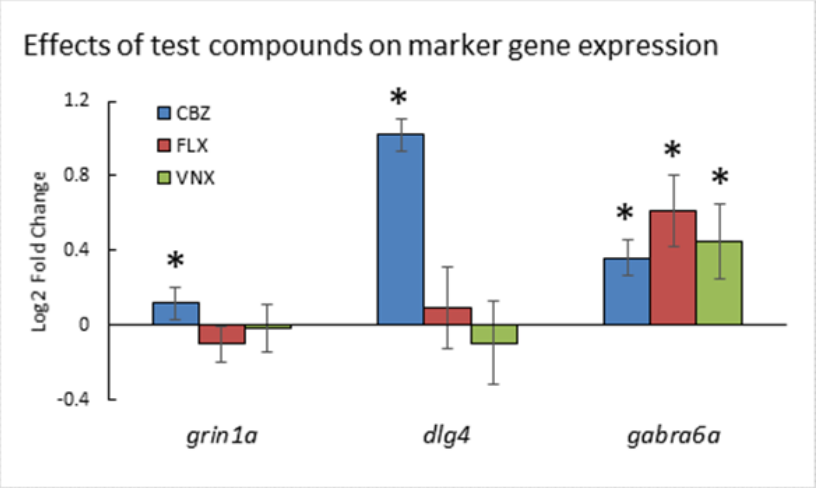


**Figure 12** Gene expression analysis after 120 h of exposure to 115 µM CBZ, 107 µM VNX and 6 µM FLX. The results are expressed by using the delta-delta-Cycle threshold method ($2^{-\Delta\Delta Ct}$). The asterisk indicates significance, P<0.05. Error bars indicate +/- SD. N=6.

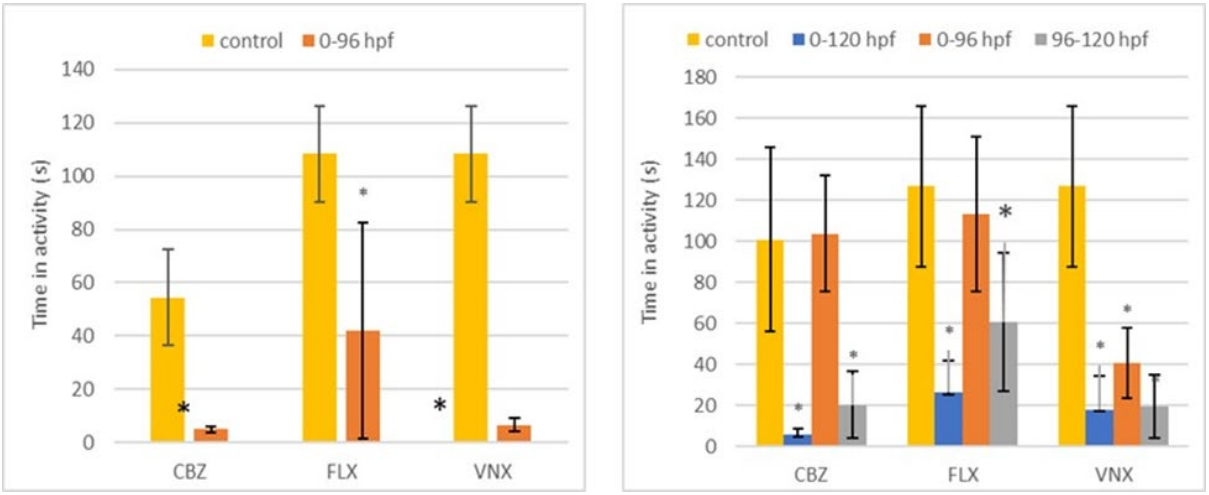


**Figure 13A-B** Effect on activity at 96 and 120 hpf, respectively. Activity measurement during the first 10 min dark periods only, at 96 and 120 hpf, following exposure to 200 µM CBZ, 10µM FLX and 300 µM VNX. Second and third dark block (not shown) provide identical results whereas the light blocks (not shown) did not show statistical differences compared to the control. The activity (Y-axis) was measured as duration of movement (in seconds) each 10 min. The X-axis represents the three different conditions (CBZ, FLX and VNX). The asterisk indicates a significant difference compared to the control group, P<0.05. N=12.

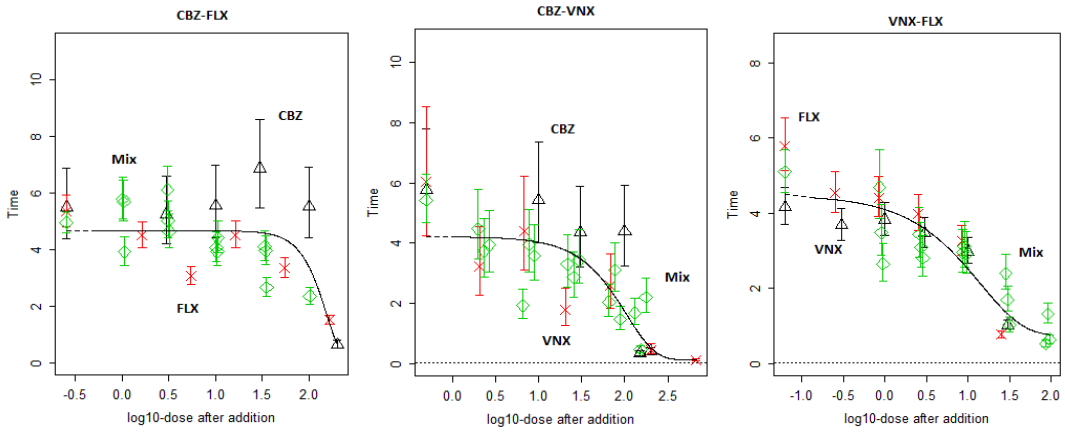


**Figure 14A-B-C** Dose-response mixture effects on swimming activity in zebrafish embryos, with the reference compound (black triangles/lines) combined with the second compound (red crosses/lines). The green diamonds represent the corresponding mixtures.

