toxics

Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants

Edited by

Demetrio Raldúa, Carlos Barata and Melissa Faria

Printed Edition of the Special Issue Published in *Toxics*

www.mdpi.com/journal/toxics

MDPI

Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants

Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants

Editors

Demetrio Raldúa
Carlos Barata
Melissa Faria

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade • Manchester • Tokyo • Cluj • Tianjin

Editors

Demetrio Raldúa
IDAEA
CSIC
Barcelona
Spain

Carlos Barata
IDAEA
CSIC
Barcelona
Spain

Melissa Faria
IDAEA
CSIC
Barcelona
Spain

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Toxics* (ISSN 2305-6304) (available at: www.mdpi.com/journal/toxics/special_issues/behavioral_Impairment_pollutants).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-0365-4260-7 (Hbk)
ISBN 978-3-0365-4259-1 (PDF)

Contents

About the Editors

Demetrio Raldúa

Dr. Demetrio Raldúa specializes in several aspects of vertebrate physiology and toxicology. His current work at IDAEA-CSIC is devoted to increasing the understanding of the molecular bases behind the neurotoxic effects of environmental pollutants and drugs. By using zebrafish as a vertebrate model, Raldua's lab is trying to decipher the adverse outcome pathways of neurotoxicants by linking toxic effects at different levels of organization, from molecular to behavioral. They use zebrafish to assess the effect of environmental concentrations of neurotoxic or neuroactive compounds in aquatic ecosystems. They also develop and validate models of different human neurotoxidromes in larval or adult zebrafish.

Carlos Barata

Dr. Carlos Barata is a senior ecotoxicologist. His main scientific activities are in the field of water quality, in the development of cost-effective toxicity tests, and in the study of biological responses to toxic stressors. His research career expands over 20 years of working in different aspects of environmental risk assessment (ERA) of pollutants in aquatic habitats. His expertise includes analytical chemistry, aquatic toxicology, ERA, and toxicogenomics. His current interest is the application of omic technologies to ERA and the study of emerging ecotoxicological effects in biota using lab and field ecotoxicological approaches.

Melissa Faria

Dr. Melissa Faria is an ecotoxicologist working at the IDAEA institute. She is interested in detecting and solving environmental problems related to surface water quality. Her research is focused on the development of new methods for detecting and deciphering apical modes of action of environmental contaminants in aquatic organisms and linking those responses with changes at the cellular, metabolic, and molecular levels. Her research fields include environmental chemistry, system biology, ecotoxicology, stress biology, and animal physiology. She has worked with a wide range of aquatic species, including invertebrates (bivalves, crustaceans, and insects) and vertebrates (fish) in both laboratory and field studies.

toxics

MDPI

Editorial

Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants

Melissa Faria *, Carlos Barata * and Demetrio Raldúa *

Institute for Environmental Assessment and Water Research (IDAEA), CSIC, Jordi Girona 18, 08034 Barcelona, Spain

* Correspondence: mdfqam@cid.csic.es (M.F.); carlos.barata@idaea.csic.es (C.B.); drpqam@cid.csic.es (D.R.)

Citation: Faria, M.; Barata, C.; Raldúa, D. Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants. *Toxics* **2022**, *10*, 243. https://doi.org/10.3390/toxics10050243

Received: 5 May 2022
Accepted: 7 May 2022
Published: 10 May 2022

Neuroactive chemicals are compounds that can modulate, at very low concentrations, the normal function of the central nervous systems of an organism through various primary modes of action (MoA). It has been estimated that around 13% of all detected chemicals in European Rivers have neuroactive potential [1]. This group of compounds includes pesticides (organophosphates, neonicotinoids insecticides, carbamates, organochlorines and pyrethroids), stimulants, CNS-acting pharmaceuticals (including, but not restricted, antidepressants, anxiolytics and antipsychotics) and illicit drugs. Globally, the use of neuroactive compounds is increasing due to growing of urban population. The development of modern chemical screening approaches have allowed for the detection and quantification of many of these chemicals in parallel, confirming the co-occurrence in mixtures of many chemicals with similar or different MoA, which in addition rises the concern about their potential combined effects. It is a known fact that such neuroactive chemicals affect wildlife behavior, with the prospective to cause detrimental effects on individual, population and community levels of ecological organization [2]. In this special issue on "Behavioral Impairment in Aquatic Organisms Exposed to Neurotoxic Pollutants", original research and review articles addressing behavioral impairment induced by the exposure of different invertebrate and vertebrate aquatics species to neuroactive chemicals, are presented. In these studies, different methodological approaches are used, including multi-compartment systems, automated plug and play systems granting medium- and high-throughput screening, as well as, homemade setups systems. Furthermore, association to changes at lower levels of biological organization, such as, gene expression, biochemical activities, neurochemical signaling and macromolecules damage, are also described.

Aiming to increase our current understanding on the ecological and toxicological dimension of environmental occurrence of psychoactive pharmaceuticals in aquatic ecosystems, the review by Stumper and Margiotta-Casaluci 2022 [3] identified 210 CNS-acting pharmaceuticals currently prescribed in the UK. Through the analysis of the PHARMS-UBA database, authors found that presence of 84 of these pharmaceuticals had already been reported in surface waters around the world, of which 33 belong to the list of the 50 most prescribed in the region. Moreover, authors calculated the Predicted Environmental Concentrations (PECs) and then, using the Fish Plasma Model approach, the Predicted Fish steady state Plasma Concentration ($F_{ss}PC$) for all the identified pharmaceuticals. By comparing $F_{ss}PC$ with the Human Therapeutic Plasma Concentration (HTPC), expressed a C_{max}, authors estimated the Predicted Pharmacological Risk for each pharmaceutical. Finally, by using this approach, 32 of the pharmaceuticals were classified as exhibiting potential high and medium risk of eliciting pharmacological effects at their PECs in fish. The results presented in this review should be extremely useful to guide future research on the risk of the environmental risk of neuroactive chemicals in aquatic ecosystems.

When it comes to the quality of the information provided by different ecotoxicological approaches, all of them have their advantages and limitations. The review by Araújo et al.

2020 [4] discusses the limitations of traditional and standardized forced exposure approaches in predicting the ecological relevance of the presence of chemicals stressor in the environment. Authors emphasize that, whereas a force exposure approach considers that the environments are chemically homogeneous and that the option to avoid exposure is inexistent, the natural environment is clearly heterogeneous. Therefore, one must consider that when organisms are confronted with contaminants, three different reactions should be considered: conformity, regulation and avoidance. In this review, the need to integrate a more direct assessment of ecological implications of behavioral changes due to the presence of a chemical stressor is highlighted. The authors propose an approach in which Stress and Landscape Ecology could be integrated in order to better understand the true effect of a contaminant on the structure and function of an ecosystem. This would be possible by the combination of non-forced multi-compartment approach, also known as "avoidance method", with the traditional and standardized forced exposure approach.

While there is widespread agreement that analysis of behavioral responses provides a more sensitive endpoint for assessing the environmental effects of neuroactive chemicals than lethality, environmental regulatory agencies have yet to include behavioral analysis among the endpoints to be analyzed in ecotoxicology. One assumption is that this may be due to the absence of optimized and standardized behavioral assays [5]. Aquatic invertebrates such as *Daphnia* spp. and *Artemia* spp. are commonly used model species to analyze different endpoints of ecotoxicological assessments, including behavior. Behavioral studies with such species have high-throughput potential, however methodological discrepancies make it difficult to be able to compare results from different studies. Two factors easily controlled that may have important implications on the response outcome are the arena size and light intensity. For *Artemia franciscana* a medium to large arena size (12 and 6 well plates) and not light intensity was crucial for a stable swimming speed response, indicating that there could be a compromise between increasing the throughput of the analyses and providing enough space for an even behavior [5].

Pharmaceuticals are a major emerging category of chemicals that pose real concern for the health of aquatic ecosystems. Invertebrate species play an essential role in the stability and well being of the ecosystems and are most threatened by the presence of these chemicals. The anti-depressant drug fluoxetine, at environmental relevant concentrations, increased swimming speed of *A. franciscana* [5]. On another study from this SI, the MoA of deprenyl was assessed for the first time in *Daphnia magna*. Deprenyl is a drug prescribed to treat major depressive disorders and Parkinson's Disease, increasing serotonin signaling through inhibition of monoamine oxidase (MAO) [6], the enzyme responsible for its breakdown. Behavioral changes observed in *D. magna* exposed to deprenyl included low basal locomotor activity and reduction in the habituation light stimuli. Furthermore, *D. magna* exposed to deprenly exhibited inhibition of MAO-activity and a concomitant increase in the serotonin and dopamine levels, suggesting the presence of vertebrate MAO-like activity in this species. Finally, as proof of concept, behavior and molecular changes caused by deprenyl were found contrary with those observed for serotonin antagonistic drug, 4-Chloro-DL-phenylalanine (PCPA).

An analogous study was executed using *Danio rerio* (zebrafish) larvae exposed to the serotonin signaling stimulants deprenyl and fluoxetine and to the serotonin synthesis inhibitor PCPA [7]. Similar behavioral outcomes were observed for both anti-depressants, including hypolocomotion, reduced escape responses evoked by vibrational and visual stimuli and increased habituation to the vibrational stimuli, which contrasted with those observed for PCPA. At lower levels of organization, deprenyl's effect was more potent, abolishing MAO activity, downregulating serotonin synthesis and transporter genes and augmenting serotonin and dopamine levels. Moreover, co-exposure of opposed serotonin signaling drugs revealed full recovery of several impaired responses. It is also interesting to highlight the homology of responses observed between *D. magna* and *D. rerio* to acute deprenyl exposure.

It is a well-known that the developing brain is more sensitive to the effect of chemicals than the adult brain [8], and that developmental exposure may result in subtle effects but can have a profound impact when amortized across the life span of and organism, permanently altering normal biological processes, which can be reflected in the organism behavior. When testing new chemicals, conventional OECD toxicity tests may not reflect their true hazardous impact to organism in the natural environment, therefore multiple experimental approaches should be applied for proper risk assessment. Anti-fugal natural extracts *Equisetum arvense*, *Mimosa tenuiflora* and Thymol, are suggested as a safer alternative for synthetic fungicides. Zebrafish developmental exposure to sublethal concentrations, up to 200 times lower than the reported 50% lethal concentrations (LC50s), showed that the first two extracts could be safe to use due to mild or absence of biological significance, however, Thymol showed to be lethal, teratogenic, alter antioxidant defenses and induce fear- and anxiety-like disorders in zebrafish eleutheroembryos [9].

Risk assessment of chemicals is usually conducted for individual chemicals whereas mixtures of chemicals occur in the environment. The different combinations of chemicals are associated with significantly different effects on communities of aquatic ecosystems. Considering that neuroactive chemicals are a group of contaminants that dominate the environment, it is then imperative to understand the combined effects of mixtures [10]. The commonly used models to predict mixture effects, namely concentration addition (CA) and independent action (IA), are thought to be suitable for mixtures of similarly or dissimilarly acting components, respectively. Furthermore, CA and IA models may be used to evaluate observations as antagonistic (less effect than predicted) or synergistic (higher effect than predicted). However, these predictions are mainly based for survival as endpoint, and it is unclear whether they can be implemented for mixture studies addressing behavioral endpoints. One challenge for the application of these predictive models (CA and IA), is that not always neuroactive substances based on similar MoA may have similar behavioral responses, so the question leis whether these models can be used to predict combined effects for neuroactive chemicals mixtures with different MoA but similar behavioral responses. Another issue that rises is whether chemicals with opposing effects can be predicted as antagonistic. In this special issue, Ogungbemi et al. 2021 [10] addressed these questions by investigating the effect over zebrafish embryos spontaneous tail coiling (STC) following exposure to mixtures of pesticides with different MoAs. Indeed, authors found that neuroactive substance with different MoA, such as propafenone and abamectin as well as chlorpyrifos and hexaconazole giving a similar direction of response outcome (hyper- or hypoactivity) seemed to be additive and therefore could be predicted using the CA and IA models. On the other hand, results that showed mixtures with both hyper- and hypoactivity-inducing components lead to an antagonistic interaction, and therefore, to qualitatively predict mixture outcomes of multi-complex mixtures as well as to understand deviations form additivity, the authors recommend considering information on common adverse outcomes of the chemicals.

Another approach to assess effects of mixtures of neuroactive chemicals, is to use their recorded concentrations in the environment. Santos et al. 2021 [11] analyzed the impact of mixtures of relevant concentrations of three common pesticides, glyphosate, chlorpyrifos and copper, over developmental stages of rainbow trout (*Oncorhyncus mykiss*). The authors found antagonistic effects over fish swimming activity when exposed to chemicals with opposing effects. It was suggested that the presence of copper and chlorpyrifos could have antagonized or reduced the effects of glyphosate on larvae swimming activity. When looking at responses at lower levels of organization, authors found additive or synergistic effects of the joint action of these pollutants, interestingly, the observed upregulation of genes involved in detoxification, mitochondrial metabolism and DNA repair suggested an adaptive response triggered to deal with toxic exposure.

In summary, this collection of original research and review works provides vital and updated information regarding research and challenges on behavior ecotoxicity of

invertebrate and vertebrate aquatic organisms as well as the molecular mechanisms behind the effects.

Funding: This work was supported by "Agencia Estatal de Investigación" from the Spanish Ministry of Science and Innovation (project PID2020-113371RB-C21) and IDAEA-CSIC, Severo Ochoa Centre of Excellence (CEX2018-000794-S), which financed M.F. with Severo Ochoa funds.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Busch, W.; Schmidt, S.; Kühne, R.; Schulze, T.; Krauss, M.; Altenburger, R. Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. *Environ. Toxicol. Chem.* **2016**, *35*, 1887–1899. [CrossRef] [PubMed]
2. Ford, A.T.; Ågerstrand, M.; Brooks, B.W.; Allen, J.; Bertram, M.G.; Brodin, T.; Dang, Z.; Duquesne, S.; Sahm, R.; Hoffmann, F.; et al. The Role of Behavioral Ecotoxicology in Environmental Protection. *Environ. Sci. Technol.* **2021**, *55*, 5620–5628. [CrossRef] [PubMed]
3. Sumpter, J.P.; Margiotta-Casaluci, L. Environmental occurrence and predicted pharmacological risk to freshwater fish of over 200 neuroactive pharmaceuticals in widespread use. *Toxics* **2022**, *10*, 233. [CrossRef]
4. Araújo, C.V.M.; Laissaoui, A.; Silva, D.C.V.R.; Ramos-Rodríguez, E.; González-Ortegón, E.; Espíndola, E.L.G.; Baldó, F.; Mena, F.; Parra, G.; Blasco, J.; et al. Not Only Toxic but Repellent: What Can Organisms' Responses Tell Us about Contamination and What Are the Ecological Consequences When They Flee from an Environment? *Toxics* **2020**, *8*, 118. [CrossRef] [PubMed]
5. Kohler, S.A.; Parker, M.O.; Ford, A.T. High-Throughput Screening of Psychotropic Compounds: Impacts on Swimming Behaviours in *Artemia franciscana*. *Toxics* **2021**, *9*, 64. [CrossRef] [PubMed]
6. Bellot, M.; Faria, M.; Gómez-Canela, C.; Raldúa, D.; Barata, C. Pharmacological Modulation of Behaviour, Serotonin and Dopamine Levels in *Daphnia magna* Exposed to the Monoamine Oxidase Inhibitor Deprenyl. *Toxics* **2021**, *9*, 187. [CrossRef] [PubMed]
7. Faria, M.; Prats, E.; Bellot, M.; Gomez-Canela, C.; Raldúa, D. Pharmacological Modulation of Serotonin Levels in Zebrafish Larvae: Lessons for Identifying Environmental Neurotoxicants Targeting the Serotonergic System. *Toxics* **2021**, *9*, 118. [CrossRef] [PubMed]
8. Rice, D.; Barone, S. Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environ. Health Perspect.* **2000**, *108*, 511–533. [PubMed]
9. Vieira, R.; Venâncio, C.; Félix, L. Teratogenic, Oxidative Stress and Behavioural Outcomes of Three Fungicides of Natural Origin (*Equisetum arvense*, *Mimosa tenuiflora*, Thymol) on Zebrafish (*Danio rerio*). *Toxics* **2021**, *9*, 8. [CrossRef] [PubMed]
10. Ogungbemi, A.O.; Massei, R.; Altenburger, R.; Scholz, S.; Küster, E. Assessing Combined Effects for Mixtures of Similar and Dissimilar Acting Neuroactive Substances on Zebrafish Embryo Movement. *Toxics* **2021**, *9*, 104. [CrossRef] [PubMed]
11. Weeks Santos, S.; Cachot, J.; Cormier, B.; Mazzella, N.; Gourves, P.-Y.; Clérandeau, C.; Morin, B.; Gonzalez, P. Environmentally Relevant Mixture of Pesticides Affect Mobility and DNA Integrity of Early Life Stages of Rainbow Trout (*Oncorhynchus mykiss*). *Toxics* **2021**, *9*, 174. [CrossRef] [PubMed]

toxics

MDPI

Article

Environmental Occurrence and Predicted Pharmacological Risk to Freshwater Fish of over 200 Neuroactive Pharmaceuticals in Widespread Use

John P. Sumpter [1] and Luigi Margiotta-Casaluci [2,*]

[1] Department of Life Sciences, College of Health, Medicine and Life Sciences, Brunel University London, London UB8 3PH, UK; john.sumpter@brunel.ac.uk
[2] Department of Analytical, Environmental and Forensic Sciences, School of Cancer and Pharmaceutical Sciences, King's College London, London SE1 9NQ, UK
* Correspondence: luigi.margiotta-casaluci@kcl.ac.uk

Abstract: There is a growing concern that neuroactive chemicals released into the environment can perturb wildlife behaviour. Among these chemicals, pharmaceuticals such as antidepressants and anxiolytics have been receiving increasing attention, as they are specifically prescribed to modify behavioural responses. Many laboratory studies have demonstrated that some of these compounds can affect various aspects of the behaviour of a range of aquatic organisms; however, these investigations are focused on a very small set of neuroactive pharmaceuticals, and they often consider one compound at a time. In this study, to better understand the environmental and toxicological dimension of the problem, we considered all pharmaceuticals explicitly intended to modulate the central nervous system (CNS), and we hypothesised that these compounds have higher probability of perturbing animal behaviour. Based on this hypothesis, we used the classification of pharmaceuticals provided by the British National Formulary (based on their clinical applications) and identified 210 different CNS-acting pharmaceuticals prescribed in the UK to treat a variety of CNS-related conditions, including mental health and sleep disorders, dementia, epilepsy, nausea, and pain. The analysis of existing databases revealed that 84 of these compounds were already detected in surface waters worldwide. Using a biological read-across approach based on the extrapolation of clinical data, we predicted that the concentration of 32 of these neuroactive pharmaceuticals in surface waters in England may be high enough to elicit pharmacological effects in wild fish. The ecotoxicological effects of the vast majority of these compounds are currently uncharacterised. Overall, these results highlight the importance of addressing this environmental challenge from a mixture toxicology and systems perspective. The knowledge platform developed in the present study can guide future region-specific prioritisation efforts, inform the design of mixture studies, and foster interdisciplinary efforts aimed at identifying novel approaches to predict and interpret the ecological implications of chemical-induced behaviour disruption.

Keywords: pharmaceuticals in the environment; environmental risk assessment; behaviour; fish; ecotoxicology; mixture toxicology; predictive toxicology; pollution

Citation: Sumpter, J.P.; Margiotta-Casaluci, L. Environmental Occurrence and Predicted Pharmacological Risk to Freshwater Fish of over 200 Neuroactive Pharmaceuticals in Widespread Use. *Toxics* **2022**, *10*, 233. https://doi.org/10.3390/toxics10050233

Academic Editors: Demetrio Raldúa, Carlos Barata and Melissa Faria

Received: 1 April 2022
Accepted: 29 April 2022
Published: 3 May 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

The sustainability of animal populations relies on the evolution and display of complex behavioural responses aimed at meeting the basic needs of the organism, such as finding resources—including food and water—surviving, and reproducing successfully. Human domination of the planet, especially recently, has led to profound changes to all ecosystems, which has often necessitated animals to rapidly adapt and change their behaviour in order to survive. A rapidly growing number of studies have reported the impact of human activities on wildlife behaviour in both aquatic and terrestrial ecosystems (recently reviewed by Wilson et al. (2020)) [1]. The range of behavioural effects is wide and includes

the disruption of movement, foraging, risk-taking behaviour, communication, and breeding. For example, a meta-analysis of 208 studies on 167 aquatic and terrestrial species carried out by Doherty et al. (2021) [2] showed that disturbance by humans has widespread impacts on the movements of birds, mammals, reptiles, amphibians, fish, and arthropods. The mechanisms via which humans disrupt wildlife behaviour are also numerous and include the active modification of population densities (e.g., via fishing, hunting, etc.) and habitat structure, and the introduction of sensory pollution [1]. For example, the noise generated by human activities has well-established detrimental effects on wildlife [3], such as the disruption of respiratory and resting behaviour of humpback whales in response to whale-watching vessel noise emissions [4]. On the other hand, light pollution is known to affect both nesting behaviour of turtles and the subsequent risk of predation of the nests and of hatchlings [5,6].

Among the many anthropogenic stressors, chemical pollution is one of the greatest global threats for both humans [7] and wildlife [8]. There is a growing concern that chemicals released into the environment so far have modified the behaviour of wild organisms [9,10]. Although demonstrating the causal effects of chemicals on the behaviour of wildlife is very challenging, it is known that some of those chemicals have already elicited such effect. For example, behaviour-modifying chemicals are widely used for large-scale pest control and management (e.g., insect repellents, semiochemicals) [11]. In the last two decades, a specific class of chemicals has sparked a renewed interest in behavioural ecotoxicology. That is the class of psychoactive pharmaceuticals, such as antidepressants and anxiolytics. The use of psychoactive drugs in Western countries has been growing steadily in the last few decades [12,13]. One of the consequences of this increased consumption is that low concentrations of these pharmaceuticals can often be detected in the aquatic environment [14]. Many pharmacological targets of psychoactive drugs are also evolutionarily conserved in fish species; therefore, these drugs may cause behavioural alterations of aquatic wildlife as they do in humans [15]. As appropriate behavioural responses are critical for virtually any key aspect of individual survival and population sustainability, drug-induced behavioural alterations may lead to profound, non-linear, and perhaps unpredictable ecological effects [16]. The importance of this issue was first brought to light in the early 2000s with the detection of the antidepressant fluoxetine in American rivers [17,18]. Brooks et al. were the first scientists to raise the possibility that some anti-depressants acting as selective serotonin transport inhibitors (SSRIs) could be present in the aquatic environment at concentrations high enough to affect the behaviour of fish and other aquatic species [19–21]. Since that discovery, significant efforts have been allocated to characterize the environmental risk of fluoxetine. These efforts (and relative controversies) still persist 20 years later, with more than 140 studies on various aspects of fluoxetine environmental risk published up to 2021. Following the scientific and media attention on the problem, the effects of a few other psychoactive drugs on aquatic species were studied in the following years, including the antidepressant sertraline [22] and the anxiolytic oxazepam [23]. The latter work contributed to raising the profile and the degree of concern of the issue further.

Despite the undoubted challenges of both recording and then interpreting behavioural data, there are now many reports from many scientists that psychoactive drugs, particularly anti-depressants, can affect various aspects of the behaviour of a range of aquatic organisms. However, nearly all of these claims are based on the results of laboratory investigations; their extrapolation to the natural environment is much less certain. Moreover, these laboratory experiments have almost all involved exposing aquatic organisms, in particular, fish, to single psychoactive pharmaceuticals. Yet there is now a very substantial body of evidence showing that the aquatic environment is contaminated with many different neuroactive drugs (see later for details), as well as non-pharmaceutical pollutants potentially able to perturb animal behaviour. Thus, it is the potential behavioural effects of these complex mixtures of drugs that is the ecologically relevant scenario.

In the present study, to better understand the environmental and toxicological dimension of the problem, we expanded our focus beyond antidepressants and anxiolytics. Specifically, we considered all pharmaceuticals explicitly intended to modulate the central nervous system (CNS), and we hypothesised that CNS-acting drugs have higher probability of perturbing animal behaviour. Using the UK pharmaceutical market as the case study, we generated a first comprehensive assessment of the pharmacological risk posed by neuroactive pharmaceuticals to wild fish. By defining the current eco-pharmacological landscape, our results provide an initial knowledge platform to guide future research efforts aimed at predicting and interpreting the ecological implications of chemical-induced behaviour disruption using a systems perspective.

2. Materials and Methods

2.1. Identification of Neuroactive Drugs Prescribed in England and Calculation of the Amount of Each Prescribed Annually

The annual prescription data used in this article were retrieved from the Prescription Cost Analysis (PCA) carried out by the National Health Services (NHS) of the United Kingdom and published by the NHS Business Services Authority (https://www.nhsbsa.nhs.uk/statistical-collections/prescription-cost-analysis-england, accessed on 1 November 2020). The NHS PCAs provide details of the number of items and cost of all prescriptions dispensed in the community, that is, by community pharmacists, appliance contractors, dispensing doctors, and items personally administered by doctors. The present work was based on prescriptions dispensed in England in 2019. These data do not include pharmaceuticals prescribed in hospitals, by private doctors, or purchased via the internet, nor drugs taken or dispensed illegally. Each pharmaceutical included in the PCA is classified within specific chapters of the British National Formulary (BNF). The latter is an annual joint publication of the British Medical Association and the Royal Pharmaceutical Society, and provides up-to-date key information on the selection, prescribing, dispensing, and administration of medicines in the UK. The BNF includes 23 chapters used to classify pharmaceuticals according to their clinical applications. Here we define neuroactive pharmaceuticals as any compound explicitly intended to modulate the central nervous system (CNS), and we propose that CNS-acting drugs have higher probability of perturbing animal behaviour. Hence, to generate a comprehensive assessment of the number and quantity of neuroactive pharmaceuticals beyond antidepressants and anxiolytics, we extracted data for all compounds classified in BNF Chapter 4, "Central Nervous System." In addition, antihistamines were also included in the analysis due to their well-known ability to modify both fish behaviour [24] and human behaviour [25]. The total amount of active principle prescribed was calculated for each individual preparation as described by [26]. The OpenPrescribing database (https://openprescribing.net, accessed on 1 February 2022) was used to evaluate the regional differences in the prescription of selected classes of neuroactive pharmaceuticals (i.e., antidepressants, anxiolytics, opioid analgesics).

2.2. Calculation of Predicted Environmental Concentrations (PECs) in England

Annual prescription data were used to derive predicted environmental concentrations (PECs) (i.e., for surface waters in England, considering a worst-case scenario with 0% removal) as described by the UK Environmental Agency Research and Development Technical Report P390 [27], using the following equation:

$$\text{Aquatic PEC}_{\text{Surface Waters}}\ (\text{g/L}) = \text{A} \times (100 - \text{R})/365 \times \text{P} \times \text{V} \times \text{D} \times 100 \quad (1)$$

where

- A (kg) = predicted amount used per year in England;

- R (%) = removal rate (set to 0 to simulate the worst-case scenario);
- P = number of inhabitants of the country (set to 56,287,000, as indicated by the UK Office for National Statistics-https://www.ons.gov.uk, accessed on 1 December 2020);
- V (m^3) = volume of wastewater per capita and day (set to 200—default value EMA guideline);
- D = factor for dilution of wastewater by surface water flow (set to 10—default value EMA guideline);
- 100 = conversion factor for percentage.

2.3. Prediction of Drug Uptake and Concentration in Fish Plasma

The PEC values for each compound were used to calculate the concentrations of drugs expected to be present in the plasma of fish exposed to those PECs. Predicted drug plasma concentrations were calculated using the theoretical partition coefficient between water and fish blood based on chemical lipophilicity, as described by Margiotta-Casaluci et al. (2014) [15,28], using the following equations:

$$\text{Log } P_{\text{Blood:Water}} = 0.73 \times \text{Log } K_{OW} - 0.88 \quad (2a)$$

$$\text{Log } P_{\text{Blood:Water}} = 0.73 \times \text{Log } D_{(\text{pH } 7{\cdot}4)} - 0.88 \quad (2b)$$

$$\text{Fish Steady State Plasma Concentration } (F_{SS}PC, \mu g/L) = \text{PEC } (\mu g/L) \times P_{\text{Blood:Water}} \quad (3)$$

Log K_{OW} and Log $D_{7.4}$ values for each chemical were retrieved from the ChemSpider database (http://www.chemspider.com, accessed between 1 January 2021 and 1 July 2021) and calculated using the ACD/Labs Percepta Platform-PhysChem Module.

2.4. Estimation of the Pharmacological Risk for Freshwater Fish Species

The pharmacological risk of each compound was estimated by comparing the predicted concentrations of pharmaceuticals in fish plasma (ng/mL) and the human therapeutic plasma concentrations (HTPC), expressed as C_{max} (ng/mL), using the following Equation (4):

$$\text{Predicted Pharmacological Risk} = F_{SS}PC/HTPC \quad (4)$$

The closer $F_{SS}PC$ is to HTPC, the higher the risk that the drug may elicit mode-of-action-specific effects in fish comparable to those observed in humans. The risk was classified using the following criteria:

- High risk—$F_{SS}PC/HTPC \geq 1$
- Medium risk—$F_{SS}PC/HTPC$ between 0.1 and 1
- Low risk—$F_{SS}PC/HTPC < 0.1$

These criteria were set using an arbitrary approach informed by pharmacological considerations and were considered as a first-tier interpretation to compare the risk of a high number of compounds. A more refined and advanced risk evaluation using drug-specific considerations was beyond the scope of the present work and was not performed. C_{max} values were retrieved from Schulz et al. (2012) [29] and Berninger et al. (2016) [30], with a few exceptions (as indicated in the Supplementary Data file).

2.5. Evaluation of the Environmental Occurrence of Each Drug

The environmental occurrence of each pharmaceutical was assessed by examining its presence in PHARMS-UBA, a publicly available database curated by the German Environment Agency (Umweltbundesamt–UBA) (https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-0, accessed on 1 February 2022). On the date of access (February 2022), the database contained environmental concentrations of human and veterinary pharmaceutical residues in 61 different types of environmental matrices from 89 countries, extracted from 2062 publications and 240 review articles. The database was also used to extract the measured concentrations of the top 50 most prescribed pharmaceuticals in our list. Specifically, we considered measured concentrations in surface

waters reported by publications characterised by "good literature credibility." The latter is a quality flag (assigned by the database managers to each data entry) that refers to the reliability, plausibility, and applied analytical standards of each publication. Reports associated with poor or unknown credibility were excluded from the analysis. To enhance the source coverage of the analysis, the UBA data were integrated with the assessment of 100 recent papers covering the issue of pharmaceuticals in the aquatic environment, including some with a specific focus on neuroactive pharmaceuticals. These papers were used to evaluate the environmental occurrence (i.e., in rivers) of the 50 most prescribed pharmaceuticals on our list.

3. Results

3.1. Prescription of Neuroactive Pharmaceuticals in England

The analysis of the annual prescription data published by the National Health Services (NHS) of England (UK) revealed that 210 different pharmaceuticals acting on the CNS are prescribed to treat a variety of CNS-related conditions, including mental health and sleep disorders, dementia, epilepsy, nausea, and pain. Prescription volumes vary greatly among active pharmaceutical ingredients (Figure 1, Supplementary Data file). Unsurprisingly, the painkillers ibuprofen and paracetamol were the most dispensed compounds in England in 2019, with 2974 and 2122 tonnes, respectively. The third, fourth, and fifth most dispensed compounds were the anticonvulsants gabapentin (208 tonnes), valproate (~85 tonnes), and levetiracetam (~66 tonnes). The most prescribed SSRI antidepressant was sertraline (40.6 tonnes). As a term of comparison, the SSRI fluoxetine (intensively investigated in ecotoxicological studies) was dispensed in much lower volumes (~6.2 tonnes) and was preceded by other antidepressants such as venlafaxine (16.7 tonnes), amitriptyline (11.9 tonnes), and citalopram (9.2 tonnes). Overall, the prescription of 43 neuroactive pharmaceuticals out of 210 exceeded 1 tonne (20%); 50 compounds (24%) were in the range of 100–999 kg, 49 compounds in the range of 10–99 kg (23%), and 36 compounds (17%) in the range of 1–9 kg. Finally, the prescription of 32 compounds (15%) was lower than 1 kg (Supplementary Data file).