# 4. Discussion

Nowadays, although humans and environment are daily exposure to a multitude of substance, the chemical risk assessment mostly based on single-compound safety which does not include possible adverse effects resulting from chemical combinations. Also, rodent studies are still mandatory tests within the chemical risk assessment leading to a slow, expensive and not suitable process for the evaluation of a large number of compounds. Given that, the development of alternative methods/tools toward a better estimation of the risk of chemical mixtures appears a necessity. In the present PhD work, an analytical screening of pesticide multiresidue on real-life samples was assessed as a support of the chemical mixtures pollution. Consecutively, the zebrafish embryo model was evaluatead as potential tool for assessing the risk of chemical mixtures in DNT. An overview of the results shows that, the UHPLC-MS/MS-MRM method allowed the quantification of pesticide residues in concentrations of $\mu g\ kg^{-1}$ and the detection of traces in the range of nanograms. The target neuroactive compounds are capable to decrease the swimming activity of 5dpf zebrafish either individually or in their binary combinations at high experimental concentrations. Of the compounds tested FLX resulted the most potent. Given the persistence of the inhibitory effect, the correlation to DNT can be assumed only for VNX. The gene expression analysis suggests a potential disturbance of the GABAergic system by all the studied compounds.

### 4.1. Analytical assessment of pesticides multiresidue

The development and the validation of the current UHPLC-MS/MS-MRM method coupled with a modified QuEChERS extraction showed, on average, LOQs and a LODs values equal to half and ten times lower than those established during the validation processes, respectively. Therefore, it allowed the qualitative and quantitative assessment of pesticides multiresidue on raw tomatoes. Moreover, since also the processed tomatoes were analysed, the behaviour of pesticides residues following industrial processing was studied. The analytical screening on a

total of 759 samples and 116 target pesticides showed pesticide residues well below the maximum residue levels (MRLs) which, as default, is set at 0.01 mg/kg unless lower legal limits are defined in **Directives 2006/125/EC** and **2006/141/EC6/5/6**. All the detected pesticides resulted in concentrations ranging from μg $kg^{-1}$ to roughly ng $kg^{-1}$. In details, the analyses on raw tomatoes revealed the presence of 46 pesticides, among which five (i.e., carbendazim, atrazine-desethyl, carbofuran, phosalone and fenarimol) are non-authorized to be used on the tomato crop. Given the integrated production strategy applied, their detection may be due to soil or water pollution resulting from a previous crops grown in the same field. Industrial processing seems to influence the final concentration of the detected pesticide residues. Indeed, processed tomato products pointed out a decrease of pesticide residues up to range concentration of roughly ng $Kg^{-1}$. Different authors deal with the overall reduction of pesticides residues following tomato processing (**Rodrigues et al., 2017**; **Angioni et al., 2004**). This may be due to thermal degradation, evaporation, and co-distillation (blanching, steaming and boiling), dissolving (water or solvents washing), mechanical removal (peeling of vegetable skin) hydrolysis, photolysis, volatilization, (**Holland et al., 1994**; **Chavarri et al., 2005**). These outcomes highlighted the importance of applying the good agriculture practices (GAP), which led the final products of the supply chain of tomato to reach residue values lower than those established for baby food **(Corrias F. et al., 2020).** Overall, a better accomplishment for human and environmental safety.

### 4.2. The target compounds inhibit the locomotor activity of zebrafish embryos

All the studied compounds, except for CBZ 10,11E, induce an inhibitory effect of the zebrafish motor activity at high concentrations. Our findings are in agreement with previous studies aimed to assess the locomotory effect of the target compounds during early developmental stages of zebrafish embryos. With regard to CBZ exposure, van Woudenberg et al., (2014)

observed a reduced activity in 5 dpf zebrafish embryos chronically exposed to ≥ 180 µM CBZ which is in accord to the CBZ-$BMC_{50}$ found in our study (Table 2). Comparable effect has been reported also in other aquatic organisms (i.e. *Oryzias latipes* and *Jenynsia multidentate*) exposed to high CBZ dose (**Calcagno et al., 2016**; **Nassef et al., 2010**). Likewise, the literature supports the locomotor inhibitory effect following exposure to high FLX dose. Roughly 5 µM FLX produced a significant decrease of the spontaneous swimming activity (SSA) in 6 dpf zebrafish (**Airhart et al., 2007**; **Prieto et al., 2012**). In agreement, the FLX-$BMC_{50}$ obtained in the present study ranges from 1.41 to 5.40 µM FLX. Moreover, a comparable effect has been described at relative low FLX concentrations (0.025, 0.046 µM) in zebrafish embryos (**de Farias et al., 2019**) as well as in other aquatic organisms (**Winder et al., 2012**). Recent studies also support the hypoactivity effect of the major FLX metabolite. Indeed, daily and late acute exposure to 100 µg/L norFLX led to a decreased swimming activity in 6 dpf zebrafish (**Huang et al., 2019**). Concerning VNX treatment, **Sehonova et al. (2019)** observed a significant decrease in swimming distance of 6 dpf zebrafish exposed to 3000 µg/L (11 µM), which is still in agreement to our VNX-$BMC_{50}$ values (Table 2). In addition, an equal locomotor inhibitory effect has been reported at lower VNX doses (**Huang et al., 2019**). The same effect seems not to be induced following daily exposure to 100 µg/L desVNX (**Huang et al., 2019**) while, in the current work, the observed effect with desVNX may be explained by the application of 16x higher concentrations ($BMC_5$= 1600 µg/L) than those used by Huang et al.

### 4.3. MOA of target compound, potential relation to DNT

At 5 days' post fertilization (dpf), the CNS of the zebrafish displays the main therapeutic targets of the studied compounds, therefore allowing to investigate the role of their major mechanism of action in DNT (**Figure 15**). CBZ is a well-known voltage-gate sodium channels blocker, which also works as GABA agonist and by increasing the concentration of serotonin at

neuronal synapses (**Harkin and Hopkinson, 2010**). The sequential CBZ-dependent block of the voltage sodium channels leads to a decrease of the action potential up to the achievement of a specific threshold value (millivolt). Once the AP is below that threshold, the collective sodium flux through still available open channels is not enough to activate further steps, inhibiting signal propagation and, causing a sudden collapse of the zebrafish motor activity. This seems reflected in the CBZ dose-response curve characterized by a high steepness value (d=4) (Fig.1B). The dose-response curve of phenytoin (PHT) showed the same steepness (d=4), supporting that the key MOA of antiepileptic drugs in zebrafish motor behaviour modulation may be the voltage-gate sodium channel blocking. In the developing zebrafish, the GABAergic nervous system is one of the first to be established and it plays an important role during the early brain development (**Tufi et al., 2016**). CBZ can increase the activity of the GABA receptor, thereby stimulating the inhibitory effect of GABA on the CNS (**Monesson-Olson et al., 2018**). FLX is a SSRI, therefore the inhibitory locomotor effect may be due to the serotonergic modulation that in zebrafish embryos starts at 4 dpf (**Airhart et al., 2007**). The accumulation of serotonin in the synaptic cleft caused by the pharmacological inhibition of its reuptake, can over-stimulate the 5-HT receptors resulting in a downregulation of these receptors (**Kaushik et al., 2016**). Since serotonin plays an important role in modulating the motor output, the alteration of the serotonergic system may disrupt the motor activity control (**Prieto M.J., 2012**). This hypothesis is supported by the study of Airhart et al. (2007) in which the decrease locomotor activity observed in zebrafish larvae was correlated with a reduction in two serotonin receptor transcripts (SERT and 5-HT1A) in the spinal cord after exposure to FLX. However, alternative MOAs of FLX such as suppression either on glutamatergic or GABAergic neurons, can not be excluded (**Lazaveric et al., 2019**; **Wang et al., 2003**). VNX is a SNRI, therefore slightly differs from the FLX therapeutic purpose. Indeed, in addition to the serotonergic modulation, VNX may act by blocking norepinephrine (NE) and dopamine

(DA) reuptake. Data from literature pointed out the decreased behavioural response by VNX may be due to changes in brain monoamine concentrations (**Melnyk-Lamont et al., 2014**; **Bisesi et al., 2014**). **Rodrigues et al. (2020)** reported changes in the expression of 80 hpf zebrafish genes belonging to the serotonergic, noradrenergic and dopaminergic pathways after chronic exposure to VNX. Despite that, the inhibitory effect on NE and DA occurs at concentrations much higher than those applied in the current study (**Ben-Ari, 2002**; **Muth et al., 1986**). Alternatively, altered activity levels in zebrafish embryos have been linked to changes in neurogenesis following contaminant exposure (**Kinch et al., 2015**). **Thompson et al. (2017)** suggested that VNX may disrupt the early developmental events of the zebrafish brain as proved by the enhanced neurogenesis in the hypothalamus, dorsal thalamus, and preoptic area in zebrafish. Our study outcomes are in agreement with this evidence. Indeed, the lack of locomotor recovery observed in 5 dpf zebrafish exposure to VNX, supports a DNT involvement for this compound. On the other hand, removal of pharmaceuticals at 4 dpf led to a recovery of locomotion in 5 dpf zebrafish exposed to CBZ and FLX, thus not providing evidence for a persistent disturbance of these two compounds with development of the CNS at that stage. However, to better explore the role of CBZ and FLX in DNT, prolonged and continued locomotory test are needed. For instance, **Airhart et al. (2007)** exposed zebrafish embryos to 24 h periods up to 4 dpf, observing a normal development of swimming stages. However, when the embryos were exposed to FLX between 4 and 6 dpf, 6 dpf embryos exhibited a significant swimming decrease persistent up to 14 dpf. This result suggests that 4-6 dpf seems to represent a critical period for the development of spontaneous swimming activity, therefore further studies should consider assessing the FLX persistence effect following exposure to this temporal window. The same approach should be applied for CBZ. The DNT assessment was supported by the gene expression of three DNT markers, *grin1a*, *dlg4* and *gabra6a*, derived from the study of Thomas et al. (2012). A relative time-matched

control upregulation was observed by all the three pharmaceuticals only in the expression of *gabra6a* whereas *dlg4* was exclusively upregulated by CBZ. Our outcomes partially match with those reported by Thomas et al. (2012) in which all the target compounds altered the gene expression of the studied DNT markers. However, Thomas et al. carried out a gene expression only in brain of 75 days old fathead minnows, whereas in the current study, we investigated the entire zebrafish embryo at 5 dpf. Therefore, it is possible that mRNA of other tissues in the embryo concealed the specific gene expression alteration in the brain. In zebrafish, *gabra6a* is mostly expressed in the photoreceptor cell layer of the retina and the cerebellum and can be detected only after 96 hpf (**Monesson-Olson et al., 2018**). The *gabra6a* gene encodes for the GABA receptor subunit 6α (Gabra6) which, together with subunit α4 and α5, plays a prominent role in $GABA_{\alpha}$ receptor function. Receptors containing these α subunits are mostly extra-synaptic and mediate tonic inhibition (**Monesson-Olson et al., 2018**). Within the cerebellum, the *gabra6a* expression occurs along with development of GABAergic synapse formation, tonic conductance, motor control and learning. Thus, even a temporal upregulation of *gabra6a* expression may lead to functional deficits of the GABAergic synapse formation in the less active embryos (**Wang et al., 2011**). Regarding *dlg4* gene, it encodes for postsynaptic density 95 (PSD-95) protein, which is a membrane-associated guanylate kinase (MAGUK) that contains multiple protein-protein interaction domains capable to induce clustering of postsynaptic receptors at excitatory synapses, such as the glutamatergic synapse (**Horzmann et al., 2016**; **Meyer et al., 2005**). From 72 hpf, PSD-95 protein is expressed in the zebrafish nervous system, specifically within the developing visual and olfactory system (**Meyer et al., 2005**; **Niell et al., 2004**). Since *dlg4* is strongly upregulated in the CBZ exposed embryos, we may assume that the clustering of receptors at excitatory synapses is disturbed in the less active embryos.