3.2. Regional Prescription Trends

The prescription volume of each pharmaceutical plays an important role in determining environmental occurrence and drug concentration in surface waters. To evaluate the significance of regional prescription trends for the interpretation of the environmental risk of pharmaceuticals, we used the OpenPrescribing database to assess the regional differences in prescription volumes in England for three major classes of interest: antidepressants, anxiolytics, and opioid analgesics. As an example, we considered the items dispensed in April 2021. The analysis revealed important region-specific scenarios (Figure 2). For example, the prescription of antidepressants in the North East and Yorkshire Commissioning region (1,370,716 items) and the Midlands Commissioning region (1,297,943 items) appeared to be higher than in the rest of England (e.g., 624,407 items in the London Commissioning region; 744,468 items in the South West Commissioning region). On the other hand, the prescription of anxiolytics was higher in the Midlands region (99,644 items) and lower, but homogenous, in all other areas. Finally, the prescription of opioid analgesics was higher in the Midlands and North England (i.e., a total number of 1,133,000 dispensed items) than in the South England regions (i.e., a total number of 778,050 dispensed items).

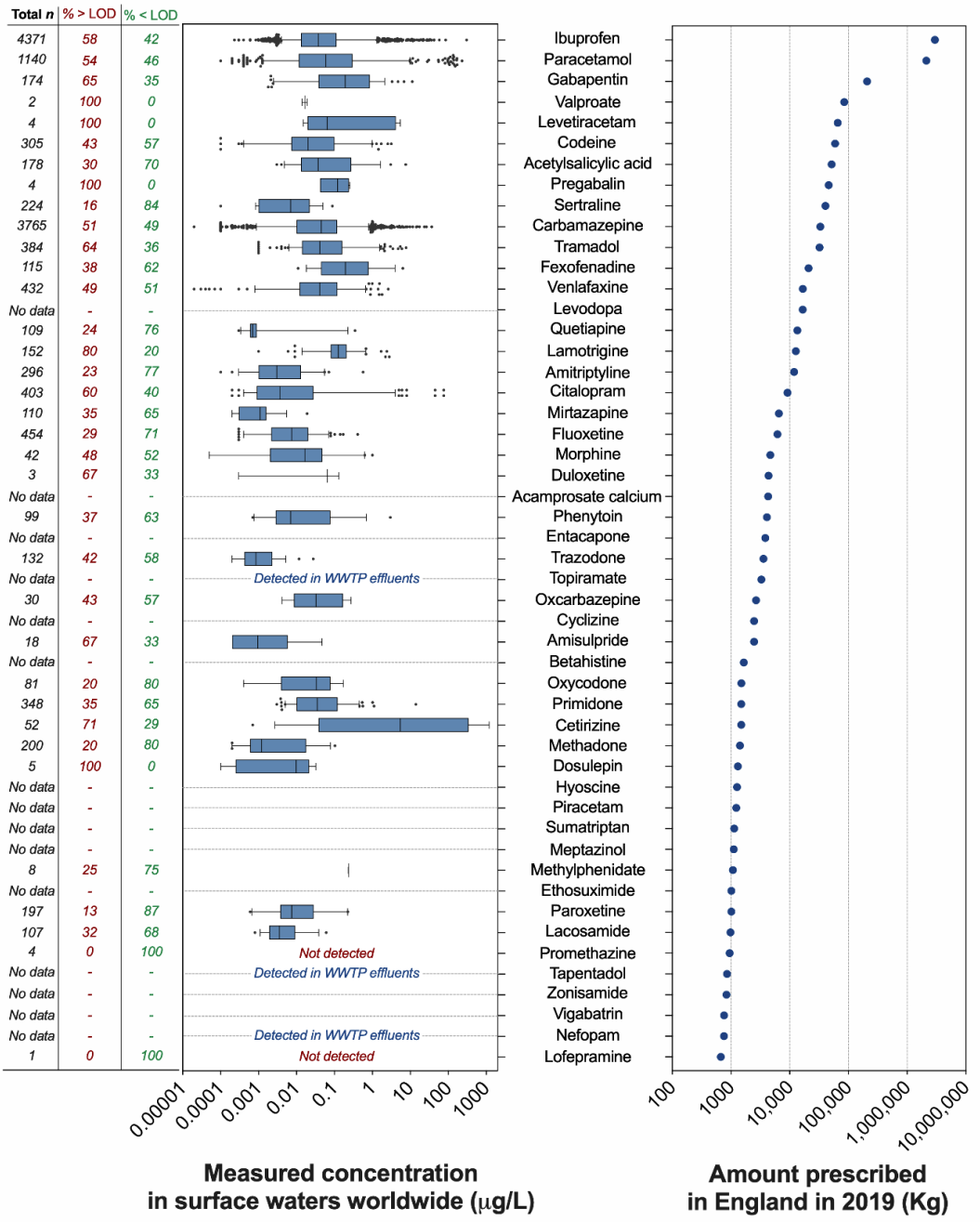

Figure 1. Top 50 neuroactive pharmaceuticals dispensed in England and their concentrations in surface waters worldwide. (**Left panel**) Measured concentrations of neuroactive pharmaceuticals in surface waters worldwide (μg/L). The range of concentrations is visualised as box plots, where the limits indicate the 5th and 95th percentiles of the data distribution. Data points outside this range are visualised as individual dots. The vertical line in each box indicates the median value. The data were extracted from the PHARMS-UBA database curated by the German Environment Agency (Umweltbundesamt–UBA) and represent only the values generated by scientific reports classified as "good literature credibility" by the database curators. For each pharmaceutical, the figure indicates the number of available datapoints in the database (first column), the percentage of data above the limit of detection (second column), and the percentage of data below the limit of detection (third column). (**Right panel**) Top 50 neuroactive pharmaceuticals prescribed in England in 2019 and ranked by dispensed amount (kg). The data were generated by analysing the Prescription Cost Analysis (PCA) report (year 2019) provided by the National Health Services (NHS) of England (United Kingdom) and published by the NHS Business Services Authority.

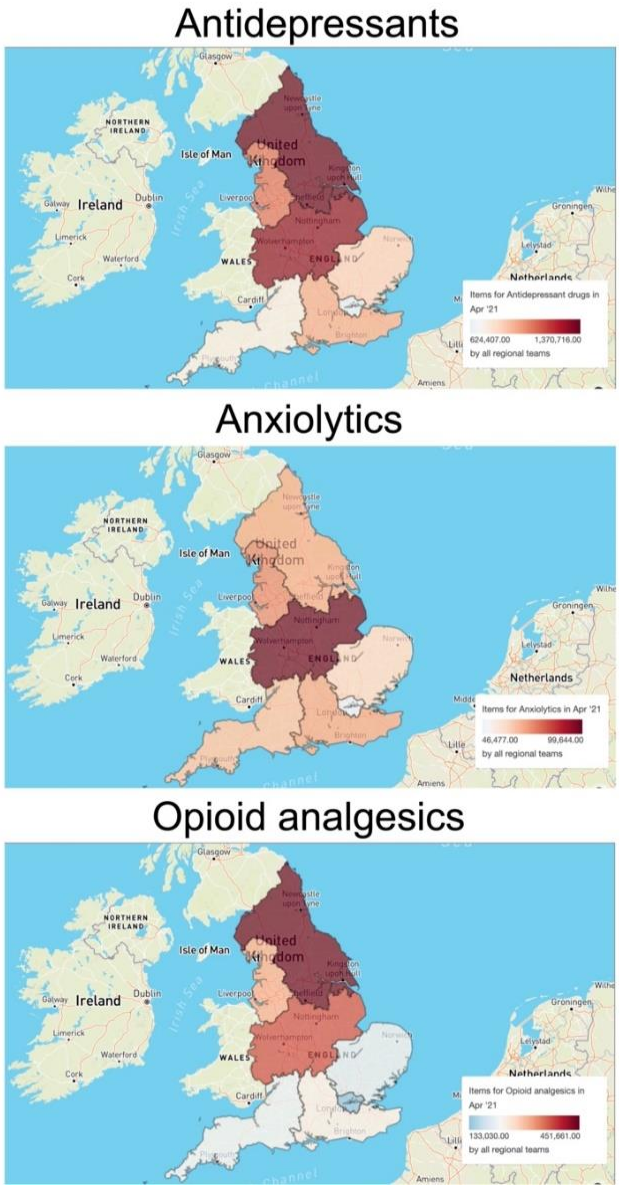

Figure 2. Regional differences in the prescription volumes of three selected classes of neuroactive pharmaceuticals (antidepressants, anxiolytics, opioid analgesics) in England in April 2021. The maps and related data were generated using the OpenPrescribing database (https://openprescribing.net/, accessed on 1 February 2022). The volume of each class of pharmaceuticals is expressed as number of items dispensed in April 2021.

3.3. Environmental Occurrence of the 50 Most Prescribed Neuroactive Pharmaceuticals

To evaluate the occurrence of the neuroactive compounds identified in our analysis in worldwide surface waters, we extracted relevant data from the PHARMS-UBA database curated by the German Environment Agency, and we integrated this evaluation with the analysis of 100 papers recently published in the field of pharmaceuticals in the environment. A detailed analysis was carried for the 50 most prescribed neuroactive pharmaceuticals (Figure 1), whereas the simple presence or absence in the database was evaluated for the remaining 161 compounds in the list.

No surface water occurrence data were available in the database for 15 out of the 50 most prescribed neuroactive pharmaceuticals. Three of these 15 compounds were detected in WWTP effluents (topiramate, tapentadol, nefopam). Some of the drugs that have not, as far as we are aware, yet been reported to be present in the aquatic environment

are new drugs that have only been in use in the last few years (e.g., nefopam, vigabatrin, zonisamide). Researchers may not have been aware of these drugs when they conducted their analytical studies, and even if they had been, the drugs may not have been present in the water samples they analysed because the drugs were not in use at the time. It is also very likely that some of the drugs in use in the UK in 2019 were not in use in other countries, and hence, water samples collected from rivers in those countries could not have contained those drugs. On the other hand, 33 of the 50 most prescribed neuroactive pharmaceuticals were detected in surface waters worldwide in a wide range of concentrations. In most cases, the reported concentrations were in the ng/L range, and often in the low ng/L range. The median measured surface water concentration exceeded 0.1 μg/L only for cetirizine (5.4 μg/L), fexofenadine (0.19 μg/L), gabapentin (0.19 μg/L), lamotrigine (0.13 μg/L), methylphenidate (0.23 μg/L), and pregabalin (0.12 μg/L). Two compounds, promethazine and lofepramine, were targeted in a small number of samples but were not detected. There were relatively few reports of drugs being present in the μg/L range. However, extremely high concentrations of some of the 50 most prescribed compounds (e.g., carbamazepine, fexofenadine, paracetamol, and tramadol) were reported from rivers in Nigeria [31], where concentrations of carbamazepine and paracetamol were not far below 100 μg/L in some river water samples.

The number of data points available for each pharmaceutical was highly variable and ranged from the 4371 measurements available for ibuprofen to the very few measurements (<5) available for valproate, levetiracetam, pregabalin, duloxetine, promethazine, and lofepramine (Figure 1). In addition to the measured concentrations reported for each compound, we also analysed how frequently each compound was targeted but not detected in the analysed surface water samples. This analysis revealed that the frequency of non-detections was considerable in most cases. Considering the pharmaceuticals associated with 10 or more measurements, the frequency of non-detections ranged from 20% (lamotrigine) to 87% (paroxetine).

Expanding the evaluation of the environmental occurrence to the full list of 210 neuroactive compounds identified in our analysis, 84 were associated with measured surface water concentrations in the PHARMS-UBA database.

3.4. Prediction of the Pharmacological Risk for Fish

Although the concentration of pharmaceuticals in surface waters is a key driver of the environmental risk assessment process, it is the concentration of the compound inside the organism (i.e., fish) that determines the pharmacological and toxicological risk. Hence, given two compounds with comparable in vitro pharmacological potency, their comparative in vivo pharmacological risk is determined by their differential tendency to be taken up by the organism, distributed, metabolised, and excreted. To predict the pharmacological risk of each neuroactive compound in our list, here we applied an integrated analysis that involved the following steps. Firstly, we used the annual amount of pharmaceuticals dispensed in England to calculate the related PECs in surface waters. Successively, we used the Fish Plasma Model to predict the drug plasma concentrations resulting from the exposure of fish to those PECs. Finally, the predicted fish plasma concentrations were compared to human C_{max} values to interpret the pharmacological risk posed by each compound. This analysis revealed that nine out of 210 neuroactive pharmaceuticals may reach plasma concentrations in wild fish high enough (i.e., equal to or higher than the human C_{max}) to elicit pharmacological effects comparable to those observed in humans in a clinical setting (Figure 3). These drugs were classified as "high risk" and included lofepramine, loratadine, sertraline, desloratadine, amitriptyline, fexofenadine, fluoxetine, nortriptyline, and rotigotine. On the other hand, 23 out of 210 neuroactive compounds were classified as "medium risk", as they predicted plasma concentrations in wild fish between 10% and 100% of human C_{max} (Figure 3). These predicted sub-therapeutic levels suggest a lower risk of phenotypically observable effects, but they may still be high enough to induce target-mediated effects, especially under conditions of chronic exposure. The classification of the

medium-/low-risk threshold was arbitrary and based on expert judgment. More complex drug-specific considerations will be needed to refine the prediction of the pharmacological risk in future studies.

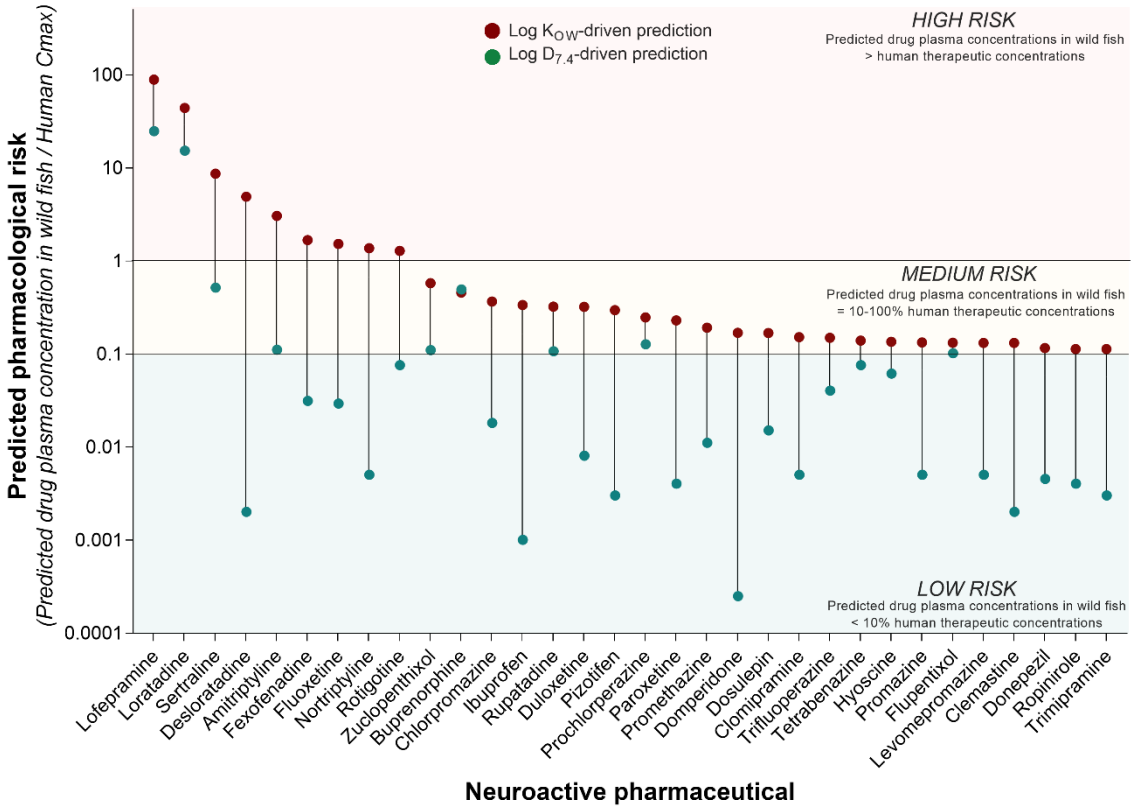

Figure 3. Predicted pharmacological risk to freshwater fish of neuroactive pharmaceuticals. The pharmacological risk of the 210 neuroactive pharmaceuticals identified in the present study was estimated by comparing the predicted concentrations of pharmaceuticals in fish plasma ($F_{SS}PC$, ng/mL) and the human therapeutic plasma concentrations (HTPC) expressed as C_{max} (ng/mL). Considering the ratio $F_{SS}PC/HTPC$, values ≥ 1 were classified as "high risk", values between 1 and 0.1 as "medium risk", and values < 0.1 as "low risk". The figure displays all neuroactive pharmaceuticals predicted to have medium and high risk, based on the use of $LogK_{OW}$ for the prediction of drug uptake (red dots). To understand the impact of the use of different partitioning factors on the overall pharmacological risk, the same prediction was also performed using Log $D_{7.4}$ (green dots).

The predictions of this analysis were generated considering two different partitioning factors for each pharmaceutical, $LogK_{OW}$ and $LogD_{7.4}$. The risk classification described above was based on the consideration of the use of $LogK_{OW}$ as a key parameter for the prediction of drug uptake in fish. However, the analysis revealed that the predicted pharmacological risk is highly sensitive to the use of different partitioning coefficients, so the predicted risk is lower when the $LogD_{7.4}$ is used (Figure 3). Considering this scenario, the pharmacological risk of lofepramine and loratadine remained high. The predicted pharmacological risk of the other compounds decreased to a "low risk" classification, with the exception of sertraline, amitriptyline, zuclopenthixol, buprenorphine, rupatadine, prochlorperazine, and flupentixol, which all retained a "medium risk" classification.

The driving role played by partitioning factors implies that the outcome of modelling exercises based on the Fish Plasma Model should be interpreted with caution, as more sophisticated drug-specific considerations are required for a more rigorous analysis. For example, it is important to note that the two compounds with the highest predicted pharmacological risk are also very hydrophobic (i.e., lofepramine $LogK_{OW}$ = 6.96; loratadine

$LogK_{OW}$ = 5.94). Of the 32 compounds with a predicted medium/high pharmacological risk, 12 have a $LogK_{OW}$ between 5 and 6.96, 19 between 3 and 5, and only 1 compound has a $LogK_{OW}$ below 1 (i.e., hyoscine). Prior to the interaction with the biological target (i.e., wild fish), the hydrophobicity of each compound determines its behaviour in the environmental matrix of interest (e.g., in wastewater treatment plants or in rivers), and ultimately its concentration in the different exposure compartments (e.g., water column vs. sediment). Here it possible to observe that the compound with the highest predicted pharmacological risk (i.e., lofepramine) has yet to be detected in surface waters. Hence, despite the predicted pharmacological risk being high, the actual environmental risk in surface waters may still be low.

3.5. Comparison of Predicted versus Measured Concentrations of Pharmaceuticals in UK Surface Waters and Implications for the Prediction of the Pharmacological Risk

The prediction of the pharmacological risk presented in this study is based on the assumption that the predicted concentration of pharmaceuticals in surface waters (i.e., in England; $PEC_{England}$) is representative of the actual concentrations measured in the rivers (i.e., MECs). A significant discrepancy between PEC and MECs would directly affect the accuracy of the predictive model. To evaluate the concordance between the two types of value, we extracted all available UK-specific concentrations measured in surface waters ($MEC_{United\ Kingdom}$) from the PHARMS-UBA database and compared them with the predicted values (i.e., $PEC_{England}$) (Figure 4). It is important to note that the database does not specify whether the UK values were generated in England or in other regions of the UK. However, we estimated that the majority of those values are likely to refer to water samples collected in England.

UK-specific data were available for 21 out of the 84 neuroactive pharmaceuticals associated with measured surface water concentrations worldwide. The comparison of PEC and MECs indicated that PECs often overestimate MECs; however, this is not always the case. For example, the PEC of cetirizine was lower than the concentration measured in the environment. Moreover, the PECs of eight compounds (duloxetine, tramadol, quetiapine, fexofenadine, carbamazepine, morphine, citalopram, dosulepin) were within the range of MECs reported in the UK (Figure 4A).

To better understand the degree of concordance between PECs and MECs, we calculated the ratio between PEC and the average MEC for each compound (Figure 4B). It is important to note that the latter value does not represent a true average of UK MEC, but only the average of the values reported in the PHARMS-UBA database, which include single measurements as well as average, minimum, and maximum values. The analysis revealed a very good concordance for duloxetine, tramadol, and quetiapine. Overall, PECs were within 10-fold the average $MEC_{United\ Kingdom}$ for 11 out of 21 compounds, whereas they exceeded the 10-fold margin for 10 compounds (i.e., from more to less discrepancy: ibuprofen, acetylsalicylic acid, paracetamol, sertraline, mirtazapine, amitriptyline, venlafaxine, oxycodone, fluoxetine, codeine).

Overall, these results indicate that, despite the overestimation, PEC values for pharmaceuticals can offer a useful first-tier estimation for downstream applications (e.g., the predictive model described in this study), especially when there is a need to compare a large number of compounds. The analytical approach displayed in Figure 4 can be used to refine the estimation of the uncertainty for specific compounds and set ranges of uncertainty tolerability for specific applications.

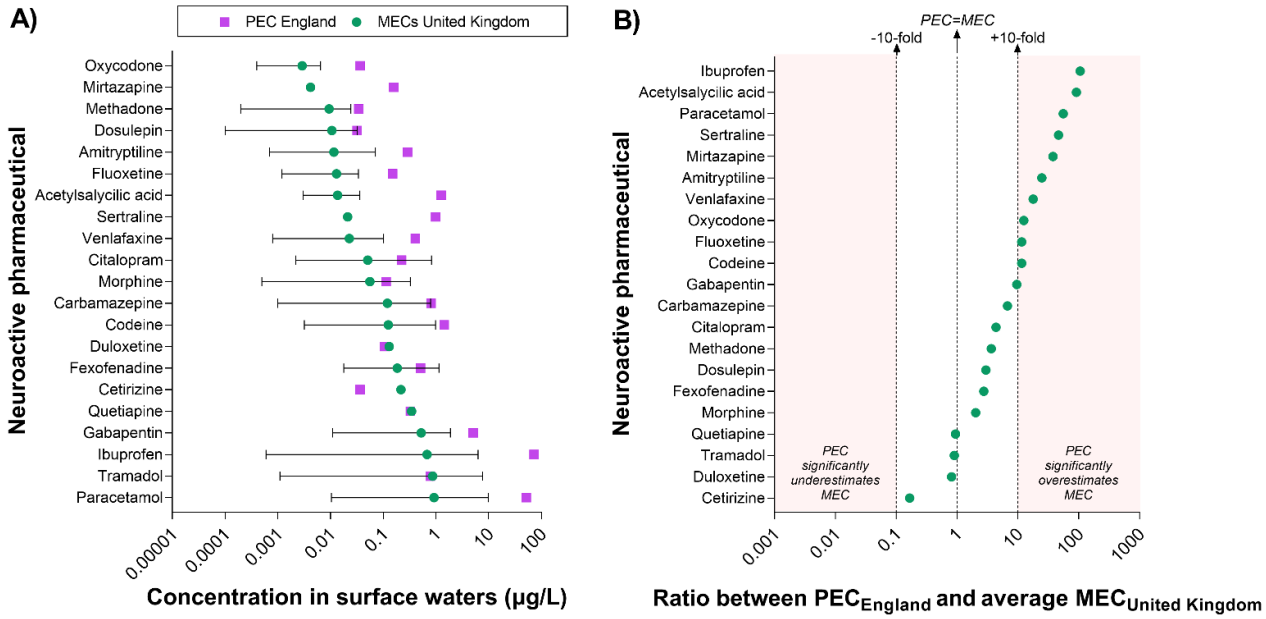

Figure 4. Comparison between predicted and measured concentrations of neuroactive pharmaceuticals in surface waters in the United Kingdom. Measured concentrations of neuroactive pharmaceuticals in UK surface waters (MECs)—extracted from the PHARMS-UBA database—were available for 21 compounds. (**A**) This panel displays the range of UK MECs reported for each compound and their average value (i.e., green dots). It is important to note that the latter is not a "true" average MEC, but only the average of the values available in the database, which include single measurements as well as average, minimum, and maximum values. England-specific PECs are indicated by purple squares. (**B**) This panel displays the ratio between $PEC_{England}$ and the average $MEC_{United\ Kingdom}$ and provides an estimation of the discrepancy between predicted and measured values. To facilitate the interpretation of the data, the vertical dotted lines indicate the level of maximum accuracy (i.e., Ratio PEC/MEC = 1) and the +10-fold and −10-fold range. The red areas indicate when a PEC value overestimates or underestimates the average MEC by more than 10-fold.

4. Discussion

There is now considerable interest in including behavioural effects in ecotoxicity testing of chemicals [10,32]. If their inclusion is to be of significant use in protecting the aquatic environment from any chemicals that could potentially affect the behaviour of aquatic organisms, the following factors need to be addressed. It is necessary to know which chemicals with the potential to affect behaviour are present in the aquatic environment, and in what concentrations. It is also necessary to know which specific behaviours could be affected by which chemicals, in which organisms, and at which concentrations. Furthermore, ideally the consequences of any behavioural changes would be known. Currently, we are a long way from meeting any of these objectives. Not only is the current relevant literature incomplete, but it is also often contradictory [33]. In this study, we make an initial attempt at identifying the complete repertoire of neuroactive pharmaceuticals likely or already shown to be present in the aquatic environment. We accept that other groups of pollutants (e.g., metals, pesticides) may contain components able to affect behaviour. We also accept that some neuroactive pharmaceuticals may not affect behaviour, and that those that have the potential to do so may affect different behaviours, possibly in different ways.

4.1. Our Findings and Their Implications

The most important result of our study is the finding that a large number (more than 200) of neuroactive pharmaceuticals are in use clinically, and that many of these drugs (n = 84) have already been reported to be present in rivers throughout the world. However, this high number is likely an underestimate of the total number of neuroactive substances in

use legally and illegally and present in the aquatic environment. This is because our analysis is based only on neuroactive pharmaceuticals prescribed by the National Health Service of the UK, which is just one source of the neuroactive drugs in use. Other sources include over-the-counter painkillers bought from pharmacies or shops without the requirement of a prescription, neuroactive pharmaceuticals prescribed by private medical practitioners, recreational use of neuroactive (illicit) substances, and neuroactive substances formed by metabolism and environmental transformation of parent pharmaceuticals and illicit drugs.

It is very difficult, if not impossible, to estimate the number and amounts of neuroactive substances entering the aquatic environment from these additional sources. It is plausible that the legal additional use, via over-the-counter purchases or private medical practitioners, would add few, if any, pharmaceuticals that are not also prescribed through the NHS. However, the amounts from these additional sources could be substantial, especially for drugs such as ibuprofen, paracetamol, and codeine. In contrast, the situation with illicit recreational drugs is completely different. This is because nearly all illicit drugs are not available in the NHS, and hence they increase the number of neuroactive substances in use and in the environment. These illicit neuroactive substances include cocaine, crack cocaine, MDMA (ecstasy), heroin, various amphetamines, cannabis, various tranquillisers, and ketamine. In addition to those "classic" illicit drugs, new psychoactive substances are constantly appearing [34]. Concentrations of many of these illicit neuroactive substances in the aquatic environment can be in the same range as the concentrations of neuroactive pharmaceuticals taken for medical reasons [35–37]. This is readily understood when it is realised that the UK's National Crime Agency reported that British people consumed 117 tonnes (nearly 120,000 kg) of cocaine in 2019 alone. Others have estimated that 23 kg of cocaine (half a million doses) is taken every day in London, equating to more than 8 tonnes of pure cocaine annually. Whereas much use of illicit drugs is probably spread relatively evenly both spatially and temporally throughout a country such as the UK, special events, such as music festivals, can lead to very irregular "hot spots" of contamination of the aquatic environment [38].

The contribution of neuroactive transformation products, formed either in the patient (metabolites) or wastewater systems and the aquatic environment, is also very difficult to estimate with any confidence, but could be significant. It is undoubtedly the case that at least some of the major neuroactive pharmaceuticals, such as fluoxetine and venlafaxine, and some of the major illicit drugs, such as cocaine, are readily and rapidly transformed (reviewed in Maculewicz et al. (2022)) [39]. Hence, they are present in the aquatic environment [35–41], often at concentrations similar to, or even exceeding, those of the parent substance. Some of these transformation products definitely possess significant biological activity, although their potencies and specificities are often different to those of the parent substances.

The presence of these neuroactive substances in the aquatic environment would not be of concern if they did not get into aquatic organisms at concentrations high enough to elicit pharmacological effects [15,42,43]. However, most do get into aquatic organisms to some extent, primarily as a consequence of them being hydrophobic [42,44]. A wide variety of human pharmaceuticals have been found in fish [45,46], including a number of neuroactive drugs [21,45], some of which have been found in the blood of wild fish [37,45,47]. A few may even be present in wild fish at concentrations close to, or even at, the human therapeutic concentrations [47]. Our predictive approach based on the integration of pharmacokinetics and pharmacodynamics considerations appears to confirm some of the experimental data, although a more sophisticated and geographically restricted set of predictions would be needed for a more rigorous comparison. For example, Cerveny et al. (2021) [47] identified the antipsychotic flupentixol in the plasma of wild fish (in the Czech Republic) and classified this compound as high risk, as it exceeded human therapeutic concentrations. In our analysis, the same compound was predicted to have medium risk in England. The same authors detected other neuroactive compounds that were predicted to have medium/high pharmacological risk in our analysis, including desloratadine, clomipramine,

and pizotifen (in both England and the Czech Republic). Some of the risk classification discrepancies between our analyses and the experimental work of Cerveny et al. (2021) [47] can be explained by the different use of reference C_{max} values. For example, the human C_{max} of desloratadine used in our predictive analysis was 2 ng/mL [29] and led to a medium risk prediction. On the other hand, Cerveny et al. (2021) [47] calculated the pharmacological risk of the same compound using a higher C_{max} of 10 ng/mL, classifying the resulting (experimental) risk as low. Setting the C_{max} value to 2 ng/mL for both studies would have led to a concordant medium risk classification. The list of compounds predicted by our analysis and validated experimentally in the field is further expanded by the work of Malev et al. (2020) [37], who detected four compounds in the blood of wild fish in Croatia that are also associated with high/medium pharmacological risk in the present work (i.e., buprenorphine, loratadine, ibuprofen, sertraline). Overall, this comparison indicates that our predictive approach based on simple drug uptake modelling and human therapeutic considerations confirms it to be a useful strategy for a first-tier risk interpretation and prioritisation exercise. This approach, based on the PECs of parent compounds, may lead to potential overestimations of the risk (Figure 4) [48]. However, the model can easily be refined by incorporating additional parameters, such us human metabolism and excretion, and linked to existing hydrogeological modelling of drug surface water concentrations to achieve a higher spatio-temporal resolution and a more realistic estimation of the risk.

Although, as our results demonstrate, regional differences in neuroactive drug use both within and between countries need to be considered, the basic finding that very many neuroactive substances are present simultaneously in the aquatic environment will be true in all rivers receiving wastewater effluent, as most do. The consequence of that realisation is that, to determine the risk posed by the presence of neuroactive substances, mixture toxicity assessment is required. Appropriate methodology has been developed [49] to enable worthwhile, informative experiments to be designed and their data correctly analysed and interpreted. In addition, Marmon et al. (2021) [50] demonstrated the high potential of using network pharmacology concepts integrated with pharmacokinetics considerations to predict the environmental risk posed by a complex mixture of pharmaceuticals (i.e., 25 NSAIDs). However, formidable obstacles still need to be overcome before it is possible to know whether the presence of complex mixtures of neuroactive substances representative of those present in the aquatic environment pose a significant risk to aquatic organisms. The main current obstacles are identifying the neuroactive substances of greatest concern, the lack of any ecotoxicological data for many of the neuroactive substances known to be present in the aquatic environment, and the limited reproducibility of much of the ecotoxicological data that are available. We discuss each of these three obstacles below.