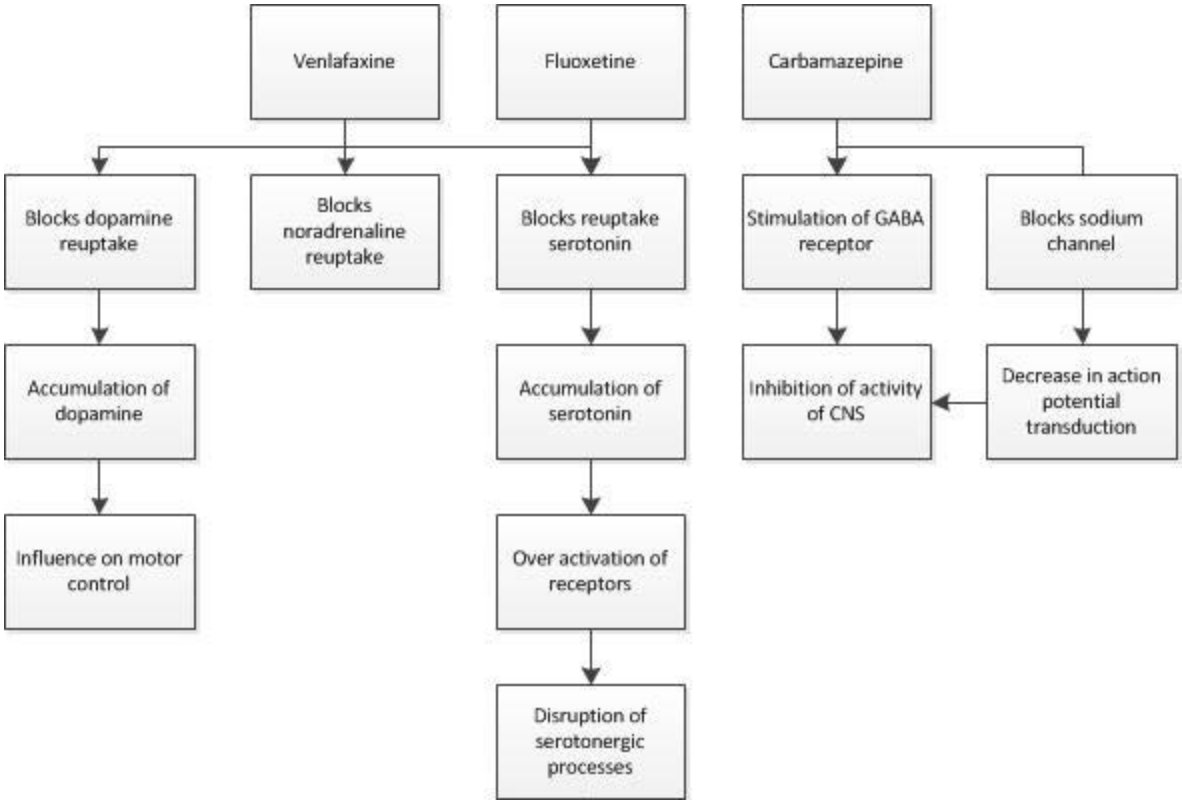


**Figure 15** Major mechanism of action the target compounds in DNT.

### 4.4. Prenatal exposure to neuroactive compounds may lead to long-term neurobehavioral outcomes

In the present Ph.D. thesis, the study hypothesis that prenatal exposure to environmental pollutants may increase the incidence of neurodevelopmental disorders (NDDs) was investigated. According to several epidemiologic studies, NDDs show an increasing incidence worldwide during the last two decades (**EPA, America's Children and the Environment, 2019**; **Chiarotti and Veronesi, 2020**; **Štuhec et al., 2015**; **Perez-Crespo et al., 2019**). Given the heterogeneity of these disturbances and their multifactorial origin (i.e. genetic, biological, psychosocial and environmental risk factors) motivate the rising trend of incidence with one clear cause is problematic. However, evidence pointed out the potential role of environmental exposure in developmental neurotoxicity (**Grandjean and Lanigan, 2006, 2014**). Environmental exposure comprises many pesticides as well as pharmaceuticals and the

neuroactive components often share mechanism **(Bjørling-Poulsen M et al., 2006)**. In the current PhD project, the attention was focused on three neuroactive compounds (CBZ, FLX and VNX) as well as their major metabolites (CBZ 10,11E, norFLX and desVNX). The target compounds are commonly used during pregnancy (**Wen et al., 2015**; **Einarson et al., 2004**) therefore increasing the risk of adverse neurodevelopmental outcomes. Specifically, the prenatal use of first-generation antiepileptic drugs (AEDs) such as phenytoin, phenobarbital, carbamazepine and valproate, has been associated to the major congenital malformations (**Ban et al., 2015**) whit valproate widely recognized as the AED most negatively affecting the developing CNS. About 63% to 85% of pregnant women with exposure to antidepressant are treated with SSRIs due to their high degree of selectivity and minimal side effects compared with tricyclic and monoamine oxidase inhibitor antidepressant (**Taouk et al., 2018**). Despite that, the prenatal exposure to SSRIs has been related to congenital malformations (**Wogelius et al., 2006**; **Kornum et al., 2010**; **Huybrechts et al., 2016**; **Jordan et al., 2016**) pregnancy complications (**Diav-Citrin et al., 2008**; **Johansen et al., 2015**), poor postnatal adaption **(Levinson-Castiel et al., 2006; Nörby et al., 2016)**. However, several human studies reported contradictory and inconclusive evidences about the potential role of the target compounds in long-term neurobehavioral outcomes (**Castro et al., 2016**; **Sujan et al., 2017**; **Nulman et al., 2015**). Inconsistencies have been noted either for early cognitive and psychomotor development (**Baker et al., 2015**; **Casper et al., 2003**; **Johnson et al., 2016**) as well as for more sensitive DNT endpoints such as social behavior, language and motor skills at school age (**Nadebaum et al., 2011**; **Veiby et al., 2013**; **Brown et al., 2016**). Varying outcomes have been obtained also in the assessment of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) (**Harrington et al., 2013, 2014**; **Boukhris et al., 2016**; **Man et al., 2017, 2018**). At the human receptor level, DLG4 has been previously associatied with neuropsychiatric diseases (**Feyissa AM et al., 2009**;

**Kristiansen and Meador-Woodruff, 2009)** as well as GRIN1 has been mostly involved in schizophrenia (**Ohtsuki et al., 2001**; **Begni et al., 2003**). For the study purpose, the locomotor activity of 5 days' post fertilization (dpf) zebrafish embryos after continuous exposure to the pharmaceuticals was measured at both environmental and laboratory levels and as single substances and as binary combinations. Although locomotor activity may be affected by other than neurological effects, is a sensitive endpoint for DNT assessment because it depends on the integrity of brain function and nervous system development (**Bilotta et al., 2002**). The risk for human health may also occur by the prenatal exposure to environmental concentrations of the target compounds. Indeed, they are frequently detected in matrices such as surface and drinking water, fish tissues (i.e. brain, liver and muscle) (**Togola and Budzinski, 2008**; **Weinberger and Klaper, 2014**; **Metcalfe et al., 2010**) which easily end up in the food chain destinated for human consumption. Actual environmental concentrations are several orders of magnitude below the effective doses observed in our exposure studies (**Metcalfe et al.,2010**; **Schultz et al., 2010**). However, in view of simultaneous presence of pharmaceutical compounds and other neuro-active compounds such as pesticides in the environment, cumulative effects should be considered across chemical classes. For cumulative risk assessment, EFSA suggested to apply dose addition as a default way to model mixture effects (**EFSA, 2013**). To test the validity of the dose-addition assumption for the compounds under study, the locomotor activity of the zebrafish embryos was also assessed after chronic exposure to the binary mixtures of the three pharmaceuticals. Our outcomes confirm dose-addition as a consistent model for predicting the mixture responses, both in combinations of compounds of similar and dissimilar MOA. This confirms the emerging concern regarding the daily human and environmental exposure to chemical combinations. Indeed, although our results seem reassuring about the toxicity of the target compounds at environmental levels, their sum might achieve higher concentrations potentially capable to damage key CNS developmental

pathways. For instance, among the studied compounds, FLX resulted the most potent showing a $BMC_5$ between 0.17 μM and 0.65 μM whereas, in aquatic environmental samples, FLX has been reported at concentrations ranging from 0.00003 μM to 0.03 μM (**Metcalfe et al., 2003**; **Weinberger and Klaper, 2014**). Consequently, a factor of roughly 27x separates the current FLX environmental concentrations from the doses affecting the locomotor activity of zebrafish embryos. Therefore, knowing that the number of potential combinations present in the environment is nearly uncountable and definitely more complex than the tested binary mixtures, the added doses might easily reach an effective value. In addition, literature data partially supports the safety of the target compounds at environmental doses. Indeed, environmental concentrations of FLX (**Parolini et al., 2019**) and VNX (**Thompson et al., 2017**) led to a decrease in locomotion in 4-5 dpf zebrafish. Furthermore, **Thomas et al. (2012)** observed that 423 nM CBZ, 29 nM FLX and 159 nM VNX did alter the behaviour of 75 days old fathead minnows in the predator escape test. However, this incongruity may be explained by critical variations in study design such as different sensitive window, analytical method and species. In contrast to these findings, many other authors did not detect behavioural impairment resulting from exposure to environmental levels of the target compounds (**Sehonova et al., 2019**; **Huang et al. 2019**; **Thompson et al., 2020**). The concern about the environmental chemical mixtures increases when considering added activity of the major metabolites. Indeed, with regard to those of the target compounds, they are commonly detected in the environment (**Metcalfe et al., 2010**; **Lajeunesse et al., 2013**; **Gurke et al., 2015**; **Alygizakis et al., 2016**), sometimes at concentrations higher than the parent compounds. Additionally, some of them are equipotent to the parent compound, thereby they strongly contribute to the overall effect.

# 5. Conclusions

The analytical screening of multiresidue pesticides on raw and processed tomatoes supports the necessity of including the chemical mixture in the risk assessment. Indeed, although all the detected pesticide residues were far below from the MRLs values establish by the EU directives and the industrial processing led to an overall decrease of pesticides concentrations, the adverse effects resulting from their potential combinations may still represent a risk for human health safety. The zebrafish model displayed a definite behavioural phenotype either following exposure to single compounds or binary mixtures. In fact, the apical adverse outcome on the developing zebrafish (locomotor inhibitory effect) showed a strong reproducibility between independent replicates and in interlaboratory comparisons with comparable experimental conditions. No differences could be identified between the outcomes of this study and those earlier obtained at the University of Amsterdam. This is supported by the literature where the inhibitory effect following exposure to the studied compounds is well described across different authors and on a variety of aquatic organism. Given the current lack of adequate DNT testing strategies, relatively few substances are recognized as major developmental neurotoxicants. At regulatory level, the detection of chemicals with DNT potential is mainly based on developmental *in vivo* studies in rats, *i.e.* the OECD TG 426 or the DNT cohort of the OECD TG 443, which are time-consuming, involving a large number of animals and costly. Moreover, DNT test occurs only if there is a trigger for DNT in other obligatory regulatory tests. Therefore, the proposed *in vitro* model represents a reliable tool for the first screening of a large number of chemicals potentially capable to interfere with the developing CNS. Indeed, it allows to observe developmental delays, malformations and effects on behaviour within a few days' post fertilization. Moreover, the high reproduction rates, do not only reduce the number of animals needed and the entire costs of the analysis, but, it allows to simultaneously assess different experimental conditions such as the application of large dose-range. Within a test strategy framework aimed to support the 3Rs perspective, the use of this behavioural

pattern may result in a general acceleration of the risk assessment process towards to prioritization of more sensitive DNT test. As previously mentioned, the outcomes of the present study partially support the application of this model in the DNT assessment. In fact, the persistence of the inhibitory effect on the zebrafish locomotor activity was observed only for VNX. Therefore, a follow-up project (see the paragraph below) has already been designed with the intent to better investigate the role of CBZ and FLX in developmental neurotoxicity and the potential persistence of the effects. Depending on the outcomes of this project, we may further support the use of the proposed model as DNT test. The current research project did not aim to assess the potential (developmental) neurotoxicity of the target pharmaceuticals exclusively as a single substance, but, it was planned to investigate the contribution of their binary combinations to (D)NT using the zebrafish embryo as a model. The inhibitory effect on the zebrafish locomotor activity was also observed in all the studied mixtures in a dose-additive manner, both in equipotent and near-equipotent ratios and regardless MOAs. Consequently, many other combinations of neuroactive compounds may adversely affect the locomotor activity of the zebrafish, increasing the DNT risk. The risk assessment of chemical mixtures is decisive to recognize the real DNT hazard of many inactive doses (of neuroactive compounds and not only) spread in the environment. Indeed, the environmental detection of individual doses does not provide adequate safety for human health. In this framework, the experimental data of the current Ph.D. study substantiate the consistency of the zebrafish model for studying the combined effect of chemical mixtures in DNT. Taking into account everything, the current Ph.D. study confirms the unique properties of the zebrafish model which render the model particularly suitable for assessment of DNT. This includes the possibility to investigate CNS development in vitro whole-organism that displays a high homology with the key neurodevelopment mechanisms of human and other vertebrate models.