4.2. Current Issues Preventing Significant Progress

At present, it is not possible to know which of the neuroactive substances present in the aquatic environment poses the greatest risk. Although a large number of different neuroactive substances are undoubtedly present, it is quite possible that only a few of them (out of 200+ compounds) contribute the majority of the overall risk posed by the mixture of 210 compounds considered here (see Gustavsson et al. (2017) [51] for an example of this concept based on pesticides). Identifying the toxicity drivers would allow scientists to reduce the complexity of the mixture to an experimentally tractable level and facilitate the regulatory interpretation of the risk. But how do we identify the neuroactive compounds that drive the overall toxicity risk? The predictive integrated approach used in the present study appears to be promising. However, evaluating the accuracy of those predictions would require experimental data. The current ecotoxicological literature is dominated by research on just a few neuroactive pharmaceuticals, including compounds such as fluoxetine and oxazepam, yet as our analysis demonstrates (see Figure 3), some of these may not be the neuroactive substances of greatest concern (e.g., oxazepam). A further complication arises in that the neuroactive substances of most concern in one location may

not be those of most concern in another. In this context, mode-of-action-driven grouping of neuroactive compounds may facilitate both mixture toxicity evaluations and read-across approaches, even when experimental data are not available for all the chemicals within the same group.

The majority of the neuroactive substances in use presently, many of which have been shown to be present in the aquatic environment, have not been studied for their fish ecotoxicity. As stated above, a few neuroactive substances have been relatively well studied (e.g., some antidepressants and anxiolytics—see Gould et al. (2021) for a recent review) [52] —although the results of those studies are in some cases inconsistent (see below)—but many are poorly studied or have not been studied at all. This observation may not be surprising, as recent studies have shown that comprehensive environmental toxicity data are lacking for 88% of drugs targeting human proteins [53]. For example, regulatory-relevant fish toxicity data (extracted by Gunnarson et al. (2019)) [53] are available only for five out of the 32 neuroactive compounds predicted to have high–medium pharmacological risk in our predictive analysis (i.e., loratadine, desloratadine, fluoxetine, ibuprofen, duloxetine). This coverage increases (to a limited extent) if we consider non-regulatory relevant academic ecotoxicology studies focused on the characterization of drug-induced behavioural effects in laboratory settings (e.g., sertraline) [22] and biomedical studies. However, the latter are dominated by exposure experiments involving embryo-larvae, and the interpretation of their ecotoxicological relevance remains challenging. On the other hand, chronic exposure studies remain limited.

The last of the three obstacles that requires discussion is the reproducibility of the available ecotoxicity data, which overlaps with the difficulty to interpret complex behavioural data in a regulatory and decision-making context. It is obvious that it will never be possible to gauge how great the threat that neuroactive substances pose to aquatic organisms is until robust, reliable, repeatable ecotoxicity data are available. Yet the present situation is that there is no agreement on the degree of risk posed by even the most studied neuroactive pharmaceuticals, such as fluoxetine (see Sumpter et al. (2014)) [33]. Some studies report apparent effects when animals are exposed to extremely low, environmentally relevant concentrations of drugs such as fluoxetine; others report effects of low concentrations that are not observed at higher concentrations, e.g., [9,54]; and others report effects only at high concentrations that are well above the environmental range, e.g., [15]. This issue is very well illustrated by the studies published on the possible effects of oxazepam on fish. The same research group has reported that this anxiolytic drug causes behavioural changes in both the laboratory and the field [55] and that it does not [56,57]. We accept that the regulation of behavioural responses is an extremely complex process likely to be modified by many different environmental factors, but nevertheless, if behavioural endpoints are to be utilised in the regulation of chemicals, as some have proposed (e.g., [10]), it is necessary to first substantially improve our understanding of normal behaviour so that any effects of chemicals can be correctly identified. This interpretative challenge is further exemplified by the exercise carried out by Tanoue et al. (2019) [58], where 37 UK and Japan ecotoxicology experts were asked to interpret the significance of a dataset concerning the behavioural effects of tramadol on fish following chronic exposure. Also in that case, the experts reached different conclusions based on the same results. A further interpretative challenge resides in the extrapolation of behavioural effects from the laboratory to the field, as the ecological relevance of typical laboratory-based behavioural testing is currently unclear.

4.3. A Possible Way Forward

What would be an appropriate way to proceed? It is clear that we need to know whether neuroactive substances present in the aquatic environment are adversely affecting aquatic organisms, and if so, which ones. The present *ad hoc* approach based on the behavioural ecotoxicity assessment of one (or very few) neuroactive compound at a time—often selected without an explicit rationale—is too fragmented and seems very unlikely to provide the answer(s) needed. By defining the current eco-pharmacological landscape,

our results could be used to inform the design of future research using a pharmacological rationale. Nonetheless, international coordination and cooperation is essential to tackle this scientific challenge in a timely and effective manner. Fostering a wider, international discussion on the best way forward would probably be very beneficial, and it could facilitate the development of coordinated interdisciplinary research initiatives that involve relevant stakeholders in academia as well as industry and regulatory sectors. Positive examples of such ambition are provided by the discussions emerging from recent dedicated workshops and symposia, e.g., Peterson et al. (2017) and Ford et al. (2021) [10,59]. The latter provided, for the first time, a series of consensus statements and useful recommendations aimed at accelerating the regulatory uptake of future behavioural ecotoxicology research. Moreover, a recent review by Bertram et al. (2022) [32] discussed some of the major outstanding questions in behavioural ecotoxicology and proposed a possible way forward. These examples indicate that many scientists around the world are now recognizing the limitation of current practices and are calling for new initiatives aimed at advancing the field in a more organic and coherent manner.

Assessing the ecotoxicity of neuroactive substances using experimental methods remains the biggest challenge. The field of fish behavioural ecotoxicology is currently experiencing an intersection of multiple independent issues (scientific, regulatory, ethical, financial, political) that significantly increases the complexity of the problem. Destructuring such complexity is essential to ensuring progress. The first layer of complexity concerns the ambition to quantify chemical-induced behavioural effects in a reproducible manner. High-throughput multi-dimensional zebrafish behavioural profiling is an established method to identify neuroactive chemicals for drug discovery purposes [60]. This approach is much more complex than the zebrafish behavioural tests commonly used in ecotoxicology research and could be applied to profile the behavioural effects (and the dose response) of hundreds of neuroactive compounds for ecotoxicology applications and generate fish-specific data. However, a limitation of this approach is that it is based on the use of zebrafish embryo-larvae exposed to the test compound for a short period time. We foresee that this approach could be adapted to quantify the behavioural effects of larvae exposed to the drug for longer periods. However, zebrafish larvae acquire a protected status at 120 h post fertilisation; thus, longer exposure times would be associated with much higher ethical costs. More ecologically relevant chronic exposure studies remain scarce, e.g., [15,22,61]. However, even if such studies would be technically feasible, the overall financial and ethical costs would likely be unsustainable or unacceptable. This scenario suggests two possible tractable solutions: (a) to limit chronic exposure studies only to priority compounds (e.g., identified using any prioritization approach, such as the one used here), and (b) to integrate the quantification of behavioural endpoints in current regulatory-relevant chronic toxicity testing, whenever relevant, in order to maximise the amount of information extracted from those in vivo experiments.

The previous points lead us to the second element of complexity, which is the uncertainty surrounding the interpretation of fish behavioural data from a regulatory perspective. To enhance their regulatory relevance, many aspects of laboratory-based in vivo fish behavioural testing require further development and standardisation (e.g., study design, use of positive controls, environmental parameters, ecological relevance of measured endpoints, inter- and intra-laboratory reproducibility, characterisation of baseline behaviour, translation from the laboratory to the field, etc.). On the other hand, behavioural observations of fish in the field can be influenced by numerous confounding factors that hamper the assessment of the causal relationship between drug exposure and effect. If it is necessary, as seems highly likely, to prioritise research in this area, an international discussion on the regulatory and scientific aspects of in vivo behavioural testing (for both adult fish and larvae) should be a high priority; otherwise, research effort will be largely wasted [10,32,56].

Laboratory-based fish in vivo testing represents the gold standard to detect chemical-induced behavioural effects, due to the integrated, complex, and dynamic nature of animal behaviour. The considerations provided above are focused on the optimisation and im-

provement of in vivo fish behavioural testing to enhance its scientific and regulatory value. However, such an in vivo testing strategy would rapidly become incompatible with the recently announced ambition of the US Environmental Protection Agency and European Commission to phase out vertebrate in vivo testing in the next decade or so (i.e., by 2035 in the US) [62]. This political consideration highlights the urgency of supporting research initiatives aimed at understanding the mechanistic basis of chemical-induced behavioural perturbation in fish (and any other relevant vertebrate species). This understanding will be critical to support the identification of a suitable set of new approach methodologies (NAMs) that could be deployed to predict the risk of chemical-induced behavioural alterations without the need to perform animal testing. In this context, the consideration of drug-specific comparative pharmacology, target conservation across species, the in vitro bioactivity profile, and comparative pharmacokinetics (PK) may provide valuable tools to address this challenge [15,50]. In the case of neuroactive pharmaceuticals, this effort can be facilitated by the (generally) advanced understanding of the PK and pharmacodynamic (PD) properties of these compounds in mammals. Based on this understanding, the development and application of multi-dimensional predictive models that integrate both PK and PD (like the one described in this study) can support an effective pharmacology-informed prioritisation and risk assessment of both single compounds and complex mixtures while minimising the reliance of in vivo testing. Thus, the development of predictive in silico/in vitro mechanistic approaches should represent an essential element of any future research strategy in the field of behavioural ecotoxicology.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/toxics10050233/s1, Supplementary Data File.

Author Contributions: Both authors (J.P.S. and L.M.-C.) contributed equally to the conceptualization, data collection and analysis, data visualisation and interpretation analysis, writing—original draft preparation, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: J.P.S. is grateful for the funding from the UK Natural Environment Research Council (grant NE/S000100/1) for the ChemPop project.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the results presented in this paper can be found in the Supplementary Data file and on the public databases and repositories indicated in the Section 2.

Acknowledgments: We thank the German Environment Agency (Umweltbundesamt—UBA) and National Health Services (NHS) of the United Kingdom for compiling their extensive database on concentrations of pharmaceuticals in the environment and annual prescriptions of pharmaceuticals, respectively, and for making access to it freely available to anyone.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wilson, M.W.; Ridlon, A.D.; Gaynor, K.M.; Gaines, S.D.; Stier, A.C.; Halpern, B.S. Ecological impacts of human-induced animal behaviour change. *Ecol. Lett.* **2020**, *23*, 1522–1536. [CrossRef] [PubMed]
2. Doherty, T.S.; Hays, G.C.; Driscoll, D.A. Human disturbance causes widespread disruption of animal movement. *Nat. Ecol. Evol.* **2021**, *5*, 513–519. [CrossRef] [PubMed]
3. Shannon, G.; McKenna, M.F.; Angeloni, L.M.; Crooks, K.R.; Fristrup, K.M.; Brown, E.; Warner, K.A.; Nelson, M.D.; White, C.; Briggs, J.; et al. A synthesis of two decades of research documenting the effects of noise on wildlife. *Biol. Rev.* **2016**, *91*, 982–1005. [CrossRef] [PubMed]
4. Sprogis, K.R.; Videsen, S.; Madsen, P.T. Vessel noise levels drive behavioural responses of humpback whales with implications for whale-watching. *eLife* **2020**, *9*, e56760. [CrossRef] [PubMed]
5. Kamrowski, R.L.; Limpus, C.; Jones, R.; Anderson, S.; Hamann, M. Temporal changes in artificial light exposure of marine turtle nesting areas. *Glob. Chang. Biol.* **2014**, *20*, 2437–2449. [CrossRef]

6. Silva, E.; Marco, A.; da Graça, J.; Pérez, H.; Abella, E.; Patino-Martinez, J.; Martins, S.; Almeida, C. Light pollution affects nesting behavior of loggerhead turtles and predation risk of nests and hatchlings. *J. Photochem. Photobiol. B Biol.* **2017**, *173*, 240–249. [CrossRef]
7. Landrigan, P.J.; Fuller, R.; Acosta, N.J.R.; Adeyi, O.; Arnold, R.; Basu, N.; Baldé, A.B.; Bertollini, R.; Bose-O'Reilly, S.; Boufford, J.I.; et al. The Lancet Commission on pollution and health. *Lancet* **2018**, *391*, 462–512. [CrossRef]
8. Diamond, M.L.; de Wit, C.A.; Molander, S.; Scheringer, M.; Backhaus, T.; Lohmann, R.; Arvidsson, R.; Bergman, Å.; Hauschild, M.; Holoubek, I.; et al. Exploring the planetary boundary for chemical pollution. *Environ. Int.* **2015**, *78*, 8–15. [CrossRef]
9. Saaristo, M.; McLennan, A.; Johnstone, C.P.; Clarke, B.O.; Wong, B.B.M. Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (Poecilia reticulata). *Aquat. Toxicol.* **2017**, *183*, 38–45. [CrossRef]
10. Ford, A.T.; Ågerstrand, M.; Brooks, B.W.; Allen, J.; Bertram, M.G.; Brodin, T.; Dang, Z.; Duquesne, S.; Sahm, R.; Hoffmann, F.; et al. The Role of Behavioral Ecotoxicology in Environmental Protection. *Environ. Sci. Technol.* **2021**, *55*, 5620–5628. [CrossRef]
11. Lucas, J.A. Advances in plant disease and pest management. *J. Agric. Sci.* **2011**, *149*, 91–114. [CrossRef]
12. Mars, B.; Heron, J.; Kessler, D.; Davies, N.M.; Martin, R.M.; Thomas, K.H.; Gunnell, D. Influences on antidepressant prescribing trends in the UK: 1995–2011. *Soc. Psychiatry Psychiatr. Epidemiol.* **2016**, *52*, 193–200. [CrossRef] [PubMed]
13. Lalji, H.M.; McGrogan, A.; Bailey, S.J. An analysis of antidepressant prescribing trends in England 2015–2019. *J. Affect. Disord. Rep.* **2021**, *6*, 100205. [CrossRef] [PubMed]
14. Grabicová, K.; Grabic, R.; Fedorova, G.; Kolářová, J.; Turek, J.; Brooks, B.W.; Randák, T. Psychoactive pharmaceuticals in aquatic systems: A comparative assessment of environmental monitoring approaches for water and fish. *Environ. Pollut.* **2020**, *261*, 114150. [CrossRef] [PubMed]
15. Margiotta-Casaluci, L.; Owen, S.F.; Cumming, R.I.; Polo, A.; Winter, M.J.; Panter, G.H.; Rand-Weaver, M.; Sumpter, J.P. Quantitative Cross-Species Extrapolation between Humans and Fish: The Case of the Anti-Depressant Fluoxetine. *PLoS ONE* **2014**, *9*, e110467. [CrossRef] [PubMed]
16. Arnold, K.E.; Brown, A.R.; Ankley, G.T.; Sumpter, J.P. Medicating the environment: Assessing risks of pharmaceuticals to wildlife and ecosystems. *Philos. Trans. R. Soc. B Biol. Sci.* **2014**, *369*, 20130569. [CrossRef]
17. Weston, J.J.; Hugget, D.B.; Rimoldi, J.; Foran, C.M.; Stattery, M. Determination of fluoxetine (ProzacTM) and norfluoxetine in the aquatic environment. In Proceedings of the Annual Meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, MD, USA, November 2001.
18. Brooks, B.W.; Chambliss, C.K.; Stanley, J.K.; Ramirez, A.; Banks, K.E.; Johnson, R.D.; Lewis, R.J. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* **2005**, *24*, 464–469. [CrossRef]
19. Brooks, B.W.; Turner, P.K.; Stanley, J.K.; Weston, J.J.; Glidewell, E.A.; Foran, C.M.; Slattery, M.; La Point, T.W.; Huggett, D.B. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* **2003**, *52*, 135–142. [CrossRef]
20. Brooks, B.W.; Foran, C.M.; Richards, S.M.; Weston, J.; Turner, P.K.; Stanley, J.K.; Solomon, K.R.; Slattery, M.; La Point, T.W. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* **2003**, *142*, 169–183. [CrossRef]
21. Brooks, B.W. Fish on Prozac (and Zoloft): Ten years later. *Aquat. Toxicol.* **2014**, *151*, 61–67. [CrossRef]
22. Valenti, T.W.; Gould, G.G.; Berninger, J.P.; Connors, K.A.; Keele, N.B.; Prosser, K.N.; Brooks, B.W. Human Therapeutic Plasma Levels of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline Decrease Serotonin Reuptake Transporter Binding and Shelter-Seeking Behavior in Adult Male Fathead Minnows. *Environ. Sci. Technol.* **2012**, *46*, 2427–2435. [CrossRef] [PubMed]
23. Brodin, T.; Fick, J.; Jonsson, M.; Klaminder, J. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science* **2013**, *339*, 814–815. [CrossRef] [PubMed]
24. Berninger, J.P.; Du, B.; Connors, K.A.; Eytcheson, S.A.; Kolkmeier, M.A.; Prosser, K.N.; Valenti, T.W.; Chambliss, C.K.; Brooks, B.W. Effects of the antihistamine diphenhydramine on selected aquatic organisms. *Environ. Toxicol. Chem.* **2011**, *30*, 2065–2072. [CrossRef] [PubMed]
25. Zannat, R.; Uddin, M.M.N.; Rahman, M.A.; Aklima, J.; Amin, M.M.A. Antihistamines considerably modulate the cognitive and psychomotor performance of human volunteers. *Cogent Psychol.* **2016**, *3*, 1216242. [CrossRef]
26. Runnalls, T.J.; Margiotta-Casaluci, L.; Kugathas, S.; Sumpter, J.P. Pharmaceuticals in the Aquatic Environment: Steroids and Anti-Steroids as High Priorities for Research. *Hum. Ecol. Risk Assess. Int. J.* **2010**, *16*, 1318–1338. [CrossRef]
27. Ayscough, N.J.; Fawell, J.; Franklin, G.; Young, W. *Review of Human Pharmaceuticals in the Environment*; R&D Report P390; UK Environment Agency (Horizon House): Bristol, UK, 2000; p. 114.
28. Fitzsimmons, P.N.; Fernandez, J.D.; Hoffman, A.D.; Butterworth, B.C.; Nichols, J.W. Branchial elimination of superhydrophobic organic compounds by rainbow trout (Oncorhynchus mykiss). *Aquat. Toxicol.* **2001**, *55*, 23–34. [CrossRef]
29. Schulz, M.; Iwersen-Bergmann, S.; Andresen, H.; Schmoldt, A. Therapeutic and toxic blood concentrations of nearly 1000 drugs and other xenobiotics. *Crit. Care* **2012**, *16*, R136. [CrossRef]
30. Berninger, J.P.; LaLone, C.A.; Villeneuve, D.L.; Ankley, G.T. Prioritization of pharmaceuticals for potential environmental hazard through leveraging a large-scale mammalian pharmacological dataset. *Environ. Toxicol. Chem.* **2016**, *35*, 1007–1020. [CrossRef]
31. Ogunbanwo, O.M.; Kay, P.; Boxall, A.B.; Wilkinson, J.; Sinclair, C.J.; Shabi, R.A.; Fasasi, A.E.; Lewis, G.A.; Amoda, O.A.; Brown, L.E. High Concentrations of Pharmaceuticals in a Nigerian River Catchment. *Environ. Toxicol. Chem.* **2022**, *41*, 551–558. [CrossRef]
32. Bertram, M.G.; Martin, J.M.; McCallum, E.S.; Alton, L.A.; Brand, J.A.; Brooks, B.W.; Cerveny, D.; Fick, J.; Ford, A.T.; Hellström, G.; et al. Frontiers in quantifying wildlife behavioural responses to chemical pollution. *Biol. Rev. Camb. Philos. Soc.* **2022**. [CrossRef]

33. Sumpter, J.P.; Donnachie, R.L.; Johnson, A.C. The apparently very variable potency of the anti-depressant fluoxetine. *Aquat. Toxicol.* **2014**, *151*, 57–60. [CrossRef]
34. Castiglioni, S.; Salgueiro-González, N.; Bijlsma, L.; Celma, A.; Gracia-Lor, E.; Beldean-Galea, M.S.; Mackuľak, T.; Emke, E.; Heath, E.; Kasprzyk-Hordern, B.; et al. New psychoactive substances in several European populations assessed by wastewater-based epidemiology. *Water Res.* **2021**, *195*, 116983. [CrossRef] [PubMed]
35. Baker, D.R.; Kasprzyk-Hordern, B. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: New developments. *Sci. Total Environ.* **2013**, *454–455*, 442–456. [CrossRef] [PubMed]
36. Baker, D.R.; Barron, L.; Kasprzyk-Hordern, B. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates. *Sci. Total Environ.* **2014**, *487*, 629–641. [CrossRef]
37. Malev, O.; Lovrić, M.; Stipaničev, D.; Repec, S.; Martinović-Weigelt, D.; Zanella, D.; Ivanković, T.; Sindičić Đuretec, V.; Barišić, J.; Li, M.; et al. Toxicity prediction and effect characterization of 90 pharmaceuticals and illicit drugs measured in plasma of fish from a major European river (Sava, Croatia). *Environ. Pollut.* **2020**, *266*, 115162. [CrossRef]
38. Maasz, G.; Molnar, E.; Mayer, M.; Kuzma, M.; Takács, P.; Zrinyi, Z.; Pirger, Z.; Kiss, T. Illicit Drugs as a Potential Risk to the Aquatic Environment of a Large Freshwater Lake after a Major Music Festival. *Environ. Toxicol. Chem.* **2021**, *40*, 1491–1498. [CrossRef]
39. Maculewicz, J.; Kowalska, D.; Świacka, K.; Toński, M.; Stepnowski, P.; Białk-Bielińska, A.; Dołżonek, J. Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. *Sci. Total Environ.* **2022**, *802*, 149916. [CrossRef]
40. Metcalfe, C.D.; Chu, S.; Judt, C.; Li, H.; Oakes, K.D.; Servos, M.R.; Andrews, D.M. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environ. Toxicol. Chem.* **2010**, *29*, 79–89. [CrossRef]
41. Schlüsener, M.P.; Hardenbicker, P.; Nilson, E.; Schulz, M.; Viergutz, C.; Ternes, T.A. Occurrence of venlafaxine, other antidepressants and selected metabolites in the Rhine catchment in the face of climate change. *Environ. Pollut.* **2015**, *196*, 247–256. [CrossRef] [PubMed]
42. Rand-Weaver, M.; Margiotta-Casaluci, L.; Patel, A.; Panter, G.H.; Owen, S.F.; Sumpter, J.P. The Read-Across Hypothesis and Environmental Risk Assessment of Pharmaceuticals. *Environ. Sci. Technol.* **2013**, *47*, 11384–11395. [CrossRef] [PubMed]
43. Huerta, B.; Margiotta-Casaluci, L.; Rodríguez-Mozaz, S.; Scholze, M.; Winter, M.J.; Barceló, D.; Sumpter, J.P. Anti-anxiety drugs and fish behavior: Establishing the link between internal concentrations of oxazepam and behavioral effects. *Environ. Toxicol. Chem.* **2016**, *35*, 2782–2790. [CrossRef] [PubMed]
44. Duarte, I.A.; Fick, J.; Cabral, H.N.; Fonseca, V.F. Bioconcentration of neuroactive pharmaceuticals in fish: Relation to lipophilicity, experimental design and toxicity in the aquatic environment. *Sci. Total Environ.* **2022**, *812*, 152543. [CrossRef] [PubMed]
45. Tanoue, R.; Nomiyama, K.; Nakamura, H.; Kim, J.; Isobe, T.; Shinohara, R.; Kunisue, T.; Tanabe, S. Uptake and Tissue Distribution of Pharmaceuticals and Personal Care Products in Wild Fish from Treated-Wastewater-Impacted Streams. *Environ. Sci. Technol.* **2015**, *49*, 11649–11658. [CrossRef] [PubMed]
46. Huerta, B.; Rodriguez-Mozaz, S.; Lazorchak, J.; Barcelo, D.; Batt, A.; Wathen, J.; Stahl, L. Presence of pharmaceuticals in fish collected from urban rivers in the U.S. EPA 2008–2009 National Rivers and Streams Assessment. *Sci. Total Environ.* **2018**, *634*, 542–549. [CrossRef] [PubMed]
47. Cerveny, D.; Grabic, R.; Grabicová, K.; Randák, T.; Larsson, D.G.J.; Johnson, A.C.; Jürgens, M.D.; Tysklind, M.; Lindberg, R.H.; Fick, J. Neuroactive drugs and other pharmaceuticals found in blood plasma of wild European fish. *Environ. Int.* **2021**, *146*, 106188. [CrossRef]
48. Sumpter, J.P.; Runnalls, T.J.; Donnachie, R.L.; Owen, S.F. A comprehensive aquatic risk assessment of the beta-blocker propranolol, based on the results of over 600 research papers. *Sci. Total Environ.* **2021**, *793*, 148617. [CrossRef]
49. More, S.J.; Bampidis, V.; Benford, D.; Bennekou, S.H.; Bragard, C.; Halldorsson, T.I.; Hernández-Jerez, A.F.; Koutsoumanis, K.; Naegeli, H.; Schlatter, J.R.; et al. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. *EFSA J.* **2019**, *17*, e05634.
50. Marmon, P.; Owen, S.F.; Margiotta-Casaluci, L. Pharmacology-informed prediction of the risk posed to fish by mixtures of non-steroidal anti-inflammatory drugs (NSAIDs) in the environment. *Environ. Int.* **2021**, *146*, 106222. [CrossRef]
51. Gustavsson, M.; Kreuger, J.; Bundschuh, M.; Backhaus, T. Pesticide mixtures in the Swedish streams: Environmental risks, contributions of individual compounds and consequences of single-substance oriented risk mitigation. *Sci. Total Environ.* **2017**, *598*, 973–983. [CrossRef]
52. Gould, S.L.; Winter, M.J.; Norton, W.H.J.; Tyler, C.R. The potential for adverse effects in fish exposed to antidepressants in the aquatic environment. *Environ. Sci. Technol.* **2021**, *55*, 16299–16312. [CrossRef]
53. Gunnarsson, L.; Snape, J.R.; Verbruggen, B.; Owen, S.F.; Kristiansson, E.; Margiotta-Casaluci, L.; Österlund, T.; Hutchinson, K.; Leverett, D.; Marks, B.; et al. Pharmacology beyond the patient—The environmental risks of human drugs. *Environ. Int.* **2019**, *129*, 320–332. [CrossRef] [PubMed]
54. Al Shuraiqi, A.; Al-Habsi, A.; Barry, M.J. Time-, dose- and transgenerational effects of fluoxetine on the behavioural responses of zebrafish to a conspecific alarm substance. *Environ. Pollut.* **2021**, *270*, 116164. [CrossRef] [PubMed]
55. Klaminder, J.; Hellström, G.; Fahlman, J.; Jonsson, M.; Fick, J.; Lagesson, A.; Bergman, E.; Brodin, T. Drug-Induced Behavioral Changes: Using Laboratory Observations to Predict Field Observations. *Front. Environ. Sci.* **2016**, *4*, 81. [CrossRef]

56. Lagesson, A.; Brodin, T.; Fahlman, J.; Fick, J.; Jonsson, M.; Persson, J.; Byström, P.; Klaminder, J. No evidence of increased growth or mortality in fish exposed to oxazepam in semi-natural ecosystems. *Sci. Total Environ.* **2018**, *615*, 608–614. [CrossRef] [PubMed]
57. Fahlman, J.; Hellström, G.; Jonsson, M.; Fick, J.B.; Rosvall, M.; Klaminder, J. Impacts of Oxazepam on Perch (Perca fluviatilis) Behavior: Fish Familiarized to Lake Conditions Do Not Show Predicted Anti-anxiety Response. *Environ. Sci. Technol.* **2021**, *55*, 3624–3633. [CrossRef] [PubMed]
58. Tanoue, R.; Margiotta-Casaluci, L.; Huerta, B.; Runnalls, T.J.; Eguchi, A.; Nomiyama, K.; Kunisue, T.; Tanabe, S.; Sumpter, J.P. Protecting the environment from psychoactive drugs: Problems for regulators illustrated by the possible effects of tramadol on fish behaviour. *Sci. Total Environ.* **2019**, *664*, 915–926. [CrossRef]
59. Peterson, E.K.; Buchwalter, D.B.; Kerby, J.L.; LeFauve, M.K.; Varian-Ramos, C.W.; Swaddle, J.P. Integrative behavioral ecotoxicology: Bringing together fields to establish new insight to behavioral ecology, toxicology, and conservation. *Curr. Zool.* **2017**, *63*, 185–194. [CrossRef]
60. Kokel, D.; Bryan, J.; Laggner, C.; White, R.; Cheung, C.Y.J.; Mateus, R.; Healey, D.; Kim, S.; Werdich, A.A.; Haggarty, S.J.; et al. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat. Chem. Biol.* **2010**, *6*, 231–237. [CrossRef]
61. Thoré, E.S.J.; Steenaerts, L.; Philippe, C.; Grégoir, A.F.; Brendonck, L.; Pinceel, T. Improving the reliability and ecological validity of pharmaceutical risk assessment: Turquoise killifish (Nothobranchius furzeri) as a model in behavioral ecotoxicology. *Environ. Toxicol. Chem.* **2019**, *38*, 262–270. [CrossRef]
62. Grimm, D. USA EPA to eliminate all mammal testing by 2035. *Science* **2019**, *365*, 1231. [CrossRef]

toxics

MDPI

Review

Not Only Toxic but Repellent: What Can Organisms' Responses Tell Us about Contamination and What Are the Ecological Consequences When They Flee from an Environment?

Cristiano V. M. Araújo [1,*], Abdelmourhit Laissaoui [2], Daniel C. V. R. Silva [3], Eloisa Ramos-Rodríguez [4], Enrique González-Ortegón [1], Evaldo L. G. Espíndola [5], Francisco Baldó [6], Freylan Mena [7], Gema Parra [8], Julián Blasco [1], Julio López-Doval [9,10], Marta Sendra [11], Mohamed Banni [12], Mohammed Ariful Islam [13] and Ignacio Moreno-Garrido [1]

1 Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (CSIC), Puerto Real, 11519 Cadiz, Spain; quique.gonzalez@icman.csic.es (E.G.-O.); julian.blasco@csic.es (J.B.); ignacio.moreno@icman.csic.es (I.M.-G.)
2 National Centre for Nuclear Energy, Science and Technology, BP 1381, Rabat RP 10001, Morocco; laissaoui@cnesten.org.ma
3 Institute of Xingu Studies, Federal University of Southern and Southeastern Pará, São Félix do Xingu, PA 68507-590, Brazil; daniel.clemente@unifesspa.edu.br
4 Department of Ecology and Institute of Water Research, University of Granada, 18010 Granada, Spain; eloisa@ugr.es
5 NEEA/CRHEA/SHS, São Carlos Engineering School, University of São Paulo, Av. Trabalhador São Carlense, 400, São Carlos, SP 13.560-970, Brazil; elgaeta@sc.usp.br
6 Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Cádiz, 11006 Cádiz, Spain; francisco.baldo@ieo.es
7 Regional Institute for Studies on Toxic Substances (IRET), Universidad Nacional, 86-3000 Heredia, Costa Rica; freylan.mena.torres@una.cr
8 Animal Biology, Plant Biology and Ecology Department, University of Jaén, 23071 Jaén, Spain; gparra@ujaen.es
9 Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, H2O Building, C/Emili Grahit, 101, 17003 Girona, Spain; jclopez@icra.cat
10 Faculty of Sciences, University of Girona, Campus de Montilivi, 17003 Girona, Spain
11 Institute of Marine Research (IIM), National Research Council (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain; msendra@iim.csic.es
12 Laboratory of Biochemistry and Environmental Toxicology, Higher Institute of Agronomy, 1349-017 Chott-Mariem, Tunisia; m_banni@yahoo.fr
13 Department of Aquatic Resource Management, Faculty of Fisheries, Sylhet Agricultural University, Sylhet 3100, Bangladesh; arif19df1.sau@gmail.com
* Correspondence: cristiano.araujo@icman.csic.es

Received: 21 October 2020; Accepted: 10 December 2020; Published: 12 December 2020

Abstract: The ability of aquatic organisms to sense the surrounding environment chemically and interpret such signals correctly is crucial for their ecological niche and survival. Although it is an oversimplification of the ecological interactions, we could consider that a significant part of the decisions taken by organisms are, to some extent, chemically driven. Accordingly, chemical contamination might interfere in the way organisms behave and interact with the environment. Just as any environmental factor, contamination can make a habitat less attractive or even unsuitable to accommodate life, conditioning to some degree the decision of organisms to stay in, or move from, an ecosystem. If we consider that contamination is not always spatially homogeneous and that many organisms can avoid it, the ability of contaminants to repel organisms should also be of concern.