# 6. Future outlook

The persistence of the effects is a key point to assess the involvement of a compound in developmental neurotoxicity. Indeed, damage occurring during early developmental stage of the CNS is commonly considered irreversible. To obtain experimental support, a follow-up project in collaboration with the Center for Health Protection (RIVM) will be conducted in the coming months. Here, the locomotor activity test will be extended up to 14 days post fertilization (dpf) assessing different exposure windows in agreement with the brain development in zebrafish. Doing that, we aim at finding out which developmental window/s is/are more sensitive for DNT in addition to suppose with more accuracy the mode of action/s of each studied compound. Moreover, in order to support the mechanistic assessment, additional compounds that display a similar or dissimilar MOA of the studied pharmaceuticals will be investigated. This includes two acetylcholinesterase inhibitors insecticides. From the outcomes of this project we aim to build a strong case for the possibilities of developmental neurotoxicity testing in zebrafish. Further studies should investigate additional behavioral endpoints that strongly characterized the major neurodevelopmental disturbances, the irreversibility of the effects and the relevance for adversity (i.e. learning, memory and social behaviors). Improvements are also required in terms of experimental protocols and data analysis strategies. Indeed, whether interlaboratory experimental designs differ widely, the comparison between zebrafish outcomes may results unreliable. For instance, the protocol for the light-dark transition test calls for uniformity in parameters such as the age of the fish, time of the day, acclimatization time, the number and the duration of the light and dark periods. At zebraBox level, setting parameters such as threshold and frequency of measurement need to be standardized. In addition, it should be decided, which dataset better reflect the chemical-mediated changes in the locomotor activity. In this study, the entire pool of data belonging from light and dark periods was used. However, in an activity period of 10 minutes, the first and the last two minutes may represent a time of acclimatization and a time of habit to the new

light condition, respectively, which therefore might be excluded from the benchmark dose analysis. In general, it should be chosen an experimental fish strain or, it should be assessed whether the use of different strains allow or not a reliable comparison between interlaboratory outcomes. Standardization of culture material used, the well-plate size is needed as well. In the current study the embryos locomotion was only affected by high concentrations of the compound. This could be due to a low absorption and/or high elimination of the compound by the embryos. To better investigate this assumption, the actual concentrations of the pharmaceuticals in the embryos should be measured by analytical methods such as LC-MS/MS analysis. At level of gene expression analysis, the assessment of further genes potentially involved in the early development processes of the CNS is needed. Furthermore, it might be better to investigate the gene expression in specific areas of the CNS such as brain and spine instead of the entire organism. Overall, keeping in mind that the developmental neurotoxicity represents one of the most complex areas in toxicology, the zebrafish model should be intended as a reliable tool within a battery of tests which aim to improve the current DNT knowledge. Indeed, the lack of data resulting from the exclusive use of animal testing cannot be filled by one-to-one replacement with the zebrafish model. Consequently, additional DNT testing such as -omic analyses in gene and/or protein levels and mechanistic models (i.e. QSARs) should always support the zebrafish data.

# 7. Supplementary data

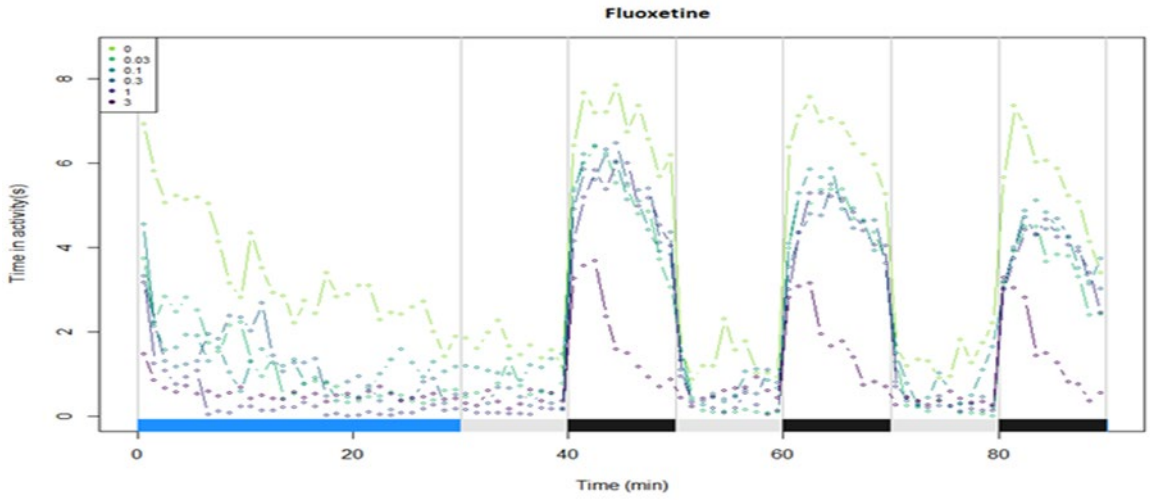


**Figure S1** Plot overview showing a complete light-dark transition test applied for the FLX experiment, after 0-120hpf exposure. This experiment was performed at the RIVM.

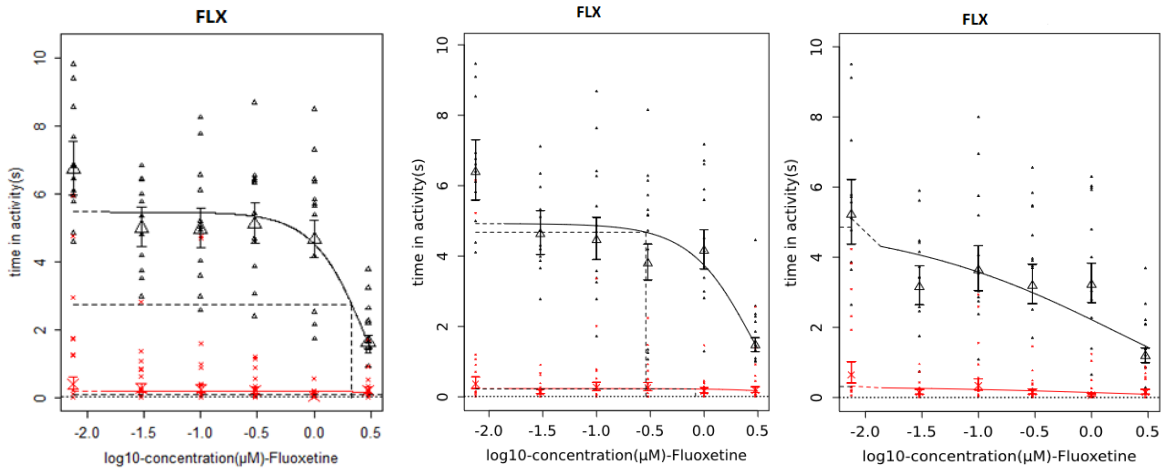


**Figure S2** Dose-response curves of an individual FLX experiment. The data set belong to the first, second and third light-dark block, respectively.