Thus, in this critical review, we have discussed the dual role of contamination: toxicity (disruption of the physiological and behavioral homeostasis) vs. repellency (contamination-driven changes in spatial distribution/habitat selection). The discussion is centered on methodologies (forced exposure against non-forced multi-compartmented exposure systems) and conceptual improvements (individual stress due to the toxic effects caused by a continuous exposure against contamination-driven spatial distribution). Finally, we propose an approach in which Stress and Landscape Ecology could be integrated with each other to improve our understanding of the threat contaminants represent to aquatic ecosystems.

Keywords: avoidance; behavior; habitat selection; multi-compartmented systems; non-forced exposure; repellency

---

1. Introduction

The concept of the risk linked to contaminants in ecotoxicology is strongly associated with the toxic effects they might produce. Therefore, the more toxic a contaminant is, the more dangerous it is [1]. Initially, this specific focus on toxicity, at the expense of a more ecological approach, was not a problem since toxicity was the driving force that drove the emergence of ecotoxicology [2]. However, it should not be the unique focus. The inclusion of more ecological approaches, beyond just toxicity, has long been called for [1,3–8]. This need to integrate ecological concepts into ecotoxicology has, in fact, led to newer approaches such as Stress Ecology (and its subdomain Chemical Stress Ecology): the study of contamination-driven alterations to biological systems [9,10]. According to these authors, this approach should cover not only the effects at the individual level, considering the entire life cycle, but also the intra- and interspecies interactions as well as their relationship with the environment [6]. Possibly, this historic trend of ecotoxicologists to apply a more toxicological approach instead of moving towards ecology comes from the origin of this science, initially defined as a branch of toxicology, due to the relatively few ecologists working in this area [2]. However, other approaches are emerging in ecology and include different stressors to study their effects when acting simultaneously on biota. Undoubtedly, information about the toxicity of chemicals for as many species as possible is crucial for environmental risk assessments (ERAs), but a more ecological view that would broaden the perspective of contaminant-driven environmental damage is urgently required [1,11,12].

The ecotoxicological approaches with the most ecological implications are mainly based on indirect effects (reaching the higher levels of biological organization) and seek to cover broader spatial scales, for instance: the structure and functioning of ecosystems (including the concepts of functional redundancy, resistance and resilience), metapopulation and community ecology, landscapes in spatially connected and heterogeneous (patchy) environments, (re)colonization, ecosystem functions and services, etc. [1,5,13–18]. Although it is widely known that organisms select their place to live according to their limits of tolerance, food availability, mating success, protection from predators and etc., under this ecological umbrella, the capacity of contaminants to repel organisms and modify their behavioral fitness and spatial distribution is a subject that should be taken into account (see the reviews by De Lange et al. [7]; Araújo et al. [19], Araújo and Blasco [20] and Moreira-Santos et al. [11]), mainly as an early warning signal [21]. The concept of repellency in ecotoxicology is linked to the avoidance behavior triggered by chemicals under conditions in which organisms are given multi-choice experiments, containing at least two chemically different environments [22–26]. The possibility of simulating scenarios in which organisms can move freely among chemically different environments allows us to assess any differences in the level of repellency of the contaminants and understand how this repellency drives the spatial distribution of organisms [27,28]. This approach changes the focus of the effects of the contaminants from toxicity to concepts related to dispersion, migration, and habitat selection processes [18,29]. Although no effect is expected to occur on individuals (avoiders might

only be in contact with the contaminant for a very short time), the migration of part of or even the entire population could be considered just as disastrous as the death of the organisms at the local scale [18,21,30]. Even a partial disappearance of populations might cause a reduction in biodiversity, affect the ecosystem's structure and functionality as well as its resilience, and the capacity to withstand other stressors (e.g., environmental changes and other anthropogenic impacts) [6,18,31]. Therefore, the environmental disturbance caused by contamination should also include the way in which chemicals repel organisms, changing their habitat selection processes and then their spatial distribution patterns. Another important mechanism used by many planktonic invertebrates (e.g., cladocerans, copepods, ostracods, and rotifers) to escape stressful conditions is temporal avoidance by entering dormant stages [32,33]. This adaptation allows species: to remain in highly unpredictable and variable environments, favors the dispersion to, and colonization of, new habitats and provides higher resilience to the ecosystem [32–35]. In spite of the importance of this adaptive mechanism and the little knowledge of its role in contaminated environments [36], the current review is exclusively focused on spatial avoidance (repellency).

The repellent character of a substance is probably not necessarily directly related to its toxicity, and so a highly repellent contaminant could have a low toxicity. In fact, in some cases a biphasic response (initial attraction at low concentrations and avoidance at higher concentrations), described as behavioral hormesis, has been observed [37,38]. The aim of the current critical review is to present a discussion on the avoidance response of organisms to escape from continuous exposure and the ecological consequences of this response compared to the traditional approach based on the toxic effects of the contaminants. The discussion focuses on the dichotomy between toxicity and repellency (Figure 1), considering their major differences, both methodological (forced exposure against non-forced multi-compartmented exposure systems) and conceptual (individual stress due to the toxic effects caused by a continuous exposure against contamination-driven spatial distribution). Regarding the exposure approach to assess repellency, we have exclusively focused on non-forced multi-compartmented exposure systems because they are a more complex method capable of simulating environmental heterogeneity, either as gradients or patches of contamination [22,30,39]. Secondly, a brief comparison between the repellency and toxicity of some chemicals is provided. Finally, we discuss the ecological implications of avoidance in multi-compartmented systems and the conceptual improvements that this approach might provide to ERAs in the light of spatial displacement (extinction at the local level, re-colonization of environments, chemical fragmentation of habitats and habitat connectivity, metapopulation, metacommunity, and meta-ecosystem). A summarized schematic representation of the concepts discussed in the current review, as well as the advantages of integrating toxicity and repellency in the environmental risk studies is shown in Figure 1. Briefly, *Toxicity* refers to the stress directly affecting the individuals with the consequent loss of (from genetic to behavioral) homeostasis due to their sensitivity or by provoking acclimation or adaptation. On the other hand, *Repellency* is here considered an indirect effect, due to the absence of damage (at any level) on individuals, as the exposure is not continuous and the response is based on the capacity of organisms to perceive contamination and avoid it: the displacement towards another area indicates the potential aversive nature of the contaminated habitat, but not a toxic effect on individuals. In this case, the loss of biodiversity at the local scale might produce problems within the ecosystems related to vulnerability and functional redundancy. The methodological differences in relation to exposure systems (forced and mandatory exposure against non-forced and multi-compartmented exposure) determine the conceptual differences between focusing on toxicity or repellency. Both approaches applied concomitantly might contribute to the integration of Stress Ecology with Landscape Ecology.

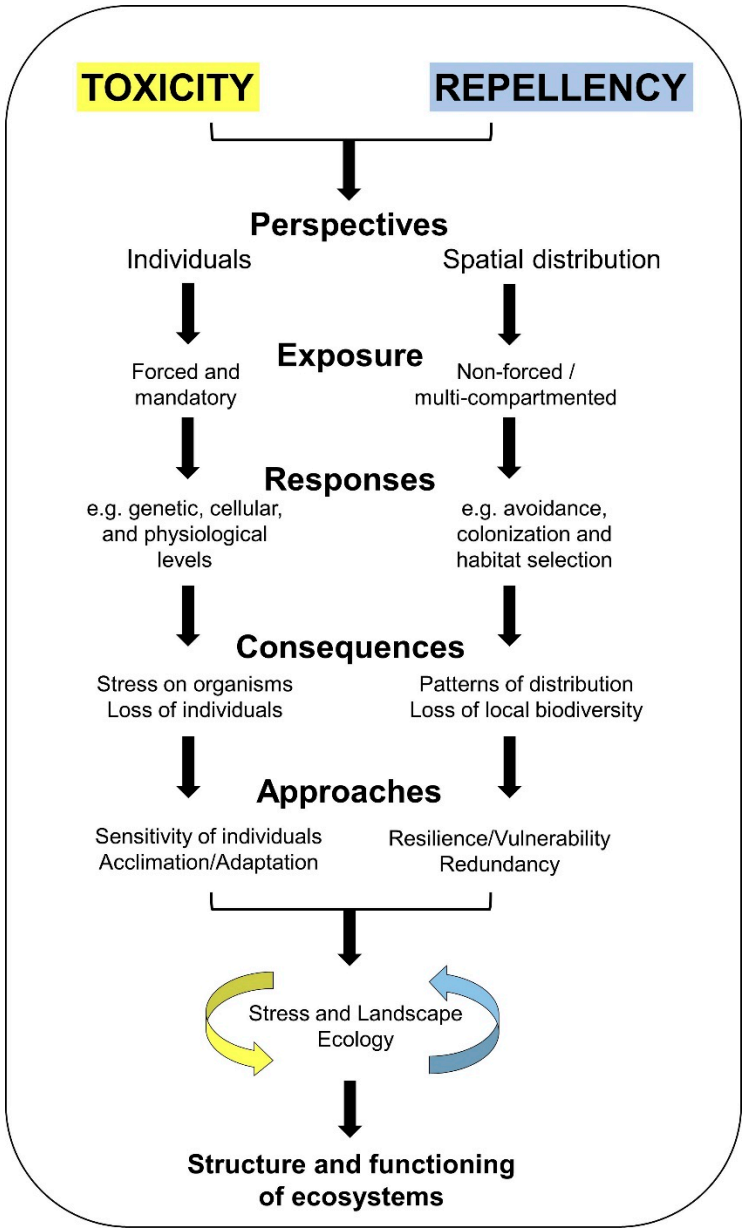

Figure 1. A schematic representation of some concepts linked to toxicity (defined according to the traditional forced exposure approach) and repellency (defined according to the non-forced multi-compartmented exposure approach) that could be integrated to understand the effects of contamination on the structure and functioning of ecosystems better. Regarding the toxicity approach, the scheme shows that the main perspective of toxicity is focused on individuals, in which the forced exposure is the more traditional exposure method. From this perspective, some classes of responses at different biological levels, the effects expected (from stress to loss of species) and the concepts that the studies focus on (sensitivity of species as well as possible mechanisms of acclimation and adaptation to face contamination) are represented. Regarding the repellency, the perspective is focused on the spatial distribution of organisms based on a non-forced exposure (as individuals are not mandatorily exposed), considering the responses related to the dispersion of species, whose effects might only be perceived due to changes in the spatial distribution of the species and possible loss of local biodiversity. The main approaches to be dealt with in the repellency-based approach include the ecosystem's capacity to resist or become more vulnerable to the changes depending on the redundancy of species (avoiders will be replaced by non-avoiders with similar or different functions). Finally, the integration of both approaches makes it possible to apply a broader approach that includes Stress and Landscape Ecology.

2. Toxicity: The Traditional Ecotoxicological Response

The main role attributed to ecotoxicology since it was launched as a science has been to provide evidence concerning the potential toxic effects of chemicals on organisms [2,40,41]. To employ a vision beyond traditional toxicology (effects of contaminants on a particular species with the aim of protecting humans), ecotoxicology has attempted to focus on effects at different levels of biological organization, from sub-organisms to community (sometimes making inferences about an ecosystem's structure and functioning) [1,5,18]. Thus, ecotoxicology is a tool to complement the information of ERA studies, previously based on chemical and ecological data. Due to this role in ERAs, ecotoxicology has begun to develop a very important legal role, which has required the standardization and regulation of procedures. Therefore, although an environmentally more realistic laboratory-scale was always desired, ecotoxicological assays moved towards prioritizing other features rather than the ecological relevance of the experiments, for instance: easy development, practicability, cost-effectiveness, and replicability [42,43]. In this sense, ecotoxicological assays progressed towards a standard method that consists in exposing organisms to different concentrations of a chemical (or environmental samples such as water and sediment) and, after a previously established exposure period, some responses/endpoints are measured and compared with a control (unexposed) population [10,44]. Throughout the exposure period, the organisms are mandatorily in continuous contact with the contaminant, allowing a direct concentration-response relationship to be established. Therefore, regardless of the level of observation, whether at the sub-individual level or higher, this type of exposure (forced and mandatory exposure) means the effects are specifically linked to toxicity.

Although the forced exposure is a standard approach used in almost all ecotoxicological studies, the endpoints employed to measure the potential toxic effect of a chemical have been described from different biological organization levels and perspectives: biochemical, cellular, molecular, physiological (e.g., growth, feeding), histopathological, and behavioral effects [1,45,46]. Whether at a low or high biological level, the toxicity comes from a cascade of events that begin with the absorption and/or adsorption of the contaminants and the consequent impairments/disruptions they may produce. This approach has helped to detect the contaminants with a very high risk to the environment due to their toxicity and to identify highly susceptible species within the various ecosystems studied. This information has been useful for environmental conservation, not only for scientists, but also for regulatory enforcement. However, when organisms are confronted with contaminants, it should be considered that three different reactions can occur: conformity, regulation, or avoidance [47]. The use of a forced exposure approach includes the conformity and the ability to regulate the contaminants, but it does not comprise the possibility of escaping. A forced exposure environment assumes that environments are chemically homogeneous and that there is no option to avoid exposure. This assumption has recently changed with the development of non-forced multi-compartmented exposure systems [30]. An avoidance behavior is no longer assessed exclusively based on changes in the swimming patterns, but rather on dispersion within a chemically heterogeneous environment. Therefore, answers to questions like "what if aquatic animals move away from contaminated habitats before suffering adverse physiological effects?" [11] seem to be easier to provide now.

3. Avoidance: A Repellency-Driven Behavioral Response

Traditionally, avoidance has been linked to behavioral changes, such as overexcitement or lethargy, that could indicate a response to flee or not from contaminants [47–50]. Since this assumption is based on a forced exposure approach, it does not allow us to know whether organisms could discriminate among different concentrations in a smoothly heterogeneous scenario, rather than only in an abruptly modified chemically heterogeneous environment. The selection of the studies under discussion in this section was based on whether they were performed in multi-compartmented exposure systems (see examples of the most used systems in Figure 2). Although many different non-forced systems can provide a contamination gradient for organisms [22], multi-compartmentalization allows the magnitude of the avoidance response to be related to all the concentrations (or water and sediment

samples from different origins) used to make up the gradient. Then, a typical concentration-response can be obtained. This also favors the comparison on how sensitive avoidance is in relation to the data with other endpoints when comparing LC*x* or EC*x* (lethal or effective concentration to *x*% of the population) values with AC*x* values (concentration eliciting an avoidance of *x*% of the population).

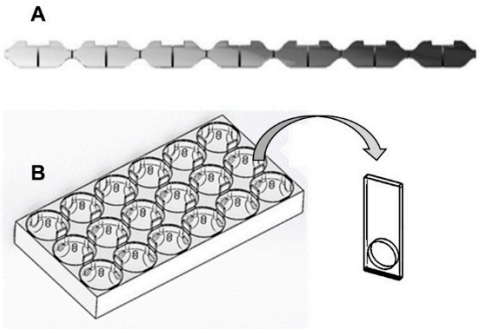

Figure 2. Schematic representation of the two most widely used non-forced multi-compartmented exposure systems in avoidance experiments: (**A**): linear system representing a contamination gradient indicated by the scale of grey and (**B**): HeMHAS (Heterogeneous Multi-Habitat Assays System) with the gate used to open or close the connections between compartments in all directions.

The development of non-forced systems has provided the possibility of confronting organisms with different scenarios to identify the more attractive or repellent zones. A pioneer flow-through multi-compartmented system, in which a smooth linear gradient (1D) of contamination can be simulated, was developed by Lopes et al. [30]. This system was later simplified by Rosa et al. [51], who turned it into a static multi-compartmented system. In recent years, a more complex system (HeMHAS—Heterogeneous Multi-Habitat Assay System) has been proposed by Araújo et al. [52]. Both systems have been used in studies with different organisms and chemicals. Although bi-compartmented exposure systems (two choice options) are also widely used to assess repellency (see review by Jutfelt et al. [22]), we briefly present data from multi-compartmented exposure systems in this section due to their ecological relevance and environmental complexity in terms of the concepts discussed here. Detailed information can be obtained in reviews by Araújo et al. [19], Araújo and Blasco [20], and Moreira-Santos et al. [11]. All comparisons with other endpoints should be made with caution, since avoidance is usually measured after a very short exposure time (between 3 and 12 h, depending on the exposure system and the maintenance of the contamination gradient).

The first evidence of avoidance in a multi-compartmented system was described for the cladoceran *Daphnia longispina* [30]. These authors observed that among the different lineages tested, the sensitivity and early reactiveness of the organisms to avoid copper was directly related to the lethal sensitivity of the lineages. Other invertebrates such as the cladoceran *D. magna* (exposed to pulp mill effluents [53]; atrazine [51]; and salinity as stress factor [54]), the freshwater copepod *Boeckella occidentalis intermedia* (crude oil as the contaminant [55]), the ostracod *Heterocypris incongruens* (salinity as the stress factor [54]), the gastropod *Peringia ulvae* (sediment spiked with cadmium [56]), the freshwater shrimp *Atyaephyra desmarestii* (exposure to copper [39,57–59]), the marine shrimp *Litopenaeus vannamei* (exposed to copper [60,61]), and the saltmarsh shrimp *Palaemon varians* (exposed to musks and sunscreens [25,62]) have been tested for avoidance. In general, the avoidance response reported in those studies was more sensitive than the lethal and some sub-lethal endpoints described by other authors (see references cited above). However, the avoidance and mortality of the copepod *B. occidentalis intermedia* was similarly sensitive [55] and the 21-day reproduction test with *D. magna* exposed to atrazine proved to be more sensitive than avoidance [51].

Regarding vertebrates, avoidance studies in multi-compartmented systems have mainly focused on amphibians and fish. Tadpoles of the amphibian *Lithobates catesbeianus* have proved to be able to avoid different chemicals such as copper [63], the fungicide pyrimethanil [64], the pesticide abamectin [65],

the 2,4-dichlorophenoxyacetic acid herbicide [66], the herbicide diuron [67], and solution containing mining tailings [68]. Avoidance by tadpoles of *Leptodactylus latrans* and *Pelophylax perezi* of contamination by copper and pyrimethanil has been also described [63,64]. In almost all these studies, avoidance was shown to be a highly sensitive response when compared with lethal or even sub-lethal (e.g., development, weight, and swimming behavior) responses (see previous citations). On the other hand, in some studies avoidance was not the most sensitive response when compared with, for instance, the responses of the: feeding rate, growth rate (SVL) and weight gain rate of tadpoles of *Xenopus laevis* exposed to gold nanorods [69] and the speed and distance responses after 16 days of exposure to mining tailings [68].

The avoidance response using the multi-compartmented approach has been mainly used for two fish species: zebrafish (*Danio rerio*) and guppy (*Poecilia reticulata*). The first avoidance study in a multi-compartmented scenario with fish was performed with zebrafish that were exposed to gradients of copper and effluent from acidic mine drainage [70]. In that study, the authors attested that avoidance is a quick response, so that the avoidance observed after 12 h exposure did not vary from exposure periods of up to 96 h. This was possible mainly because the system maintained the contamination gradient for a long time. Later, in a study also performed with zebrafish exposed to the fungicide pyrimethanil, it was shown that the exposure period to measure avoidance could be as short as 4 h [71]. This is of great importance if static systems (without peristaltic pumps) are used, as it is difficult to maintain the gradient for a long time when the fish are swimming continuously. Avoidance studies with fish have also been performed with different contaminants such as: tuna fish processing plant effluent [72], triclosan [73], atrazine [74], river samples [75,76], bisphenol [77], copper [78,79], fipronil and 2,4-D [80], dairy wastewater [76], among others. In the study by Araújo et al. [71], the avoidance response was assessed during very short exposure periods, sometimes not exceeding 4 h. In almost all cases, the avoidance initially observed (e.g., after 30 min) was similar to the avoidance at different periods during the remaining hours of the experiment. Furthermore, in some of those studies, avoidance occurred at sub-lethal concentrations and even at environmentally relevant concentrations [66,73,74,77].

The use of the multi-compartmented exposure approach to assess the ability of the organism to escape from contamination seems to be a suitable alternative to understand the environmental risks caused by the repellent characteristics of the contaminants. In addition, the avoidance response has been detected after a very short exposure time, generally not superior to 12 h [11,19,70]. However, the use of avoidance in multi-compartmented systems has some limitations, since its ecological relevance is conditioned to heterogeneous environments and the motility of the species (e.g., i. only organisms with active motility and displacement ability can be used; ii. the environmental relevance of the scenario simulated depends on the chemical heterogeneity occurring in the environment; iii. the spatial scale of the scenario simulated in the laboratory is much lower than the real spatial scale; iv. the time of experimentation is determined by how long the differences among the concentrations is maintained inside the system; v. the use of bigger species requires much bigger exposure systems and a greater quantity of chemicals). The current approach does not replace the traditional forced exposure but provides a complementary tool that could be applied to better understand the potential damages that chemicals can cause, by not only focusing on toxicity, but also on repellency. In this sense, it is important to point out that repellency can be as variable as the different chemical structure of the contaminants. In fact, experimental evidence has shown that even potentially toxic chemicals can present a certain level of attractiveness to organisms rather than repellency [25,26,37,81,82].

4. The Higher the Toxicity, the Higher the Repellency?

Although there could be a tendency to assume that the repellency of a contaminant is related to its toxicity, this relation seems not to be linearly direct, especially for chemicals with a neurotoxic action [65,67,82]. It has been shown that potentially toxic chemicals can exert some attraction to organisms, a similar phenomenon to the classical hormesis effect that might be limited by increasing concentrations [37,38]. For instance, attraction to contamination was observed: in the mud snail

Illyanassa obsolete and the amphipod *Corophium volutator* exposed to chlorothalonil [83] and in the crayfish *Orconectes virilis* exposed to the antidepressant sertraline [82]. In a study with essential oil extracted from the fruits of *Evodia lenticellata*, monoterpenes were shown to be the most toxic group of chemicals, but not the most repellent for the insects *Tribolium castaneum*, *Lasioderma serricorne* and *Liposcelis bostrychophila* [23]; on the other hand, caryophyllene oxide and β-caryophyllene were only moderately toxic, but strongly repellent.

For aquatic animals, such as fish and crustaceans, their interaction with the environment and their behavioral response to chemical signals are significantly mediated by sensory systems (e.g., gustation, chemosensory cells, olfactory epithelium at the gills, chemoreceptors in the antennulae, the olfactory nerve center of the suprapharyngeal ganglion, sensory bristles, and aesthetascs, for example) [50,84,85]. However, some contaminants like metals or pesticides can interfere with the sensorial process and affect the related behavioral response [50,86–90]. This interference can be caused by different mechanisms: direct exposure and damage to exposed olfactory neurons or the disruption in the expression of olfactory system-related genes [85,91]. Thus, the interaction of a contaminant with the sensory system of an organism can affect the behavioral response without a direct relationship with its toxicity. This becomes particularly relevant when the scenario of a mixture of pollution is considered, as the presence of one contaminant can interfere with organism's response regarding another.

Another factor that makes it difficult to link repellency and toxicity is related to any stimulative or lethargic effects. Some contaminants cause overexcitement in organisms, which indicates toxicity, but that could favor organisms fleeing from contamination. On the other hand, this same contaminant, depending on the concentrations, may induce a lethargic state, which might prevent escape [92]. An interesting pattern was observed in tadpoles exposed to a 2,4-D-based herbicide [66]: the distance-travelled response was not altered, while the speed of response to a stimulus was reduced (both using forced exposure); however, the avoidance in a non-forced system was evident at the lowest concentrations, but less marked at the highest. In another study with tadpoles exposed to sublethal concentrations of copper sulfate and ammonium nitrate, impairments in some behavioral indices (response to stimuli, distance moved and type of movement) were observed, leading to a reduction in the ability to escape [93]. Lethargy has also been observed in tadpoles exposed to copper, where at 200 µg/L the avoidance reached 80% but decreased due to moribundity [63]. Additionally, in tadpoles exposed to mining tailings, there was a tendency for individuals to avoid low concentrations, but not the compartments with highest levels of tailings [68]. In a study with the marine shrimps *L. vannamei* and *P. varians* (Redondo et al. *unpublished data*), it was observed that both are able to avoid toxic copper concentrations when exposed to a gradient; however, whereas *L. vannamei* showed signs of overexcitement when it was in a forced exposure, *P. varians* clearly displayed lethargy.

The best way to verify the relationship between toxicity and repellency is to compare the mortality and repellency data of different chemicals for the same species and then to verify whether the repellency levels of the compounds (from less to more toxic) is related to the toxicity levels. After a bibliographic search, we found little data on toxicity in forced systems and repellency in non-forced multi-compartmented systems that could be compared. However, it was found for: the saltmarsh shrimp *P. varians* (exposed to copper, galaxolide, tonalide, and triclosan), the amphibian *L. catesbeianus* (exposed to abamectin, copper, diuron and 2,4-D), and the freshwater fish *D. rerio* (exposed to Ag-NPs, copper, glyphosate, and pyrimethanil) and *P. reticulata* (exposed to atrazine, bisphenol, copper, and triclosan) (Table 1). Although we tried to consider data published for the same species, in the case of the shrimps, toxicity data for copper and triclosan were taken from other species (see details in Table 1). Before reaching a conclusion on the data, it is important to consider that ecotoxicity results may vary depending on the life stage of the organisms, the culture medium, the environmental conditions during experiments, etc. [94,95]. Therefore, comparisons of the results from different studies should be made with caution.

For the saltmarsh shrimp *P. varians*, copper seems to be the least toxic, but the most repellent contaminant. On the other hand, triclosan follows a pattern of lower lethal toxicity and lower repellency.

For the two fragrances, galaxolide seems to be highly repellent and have a low toxicity, whereas tonalide seems to present a potential toxicity very similar to its repellency (Table 1).

In the case of the amphibian *L. catesbeianus*, the pesticide abamectin was the most toxic and the second most repellent contaminant and, following a similar pattern, 2,4-D was the least toxic and least repellent chemical. Diuron deserved special attention because it presented a very high repellency, but low lethal toxicity (Table 1). In spite of this apparent low toxicity of diuron, neurological effects associated with acetylcholinesterase (AChE) activity were observed in the fish *Carassius auratus* exposed at 50 μg/L, but not at 5 μg/L [96] which was the concentration at which the avoidance of tadpoles of *L. catasbeianus* was maximum (around 90%) [67]. Interestingly, the avoidance reduced to 20% at 10 μg/L, which indicates that the increase of diuron concentration caused a reduction in the ability to avoid it [67].

For the fish *D. rerio*, Ag-NPs, copper and glyphosate presented a similar repellency, but in terms of toxicity, this similarity was observed only between copper and glyphosate; Ag-NPs seem to present a lower toxicity. Pyrimethanil seems to be the least toxic and repellent chemical among them (Table 1); in spite of this, the risk cannot be neglected, since sub-lethal effects may be recorded at lower concentrations (38 μg/L) than the AC_{50} [97]. The effects of glyphosate on zebrafish deserve special attention. Although short (96 h) forced exposure to glyphosate can cause behavioral impairments [98], in a 4 h-non-forced exposure approach, avoidance was time-dependent: an attraction was observed during the first two hours, followed by an avoidance in the remaining time (Mena et al., *unpublished data*). This response could be a clear example of time-dependent behavioral hormesis, as the possible overcompensation presented by glyphosate is clearly time-dependent. The importance of time when assessing behavioral changes (initial stimulation followed by a progressive slowdown in movement) after exposure to contaminants has also been pointed out by Ren et al. [48]. An attraction effect has also been observed for female Japanese quails (*Cortunix japonica*) that preferred glyphosate-based herbicide-contaminated food to the control food [26].

Table 1. Comparison of the toxic and repellent potential of different contaminants for four species based on data of toxicity (LC_{50}: lethal concentration to 50% of the population; in μg/L) and repellency (AC_{50}: concentration eliciting avoidance in 50% of the population; in μg/L).

Species	Contaminant	Toxicity (LC_{50})	Avoidance (AC_{50})	References for Toxicity/Avoidance
Palaemon varians (saltmarsh shrimp) [a]	Copper	660	10.4	[25,99]
	Galaxolide	401	14.1	[62]
	Tonalide	88.1	30.8	[62]
	Triclosan	154	42	[100,101]
Lithobates catesbeianus (amphibian) [b]	Abamectin	138	36	[65]
	Copper	372	101	[63]
	Diuron	31,000	±0.5 [c]	[67]
	2,4-D	574,000	242 [d]	[66]
Danio rerio (freshwater fish)	Ag-NPs	2900	9.08	[102], Sendra et al. (*unpublished data*)
	Copper	880	16.7	[78,103]
	Glyphosate	620	12.2	[104], Mena et al. (*unpublished data*)
	Pyrimethanil	2850	1100	[71,97]
Poecilia reticulata (freshwater fish)	Atrazine	4300	0.065	[74,105]
	Bisphenol A	1660	0.154	[77]
	Copper	348	15.9	[78,106]
	Triclosan	1650	8.04	[73]

[a]: Toxicity data of copper and triclosan were based on the post larvae of *Penaeus monodon* [99] and larvae of *Palaemonetes pugio* [100], respectively. [b]: Gosner stage 25. [c]: the AC_{50} value was not provided, but the authors reported an avoidance of around 50% at 0.5 μg/L. [d]: the AC_{50} value was not provided, but the authors reported an avoidance of around 50% at 242 μg/L.

Data for *P. reticulata* show that atrazine seems to have a low toxicity, but can be highly repellent, whereas copper seems to be the most toxic, but less repellent; although the AC_{50} values for copper could also be considered very sensitive. Specifically comparing bisphenol A and triclosan, the acute toxicity of both chemicals is very similar; however, bisphenol A is more repellent (Table 1).

The data presented here perhaps represent an oversimplified estimate about the relationship between toxicity (based on mortality) and repellency. Because repellency was based exclusively on studies performed in multi-compartmented exposure systems simulating a contamination gradient, the amount of data is not robust enough to allow for an extensive and more conclusive comparison. However, the data published by other authors and discussed here provide evidence that toxicity cannot be used as a surrogate for repellency. Therefore, we would like to encourage the use of non-forced, multi-compartmented approaches in order to generate a robust database that would help us to understand this relationship between toxicity and repellency better. In addition, the immediate nature of the avoidance makes its interpretation completely different from a forced and extended exposure.

5. The Decision of Avoiding or Not: A Cost-Benefits Balance

Ecological systems are very complex and difficult to simulate reliably under any experimental conditions. Many studies have pointed out how the toxicity of a compound can vary depending on the biotic and abiotic changes in the field and under the experimental conditions [107–111]). Any experimental approach in ecotoxicology could be considered environmentally reductionist, but this does not invalidate the importance of the results in terms of understanding the risk of the contamination to the environment. Even the apparently obvious avoidance response triggered by the repellency of contaminants can change if other environmental factors are included. Recent studies in multi-compartmented exposure systems have tested different scenarios by including other elements to the exposure conditions and to verify whether the magnitude of the avoidance response varies and what the level of importance that contamination might have for the habitat selection processes is. The main results found related to other relevant elements in some of these studies are described below. In spite of the factors described below, other factors such as the light should also be studied to understand how the avoidance response might vary during the circadian cycle for diurnal and nocturnal periods.