**Table S1** Fluoxetine CEDs at 5% effect level of each dark period of the L-D transition test.

| Block 1 | Block 2 | Block3 |
|---|---|---|
| $CED_{05}$(CI) | $CED_{05}$(CI) | $CED_{05}$(CI) |
| 0.48 (0.06-1.41) | 0.29 (0.01-1.29) | 0.0002 (0.00003-0.35)[a] |

[a] Block 3 response deviates from block 1-2 (similar with VNX, but different from CBZ), leading to a much lower CED but with a very wide CI. Block 3 CED is therefore less reliable.

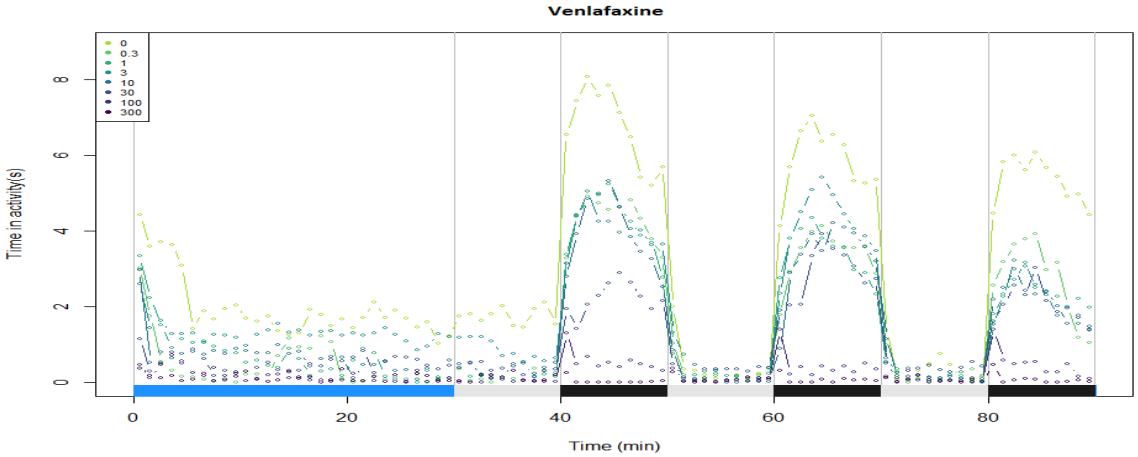


**Figure S3** Plot overview showing a complete light-dark transition test applied for the VNX experiment, after 0-120hpf exposure. This experiment was performed at the RIVM.

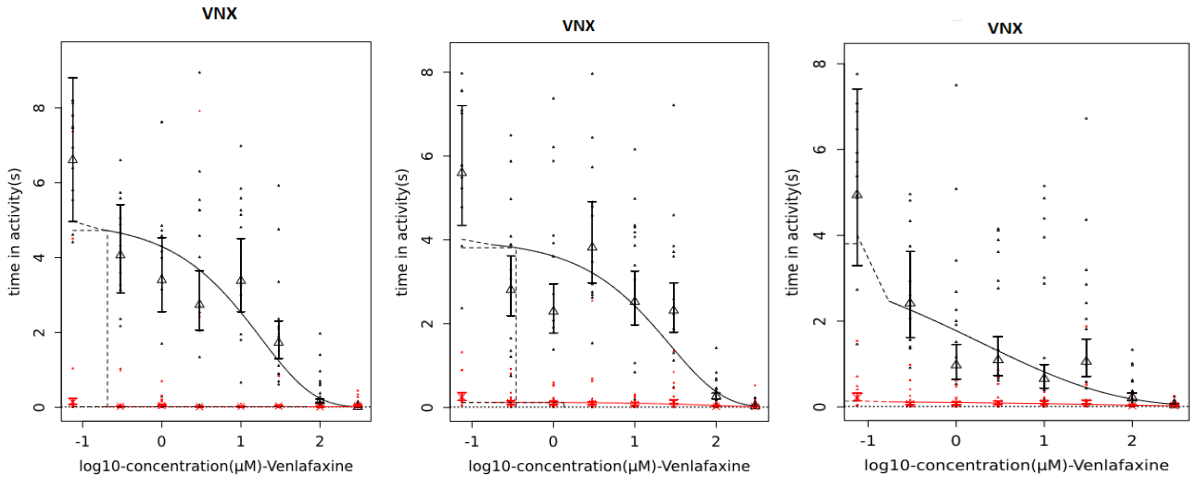


**Figure S4** Dose-response curves of an individual VNX experiment. The data set belong to the first, second and third light-dark block, respectively.

**Table S2** Venlafaxine CEDs at 5% effect level of each dark period of the L-D transition test.

| Block 1 | Block 2 | Block3 |
|---|---|---|
| $CED_{05}$(CI) | $CED_{05}$(CI) | $CED_{05}$(CI) |
| 0.21 (0.028-0.96) | 0.35 (0.05-1.35) | 0.000084 (0.0000023-0.15)[a] |

[a] Block 3 response deviates from block 1-2 (similar with FLX, but different from CBZ), leading to a much lower CED but with a very wide CI. Block 3 CED is therefore less reliable.

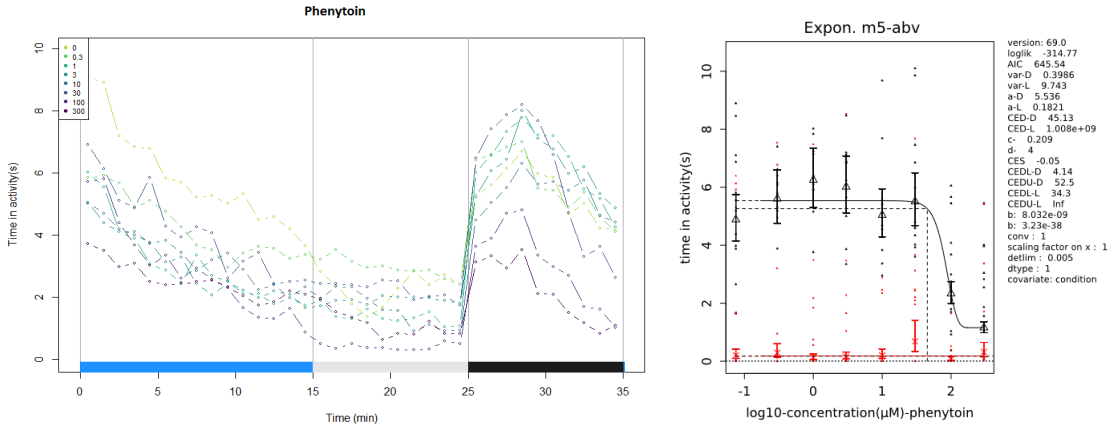


**Figure S3** Plot overview showing a short light-dark transition test applied for a PHT experiment (R1), after 0-120hpf exposure. . This experiment was performed at the RIVM.