5.1. Population Density

The effects of density on the avoidance response were tested using the freshwater shrimp *A. desmarestii* exposed to a copper gradient [57]. The authors employed three different population densities (3, 5 and 10 shrimps per compartment representing 0.5, 0.8, and 1.7 organisms per mL) in a multi-compartmented system. Avoidance was dependent on the population density, the higher the density, the lower the avoidance. Although shrimps clearly can detect and avoid copper contamination, the stress produced by a high population density (possible intra-species competition) might potentially reduce or even prevent the displacement of organisms to a less disturbed area. The response to toxicants at the population level, when intraspecific competition is present (high population density), differs from the response at the individual level. This was attested by Liess [112], who found that the direct effects of the toxicant were partly compensated by the indirect reduction in intraspecific competitive pressure, which led to a greater availability of food for those who remained.

5.2. Competition

The aim of a study performed by Silva et al. [78] with zebrafish (*Danio rerio*) and guppies (*Poecilia reticulata*) was to assess whether the avoidance of both species was affected by the other. In the monospecies experiments, both species avoided the copper gradient in a very similar way: the range of copper concentrations that triggered avoidance to 20, 50, and 80% of the populations overlapped. However, when both species were tested simultaneously (multispecies test), guppies displaced the zebrafish to concentrations that had previously been avoided by the zebrafish. Changes in the

avoidance to copper caused by interspecies interactions were also observed in a study with the shrimp *A. desmarestii* and zebrafish [113]. In the presence of fish, the avoidance by shrimps was lower and time-delayed. Both studies evidence that competition among species can change the avoidance pattern in relation to the response in monospecies tests.

5.3. Food

The search for food could easily be considered one of the most important drivers that determine the behavior of organisms, especially in conditions where it is not abundant. Based on this statement, an avoidance study was carried out with the fish tilapia (*Oreochromis* sp.) to understand the relationship between the repellency of effluents from a tuna fish processing plant and the availability of food [72]. Firstly, the tilapia fry detected the gradient of contamination and avoided raw and treated effluents. Secondly, organisms were exposed to a gradient of contamination and food simultaneously, so that the more contaminated the area was, the more food was provided. The results indicated that the fish moved intermittently towards the most contaminated areas to feed, in spite of the threat of toxicity.

In another study performed by Islam et al. [79], the effect of food was assessed in three different approaches: avoidance, recolonization and habitat fragmentation. Differently to the method used by Araújo et al. [72], the zebrafish were exposed to a copper gradient, but food was not introduced as a gradient, in the study by Islam and colleagues. In the approaches of avoidance and recolonization, the food was only available in the last and most contaminated zones, whereas in the approach using a chemical fragmentation of habitat, food was only available after the chemical barrier. Those authors found that food did not stimulate the fish to cross the barrier, probably because the trade-off was not perceived.

5.4. Predators and Shelters

In a complex environment, the organisms' decision to avoid or not a contaminated area might be evaluated according to the costs and benefits provided by the different environmental components. In this sense, a study performed with the freshwater shrimp *A. desmarestii* assessed the importance of three elements (i. contamination by copper, ii. presence of shelter that provided protection and iii. kairomones of trout as a predator signal) in the shrimp's habitat selection process [59]. When the shrimps were exposed to the three elements individually, the contaminated areas and areas with the presence of trout kairomones were avoided, whereas the zones with shelter were preferred. If the organisms were provided with a choice between a clean area with no protection and a contaminated area with protection, they preferred the clean area in spite of the lack of protection. However, when a predator signal was included in the clean area in that scenario, the shrimps moved towards a moderately contaminated area, avoiding the predation risk and the most contaminated zones. This is clear evidence of the disturbance that contamination might cause in the habitat selection process of this species.

The cost–benefit analysis that the organisms "need to carry out" in the presence of several stressors (predation and toxicants) could lead to unexpected results in a kind of compensatory outcome. That could support the hypothesis of "functional compensation" of stressor effects that has been described when an unexpected outcome occurs in a multiple stressor scenario.

5.5. Salinity

Salinity is a factor that deserves special attention, not only due to the salinization of coastal freshwater ecosystems (which causes an osmotic unbalance), but also because it is a global and growing threat that might be amplified by climate and anthropic causes [114] and a potential avoidance trigger for many organisms [54]. For instance, fluctuating salinities in estuarine areas can create a very restrictive environment that requires a high osmoregulation capacity [115], which makes salinity a primary environmental factor determinant for the spatial distribution of species [116,117]. In an experiment, Venâncio et al. [54] showed how the cladocera *D. magna*, the ostracod *Heterocypris*

incongruens, the amphibian *Xenopus laevis*, and the fish *D. rerio* detected and avoided increasing salt concentrations at much lower levels than those considered lethally dangerous. By combining salinity with contamination (in this case the insecticide diazinon), Mena et al. [61] observed that the ability of the white leg shrimp *L. vannamei* to avoid diazinon was impaired at a salinity of 30, but not at 10 and 20; at a salinity of 30 a higher effect on osmoregulation was also detected. Although salinity is itself a potential avoidance-driving element, in combination with another avoidable element it can have more serious consequences for organisms, either by potentiating avoidance or even preventing it and causing toxicity [54,61].

6. Ecological Improvements by Simulating a Chemically Heterogeneous Environment

All the approaches used in ecotoxicology have advantages and limitations regarding the information provided. If identifying ecological succession in a contaminated ecosystem is very important, it is no less important to understand the mode of action of the chemicals (especially the contaminants of emerging concern) and how genetic and physiological mechanisms are triggered in response to contamination [118]. Apart from this, it is widely recognized that ecological approaches are much less frequent than individual or sub-organism approaches. The multi-compartmented exposure approach simulating chemically heterogeneous scenarios does not definitively solve the problem of the lack of ecological relevance of ecotoxicity tests. The aim of the approach presented here is to provide a complementary approach to how the repellency of contaminants can be assessed. Although we know the intrinsic limitations of this approach, some ecological concepts can be integrated into ecotoxicological studies when the spatial chemical heterogeneity is considered, since that the avoidance response of populations might suppose changes in ecological interactions and, therefore, in the ecosystem's structure and functioning. Some of the improvements provided by the multi-compartmented exposure approach are discussed below.

6.1. Spatial Displacement: Extinction at the Local Level

The study of the repellency of the contaminants in a chemically heterogeneous spatial exposure scenario shifts the paradigm of responses and effects. Assuming that organisms could potentially detect contaminants at levels of risk and, therefore, move to more favorable areas, the concept of the stress associated to toxicity at the individual level would not necessarily be applied. When organisms flee an area, although there seems to be no direct effect on the organisms themselves, the loss of abundance of the population that fled could be a major problem at the ecosystem level [30,51] and trigger indirect effects on other species or alterations of ecosystem's functions [18]. The analysis of the avoidance response goes beyond the repellency of contaminants or even the ability of organisms to detect them, but it brings ecological implications that could lead to a local reduction in biodiversity that, at the same time, could suppose an increase in the species that are highly tolerant to a specific type of contamination, but probably less tolerant for novel stressors [18]. Other effects include restrictions in habitable areas, changes in the species' interactions (e.g., trophic relationships) in the avoided ecosystems, alterations to migratory patterns, etc. Although in situ observations of the relation between contamination and restrictions in the habitat use are scarce, some studies have evidenced the effects of contamination on the spatial distribution of fish [28,119–121].

Finally, when avoidance is associated with a short-term response that also involves the loss of organisms, such as lethality, avoidance data can be used to predict the immediate decline of a population (PID: Population Immediate Decline); a concept developed by Rosa et al. [51] that has been applied in different studies [62,71,73]. The PID calculated from the integration of avoidance (repellency in a non-forced approach) and mortality (toxicity in a forced approach) could help us to understand to what extent the population will decrease better, by considering the proportion of potential avoiders and the proportion of fatalities expected to occur in the non-avoider population. Local extinction rates are affected by spatial heterogeneity and migration rates [122]. The increase in mortality rates, due to

toxicity, together with the increase in emigrant rate, would lead to an increased local extinction rate and reduce the probability of local persistence.

6.2. Potential to Predict the (Re)Colonization of Environments

Another interesting concept to be employed in this approach is about (re)colonization and restoration of disturbed environments. Generally, ecotoxicity studies are focused on contaminated environments, and so less attention has been given to ecosystem recovery. The concept of colonization in non-forced exposure studies may be used to understand the threshold of the contamination that allows individuals to move from a clean area to an area with acceptable levels of contamination [79,123]. The idea is to identify the levels of contamination that prevent colonization, a chemical threshold from which colonization is less probable or prevented completely. According to the "avoidance-recolonisation hypothesis" [123], the capacity of an ecosystem to receive individuals could be predicted by avoidance tests as follows: if x% of the population avoids a given level of contamination, it is expected that a proportion of 100-x% of the population colonizes an environment with that level of contamination. However, it is unlikely this relation would be so linear as other factors may affect the decision to avoid or not an area ([18,79] see also the discussion in Section 5 of the current review). Furthermore, the repellency and attraction of the chemicals can present a non-linear pattern due to the hormetic effects [38,124]. We encourage the application of the colonization concept as a measure of an ecosystem's ability to recover from a disturbance, as well as of the organisms' emigration/immigration patterns. This approach could provide insights about the species that can (or not) potentially colonize an area, which would allow researchers to predict the ecological implications that colonization might represent to the ecosystem. The multi-compartmented approach is an alternative method that may be used to integrate the conceptual model of an affected community based on the dynamics of invader/remainer/escaper [18].

6.3. Chemical Fragmentation of Habitat

Habitat fragmentation occurs as result of a discontinuity of the habitat, generally linked to a physical barrier that isolates populations. However, a habitat can be chemically fragmented if the levels of chemicals present in some areas limit the displacement of organisms, even when there is no physical barrier [120,125]. To our knowledge, the application of the concept of chemical fragmentation of habitat using multi-compartmented exposure systems has only taken place in the studies by Araújo et al. [58,74] and Islam et al. [79]. In Araújo et al. [74], the authors showed that the fish *P. reticulata* avoided the herbicide atrazine and that the concentration (105 µg/L), eliciting an avoidance of 80%, produced an isolation of around 50% of the population. A similar study using the fish *D. rerio* and copper showed that a concentration (90 µg/L) that elicited an avoidance to 50% of the population led to the isolation of 41% of that population [79]. This percentage did not vary when food was provided on the clean side, probably because the organisms could not perceive it until after crossing the chemical barrier. The chemical fragmentation of the habitat was also tested with samples of water and sediment from the river Guadalete (Southwest of Spain) [58]. The authors took samples from different parts of the river and simulated the sampled points in a multi-compartmented system. The experimental results evidenced that contamination in both water and sediment might potentially cause a population isolation of the freshwater shrimp *A. desmarestii* that was unable to cross the chemical barrier formed by the most contaminated samples [58]. The possibility of using the concept of chemical barrier in ecotoxicological studies would help to understand another role of contamination that disturbs the ecosystem's equilibrium and interrupts spatial continuity. The chemical fragmentation of a habitat is a theme that deserves special attention because environmental restrictions can lead to local extinctions, due to the reduction of individuals causing genetic erosion, which may increase the vulnerability of the population [125,126].

In isolated populations, due to habitat restrictions, the risk of extinction is increased and can occur in two main ways (following the extinction vortex model by Gilpin [127]): (i) the allogenic

vortex driven by change in the environment (pollution) and (ii) autogenic vortex, driven by population genetics (population isolation leads to a smaller gene pool and the loss of adaptability to environmental change/disturbance).

6.4. Habitat Connectivity, Metapopulation, Metacommunity, and Meta-Ecosystem

One of the indirect consequences of contamination is the loss of continuity of habitats that present a patchy distribution in terms of environmental quality. In some circumstances, environmental heterogeneity induces populations to form a spatial arrangement such as metapopulation, moving among habitats with different conditions and, therefore, transferring matter and energy among them (meta-ecosystem) [5,128,129]. Initially, the strategy of avoiding contamination may be successful environmentally due to the absence of stress at the individual level as previously discussed. However, the consequences of this change in the arrangement of the populations could affect the ecosystem where the organisms moved to, since these new individuals might cause some changes in the ecological relationships in that ecosystem [18,130].

The loss of individuals due to avoidance can have important ecological implications on the structure and functioning of ecosystems. The avoidance by the most sensitive species (regarding the ability to detect a contaminant) might lead to indirect effects on other more resistant species (and even on species that cannot flee), due to creating an imbalance in the community, affecting not only the species with which the avoiders have a direct relationship (e.g., predator–prey relationship), but also ecological interactions and even biogeochemical cycles (e.g., energy flow and nutrient cycling) due to the reduction or absence of key species for some ecosystem functions. In this sense, it would be interesting to know how the avoidance of organisms belonging to different trophic levels influences the functioning of the ecosystem, although these ecological questions might require a different experimental approach.

The use of systems such as HeMHAS favors an understanding of the importance that uncontaminated zones might represent as potential areas (refuges) to protect populations against contamination and alleviate individuals from a continuous stress [39]. These authors showed experimentally that in patchy contamination scenarios, the shrimps *A. desmarestii* could present a distribution partially conditioned by copper contamination and dependent on the presence of clean areas in the environment. The complexity of the experimentation systems, such as HeMHAS [52], is crucial to simulate more chemically complex scenarios and understand a little more about the consequences caused by contamination concerning the spatial distribution of organisms and the probability of populations persisting in spite of disturbance [131]. Gilarranz and colleagues showed how a system simulating patches with different levels of disturbance could help to elucidate the effect of the discontinuity of habitats on the maintenance of the populations and how the increase in the disturbance could increase the probability of extinction [131]. Thus, within a connected and heterogeneous landscape, two important questions need to be answered: i. how determinant the differences in the ability to avoid among species of a metacommunity are to the structure the local communities and ii. to what extent the behavioral traits related to avoiding or not contamination could be explained by genetic differences and sensory abilities?

7. Final Remarks

As discussed by Ågerstrand et al. [12], the use of ecotoxicological data in regulatory assessments have been based on endpoints such as mortality, growth, reproduction, and development, basically because such responses can lead to population decline. Although this simplifies the application of ecotoxicity tests for a regulatory basis, no other evidence of stress (either to biochemical stress or behavioral alterations) is considered. However, authors and organizations are requesting the inclusion of tests with a higher ecological relevance in the risk assessment of chemical substances [132–134]. From a conceptual point of view, these endpoints (mortality, growth, reproduction, and development) are directly related to the toxicity of chemicals, following the cascade of effects [40,41] that are triggered when organisms are exposed to contaminants continuously (cascade of effects related to toxicity).

Considering that some organisms cannot avoid contamination (cascade of effects related to repellency), either because of their inability to move or because the spatial scale of contamination is spread beyond the area to which they could move, the focus on toxicity is appropriate and ecologically relevant. However, for mobile organisms and in a heterogeneous contamination scenario, the potential repellency of the chemicals should also be considered. The application of this repellency as an endpoint for regulatory application could be easily justified since the evasion of organisms might lead to a population decline at the local level [18,30]. As discussed in the current review, this displacement might cause disturbances at the structural and functional levels, not only in the ecosystems avoided, but also in the alternative chosen one. This approach extends the concept of stress to a level beyond the individual response [1,18], integrating the susceptibility of organisms (that can require adaptation or lead to a loss of the most sensitive species) and environmental vulnerability (see Figure 1 and discussion in [6,7,12,18]). In addition, the repellency response is expected to be immediate (normally after not more than a 12 h exposure) [70], which could help reduce misunderstandings related to a time-delayed effect due to a continuous and extended exposure [70].

Although the theoretical basis that could justify the use of behavioral responses has been recognized [21,135,136], the practical application of this approach is criticized mainly for the lack of standard protocols that could help minimize the errors associated to observation [12]. However, the implementation of automatic systems has contributed to increasing the validity of traditional behavioral responses [12,137]. For tests with aquatic organisms in non-forced multi-compartmented systems some attempts have been made to standardize the procedures, namely with the publication of a standard operating procedure for linear systems [123] and the development of the HeMHAS [52], but much more effort is still required.

The final reason why behavioral ecotoxicology is not employed for a regulatory basis can be sustained by the lack of results using behavioral endpoints. In the case of avoidance measured in a multi-compartmented exposure system, this lack is even greater. Therefore, with this review we have intended not only to demonstrate that repellency can trigger an ecologically relevant response such as spatial avoidance, but also to encourage studies using the non-forced multi-compartmented approach to improve our understanding of the spatial distribution of organisms driven by contamination. The aim of the approach presented here is to integrate Stress Ecology and Landscape Ecology, considering contaminants as one more element of ecosystems, from a more ecological perspective (habitat selection processes and the potential interactions with biotic and abiotic factors to "take the decision" of staying or avoiding a habitat), and broadening the spatial scale (landscape) of the observation, considering not only the contaminated and avoided ecosystem, but also the surrounding areas receiving the avoiders (environmental heterogeneity). Finally, such as indicated in Figure 1, we strongly support the integration of toxicity (when the effects are based on the sensitivity of organisms) and repellency (when the organisms change the habitat selected according to the levels of contamination) to achieve a conceptually broader environmental assessment.

Author Contributions: All authors contributed substantially for the current review. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by the Spanish Ministry of Science and Innovation.

Acknowledgments: C.V.M.A. received the Ramón y Cajal contract (RYC-2017-22324) from the Spanish Ministry of Science and Innovation. This study was performed within the framework of the projects: MultiCecotox project (i-COOP2019 program from CSIC: #COOPB20444) and BrEStress project (PID2019-105868RA-I00) and CaPETHaS project (Explora call: #CGL2017-92160-EXP)—both funded by the Spanish Ministry of Science and Innovation). We thank Jon Nesbit for his revision of the English text.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Beketov, M.A.; Liess, M. Ecotoxicology and macroecology—Time for integration. *Environ. Pollut.* **2012**, *162*, 247–254. [CrossRef] [PubMed]
2. Truhaut, R. Ecotoxicology: Objectives, principles and perspectives. *Ecotoxicol. Environ. Saf.* **1977**, *1*, 151–157. [CrossRef]
3. Cairns, J., Jr. Are single species toxicity tests alone adequate for estimating environmental hazard? *Environ. Monitor. Assess.* **1984**, *4*, 259–273. [CrossRef] [PubMed]
4. Chapman, P.M. Integrating toxicology and ecology: Putting the "eco" into ecotoxicology. *Mar. Pollut. Bull.* **2002**, *44*, 7–15. [CrossRef]
5. Fleeger, J.W.; Carman, K.R.; Nisbet, R.M. Indirect effects of contaminants in aquatic ecosystems. *Sci. Total Environ.* **2003**, *317*, 207–233. [CrossRef]
6. Clements, W.H.; Rohr, J.R. Community responses to contaminants: Using basic ecological principles to predict ecotoxicological effects. *Environ. Toxicol. Chem.* **2009**, *28*, 1789–1800. [CrossRef]
7. De Lange, H.J.; Sala, S.; Vighi, M.; Faber, J.H. Ecological vulnerability in risk assessment—A review and perspectives. *Sci. Total Environ.* **2010**, *408*, 3871–3879. [CrossRef]
8. Rico, A.; Van den Brink, P.J.; Gylstra, R.; Focks, A.; Brock, T.C.M. Developing ecological scenarios for the prospective aquatic risk assessment of pesticides. *Integr. Environ. Assesss.* **2015**, *12*, 510–521. [CrossRef]
9. Van Straalen, N.M. Ecotoxicology becomes Stress Ecology. *Environ. Sci. Technol.* **2003**, *37*, 324A–330A. [CrossRef]
10. Van den Brink, P.J. Ecological risk assessment: From book-keeping to chemical stress ecology. *Environ. Sci. Technol.* **2008**, *42*, 8999–9004. [CrossRef]
11. Moreira-Santos, M.; Ribeiro, R.; Araújo, C.V.M. What if aquatic animals move away from pesticide-contaminated habitats before suffering adverse physiological effects? A critical review. *Crit. Rev. Environ. Sci. Technol.* **2019**, *49*, 989–1025. [CrossRef]
12. Ågerstrand, M.; Arnold, K.; Balshine, S.; Brodin, T.; Brooks, B.W.; Maack, G.; McCallum, E.S.; Pyle, G.; Saaristo, M.; Ford, A.T. Emerging investigator series: Use of behavioural endpoints in the regulation of chemicals. *Environ. Sci. Process. Impacts* **2020**, *22*, 49–65. [CrossRef] [PubMed]
13. Cairns, J., Jr.; Niederlehner, B.R. Developing a field of landscape ecotoxicology. *Ecol. Appl.* **1996**, *6*, 790–796. [CrossRef]
14. Spromberg, J.A.; John, B.M.; Landis, W.G. Metapopulation dynamics: Indirect effects and multiple distinct outcomes in ecological risk assessment. *Environ. Toxicol. Chem.* **1998**, *17*, 1640–1649. [CrossRef]
15. Johnson, R.A. Landscape ecotoxicology and assessment of risk at multiple scales. *Hum. Ecol. Risk Assess.* **2002**, *8*, 127–146. [CrossRef]
16. Rohr, J.R.; Kerby, J.L.; Sih, A. Community ecology as a framework for predicting contaminant effects. *Trends Ecol. Evol.* **2006**, *21*, 606–613. [CrossRef] [PubMed]
17. Schmitt-Jansen, M.; Veit, U.; Dudel, G.; Altenburger, R. An ecological perspective in aquatic ecotoxicology: Approaches and challenges. *Basic Appl. Ecol.* **2008**, *9*, 337–345. [CrossRef]
18. Moe, S.J.; De Schamphelaere, K.; Clements, W.H.; Sorensen, M.T.; Van Den Brink, P.J.; Liess, M. Combined and interactive effects of global climate change and toxicants on populations and communities. *Environ. Toxicol. Chem.* **2013**, *32*, 49–61. [CrossRef]
19. Araújo, C.V.M.; Moreira-Santos, M.; Ribeiro, R. Active and passive spatial avoidance by aquatic organisms from environmental stressors: A complementary perspective and a critical review. *Environ. Int.* **2016**, *92–93*, 405–415. [CrossRef]
20. Araújo, C.V.M.; Blasco, J. Spatial avoidance as a response to contamination by aquatic organisms in non-forced, multi-compartmented exposure systems: A complementary approach to the behavioral response. *Environ. Toxicol. Chem.* **2019**, *38*, 312–320. [CrossRef]
21. Hellou, J. Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. *Environ. Sci. Pollut. Res.* **2011**, *18*, 1–11. [CrossRef] [PubMed]
22. Jutfelt, F.; Sundin, J.; Raby, G.D.; Krang, A.-S.; Clark, T.D. Two-current choice flumes for testing avoidance and preference in aquatic animals. *Methods Ecol. Evol.* **2017**, *8*, 379–390. [CrossRef]

23. Cao, J.-Q.; Guo, S.-S.; Wang, Y.; Pang, X.; Geng, Z.-F.; Du, S.-S. Toxicity and repellency of essential oil from *Evodia lenticellata* Huang fruits and its major monoterpenes against three stored-product insects. *Ecotoxicol. Environ. Saf.* **2018**, *160*, 342–348. [CrossRef] [PubMed]
24. Padilha, A.C.; Piovesan, B.; Morais, M.C.; Arioli, C.J.; Zotti, M.J.; Grützmacher, A.D.; Botton, M. Toxicity, attraction, and repellency of toxic baits to stingless bees *Plebeia emerina* (Friese) and *Tetragonisca fiebrigi* (Schwarz) (Hymenoptera: Apidae: Meliponini). *Ecotoxicol. Environ. Saf.* **2019**, *183*, 109490. [CrossRef]
25. Araújo, C.V.M.; Rodríguez-Romero, A.; Fernández, M.; Sparaventi, E.; Medina, M.M.; Tovar-Sánchez, A. Repellency and mortality effects of sunscreens on the shrimp *Palaemon varians*: Toxicity dependent on exposure method. *Chemosphere* **2020**, *257*, 127190. [CrossRef]
26. Ruuskanen, S.; Rainio, M.J.; Kuosmanen, V.; Laihonen, M.; Saikkonen, K.; Saloniemi, I.; Helander, M. Female preference and adverse developmental effects of glyphosate-based herbicides on ecologically relevant traits in Japanese quails. *Environ. Sci. Technol.* **2020**, *54*, 128–135. [CrossRef]
27. Åtland, Å.; Barlaup, B.T. Avoidance of toxic mixing zones by atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) in the limed river Audna, southern Norway. *Environ. Pollut.* **1995**, *90*, 203–208. [CrossRef]
28. Hansen, J.A.; Woodward, D.F.; Little, E.E.; DeLonay, A.J.; Bergman, H.L. Behavioral avoidance: Possible mechanism for explaining abundance and distribution of trout species in a metal-impacted river. *Environ. Toxicol. Chem.* **1999**, *18*, 313–317. [CrossRef]
29. Carlsen, T.N.; Coty, J.D.; Kercher, J.R. The spatial extent of contaminants and landscape scale: An analysis of the wildlife, conservation biology, and population modeling literature. *Environ. Toxicol. Chem.* **2004**, *23*, 798–811. [CrossRef]
30. Lopes, I.; Baird, D.J.; Ribeiro, R. Avoidance of copper contamination by field populations of *Daphnia longispina*. *Environ. Toxicol. Chem.* **2004**, *23*, 1702–1708. [CrossRef]
31. Clements, W.H.; Hickey, C.W.; Kidd, K.A. How do aquatic communities respond to contaminants? It depends on the ecological context. *Environ. Toxicol. Chem.* **2012**, *31*, 1932–1940. [CrossRef] [PubMed]
32. Dahms, H.-U. Dormancy in the Copepoda—An overview. *Hydrobiologia* **1995**, *306*, 199–211. [CrossRef]
33. Radzikowski, J. Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. *J. Plankton Res.* **2013**, *35*, 707–723. [CrossRef]
34. De Stasio, B.T., Jr. The role of dormancy and emergence patterns in the dynamics of a freshwater zooplankton community. *Limnol. Oceanogr.* **1990**, *35*, 1079–1090. [CrossRef]
35. Vandekerkhove, L.; Declerck, S.; Jeppesen, E.; Conde-Porcuma, J.M.; Brendonck, L.; De Meester, L. Dormant propagule banks integrate spatio-temporal heterogeneity in cladoceran communities. *Oecologia* **2005**, *142*, 109–116. [CrossRef]
36. Portinho, J.L.; Nielsen, D.L.; Daré, L.; Henry, R.; Oliveira, R.C.; Branco, C.C.Z. Mixture of commercial herbicides based on 2,4-D and glyphosatemixture can suppress the emergence of zooplankton from sediment. *Chemosphere* **2018**, *203*, 151–159. [CrossRef]
37. Hadjinicolaou, J.; LaRoche, G. Behavioral Responses to Low Levels of Toxic Substances in Rainbow Trout (*Salmo Gairdneri*, Rich). In *Aquatic Toxicology and Hazard Assessment*; Adams, W., Chapman, G., Landis, W., Eds.; ASTM International: West Conshohocken, PA, USA, 1988; Volume 10, pp. 327–340.
38. Costantini, D.; Metcalfe, N.B.; Monaghan, P. Ecological processes in a hermetic framework. *Ecol. Lett.* **2010**, *13*, 1435–1447. [CrossRef]
39. Vera-Vera, V.C.; Guerrero, F.; Blasco, J.; Araújo, C.V.M. Habitat selection response of the freshwater shrimp *Atyaephyra desmarestii* experimentally exposed to heterogeneous copper contamination scenarios. *Sci. Total Environ.* **2019**, *662*, 816–823. [CrossRef]
40. Newman, M.C.; Unger, M.A. *Population Ecotoxicology*, 2nd ed.; Lewis Publishers: Boca Raton, FL, USA, 2001; p. 458.
41. Walker, C.H.; Hopkin, S.P.; Sibly, R.M.; Peakall, D.B. *Principles of Ecotoxicology*, 2nd ed.; Taylor & Francis: Boca Raton, FL, USA, 2001; p. 309.
42. Cairns, J., Jr. Putting eco into ecotoxicology. *Regul. Toxicol. Pharmacol.* **1988**, *8*, 226–238. [CrossRef]
43. Koivisto, S. Is *Daphnia magna* an ecologically representative zooplankton species in toxicity tests? *Environ. Pollut.* **1995**, *90*, 263–267. [CrossRef]

44. Martinez-Haro, M.; Beiras, R.; Bellas, J.; Capela, R.; Pedro, J.; Lopes, I.; Moreira-Santos, M.; Reis-Henriques, A.M.; Ribeiro, R.; Santos, M.M.; et al. A review on the ecological quality status assessment in aquatic systems using community based indicators and ecotoxicological tools: What might be the added value of their combination? *Ecol. Indic.* **2015**, *48*, 8–16. [CrossRef]
45. Johnston, E.L.; Mayer-Pinto, M.; Crowe, T.P. Chemical contaminant effects on marine ecosystem functioning. *J. Appl. Ecol.* **2015**, *52*, 140–149. [CrossRef]
46. Willmer, P.; Stone, G.; Johnston, I. *Environmental Physiology of Animals*; Blackwell Science: Osney Mead, UK, 2000; p. 644.
47. Nakamura, F. Avoidance behavior and swimming activity of fish to detect pH changes. *Bull. Environ. Contam. Toxicol.* **1986**, *37*, 808–815. [CrossRef] [PubMed]
48. Ren, Z.; Zha, J.; Ma, M.; Wang, Z.; Gerhardt, A. The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioral changes of *Daphnia magna*. *Environ. Monit. Assess.* **2007**, *134*, 373–383. [CrossRef] [PubMed]
49. Oliveira, C.; Almeida, J.R.; Guilhermino, L.; Soares, A.M.V.M.; Gravato, C. Swimming velocity, avoidance behavior and biomarkers in *Palaemon serratus* exposed to fenitrothion. *Chemosphere* **2013**, *90*, 936–944. [CrossRef]
50. Tierney, K.B. Chemical avoidance responses of fish. *Aquat. Toxicol.* **2016**, *174*, 228–241. [CrossRef]
51. Rosa, R.; Materatski, P.; Moreira-Santos, M.; Sousa, J.P.; Ribeiro, R. A scaled-up system to evaluate zooplankton spatial avoidance and population immediate decline concentration. *Environ. Toxicol. Chem.* **2012**, *31*, 1301–1305. [CrossRef]
52. Araújo, C.V.M.; Roque, D.; Blasco, J.; Ribeiro, R.; Moreira-Santos, M.; Toribio, A.; Aguirre, E.; Barro, S. Stress-driven emigration in a complex scenario of habitat disturbance: The heterogeneous multi-habitat assay system (HeMHAS). *Sci. Total Environ.* **2018**, *644*, 31–36.
53. Rosa, R.; Moreira-Santos, M.; Lopes, I.; Picado, A.; Mendonça, F.; Ribeiro, R. Development and sensitivity of a 12-h laboratory test with *Daphnia magna* Straus based on avoidance of pulp mill effluents. *Bull. Environ. Contam. Toxicol.* **2008**, *81*, 464–469. [CrossRef]
54. Venâncio, C.; Ribeiro, R.; Lopes, I. Active emigration from climate change-caused seawater into freshwater habitats. *Environ. Pollut.* **2020**, *258*, 113805. [CrossRef]
55. Araújo, C.V.M.; Moreira-Santos, M.; Sousa, J.P.; Ochoa-Herrera, V.; Encalada, A.C.; Ribeiro, R. Active avoidance from a crude oil soluble fraction by an Andean paramo copepod. *Ecotoxicology* **2014**, *23*, 1254–1259. [CrossRef] [PubMed]
56. Araújo, C.V.M.; Martínez-Haro, M.; Pais-Costa, A.J.; Marques, J.C.; Ribeiro, R. Patchy sediment contamination scenario and the habitat selection by an estuarine mudsnail. *Ecotoxicology* **2016**, *25*, 412–418. [CrossRef] [PubMed]
57. Araújo, C.V.M.; Pereira, K.C.; Blasco, J. Avoidance response by shrimps to a copper gradient: Does high population density prevent avoidance of contamination? *Environ. Toxicol. Chem.* **2018**, *37*, 3095–3101. [CrossRef] [PubMed]
58. Araújo, C.V.M.; González-Ortegón, E.; Pintado-Herrera, M.; Biel-Maeso, M.; Lara-Martín, P.; Tovar-Sánchez, A.; Blasco, J. Disturbance of ecological habitat distribution driven by a chemical barrier of domestic and agricultural discharges: An experimental approach to test habitat fragmentation. *Sci. Total Environ.* **2019**, *651*, 2820–2829. [CrossRef] [PubMed]
59. Araújo, C.V.M.; Pereira, K.C.; Sparaventi, E.; González-Ortegón, E.; Blasco, J. Contamination may induce behavioural plasticity in the habitat selection by shrimps: A cost-benefits balance involving contamination, shelter and predation. *Environ. Pollut.* **2020**, *263*, 114545. [CrossRef]
60. Araújo, C.V.M.; Cedeño-Macias, L.A.; Vera-Vera, V.C.; Salvatierra, D.; Rodríguez, E.N.V.; Zambrano, U.; Kuri, S. Predicting the effects of copper on local population decline of 2 marine organisms, cobia fish and whiteleg shrimp, based on avoidance response. *Environ. Toxicol. Chem.* **2016**, *35*, 405–410. [CrossRef] [PubMed]
61. Mena, F.; González-Ortegón, E.; Solano, K.; Araújo, C.V.M. The effect of the insecticide diazinon on the osmoregulation and the avoidance response of the white leg shrimp (*Litopeneaus vannamei*) is salinity dependent. *Ecotocol. Environ. Saf.* **2020**, *206*, 111364. [CrossRef]

62. Ehiguese, F.O.; Corada Fernandez, M.C.; Lara-Martín, P.A.; Martín-Díaz, M.L.; Araújo, C.V.M. Avoidance behaviour of the shrimp *Palaemon varians* regarding a contaminant gradient of galaxolide and tonalide in seawater. *Chemosphere* **2019**, *232*, 113–120. [CrossRef]
63. Araújo, C.V.M.; Shinn, C.; Moreira-Santos, M.; Lopes, I.; Espíndola, E.L.G.; Ribeiro, R. Cooper-driven avoidance and mortality in temperate and tropical tadpoles. *Aquat. Toxicol.* **2014**, *146*, 70–75. [CrossRef]
64. Araújo, C.V.M.; Shinn, C.; Vasconcelos, A.M.; Ribeiro, R.; Espíndola, E.L.G. Preference and avoidance responses by tadpoles: The fungicide pyrimethanil as a habitat disturber. *Ecotoxicology* **2014**, *23*, 851–860. [CrossRef]
65. Vasconcelos, A.M.; Daam, M.A.; dos Santos, L.R.A.; Sanches, A.L.M.; Araújo, C.V.M.; Espíndola, E.L.G. Acute and chronic sensitivity, avoidance behavior and sensitive life stages of bullfrog tadpoles exposed to the biopesticide abamectin. *Ecotoxicology* **2016**, *25*, 500–509. [CrossRef] [PubMed]
66. Freitas, J.S.; Girotto, L.; Goulart, B.V.; Alho, L.O.G.; Gebara, R.C.; Montagner, C.C.; Schiesari, L.; Espíndola, E.L.G. Effects of 2,4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. *Ecotoxicol. Environ. Saf.* **2019**, *182*, 109446. [CrossRef] [PubMed]
67. Moreira, R.A.; Freitas, J.S.; Pinto, T.J.S.; Schiesari, L.; Damm, M.A.; Montagner, C.C.; Goulart, B.V.; Espíndola, E.L.G. Mortality, spatial avoidance and swimming behavior of bullfrog tadpoles (*Lithobates catesbeianus*) exposed to the herbicide diuron. *Water Air Soil Pollut.* **2019**, *230*, 125. [CrossRef]
68. Girotto, L.; Espíndola, E.L.G.; Gebara, R.C.; Freitas, J.S. Acute and chronic effects on tadpoles (*Lithobates catesbeianus*) exposed to mining tailings from the dam rupture in Mariana, MG (Brazil). *Water Air Soil Pollut.* **2020**, *231*, 325. [CrossRef]
69. Costa, B.; Quintaneiro, C.; Daniel-da-Silva, A.L.; Trindade, T.; Soares, A.M.V.M.; Lopes, I. An integrated approach to assess the sublethal effects of coloidal gold nanorods in tadpoles of *Xenopus laevis*. *J. Hazard. Mater.* **2020**, *400*, 123237. [CrossRef] [PubMed]
70. Moreira-Santos, M.; Donato, C.; Lopes, I.; Ribeiro, R. Avoidance tests with small fish: Determination of the median avoidance concentration and of the lowest-observed-effect gradient. *Environ. Toxicol. Chem.* **2008**, *27*, 1576–1582. [CrossRef]
71. Araújo, C.V.M.; Shinn, C.; Mendes, L.B.; Delello-Scheneider, D.; Sanchez, A.L.; Espíndola, E.L.G. Avoidance response of *Danio rerio* to a fungicide in a linear contamination gradient. *Sci. Total Environ.* **2014**, *484*, 36–42. [CrossRef]
72. Araújo, C.V.M.; Rodríguez, E.N.V.; Salvatierra, D.; Cedeño-Macias, L.A.; Vera-Vera, V.C.; Moreira-Santos, M.; Ribeiro, R. Attractiveness of food and avoidance from contamination as conflicting stimuli to habitat selection by fish. *Chemosphere* **2016**, *163*, 177–183. [CrossRef]
73. Silva, D.C.V.R.; Araújo, C.V.M.; López-Doval, J.C.; Neto, M.B.; Silva, F.T.; Paiva, T.C.B.; Pampêo, M.L.M. Potential effects of triclosan on spatial displacement and local population decline of the fish *Poecilia reticulata* using a nonforced system. *Chemosphere* **2017**, *184*, 329–336. [CrossRef]
74. Araújo, C.V.M.; Silva, D.C.V.R.; Gomes, L.E.T.; Acayaba, R.D.; Montagner, C.C.; Moreira-Santos, M.; Ribeiro, R.; Pompêo, M.L.M. Habitat fragmentation caused by contaminants: Atrazine as a chemical barrier isolating fish populations. *Chemosphere* **2018**, *193*, 24–31. [CrossRef]
75. Araújo, C.V.M.; Griffith, D.M.; Vera-Vera, V.C.; Jentzsch, P.V.; Cervera, L.; Nieto-Ariza, B.; Salvatierra, D.; Erazo, S.; Jaramillo, R.; Ramos, L.A.; et al. A novel approach to assessing environmental disturbance based on habitat selection by zebra fish as a model organism. *Sci. Total Environ.* **2018**, *619–620*, 906–915. [CrossRef] [PubMed]
76. Silva, D.C.V.R.; Queiroz, L.G.; Marassi, R.J.; Araújo, C.V.M.; Bazzan, T.; Cardoso-Silva, S.; Silva, G.C.; Müller, M.; Silva, F.T.; Montagner, C.C.; et al. Predicting zebrafish spatial avoidance triggered by discharges of dairy wastewater: An experimental approach based on self-purification in a model river. *Environ. Pollut.* **2020**, *266*, 115325. [CrossRef] [PubMed]
77. Silva, D.C.V.R.; Araújo, C.V.M.; França, F.M.; Neto, M.B.; Paiva, T.C.B.; Silva, F.T.; Pampêo, M.L.M. Bisphenol risk in fish exposed to a contamination gradient: Triggering of spatial avoidance. *Aquat. Toxicol.* **2018**, *197*, 1–6. [CrossRef] [PubMed]
78. Silva, D.C.V.R.; Araújo, C.V.M.; Marassi, R.J.; Cardoso-Silva, S.; Neto, M.B.; Silva, G.C.; Ribeiro, R.; Silva, F.T.; Paiva, T.C.B.; Pampêo, M.L.M. Influence of interspecific interactions on avoidance response to contamination. *Sci. Total Environ.* **2018**, *642*, 824–831. [CrossRef]

79. Islam, M.A.; Blasco, J.; Araújo, C.V.M. Spatial avoidance, inhibition of recolonization and population isolation in zebrafish (*Danio rerio*) caused by copper exposure under a non-forced approach. *Sci. Total Environ.* **2019**, *653*, 504–511. [CrossRef]
80. Moreira, R.A.; Araújo, C.V.M.; Pinto, T.J.S.; Silva, L.C.M.; Goulart, B.V.; Viana, N.P.; Montagner, C.C.; Fernandes, M.N.; Espíndola, E.L.G. Fipronil and 2,4-D effects on tropical fish: Could avoidance response by explained by the changes in swimming behavior and neurotransmission impairments? *Chemosphere* **2021**, *263*, 127972. [CrossRef]
81. Tierney, K.B.; Sekela, M.A.; Cobbler, C.E.; Xhabija, B.; Gledhill, M.; Ananvoranich, S.; Zielinski, B.S. Evidence for behavioral preference toward environmental concentrations of urban-use herbicides in a model adult fish. *Environ. Toxicol. Chem.* **2011**, *30*, 2046–2054. [CrossRef]
82. Woodman, S.G.; Steinkey, D.; Dew, W.A.; Burket, S.R.; Brooks, B.W.; Pyle, G.G. Effects of sertraline on behavioral indices of crayfish *Orconectes virilis*. *Ecotoxicol. Environ. Saf.* **2016**, *134*, 31–37. [CrossRef]
83. Hellou, J.; Cook, A.; Lalonde, B.; Walker, P.; Dunphy, K.; Macleod, S. Escape and survival of *Corophium volutator* and *Ilyanassa obsoleta* exposed to freshwater and chlorothalonil. *J. Environ. Sci. Health A* **2009**, *44*, 778–790. [CrossRef]
84. Blinova, N.K.; Cherkashin, S.A. The olfactory system of crustaceans as a model for ecologo-toxicological studies. *J. Evol. Biochem. Phys.* **2012**, *48*, 155–165. [CrossRef]
85. Volz, S.; Schiwy, S.; Hollert, H. Olfactory toxicity in fish—Why we should care about it. *Interg. Environ. Assess. Manag.* **2016**, *12*, 597–598. [CrossRef] [PubMed]
86. Kasumyan, A.O. Effects of chemical pollutants on foraging behavior and sensitivity of fish to food stimuli. *J. Ichthyol.* **2001**, *41*, 76–87.
87. Scott, G.R.; Sloman, K.A. The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* **2004**, *68*, 369–392. [CrossRef] [PubMed]
88. Cherkashin, S.A.; Blinova, N.K. Effect of heavy metals on chemoreception and behavior of crustaceans (a review). *Hydrob. J.* **2011**, *47*, 83–93. [CrossRef]
89. Tierney, K.B.; Singh, C.R.; Ross, P.S.; Kennedy, C.J. Relating olfactory neurotoxicity to altered olfactory-mediated behaviors in rainbow trout exposed to three currently-used pesticides. *Aquat. Toxicol.* **2007**, *81*, 55–64. [CrossRef] [PubMed]
90. Gosavi, S.M.; Tapkir, S.D.; Kumkar, P.; Verma, C.R.; Kharat, S.S. Act now before its too late: Copper exposure drives chemo-ecology of predator-prey dynamics of freshwater common spiny loach, *Lepidocephalichthys thermalis* (Valenciennes, 1846). *Environ. Res.* **2020**, *186*, 109509. [CrossRef]
91. Tierney, K.B.; Baldwin, D.H.; Hara, T.J.; Ross, P.S.; Scholz, N.L.; Kennedy, C.J. Olfactory toxicity in fishes. *Aquat. Toxicol.* **2010**, *96*, 2–26. [CrossRef]
92. Díaz-Gil, C.; Cotgrove, L.; Smee, S.L.; Simón-Otegui, D.; Hinz, H.; Grau, A.; Palmer, M.; Catalán, I.A. Anthropogenic chemical cues can alter the swimming behaviour of juvenile stages of a temperate fish. *Mar. Environ. Res.* **2017**, *125*, 34–41. [CrossRef]
93. García-Muñoz, E.; Guerrero, F.; Parra, G. Larval escape behavior in anuran amphibians as a wetland rapid pollution biomarker. *Mar. Fresh. Behav. Physiol.* **2011**, *44*, 109–123. [CrossRef]
94. Cowgill, U.M. Critical analysis of factors affecting the sensitivity of zooplankton and the reproducibility of toxicity test results. *Wat. Res.* **1987**, *21*, 1453–1462. [CrossRef]
95. Moore, D.R.J.; Warren-Hicks, W.; Parkhurst, B.R.; Scott Teed, R.; Baird, R.B.; Berger, R.; Denton, D.L.; Pletl, J.J. Intra- and inter treatment variability in reference toxicant tests: Implications for whole effluent toxicity testing programs. *Environ. Toxicol. Chem.* **2000**, *19*, 105–112. [CrossRef]
96. Bretaud, S.; Toutant, J.P.; Saglio, P. Effects of carbofuran, diuron, and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassius auratus*). *Ecotoxicol. Environ. Saf.* **2000**, *47*, 117–124. [CrossRef] [PubMed]
97. Wang, Y.; Wu, S.; Chen, J.; Zhang, C.; Xu, Z.; Li, G.; Cai, L.; Shen, W.; Wang, Q. Single and joint toxicity assessment of four currently used pesticides to zebrafish (*Danio rerio*) using traditional and molecular endpoints. *Chemosphere* **2018**, *192*, 14–23. [CrossRef] [PubMed]
98. Bridi, D.; Altenhofen, S.; Gonzalez, G.B.; Reolon, G.K.; Bonan, C.D. Glyphosate and Roundup® alter morphology and behavior in zebrafish. *Toxicology* **2017**, *392*, 32–39. [CrossRef] [PubMed]
99. Rajkumar, J.S.I.; John Milton, M.C.; Ulthiralingam, M.; Azhaguraj, R.; Ganesh, J.; Ambrose, T. Toxic effects and bioaccumulation of cadmium, copper, lead and zinc in post larval stages of *Penaeus monodon*. *Int. J. Dev. Res.* **2011**, *1*, 1–5.

100. DeLorenzo, M.E.; Keller, J.M.; Arthur, C.D.; Finnegan, M.C.; Harper, H.E.; Winder, V.L.; Zdankiewicz, D.L. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environ. Toxicol.* **2008**, *23*, 224–232. [CrossRef]
101. Araújo, C.V.M.; Gómez, L.; Silva, D.C.V.R.; Pintado-Herrera, M.G.; Lara-Martín, P.A.; Hampel, M.; Blasco, J. Risk of triclosan based on avoidance by the shrimp *Palaemon varians* in a heterogeneous contamination scenario: How sensitive is this approach? *Chemosphere* **2019**, *235*, 126–135. [CrossRef]
102. Kovrižnych, J.A.; Sotníková, R.; Zeljenková, D.; Rollerová, E.; Szabová, E.; Wimmerová, S. Acute toxicity of 31 different nanoparticles to zebrafish (*Danio rerio*) tested in adulthood and in early life stages—Comparative study. *Interdiscip. Toxicol.* **2013**, *6*, 67–73. [CrossRef]
103. Hernandez, P.P.; Undurraga, C.; Gallardo, V.E.; Mackenzie, N.; Allende, M.L.; Reyes, A.E. Sublethal concentrations of waterborne copper induce cellular stress and cell death in zebrafish embryos and larvae. *Biol. Res.* **2011**, *44*, 7–15. [CrossRef]
104. Neškovic, N.K.; Poleksic, V.; Elezovic, I.; Karan, V.; Budimir, M. Biochemical and histopathological effects of glyphosate on carp, *Cyprinus carpio* L. *Bull. Environ. Contam. Toxicol.* **1996**, *56*, 295–302.
105. Munn, M.D.; Gilliom, R.J. *Pesticide Toxicity Index for Freshwater Aquatic Organisms; U.S. Geological Survey, Water-Resources Investigations Report 01-4077*; National Water-Quality Assessment Program: Washington, DC, USA, 2001; p. 61.
106. Shuhaimi-Othman, M.; Yakub, N.; Ramle, N.-A.; Abas, A. Comparative toxicity of eight metals on freshwater fish. *Toxicol. Ind. Health* **2013**, *31*, 773–782. [CrossRef] [PubMed]
107. Holmstrup, M.; Bindesbøl, A.-M.; Oostingh, G.J.; Duschl, A.; Scheil, V.; Köhler, H.R.; Loureiro, S.; Soares, A.M.V.M.; Ferreira, A.L.G.; Kienle, C.; et al. Interactions between effects of environmental chemicals and natural stressors: A review. *Sci. Total Environ.* **2010**, *408*, 3746–3762. [CrossRef] [PubMed]
108. González-Ortegón, E.; Blasco, J.; Le Vay, L.; Giménez, L. A multiple stressor approach to study the toxicity and sub-lethal effects of pharmaceutical compounds on the larval development of a marine invertebrate. *J. Hazard. Mat.* **2013**, *263*, 233–238. [CrossRef] [PubMed]
109. González-Ortegón, E.; Giménez, L.; Blasco, J.; Le Vay, L. Effects of food limitation and pharmaceutical compounds on the larval development and morphology of *Palaemon serratus*. *Sci. Total Environ.* **2015**, *503*, 171–178. [CrossRef] [PubMed]
110. González-Ortegón, E.; Blasco, J.; Nieto, E.; Hampel, M.; Le Vay, L.; Giménez, L. Individual and mixture effects of selected pharmaceuticals on larval development of the estuarine shrimp *Palaemon longirostris*. *Sci. Total Environ.* **2016**, *540*, 260–266. [CrossRef] [PubMed]
111. Hooper, M.J.; Ankley, G.T.; Cristol, D.A.; Maryoung, L.A.; Noyes, P.D.; Pinkerton, K.E. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. *Environ. Toxicol. Chem.* **2013**, *32*, 32–48. [CrossRef]
112. Liess, M. Population response to toxicants is altered by intraspecific interaction. *Environ. Toxicol. Chem.* **2002**, *21*, 138–142. [CrossRef]
113. Araújo, C.V.M.; Pontes, J.R.S.; Blasco, J. Might the interspecies interaction between fish and shrimps change the pattern of their avoidance response to contamination? *Ecotoxicol. Environ. Saf.* **2019**, *186*, 109757. [CrossRef]
114. Cañedo-Argüelles, M.; Hawkins, C.P.; Kefford, B.J.; Schäfer, R.B.; Dyack, B.J.; Brucet, S.; Buchwater, D.; Dunlop, J.; Frör, O.; Lazorchak, J.; et al. Saving freshwater from salts. *Science* **2016**, *351*, 914–916. [CrossRef]
115. González-Ortegón, E.; Pascual, E.; Cuesta, J.A.; Drake, P. Field distribution and osmoregulatory capacity of shrimps in a temperate European estuary (SW Spain). *Estuar. Coast. Shelf Sci.* **2006**, *67*, 293–302. [CrossRef]
116. Marshall, S.; Elliott, M. Environmental Influences on the fish assemblage of the Humber Estuary, U.K. *Estuar. Coast. Shelf Sci.* **1998**, *46*, 175–184. [CrossRef]
117. González-Ortegón, E.; Baldó, F.; Arias, A.; Cuesta, J.A.; Fernández-Delgado, C.; Vilas, C.; Drake, P. Freshwater scarcity effects on the aquatic macrofauna of a European Mediterranean-climate estuary. *Sci. Total Environ.* **2015**, *503*, 213–221. [CrossRef] [PubMed]
118. Renaud, M.; Sousa, J.P.; Siciliano, S.D. A dynamic shift in soil metal risk assessment, it is time to shift from toxicokinetics to toxicodynamics. *Environ. Toxicol. Chem.* **2020**, *39*, 1307–1308. [CrossRef] [PubMed]
119. Hare, L.; Shooner, F. Do aquatic insects avoid cadmium-contaminated sediments? *Environ. Toxicol. Chem.* **1995**, *14*, 1071–1077. [CrossRef]

120. Maes, J.; Stevens, M.; Breine, J. Poor water quality constrains the distribution and movements of twaite shad *Alosa fallax fallax* (Lacépède, 1803) in the watershed of river Scheldt. *Hydrobiologia* **2008**, *602*, 129–143. [CrossRef]
121. Gomes-Silva, G.; Cyubahiro, E.; Wronski, T.; Riesch, R.; Apio, A.; Plath, M. Water pollution affects fish community structure and alters evolutionary trajectories of invasive guppies (*Poecilia reticulata*). *Sci. Total Environ.* **2020**, *730*, 138912. [CrossRef]
122. Rodríguez, J. *Ecología*; Ediciones Pirámide: Madrid, Spain, 2010; p. 502.
123. Araújo, C.V.M.; Moreira-Santos, M.; Ribeiro, R. Stressor-driven emigration and recolonisation patterns in disturbed habitats. *Sci. Total Environ.* **2018**, *643*, 884–889. [CrossRef]
124. Calabrese, E.J.; Baldwin, L.A. Toxicology rethinks its central belief. *Nature* **2003**, *421*, 691–692. [CrossRef]
125. Fuller, M.R.; Doyle, M.W.; Strayer, D.L. Causes and consequences of habitat fragmentation in river networks. *Ann. N. Y. Acad. Sci.* **2015**, *1355*, 31–51. [CrossRef]
126. Ribeiro, R.; Lopes, I. Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: An essay. *Ecotoxicology* **2013**, *22*, 889–899. [CrossRef]
127. Gilpin, M.E. Minimal viable populations: Processes of species extinction. In *Conservation Biology: The Science of Scarcity and Diversity*; Sinauer Associates, Inc.: Sunderland, MA, USA, 1986; pp. 19–34.
128. Grimm, V.; Reise, K.; Strasser, M. Marine metapopulations: A useful concept? *Helgol. Mar. Res.* **2003**, *56*, 222–228. [CrossRef]
129. Loreau, M.; Mouquet, N.; Holt, R.D. Meta-ecosystems: A theoretical framework for a spatial ecosystem ecology. *Ecol. Lett.* **2003**, *6*, 673–679. [CrossRef]
130. Schiesari, L.; Leibold, M.A.; Burton, G.A., Jr. Metacommunities, metaecosystems and the environmentl fate of chemical contaminants. *J. Appl. Ecol.* **2018**, *55*, 1553–1563. [CrossRef]
131. Gilarranz, L.J.; Rayfield, B.; Liñán-Cembrano, G.; Bascompte, J.; Gonzalez, A. Effects of networl modularity on the spread of perturbation impact in experimental metapopulations. *Science* **2017**, *357*, 199–201. [CrossRef] [PubMed]
132. EFSA Panel on Plant Protection Products and their Residues (PPR). Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. *EFSA J.* **2013**, *11*, 3290.
133. Gessner, M.O.; Tlili, A. Fostering integration of freshwater ecology with ecotoxicology. *Fresh. Biol.* **2016**, *61*, 1991–2001. [CrossRef]
134. Nilsen, E.; Smalling, K.L.; Ahrens, L.; Gros, M.; Miglioranza, K.S.; Picó, Y.; Schoenfuss, H.L. Critical review: Grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. *Environ. Toxicol. Chem.* **2019**, *38*, 46–60. [CrossRef]
135. Hellström, G.; Klaminder, J.; Jonsson, M.; Fick, J.; Brodin, T. Upscalling behavioural studies to the field using acoustic telemetry. *Aquatic. Toxicol.* **2016**, *170*, 384–389. [CrossRef]
136. Peterson, E.K.; Buchwalter, D.B.; Kerby, J.L.; LeFauve, M.K.; Varian-Ramos, C.W.; Swaddle, J.P. Integrative behavioral ecotoxicology: Bringing together fields to establish new insight to behavioral ecology, toxicology, and conservation. *Curr. Zool.* **2017**, *63*, 185–194. [CrossRef]
137. Park, M.O. Adult vertebrate behavioral aquatic toxicology: Reliability and validity. *Aquatic. Toxicol.* **2016**, *170*, 323–329. [CrossRef]

toxics

MDPI

Article

High-Throughput Screening of Psychotropic Compounds: Impacts on Swimming Behaviours in *Artemia franciscana*

Shanelle A. Kohler [1], Matthew O. Parker [2] and Alex T. Ford [1,*]

[1] Institute of Marine Science Laboratories, Ferry Road, Eastney, Hants PO4 9LY, UK; shanelle.kohler@port.ac.uk
[2] School of Pharmacy & Biomedical Science, White Swan Road, St. Michael's Building, Portsmouth PO1 2DT, UK; matthew.parker@port.ac.uk
* Correspondence: alex.ford@port.ac.uk

Abstract: Animal behaviour is becoming increasingly popular as an endpoint in ecotoxicology due to its increased sensitivity and speed compared to traditional endpoints. However, the widespread use of animal behaviours in environmental risk assessment is currently hindered by a lack of optimisation and standardisation of behavioural assays for model species. In this study, assays to assess swimming speed were developed for a model crustacean species, the brine shrimp *Artemia franciscana*. Preliminary works were performed to determine optimal arena size for this species, and weather lux used in the experiments had an impact on the animals phototactic response. Swimming speed was significantly lower in the smallest arena, whilst no difference was observed between the two larger arenas, suggesting that the small arena was limiting swimming ability. No significant difference was observed in attraction to light between high and low light intensities. Arena size had a significant impact on phototaxis behaviours. Large arenas resulted in animals spending more time in the light side of the arena compared to medium and small, irrespective of light intensity. The swimming speed assay was then used to expose specimens to a range of psychotropic compounds with varying modes of action. Results indicate that swimming speed provides a valid measure of the impacts of behaviour modulating compounds on *A. franciscana*. The psychotropic compounds tested varied in their impacts on animal behaviour. Fluoxetine resulted in increased swimming speed as has been found in other crustacean species, whilst oxazepam, venlafaxine and amitriptyline had no significant impacts on the behaviours measured. The results from this study suggest a simple, fast, high throughput assay for *A. franciscana* and gains insight on the impacts of a range of psychotropic compounds on the swimming behaviours of a model crustacean species used in ecotoxicology studies.

Keywords: ecotoxicology; behaviour; artemia; psychotropics; behavioural ecotoxicology

Citation: Kohler, S.A.; Parker, M.O.; Ford, A.T. High-Throughput Screening of Psychotropic Compounds: Impacts on Swimming Behaviours in *Artemia franciscana*. *Toxics* **2021**, *9*, 64. https://doi.org/10.3390/toxics9030064

Academic Editors: Demetrio Raldúa, Carlos Barata and Melissa Faria

Received: 31 January 2021
Accepted: 10 March 2021
Published: 17 March 2021

1. Introduction

One of the main challenges facing regulatory risk assessment is the speed with which we can assess the sub-lethal effects of pollutants [1]. Behavioural responses have been indicated as a useful endpoint as they tend to be more sensitive than lethality and faster to assess than endpoints for growth, development and reproduction [2]. However, the use of animal behaviours in environmental risk assessment is currently hindered by a lack of optimisation and standardisation of behavioural assays [2,3]. The use of behavioural hardware mitigates some of the issues of standardisation by providing a controlled environment within which to perform behavioural assays [4]. In recent years, there have been some excellent examples of automated high-throughput behavioural assays with crustacean species. Micro-fluidic behavioural chambers were developed for amphipods (*Allorchestes compressa*) and brine shrimp (*Artemia franciscana*) [5–7] and proved to be sensitive assays for measuring alterations in swimming and locomotion in the presence of behaviour modifying compounds. Other studies on zebrafish larvae have successfully used a static, multi-well plate system for high-throughput assessment of compounds on swimming, social, and anxiety behaviours [8,9]. A multi-well plate system is desirable as

the plates have standardised dimensions, are readily available, and are compatible with commercial plug-and-play behavioural hardware. In addition to the standardisation of hardware, understanding the baseline unconditioned behaviours of a model species is also important when performing behavioural studies. It has been shown that behaviours can vary with differences in experimental design such as the shape and size of behavioural arenas both between and within species [10,11]. Performing preliminary experiments to understand the baseline behaviours of a model species would help to both optimise the experimental design and aid the interpretation of results from behavioural assays.

Brine shrimp or *Artemia spp* are small crustaceans adapted to hyper-salinity, dry or harsh conditions, and are closely related to other zooplanktons such as the freshwater *Daphnids* [12]. *Artemia spp* have been used as a model species in ecotoxicology testing for more than five decades to assess the potential impacts of environmental pollutants [12,13], and are desirable due to their rapid hatching, cost effectiveness, and commercial availability. *Artemia spp* cysts can be sourced with standardised toxicity kits and hatched under controlled conditions in the lab for fast screening of toxicity in lethality tests (LC50s). Other endpoints, including behaviour, have also proved useful in ecotoxicology testing. The Swimming Speed Alteration (SSA) test was developed by Faimali et al. in 2006 [14] with barnacle larvae, and used video tracking for high-throughput assessment of swimming behaviour. The methods outlined by Faimali et al. have since been applied to *Artemia* by Garaventa et al. in 2010 [15] and Manfra et al. in 2015 [16] who found swimming speed to be more sensitive as an endpoint than mortality.

Psychotropic compounds such as anxiolytics and antidepressants come in a range of different classes with varying modes of action (MOA). In this study, brine shrimp (*Artemia franciscana*) were exposed to environmentally relevant concentrations of fluoxetine hydrochloride, oxazepam, amitriptyline hydrochloride, and venlafaxine hydrochloride; representing the most prescribed compounds from four separate classes of antidepressants and anxiolytics. The MOA, presence in aquatic environments, and effects on animal behaviours are summarised in Table 1.

Table 1. Summary of the current literature for the four psychotropic compounds used in this study including their MOA, presence in aquatic environments, and effects on behaviours.

Compound	Class	Environment	Concentration	Source	Behaviour Impacts	Source
fluoxetine hydrochloride	Selective Serotonin Reuptake Inhibitor (SSRI)	effluents	0.001–5 µg/L	[17–20]	activity	[21,22]
		surface waters	0.012–0.02 µg/L		reproduction	[23–27]
		marine env	0.012 µg/L		aggression	[27–29]
					feeding	[30–32]
					predator avoidance	[21,32–34]
					stress/anxiety	[35–37]
					taxis	[38,39]
oxazepam	Benzodiazepine (BZD)	effluents	0.25–0.73 ug/L	[18,20,40]	activity	[41–43]
		surface waters	0.02–0.58 ug/L		feeding	[44]
					boldness	[41,45,46]
					social behaviour	[44]
					migration	[47]
amitriptyline hydrochloride	Tricyclic Antidepressant (TCA)	effluents	<2–357 ng/L	[48–51]	activity	[52]
		surface waters	<0.5–72 ng/L		reproduction	[53]
		bio-solids	263–632 ng/g		feeding	[54]
					stress/anxiety	[55]
					memory & learning	[56]
					orientation	[57]
venlafaxine hydrochloride	Selective Serotonin and Norepinephrine Reuptake Inhibitor (SNRI)	effluents	600–1454 ng/L	[49,50]	activity	[58,59]
		surface waters	187 ng/L		feeding	[60]
		bio-solids	289–499 ng/g		stress/anxiety	[61–64]

In the literature, most studies assessing the ecological effects of antidepressants and anxiolytics have focused on fluoxetine. Few have examined the effects of other psychotropic compounds, and fewer still have assessed their effects on aquatic invertebrates. Planktonic crustaceans share with vertebrates several of the neurotransmitters that are targeted by neuroactive drugs. Including serotonin, dopamine, epinephrine and GABA receptors [1], making it possible for psychotropic compounds to have effects on non-target organisms in the environment. From the research to date, there appears to be a trend of increased activity in crustaceans exposed to both anxiolytics and antidepressants including fluoxetine and oxazepam [22,30,43,65] in amphipod and decapod species. To date, the impact of psychotropic compounds on the swimming of anostraca species remains unexplored.

The main aims of the study were to develop a standardised high-throughput behavioural assay for aquatic invertebrates for use in toxicity testing, and to assess the effects of a range of psychotropic compounds with varying modes of action on crustacean behaviour. Behavioural assays were developed for *A. franciscana* and data on the baseline unconditioned swimming behaviours were collected. The swimming speed and photosensitivity was assessed under a range of arena sizes. It was thought that as has been shown in amphipods [10], that smaller arenas would limit swimming speeds. Some studies have reported that both adult and larval *Artemia spp* can switch between positive and negative phototaxis depending on the intensity of light used [66,67]. As a result of this, the phototactic response of *A. franciscana* was also assessed under different light intensities. It was hypothesised that at higher light intensities *A. franciscana* would exhibit a preference for dark areas which would be reduced or mitigated at lower intensities. Following assay development, *A. franciscana* were exposed to three antidepressants and an anxiolytic at environmentally relevant concentrations and swimming behaviours were assessed. Based on the current literature for crustaceans, it was hypothesised that psychotropic compounds would increase swimming speed in *A. franciscana*.