**Figure S4** A dose-response curve of an individual PHT experiment (R1). The data set belong to the first (and only) light-dark block.

**Table S3** Phenytoin CEDs at 5% final effect (dark period)

| **R 1** |
|---|
| $CED_{05}$(CI) |
| 45.13 (4.14-52.5) |

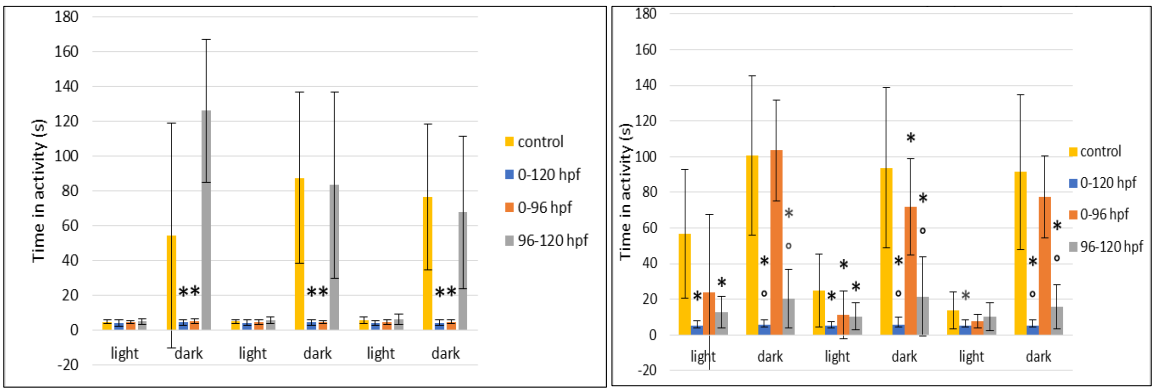


**Figure S4** Activity measurement during six intermitted 10 min periods of light and dark (three periods each) at 96 hpf (A) and 120 hpf (B) after different times of exposure to 200 µM CBZ. The activity (Y-axis) was measured (s) as duration of movement each 10 min. The X-axis represents the light and dark periods. The asterisk indicates a significant difference compared to the control group and the circle indicates significance compared to the activity of the 0-96 hpf group, P<0.05. N=12.

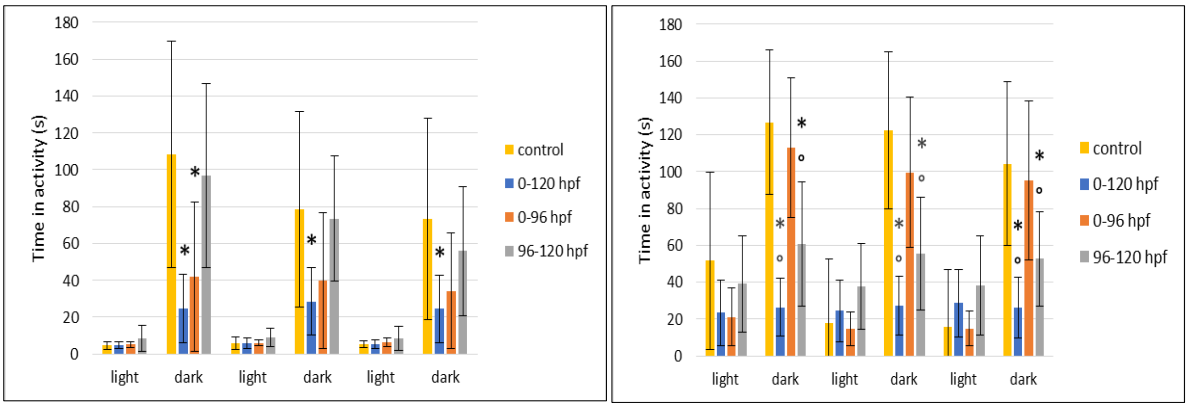


**Figure S5** Activity measurement during six intermitted 10 min periods of light and dark (three periods each) at 96 hpf (A) and 120 hpf (B) after different times of exposure to 10 µM FLX. The activity (Y-axis) was measured (s) as duration of movement each 10 min. On the X-axis represents the light and dark periods. The asterisk indicates a significant difference compared to the control group and the circle indicates significance compared to the activity of the 0-96 hpf group, P<0.05. N.

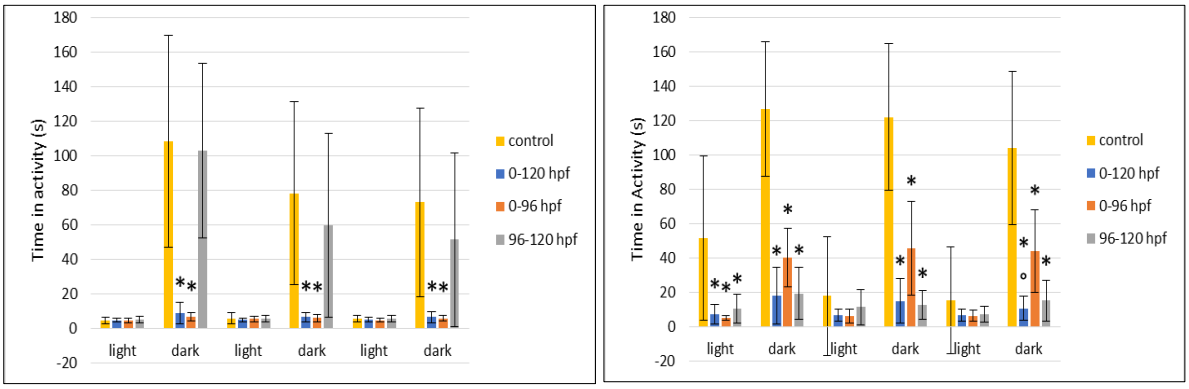


**Figure S6** Activity measurement during six intermitted 10 min periods of light and dark (three periods each) at 96 hpf (A) and 120 hpf (B) after different times of exposure to 300 µM VNX. The activity (Y-axis) was measured (s) as duration of movement each 10 min. The X-axis represents the light and dark periods. The asterisk indicates a significant difference compared to the control group and the circle indicates significance compared to the activity of the 0-96 hpf group, P<0.05. N=12.

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