2. Materials and Methods

2.1. Animal Culture and Husbandry

A. franciscana were purchased from MicroBioTests Inc (Kleimoer 15 9030 Gent, Belgium), as dried cysts and hatched in a 1 L separating funnel connected to an air pump. Following hatching, organisms were transferred to a 5 L aquarium with an air stone. The hatchery and aquaria were set up within an incubator to keep temperature and light conditions consistent. Cool white, fluorescent lamps were used, and light intensity ranged between 1665–1608 Lux (21.73–22.51 $\mu mol \cdot s^{-1} \cdot m^{-2}$) from the top to the bottom of the incubator, respectively. Hatching and growth parameters were in accordance with the MicroBioTests Artoxkit M protocol. Artificial seawater (AFSW) was used at 35 ppt and a constant temperature of 21 $\pm$ 1 °C. A 12:12 light/dark regime was used during both hatching and growth of organisms. Once nauplii were transferred from the separating funnel to the growing aquaria, a water change was performed every 3 days, and animals were fed 2–4 drops of concentrated algae solution containing *Nannochloropsis spp* and *Tetraseimis spp* (purchased from Amazon.co.uk by supplier Phyto Plus) every 1–2 days. The amount of food added was judged by eye based on the colour of the aquarium water, as per instructions on the algae solution bottle, to obtain a light green tint to the culture water. Nauplii were kept in the growing aquaria and reared to adult stage. It took between 3–4 weeks to rear *A. franciscana* from Instar stage I to trackable sized adults with a mean body length of 10 mm.

2.2. Measuring Behaviour

All behaviours were measured using DanioVision™ observation chamber (Noldus, Wageningen, The Netherlands) connected to EthoVision®XT 11.5 software (TrackSys, Nottingham, UK). The observation chamber was comprised of an external hood and internal holder for a multi-well plate. The holder is infrared backlit with an additional cold white light source which can be programmed to operate automatically. Together these provide a

controlled environment for behavioural experiments. The EthoVision®XT 11.5 software can measure a variety of parameters associated with movement and activity simultaneously and can be programmed to operate DanioVision™ hardware.

2.3. Baseline Behaviours

Prior to psychotropic exposures, preliminary tests were performed to determine the optimal arena size for behavioural assays. Standard Thermo Scientific 'Nunc' 6-well, 12-well and 24-well plates (sourced from Thermo Fisher Scientific, Waltham, MA, USA) were used to measure the baseline unconditioned behaviours of *A. franciscana*.

2.4. Velocity

To measure swimming speed, animals were gently transferred from growth tanks and loaded into multi-well plates using a plastic Pasteur pipette. The pipette was widened by cutting the tip so that *A. franciscana* could be transferred without physical damage. A single individual was placed in each well. For ease of writing, the 24-well, 12-well and 6-well plate will be henceforth referred to as small, medium, and large arenas, respectively. Each well was filled to half of its maximum volume with AFSW which allowed for free horizontal swimming but limited vertical motions. The dimensions of arenas including the volume of AFSW used and the number of replicates of *A. franciscana* are outlined in Table 2.

Table 2. Dimensions of Small (24-well), medium (12-well) and large (6-well) arenas with volume of AFSW and number of replicates of *A. franciscana* used for velocity studies.

Arena	Diameter	Area	Volume AFSW	Replicates
Small	1.5 cm	3 cm^3	1.5 mL	24
Medium	2.1 cm	6 cm^3	3 mL	24
Large	3.5 cm	16.4 cm^3	8 mL	30

Once loaded, multi-well plates were placed inside the DanioVision™ and animals were tracked for 8 min under a 2 min dark: 2 min light, cycle. The differing light phases were used as a stimuli and to assess the photosensitivity of *A. franciscana*. The cold light was set to 100% intensity equating to 4000 Lux. This was almost double the Lux used for hatching and culturing of organisms to try and combat habituation to the lighting in the behaviour trials.

Phototaxis

The small, medium and large arenas were also used to assess the effects of arena size on the baseline unconditioned phototaxis behaviour in *A. franciscana*. Animals were tracked in the DanioVision™ for 6 min with a 3 min dark phase followed by a 3 min light phase. Here, the light phase was used to measure a phototactic response. A series of custom acrylic strips were used. The strips consisted of a clear acrylic that both white light and infra-red light could pass through and a black acrylic through which only the infra-red light could pass. During the dark phase the entire arena was dark and during the light phase one half of the arena was illuminated whilst the other half remained dark and *A. franciscana* could choose to be in either the light or dark side of the arena. Zone use during the dark phase when the entire arena was dark was used to control for animals that generally preferred one side of an arena compared to another. It was expected that during dark phases animals would use all of the arena equally. During light phases, animals would exhibit phototaxis if they then showed a preference for either the light or dark side of the arena. The acrylic strips were produced in a range of sizes to provide a half-light and half-dark side of the arena for each size class, the dimensions of the light and dark zones within each arena are outlined in (Figure 1). Two plates were made for each size arena which could be interchanged during trials so that the light and dark side of the arenas could be alternated at random (Figure 1).

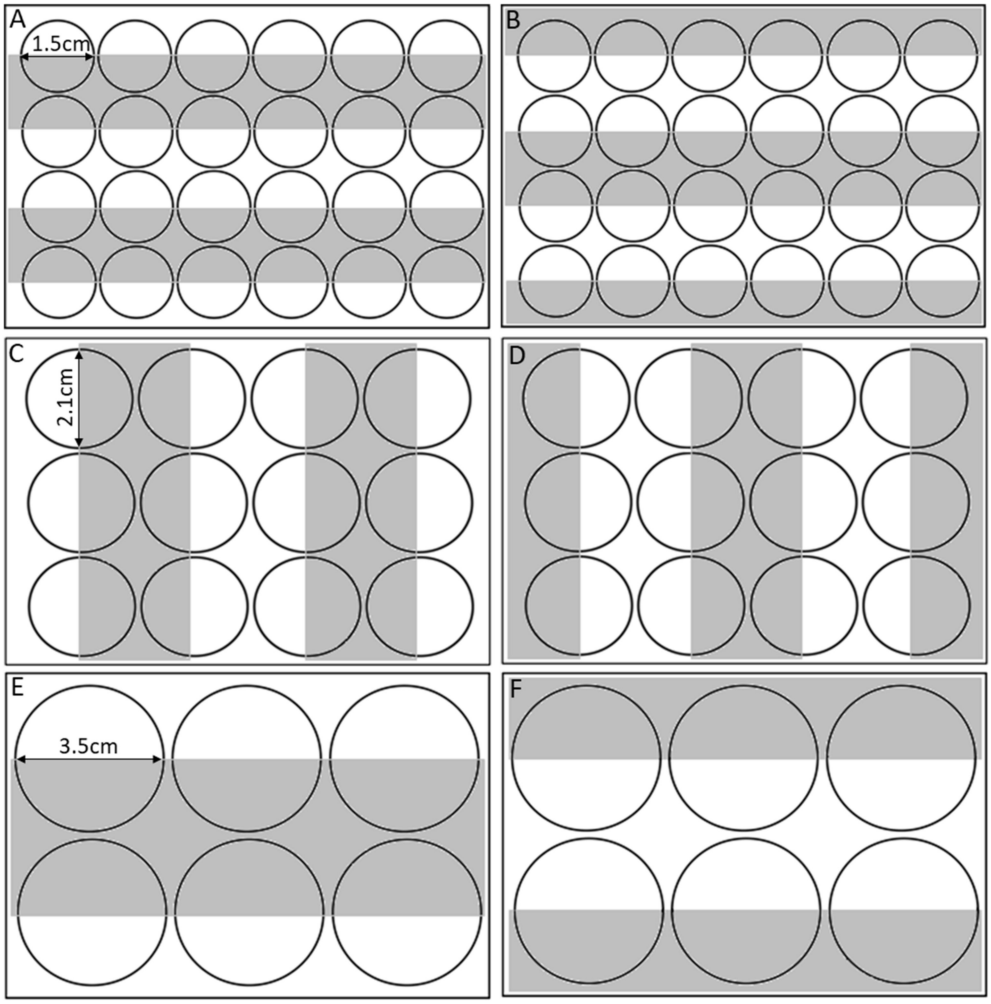

Figure 1. Dimensions and location of light and dark zones for (**A**) small arena, acrylic plate 1, (**B**) small arena, acrylic plate 2, (**C**) medium arena, acrylic plate 1, (**D**) medium arena, acrylic plate 2, (**E**) large arena, acrylic plate 1, (**F**) large arena, acrylic plate 2.

To assess the impacts of light intensity on phototactic response of *A. franciscana.* The light phase for phototaxis trials were performed under two light intensities. Two conditions 5% and 100% light intensity (200 and 4000 Lux, respectively) were used. A total of 312 animals were used for phototaxis assessment with replicates divided between arena size, acrylic plate, and light intensity. The number of replicates used for each condition are outlined in Table 3.

2.5. Psychotropic Exposures

Preparation of Solutions

Following preliminary experiments, *A. franciscana* were exposed to a range of psychotropic compounds. All compounds were sourced from Sigma-Aldrich (Saint Louis, Missouri, USA) in dry powder form including Fluoxetine hydrochloride (CAS: 56296-78-7), Oxazepam (CAS: 604-75-1), Amitriptyline hydrochloride (CAS: 549-18-8), and Venlafaxine hydrochloride (CAS: 99300-78-4). All compounds were water soluble, so solutions were made without a solvent. Due to the minimum amount of dry compound that can be accurately weighed, a stock solution of 1 mg/L was made in 2 L volumetrics for each compound. Stock solutions were stored in sealed glass vials wrapped in aluminium foil and stored in the fridge at 10 ± 1 °C to prevent degradation. A serial dilution of 10 ng/L, 100 ng/L and 100 ng/L plus an AFSW control was made for each compound, from stock solutions, into artificial seawater at 35 ppt.

Table 3. Number of replicates used for each experimental condition for assessment of phototaxis in *A. franciscana* for each of the three arena sizes and the two acrylic plates to alternate the light and dark zones between the two light intensities.

Arena Size	Acrylic Plate Used	Light Intensity	Replicates
Small	1	100%	24
		5%	24
	2	100%	24
		5%	24
Medium	1	100%	24
		5%	24
	2	100%	24
		5%	24
Large	1	100%	30
		5%	30
	2	100%	30
		5%	30

2.6. Exposures and Behavioural Analysis

The experimental design for psychotropic exposures is outlined in Figure 2. Medium arenas were used, as per results from the studies on baseline behaviours, as this provided the best trade-off between high-throughput analysis and providing 'space to behave' in this species. A single individual of *A. franciscana* was loaded into each well with water from the culture tanks. Once all animals were in the arenas, the culture water was removed from the wells with an electronic pipette and replaced with 4 mL of AFSW control or AFSW spiked with a psychotropic compound at 10 ng/L, 100 ng/L or 1000 ng/L. 12 replicates were performed per concentration providing a total of 48 animals per compound. The organisms were then placed in an incubator under a 12:12 h light:dark cycle at 21 °C ± 1. After 1 h, the well plates were removed from the incubator and placed in the DavioVision™. *A. franciscana* were tracked using EthoVision®XT software for a total of 8 min under a 2 min dark: 2 min light cycle. The light phase was set to 100% light intensity (4000 Lux) as per results from studies on baseline behaviours. The process was repeated with the same animals after 1 day and 1 week of exposure.

2.7. Statistics

When analysing baseline behaviours, statistical analysis was performed in IBM SPSS Statistics 24. Phototaxis was measured as the percent duration of time spent in the light zone of the arena. Total distance moved was included in the model as a co-variate to correct for animals that did not move during trials [3]. Velocity was measured as mean velocity in centimetres per second and was analysed in both 2-min and 10-s time bins. Extreme anomalous values generated by the loss of tracking by the EthoVision®XT software was excluded from the data analysis (as defined by values > median ± 3*IQR) and never removed more than 3% of data points. Linear mixed effects (LME) models were used for all comparisons, residuals from LME model analysis were checked for normality using Q-Q plots and Shapiro-Wilk test of normality. All datasets were normally distributed. Treatment, length of exposure and time were input as factors. Individual animal ID was included as a random effect in the model to correct for repeated measures. Post Hoc analysis was performed using Bonferroni adjustments to correct for type- II errors. p-values of < 0.05 were considered significant. When analysing data from the psychotropic exposures, statistical analysis was performed with jamovi version 1.2.27. Velocity was analysed in both 2-min and 10-s time bins. Linear mixed effects models were used for all compounds.

For the 10-s data treatment and length of exposure were input as factors whilst time was used as a covariate. Individual ID was used as a random effect in the model to correct for repeated measures. Post Hoc analysis was performed using Holmes adjustments to correct for type-II errors. For the 2-s data, treatment, length of exposure and time in the form of 2-min light or 2-min dark phases were all input as factors. Individual ID was used as a random effect in the model to correct for repeated measures. Post Hoc analysis was performed using Holmes adjustments. No differences were observed in the data output from the mixed effects models between 2-min and 10-s data. As such only the 2-min data has been presented in the results. All figures were made using the estimated marginal means from linear mixed effects models.

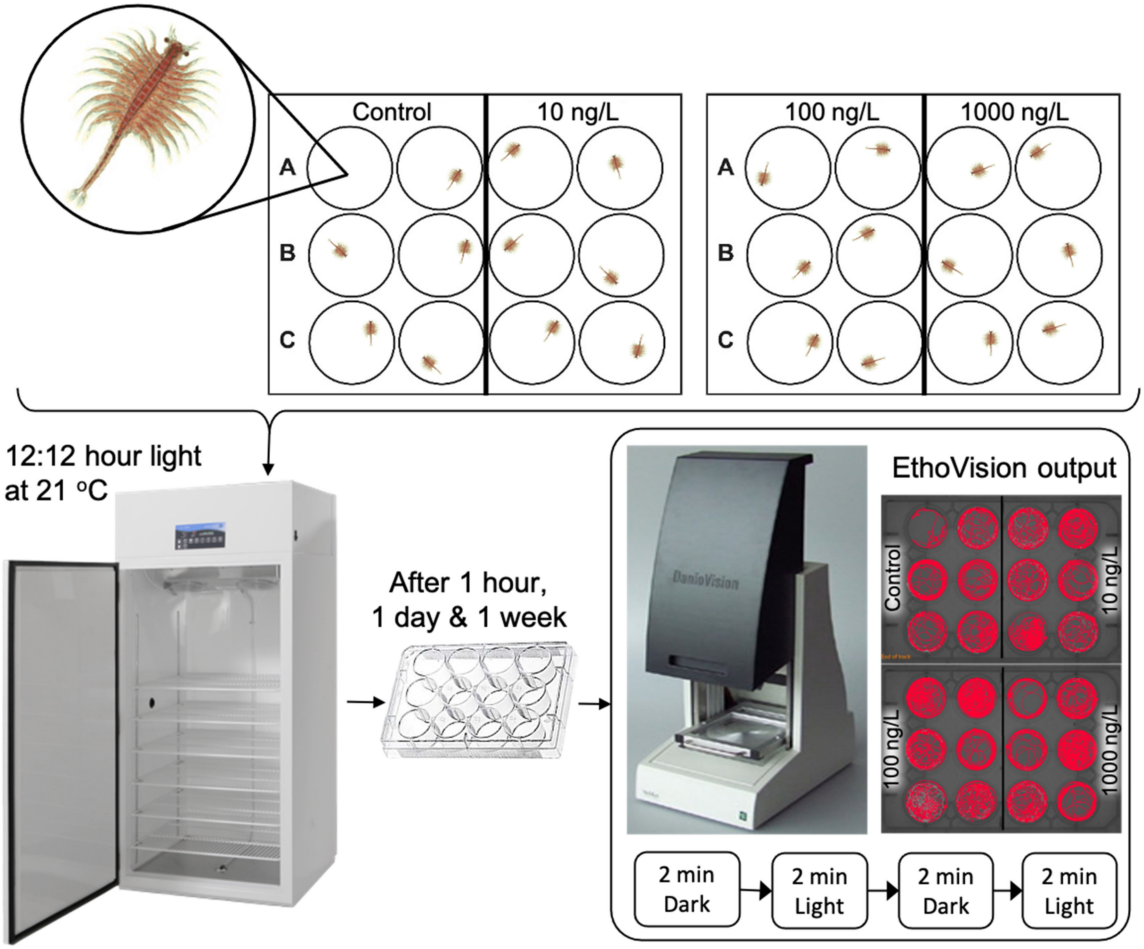

Figure 2. Experimental design for *A. franciscana* exposure and behavioural analysis. The 12-well plates were loaded in duplicate to provide 12 replicates per treatment. The procedure was repeated for each of the four compounds.

3. Results

3.1. Baseline Behaviours

Velocity

When assessing the baseline unconditioned velocity behaviour of *A. franciscana*, no statistical differences were observed between the 2-min and 10-s time bins when comparing arena sizes or light phase (Table 4). A significant effect of arena size in both 2-min and 10-s time bins was observed (Table 4). *A. franciscana* reached a greater mean velocity, in a range of 1.1–1.5 cm/s in large and medium arenas compared to small arenas where animals reached a maximum velocity of 0.9 cm/s (Figure 3). No significant effects ($p > 0.05$) were observed in velocity between large and medium arenas for 2-min time bins (Figure 3A). A significant effect of time was observed with *A. franciscana* swimming faster during dark

phases compared to light (Table 4). A significant interaction was observed between arena size and time when splitting data into 10-s time bins but not with 2-min time bins (Table 4). The significant interaction was driven by *A. franciscana* swimming faster in the large arena compared to medium and small during the second dark phase (Figure 3B).

Table 4. Output from linear mixed effects model for both 2-min and 10 s velocity data of *A. franciscana* between arena sizes. Significance level $p < 0.05$. In this model 'time' represents light phase split into 10 s time bins or 2-min time bins.

Comparison	2-min				10-s			
	N-df	D-df	F	*p*	N-df	D-df	F	*p*
arena size	2	75	16.3	<0.001	2	74	16.2	<0.001
time	3	225	13.6	<0.001	47	3522	7.0	<0.001
arena size * time	6	225	1.5	0.180	94	3522	1.7	<0.001
ICC	0.679				0.475			
R^2	0.271				0.217			

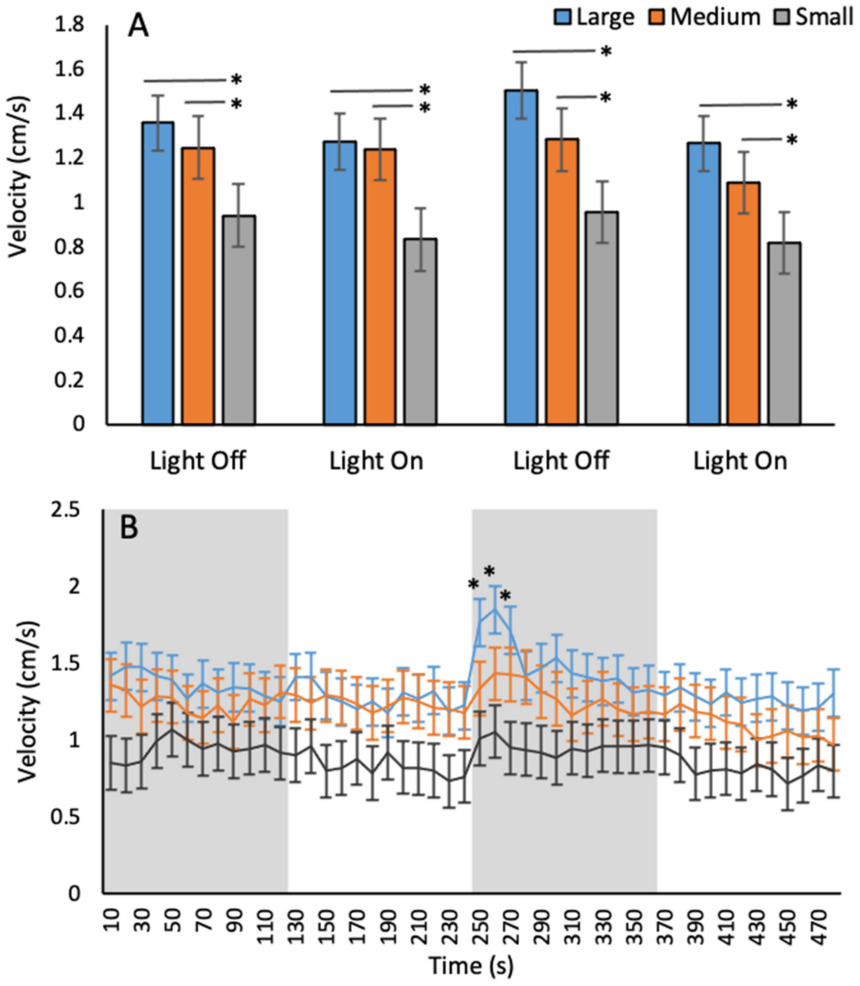

Figure 3. Mean velocity of *A. franciscana* between arena sizes in (**A**) 2-min and (**B**) 10-s time bins. Error bars represent 95% confidence. Asterisks indicate significant differences between arena sizes. For the 10-s data, asterisks indicate significant differences in velocity between large and medium arena only. Significance level * $p \leq 0.05$.

3.2. Phototaxis

When assessing the baseline unconditioned phototactic behaviour of *A. franciscana*, arena size had a significant impact on time spent in the light zone (Table 5). Animals spent significantly more time in the light zone when in large arenas compared to medium and small. Light phase also had a significant impact on phototactic response (Table 5) with animals spending more time in the light zone during light phases compared to dark. No significant effects were observed in phototactic response between the two light intensities (Table 5). There was a significant interaction between arena size and time (Table 5). There were no observed differences between arena sizes during 3-min dark phases with all animals spending ~50% of their time in the light side of the arena (Figure 4). However, during light phases *A. franciscana* spent a greater proportion (~55%) of time in the light zone when in the large arena and a smaller proportion of time (~35–50%) in the light zone when in a medium or small arena (Figure 4).

Table 5. Output from linear mixed effects model for time spent in the light side of the arena for *A. franciscana* between arena sizes and light intensity. Significance level $p < 0.05$.

Comparison	Num df	Den df	F	*p*
arena size	2	344.64	13.37	<0.001
light intensity	1	302.92	1.02	0.313
light phase	1	337.59	4.35	0.038
arena size * light intensity	2	303.77	3.72	0.025
arena size * light phase	2	305.09	54.75	<0.001
light intensity * light phase	1	303.25	8.07	0.005
arena size * light intensity * light phase	2	307.19	0.73	0.483
ICC = 0.364				
R^2 = 0.198				

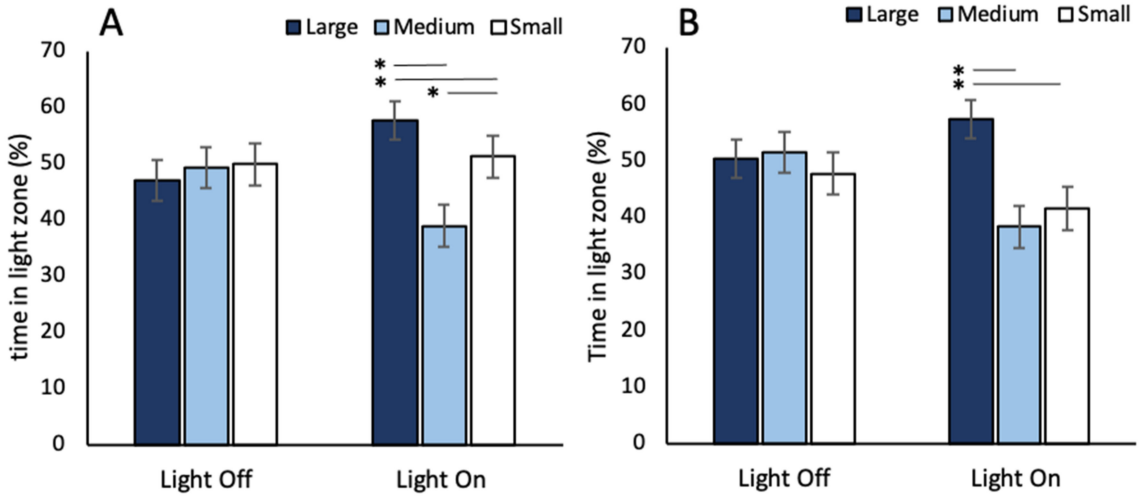

Figure 4. Percent duration *A. franciscana* spent in the light zone during 3-min dark and 3-min light phases between small, medium and large arenas when exposed to light at (**A**) 100% intensity and (**B**) 5% intensity. Error bars indicate 95% confidence. Asterisks indicate significant differences between arena sizes. Significance level * $p \leq 0.05$.

3.3. Psychotropic Exposures

Fluoxetine

When assessing the effects of fluoxetine on the velocity of *A. franciscana*, mean velocity ranged between 0.3–0.7 cm/s across all treatments and exposures (Figure 5D–F). Fluoxetine had a significant impact on swimming speed between treatments (Table 6). Animals exposed to 100 ng/L of fluoxetine had a significantly greater velocity than both control animals and those exposed to the lowest treatment group of 10 ng/L (Figure 5A). Animals

in the 1000 ng/L treatment group also reached a greater mean velocity than controls and animals exposed to 10 ng/L, but this did not reach the threshold for significance following multiple testing correction ($p = 0.072$). Light phase had a significant effect on the velocity of *A. franciscana* (Table 6) with animals generally swimming faster during dark phases compared to light (Figure 5B). The length of exposure also had a significant effect on swimming speed (Table 6) with animals swimming significantly slower time (Figure 5C). No significant interactions were found between fluoxetine treatments with length of exposure or light phase (Table 6; Figure 5D–F).

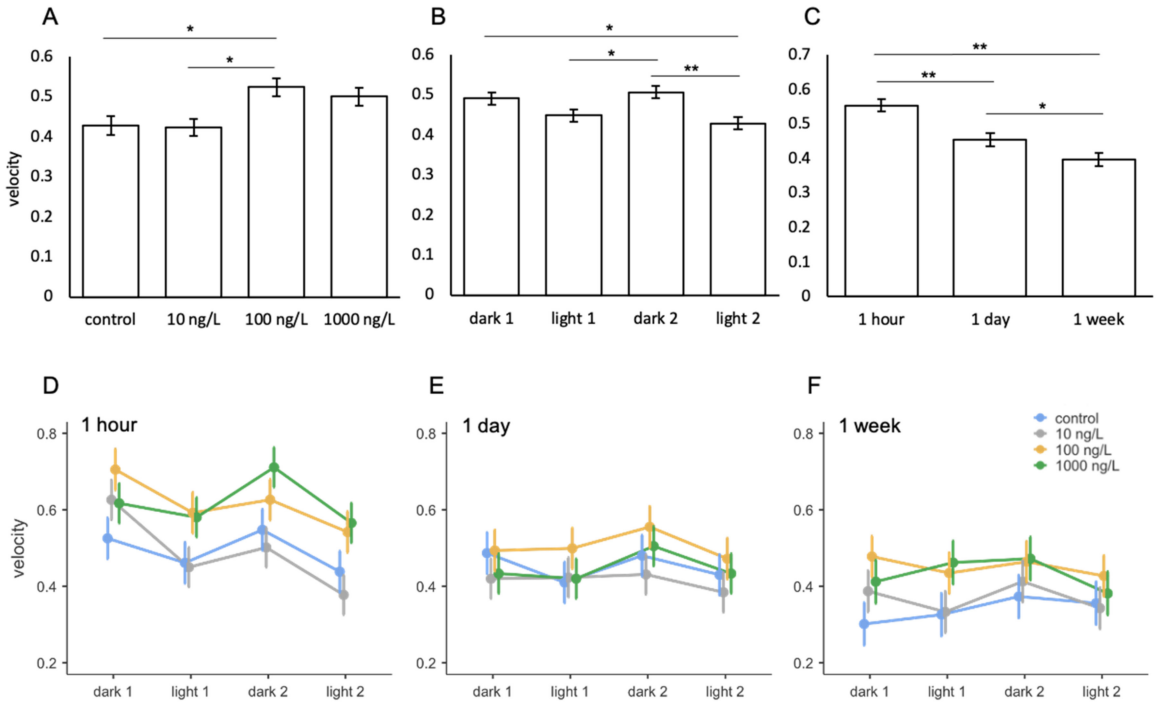

Figure 5. Mean velocity of *A. franciscana* following exposure to fluoxetine between (**A**) treatment, (**B**) light phase, (**C**) length of exposure, and (**D**–**F**) interactions between treatment and light phase across the three lengths of exposure. Error bars represent standard error. Asterisks indicate significant differences from post hoc analysis. * $p \leq 0.05$, ** $p \leq 0.001$.

Table 6. Output from linear mixed effects model for velocity of *A. franciscana* exposed to fluoxetine.

Comparison	F	Num df	Den df	*p*
treatment	5.328	3	111	0.002
light phase	8.221	3	392	<0.001
exposure period	17.400	2	111	<0.001
treatment * light phase	0.757	9	392	0.657
treatment * exposure period	0.663	6	111	0.679
light phase * exposure period	1.728	6	392	0.113
treatment * light phase * exposure period	0.520	18	392	0.949
ICC = 0.315				
$R^2 = 0.212$				

3.4. Oxazepam

For the oxazepam study, the mean velocity of *A. franciscana* ranged between 0.3–0.7 cm/s (Figure 6D–F). No significant effects of oxazepam were observed between treatments (Table 7; Figure 6A). Velocity was significantly different between light phases (Table 7) with animals swimming faster during the second dark phase compared to the two light phases (Figure 6B). Mean velocity significantly decreased with increasing of length of exposure (Table 7; Figure 6C), and a significant interaction was observed between light phase and exposure (Table 7). The significant interaction was driven by the 1-h exposure, in which

velocity was significantly greater during the two dark periods compared to the two light periods, and significantly greater than 1 day and 1-week exposures (Table 1). No significant interactions were found between oxazepam treatments with length of exposure or light phase (Table 7; Figure 6D–F).

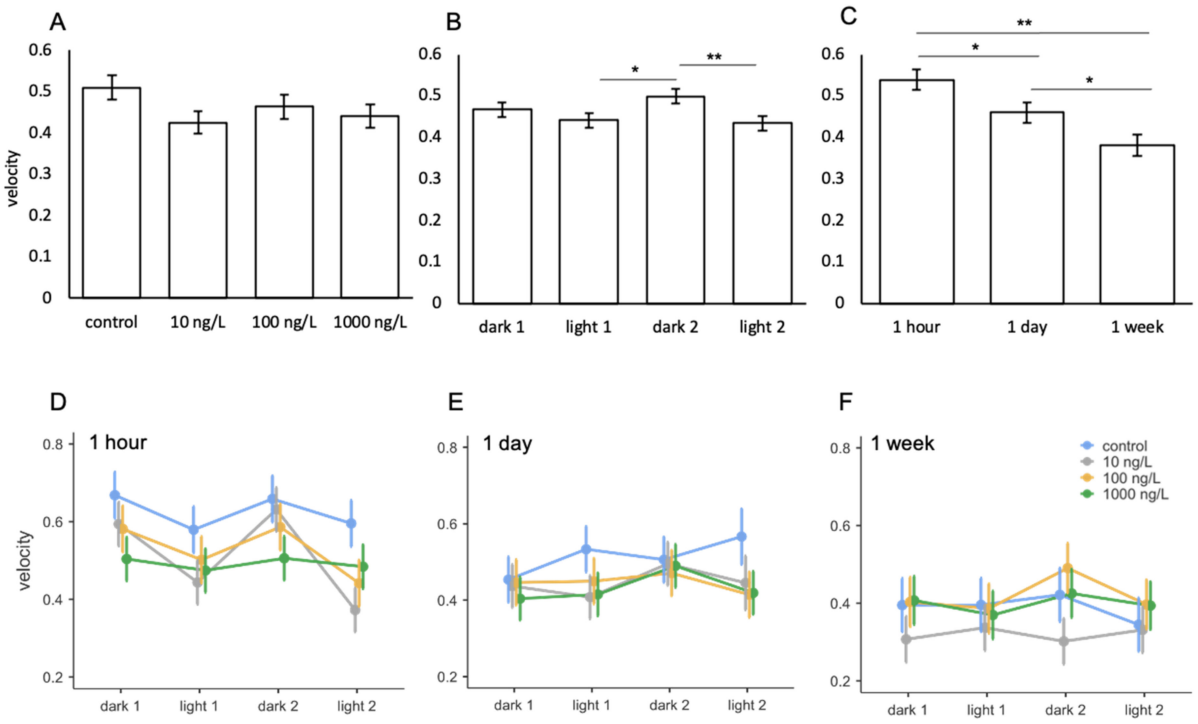

Figure 6. Mean velocity of *A. franciscana* following exposure to oxazepam between (**A**) treatment, (**B**) light phase, (**C**) length of exposure, and (**D**–**F**) interactions between treatment and light phase across the three lengths of exposure. Error bars represent standard error. Asterisks indicate significant differences from post hoc analysis. * $p \leq 0.05$, ** $p \leq 0.001$.

Table 7. Output from linear mixed effects model for velocity of *A. franciscana* exposed to oxazepam.

Comparison	F	Num df	Den df	*p*
treatment	1.602	3	114	0.193
light phase	6.490	3	367	<0.001
exposure period	9.926	2	114	<0.001
treatment * light phase	0.563	9	367	0.827
treatment * exposure period	0.534	6	114	0.782
light phase * exposure period	2.783	6	367	0.012
treatment * light phase * exposure period	1.109	18	367	0.341
ICC = 0.557				
R^2 = 0.174				

3.5. Amitriptyline

During the amitriptyline study, the swimming velocity of *A. franciscana* ranged between 0.3–0.65 cm/s (Figure 7D–F). Light phase had a significant effect on swimming behaviours (Table 8) with *A. franciscana* reaching a greater velocity during the second dark phase compared to the first and second light phase (Figure 7B). Length of exposure also had a significant effect on velocity (Table 8) with swimming speed decreasing significantly after 1 week of exposure compared to 1 h (Figure 7C). Whilst animals exposed to the highest concentrations of amitriptyline swam slower than the controls after 1 h and 1 day of exposure (Figure 7D,E), this did not meet the significance threshold. No significant effects of amitriptyline treatment were observed on Artemia velocity (Table 8; Figure 7A)

and no significant interactions were observed between treatment with light phase and/or exposure (Table 8; Figure 7D–F).

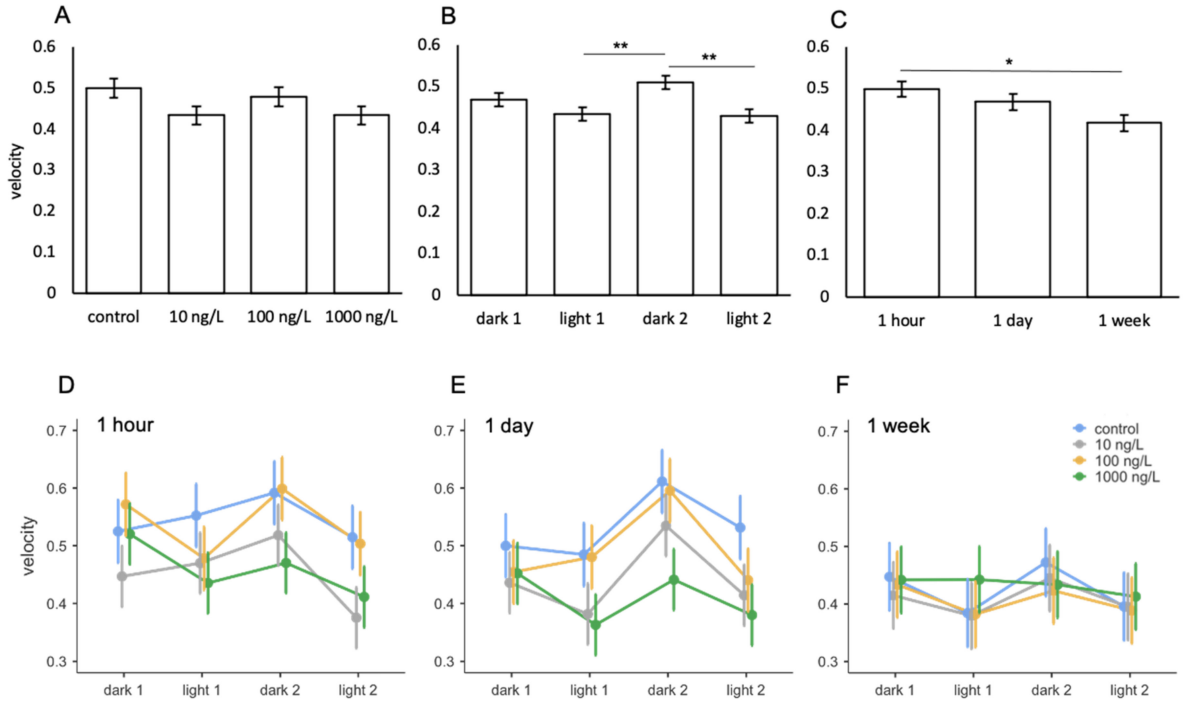

Figure 7. Mean velocity of *A. franciscana* following exposure to amitriptyline between (**A**) treatment, (**B**) light phase, (**C**) length of exposure, and (**D**–**F**) interactions between treatment and light phase across the three lengths of exposure. Error bars represent standard error. Asterisks indicate significant differences from post hoc analysis. * $p \leq 0.05$, ** $p \leq 0.001$.

Table 8. Output from linear mixed effects model for velocity of *A. franciscana* exposed to amitriptyline.

Comparison	F	Num df	Den df	*p*
treatment	2.204	3	112	0.092
light phase	8.415	3	386	<0.001
exposure period	4.258	2	112	0.017
treatment * light phase	0.537	9	386	0.847
treatment * exposure period	0.703	6	112	0.648
light phase * exposure period	0.861	6	386	0.524
treatment * light phase * exposure period	0.393	18	386	0.989
ICC = 0.318				
R^2 = 0.116				

3.6. Venlafaxine

When assessing the effects of venlafaxine on the velocity of *A. franciscana*, swimming velocity ranged between 0.3–0.6 cm/s (Figure 8D–F). Light phase had a significant effect on swimming behaviours (Table 9) with animals swimming faster during dark phases compared to light (Figure 8B). Length of exposure also had a significant effect on velocity (Table 9) with swimming speed decreasing significantly after 1 week of exposure compared to 1 day (Figure 8C). It was observed that animals in the highest treatment group swam consistently slower than control animals across all three measured durations of exposure (Figure 8D–F). However, this did not reach a level of significance and no significant impacts of venlafaxine were observed on the velocity of *A. franciscana* between treatment groups (Table 9; Figure 8A). No significant interactions were observed between treatments with light phase and/or length of exposure (Table 9; Figure 8D–F).

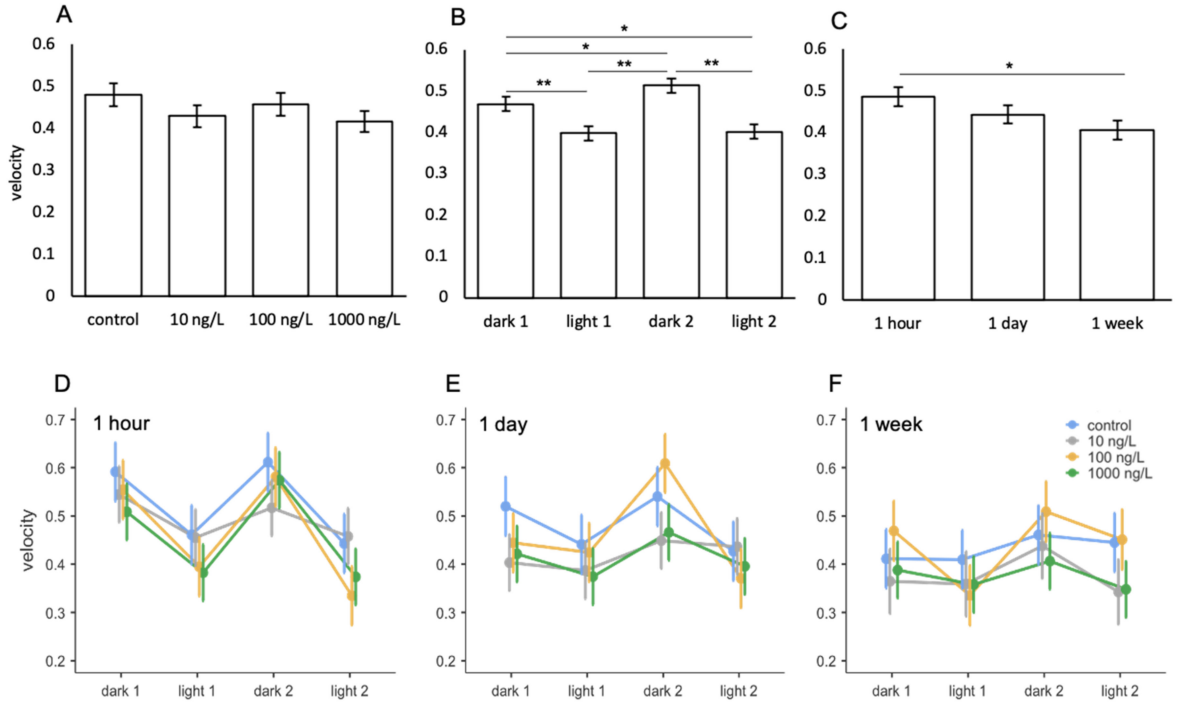

Figure 8. Mean velocity of *A. franciscana* following exposure to venlafaxine between (**A**) treatment, (**B**) light phase, (**C**) length of exposure, and (**D**–**F**) interactions between treatment and light phase across the three lengths of exposure. Error bars represent standard error. Asterisks indicate significant differences from post hoc analysis. * $p \leq 0.05$, ** $p \leq 0.001$.

Table 9. Output from linear mixed effects model for velocity of *A. franciscana* exposed to venlafaxine.

Comparison	F	Num df	Den df	*p*
treatment	1.180	3	116	0.321
light phase	18.062	3	394	<0.001
exposure period	3.091	2	116	0.049
treatment * light phase	0.873	9	394	0.550
treatment * exposure period	0.200	6	116	0.976
light phase * exposure period	2.095	6	394	0.053
treatment * light phase * exposure period	0.581	18	394	0.913
ICC = 0.410				
R^2 = 0.121				

4. Discussion

4.1. Baseline Behaviours

There is growing evidence that collection of baseline data on model species is important when conducting behavioural assays. Researchers have highlighted a lack of baseline data to be a source of variability in the results of behavioural studies in ecotoxicology [68]. This has been supported by Melvin et al. [69] who explored how fish acclimate to behavioural arenas and how different lengths of observation time impact estimates of basic swimming parameters. They concluded that researchers need to establish a basic knowledge about the baseline behavioural characteristics for a model species, as this could influence study outcomes of behavioural ecotoxicology experiments. This was further reinforced by Kohler et al. [10,11] who discovered that differences in study design could impact the baseline unconditioned behaviours in amphipods which could in turn implicate the results of ecotoxicology studies.

In this study, experiments were performed to assess the baseline unconditioned velocity and phototaxis behaviours of *A. franciscana* under a range of arena sizes to both optimise the assay for high-throughput analysis and to determine the sensitivity of the test species to the behavioural assays. Results from velocity assessments indicate a trade-off

between 'high-throughput' analysis and providing 'space to behave' with *A. franciscana* reaching a significantly greater velocity in large and medium arenas compared to small suggesting that the small arenas were limiting animals ability to reach a greater swimming speed. Analysing velocity data in 2-min time bins found no significant differences in swimming speed between the large and medium arenas, whereas the 10-s analysis found that animals reached a significantly faster swimming speed in large arenas compared to medium in the second dark phase. Similar results were also described by Kohler et al. [11] in amphipods whereby differences in swimming behaviours between a marine and freshwater amphipod were only observed when data was separated into smaller time bins. With the exception of the 30 s in the second dark phase, no significant differences were observed in swimming speed between medium and large arenas in an 8-min behaviour trial. This suggests that arena size was no longer a limiting factor on *Artemia* velocity and that any further increase in arena size would no longer impact the swimming speed that this species could reach. Increased swimming speed with increasing space to explore has been reported in a range of vertebrate and invertebrate species including the amphipod *G. pulex* [10] fruit flies *Drosophila melanogaster* [70]; rats [71]; and gerbils [72].

During swimming speed assays, light phase had a significant impact on the velocity of *A. franciscana*. Swimming speed was greater during 2-min dark phases compared to light. A transition into darkness may trigger migration behaviours or increase exploratory behaviour as a result of perceived reduction in predation risk or increased searching for light areas. In the literature it has been reported that many zooplankton, including brine shrimp, undergo nocturnal diel vertical migration (DVM) involving an ascent in the water column to feed during times of low light levels near the surface and descend to dim lit areas during the day to avoid predators [73,74]. It has been reported that *Artemia spp* can switch between positive and negative phototaxis depending on the intensity of light used [66,67]. However, in this study, no significant differences in time spent in the light zone were observed between the 2000 and 400 Lux phototaxis trials in this study. No effects of Lux on crustacean behaviours has also been reported previously in the literature for amphipod species [10]. In the study by Kohler et al. in 2018 [10], no significant effect of varying lux were observed for multiple behavioural endpoints including swimming speed and thigmotaxis behaviours. This suggests that the switch between positive and negative phototaxis which was observed by Bradley et al. (1984) and Dojmi Di Delupis et al. (1988) [66,67] was a result of something more complex than simple lux in the control of *Artemia* migration patterns and provides and area for future research. In phototaxis trials, under both light intensities, animals in large arenas exhibited a preference for the light side of the arena compared to the dark. This would support the theory of increased light searching to explain the greater velocity observed during dark phases in swimming speed assays. Interestingly the opposite was observed when assessing phototaxis behaviours in the medium and small arenas. However, this may be the result of the arenas small size resulting in bleeding of light into the dark zone from the bright half of the arena. An increase in activity when in the dark has also been observed in zebrafish. It has been observed that a sudden transition to darkness results in a significant and sudden increase in locomotor activities which have been described as a light searching behaviour and is attributed to increased stress or anxiety [75–77]. This theory may also be the case for *A. franciscana*. However, it is worth noting that the increase in activity during dark phases was neither immediate upon sudden transition to the dark, nor consistent for every dark phase which would be expected for a startle or escape response associated with anxiety or stress.

4.2. Psychotropic Exposures

Here, *A. franciscana* were exposed to environmentally relevant concentrations of four psychotropic compounds with varying modes of action, and their swimming behaviours were assessed. Fluoxetine had a significant impact on the swimming speed of *A. franciscana* between treatment groups in that velocity was greater in the two highest treatment groups compared to the lowest treatment and control animals. Pairwise comparisons revealed that

velocity in the 100 ng/L treated animals was significantly greater than the controls and the 10 ng/L treated animals. There are many studies in the literature which have reported increased activity when exposed to fluoxetine in crustaceans including amphipods [22,30]; shore crabs [65]; crayfish [39]; and *Daphnia* [78]. The results from this study appear to support the current literature and our hypothesis that fluoxetine would increase activity in *A. franciscana*.

No significant impacts on swimming behaviours were observed for oxazepam amitriptyline or venlafaxine. It was noted that both amitriptyline and venlafaxine exposure consistently reduced swimming speeds compared to controls, albeit these results failed to pass our significance threshold. Whether these represent real effects would need further investigation however effect sizes revealed through the ICC and R^2 were modest at best. It was, however, interesting to note that both of these compounds target norepinephrine receptors. Both norepinephrine and octopamine have been suggested to reduce swimmeret rhythm in crayfish [79] although the exact role of NE in crustaceans is still debated [80].

It has been reported in the literature that compounds with varying modes of action can result in significant effects being found for some compounds, while others have no impacts on behaviour. A study on crayfish reported a significant increase in activity following oxazepam exposure while no significant impacts were observed for venlafaxine [43]. Studies on *Daphnia* behaviour with multiple psychotropic compounds found that fluoxetine induced the most severe behavioural effects and impacted behaviours at the lowest concentrations compared to all other compounds tested [1,81]. It was hypothesised that all of the psychotropic compounds tested in this study could impact the behaviour of *A. franciscana* as they share many of the neurotransmitters that are targeted by neuroactive drugs in vertebrates. This hypothesis was not supported by the results found in this study which instead suggest that oxazepam, amitriptyline, and venlafaxine have no significant effects on swimming speed. This is not to say that these compounds could not influence other behaviours. It has been reported that compounds can affect organisms independently of their intended pathway. In a recent study by Rivetti et al. in 2018 [82], the genes encoding for serotonin synthesis were deleted in *D. magna* generating mutants completely deprived of serotonin. Fluoxetine altered behavioural responses in wild type *D. magna* that had serotonin but had no effect on serotonin deprived mutants as expected for compounds acting via the serotonergic-pathway. However, fluoxetine impacted fecundity and life-history responses of both mutants and wild type *D. magna* suggesting that this drug affects reproduction independently of the serotonin pathway. It has also been reported that psychotropic compounds can impact some behaviours but not others. Tierney et al. [39] found that fluoxetine significantly reduced locomotion in juvenile crayfish but had no impact on thigmotaxis or sheltering behaviours. A study by Mesquita et al. in 2011 [65] reported that crabs exposed to fluoxetine were significantly more active than unexposed crabs, but no differences were observed in their speed. Whilst this study found no significant effects of oxazepam, amitriptyline, and venlafaxine on swimming speed, experiments on other endpoints would be required to elucidate whether it is just swimming speed or whether *A. franciscana* are not sensitive to these compounds.

In this study, the length of exposure significantly impacted the swimming speed of *A. franciscana* across all experiments, independently of compound or dose, with animals swimming slower with increased length of exposure. This could be explained by the static nature of the test arena used for compound exposure. Previous studies on *A. franciscana* found that immobility and fatality of *Artemia* larvae significantly increased after 12 h in a static system, whereas under constant water flow in a microfluidic system, activity levels remained unchanged after 18 h [6]. The reduction in artemia health and mobility was attributed to a depletion of oxygen in a static system. Medium arenas were used for psychotropic exposures in this study as per results from preliminary experiments. The medium arena size allowed for a greater volume of water and surface area compared to small arenas, and a water change was performed after three days to combat the effects of oxygen depletion and compound degradation. Mortality was 12.5% after 1 week of

exposure and 29% of deceased animals came from the control groups suggesting that mortality and the decreased activity of *A. franciscana* throughout the experiment was more likely a result of oxygen depletion rather than effects of toxicity from the compounds. It is also possible that the reduction of activity of *A. franciscana* was the result of habituation to the behavioural system. Habituation to behavioural assays has also been reported in a wide range of both vertebrate and invertebrate species [39,70,83–85] and has been demonstrated in *E. marinus* and *G. pulex* [10,11]. Repeating the experiment under flow through conditions may help to elucidate weather the reduction in swimming speed observed in *A. franciscana* was a result of depleted oxygen or habituation to the behavioural assay.

5. Conclusions

In this study baseline data was collected on swimming and phototactic behaviours of A. franciscana. A trade-off was observed between high-throughput analysis and providing space to behave. Results indicate a 12-well was the minimum arena size in which swimming behaviours were not limited. Arena size also had a significant impact on phototactic behaviours, but light intensity did not. Analysing velocity data in 10-s time bins found differences between medium and large arena sizes whereas 2-min time bins did not suggesting that the increased sensitivity of the 10 s time bin may be necessary for elucidating subtle differences between treatments. Following the collection of baseline data, behavioural assays to assess swimming speed and photosensitivity were developed. Velocity proved a useful endpoint to measure swimming speed and photosensitivity in *A. franciscana*. Compounds with differing MOAs varied in their impacts on animal behaviours. The results from this study further support the evidence that fluoxetine can impact swimming behaviours at environmentally relevant concentrations. Interestingly, no significant effects were observed in the other compounds although it was noted that those that target norepinephrine as well as serotonin displayed reduced swimming activity. We suggest a simple, fast, high throughput assay for *A. franciscana* and provides a baseline on the impacts of a range of psychotropic compounds on the swimming behaviours of a model crustacean species used in ecotoxicology studies.

Author Contributions: Conceptualization, S.A.K., M.O.P. and A.T.F.; methodology, S.A.K.; formal analysis, S.A.K.; investigation, S.A.K.; writing—original draft preparation, S.A.K.; writing—review and editing, S.A.K., M.O.P. and A.T.F.; visualization, S.A.K.; supervision, M.O.P. and A.T.F.; project administration, M.O.P. and A.T.F.; funding acquisition, M.O.P. and A.T.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee AWERB of the University of Portsmouth (protocol code 218D, approved on 7 February 2018).

Informed Consent Statement: Not applicable.

Acknowledgments: We would like to acknowledge Bill Buddenberg for producing the custom acrylic strips used in phototaxis studies. S.A.K. was supported through a University of Portsmouth studentship and both A.T.F. and M.O.P. are currently supported through the EU Interreg project REDPOL.

Conflicts of Interest: The authors declare no conflict of interests.

References

1. Simão, F.C.P.; Martínez-Jerónimo, F.; Blasco, V.; Moreno, F.; Porta, J.M.; Pestana, J.L.T.; Soares, A.M.V.M.; Raldúa, D.; Barata, C. Using a new high-throughput video-tracking platform to assess behavioural changes in *Daphnia magna* exposed to neuro-active drugs. *Sci. Total Environ.* **2019**, *662*, 160–167. [CrossRef]
2. Melvin, S.D.; Wilson, S.P. The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. *Chemosphere* **2013**, *93*, 2217–2223. [CrossRef]

3. Parker, M.O. Adult vertebrate behavioural aquatic toxicology: Reliability and validity. *Aquat. Toxicol.* **2016**, *170*, 323–329. [CrossRef] [PubMed]
4. Pyle, G.G.; Ford, A.T. Behaviour revised: Contaminant effects on aquatic animal behaviour. *Aquat. Toxicol.* **2016**, *182*, 226–228. [CrossRef] [PubMed]
5. Wlodkowic, D.; Khoshmanesh, K.; Akagi, J.; Williams, D.E.; Cooper, J.M. Wormometry-on-a-chip: Innovative technologies for In Situ Analysis of Small Multicellular Organisms. *Cytom. Part. A* **2011**, *79*, 799–813. [CrossRef] [PubMed]
6. Huang, Y.; Persoone, G.; Nugegoda, D.; Wlodkowic, D. Enabling sub-lethal behavioral ecotoxicity biotests using microfluidic Lab-on-a-Chip technology. *Sens. Actuators B Chem.* **2016**, *226*, 289–298. [CrossRef]
7. Cartlidge, R.; Nugegoda, D.; Wlodkowic, D. Millifluidic Lab-on-a-Chip technology for automated toxicity tests using the marine amphipod *Allorchestes compressa*. *Sens. Actuators* **2017**, *239*, 660–670. [CrossRef]
8. Richendrfer, H.; Pelkowski, S.D.; Colwill, R.M.; Creton, R. On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. *Behav. Brain Res.* **2012**, *228*, 99–106. [CrossRef]
9. Schnörr, S.J.; Steenbergen, P.J.; Richardson, M.K.; Champagne, D.L. Measuring thigmotaxis in larval zebrafish. *Behav. Brain Res.* **2012**, *228*, 367–374. [CrossRef] [PubMed]
10. Kohler, S.A.; Parker, M.O.; Ford, A.T. Shape and size of the arenas affect amphipod behaviours: Implications for ecotoxicology. *PeerJ* **2018**, *6*, e5271. [CrossRef]
11. Kohler, S.A.; Parker, M.O.; Ford, A.T. Species-specific behaviours in amphipods highlight the need for understanding baseline behaviours in ecotoxicology. *Aquat. Toxicol.* **2018**, *202*, 173–180. [CrossRef]
12. Lu, Y.; Yu, J. A Well-Established Method for the Rapid Assessment of Toxicity Using *Artemia* spp. Model. In *Assessment and Management of Radioactive and Electronic Wastes*; IntechOpen: London, UK, 2019; pp. 1–15. [CrossRef]
13. Persoone, G.; Wells, P. Artemia in aquatic toxicology: A review. *Artemia Res. Appl.* **1987**, *1*, 259–275.
14. Faimali, M.; Garaventa, F.; Piazza, V.; Greco, G.; Corrà, C.; Magillo, F.; Pittore, M.; Giacco, E.; Gallus, L.; Falugi, C.; et al. Swimming speed alteration of larvae of *Balanus amphitrite* as a behavioural end-point for laboratory toxicological bioassays. *Mar. Biol.* **2006**, *149*, 87–96. [CrossRef]
15. Garaventa, F.; Gambardella, C.; Di Fino, A.; Pittore, M.; Faimali, M. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. *Ecotoxicology* **2010**, *19*, 512–519. [CrossRef] [PubMed]
16. Manfra, L.; Savorelli, F.; Di Lorenzo, B.; Libralato, G.; Comin, S.; Conti, D.; Floris, B.; Francese, M.; Gallo, M.L.; Gartner, I.; et al. Intercalibration of ecotoxicity testing protocols with *Artemia franciscana*. *Ecol. Indic.* **2015**, *57*, 41–47. [CrossRef]
17. Andrés-Costa, M.J.; Proctor, K.; Sabatini, M.T.; Gee, A.P.; Lewis, S.E.; Pico, Y.; Kasprzyk-Hordern, B. Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance. *Sci. Rep.* **2017**, *7*, 1–13. [CrossRef]
18. Calisto, V.; Esteves, V.I. Psychiatric pharmaceuticals in the environment. *Chemosphere* **2009**, *77*, 1257–1274. [CrossRef] [PubMed]
19. Kolpin, D.W.; Furlong, E.; Meyer, M.; Thurman, M.E.; Zaugg, S.; Barber, L.; Buxton, H. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999–2000: A National Reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202–1211. [CrossRef] [PubMed]
20. Meador, J.P.; Yeh, A.; Young, G.; Gallagher, E.P. Contaminants of emerging concern in a large temperate estuary. *Environ. Pollut.* **2016**, *213*, 254–267. [CrossRef]
21. Barry, M.J. 'Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish'. *Ecotoxicology* **2013**, *22*, 425–432. [CrossRef] [PubMed]
22. Bossus, M.C.; Guler, Y.Z.; Short, S.J.; Morrison, E.R.; Ford, A.T. Behavioural and transcriptional changes in the amphipod *Echinogammarus marinus* exposed to two antidepressants, fluoxetine and sertraline. *Aquat. Toxicol.* **2014**, *151*, 46–56. [CrossRef] [PubMed]
23. Campos, B.; Piña, B.; Fernández-Sanjuán, M.; Lacorte, S.; Barata, C. Enhanced offspring production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and food-dependent effects. *Aquat. Toxicol.* **2012**, *109*, 100–110. [CrossRef] [PubMed]
24. Campos, B.; Garcia-Reyero, N.; Rivetti, C.; Escalon, L.; Habib, T.; Tauler, R.; Tsakovski, S.; Pina, B.; Barata, C. Identification of Metabolic Pathways in *Daphnia magna* Explaining Hormetic Effects of Selective Serotonin Reuptake Inhibitors and 4-Nonylphenol Using Transcriptomic and Phenotypic Responses. *Environ. Sci. Technol.* **2013**, *47*, 9434–9443. [CrossRef] [PubMed]
25. Campos, B.; Rivetti, C.; Kress, T.; Barata, C.; Dircksen, H. Depressing antidepressant: Fluoxetine affects serotonin neurons causing adverse reproductive responses in *Daphnia magna*. *Environ. Sci. Technol.* **2016**. [CrossRef]
26. Lazzara, R.; Blázquez, M.; Porte, C.; Barata, C. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*. *Aquat. Toxicol.* **2012**, *106*, 123–130. [CrossRef]
27. Perreault, H.; Semsar, K.; Godwin, J. Fluoxetine treatment decreases territorial aggression in a coral reef fish. *Physiol. Behav.* **2003**, *79*, 719–724. [CrossRef]
28. Dzieweczynski, T.L.; Hebert, O.L. Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, *Betta splendens*. *Physiol. Behav.* **2012**, *107*, 92–97. [CrossRef]
29. Huber, R.; Delago, A. Serotonin alters decisions to withdraw in fighting crayfish, *Astacus astacus*: The motivational concept revisited. *J. Comp. Physiol. A Sens. NeuralBehav. Physiol.* **1998**, *182*, 573–583. [CrossRef]

30. De Castro-Català, N.; Muñoz, I.; Riera, J.L.; Ford, A.T. Evidence of low dose effects of the antidepressant fluoxetine and the fungicide prochloraz on the behavior of the keystone freshwater invertebrate *Gammarus pulex*. *Environ. Pollut.* **2017**, *231*, 406–414. [CrossRef]
31. Hazelton, P.D.; Cope, W.G.; Mosher, S.; Pandolfo, T.J.; Belden, J.B.; Barnhart, M.C.; Bringolf, R.B. Fluoxetine alters adult freshwater mussel behavior and larval metamorphosis. *Sci. Total Environ.* **2013**, *445*, 94–100. [CrossRef]
32. Weinberger, J.; Klaper, R. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow). *Aquat. Toxicol.* **2014**, *151*, 77–83. [CrossRef]
33. Barbosa, A.; Alves, F.L.; Pereira, A.D.S.F.; Ide, L.M.; Hoffmann, A. Behavioral characterization of the alarm reaction and anxiolytic-like effect of acute treatment with fluoxetine in piaucu fish. *Physiol. Behav.* **2012**, *105*, 784–790. [CrossRef] [PubMed]
34. Martin, J.M.; Saaristo, M.; Bertram, M.G.; Lewis, P.J.; Coggan, T.L.; Clarke, B.O.; Wong, B.B.M. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. *Environ. Pollut.* **2017**, *222*, 1–8. [CrossRef] [PubMed]
35. Egan, R.J.; Bergner, C.L.; Hart, P.C.; Cachat, J.M.; Canavello, P.R.; Elegante, M.F.; Elkhayat, S.I.; Bartels, B.K.; Tien, A.K.; Tien, D.H.; et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* **2009**, *205*, 38–44. [CrossRef] [PubMed]
36. Hamilton, T.J.; Kwan, G.T.; Gallup, J.; Tresguerres, M. Acute fluoxetine exposure alters crab anxiety-like behaviour, but not aggressiveness. *Sci. Rep.* **2016**, *6*, 19850. [CrossRef] [PubMed]
37. Wong, R.Y.; Oxendine, S.E.; Godwin, J. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genom.* **2013**, *14*, 348. [CrossRef] [PubMed]
38. Guler, Y.; Ford, A.T. Anti-depressants make amphipods see the light. *Aquat. Toxicol.* **2010**, *99*, 397–404. [CrossRef]
39. Tierney, A.J.; Hanzlik, K.N.; Hathaway, R.M.; Powers, C.; Roy, M. Effects of fluoxetine on growth and behavior in the crayfish *Orconectes rusticus*. *Mar. Freshw. Behav. Physiol.* **2016**, *49*, 133–145. [CrossRef]
40. Kosjek, T.; Perko, S.; Zupanc, M.; ZanoŠki Hren, M.; Landeka Dragičević, T.; Žigon, D.; Kompare, B.; Heath, E. Environmental occurrence, fate and transformation of benzodiazepines in water treatment. *Water Res.* **2012**, *46*, 355–368. [CrossRef]
41. Brodin, T.; Nordling, J.; Lagesson, A.; Klaminder, J.; Hellström, G.; Christensen, B.; Fick, J. Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish, (*Rutilus rutilus*). *J. Toxicol. Environ. Health Part. A Curr. Issues* **2017**, *80*, 963–970. [CrossRef]
42. Chiffre, A.; Clérandeau, C.; Dwoinikoff, C.; Le Bihanic, F.; Budzinski, H.; Geret, F.; Cachot, J. Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (*Oryzias latipes*) larvae above environmental concentrations. *Environ. Sci. Pollut. Res.* **2016**, *23*, 4964–4977. [CrossRef] [PubMed]
43. Kubec, J.; Hossain, M.; Grabicová, K.; Randák, T.; Kouba, A.; Grabic, R.; Roje, S.; Buřič, M. Oxazepam Alters the Behavior of Crayfish at Diluted Concentrations, Venlafaxine Does Not. *Water* **2019**, *11*, 196. [CrossRef]
44. Brodin, T.; Fick, J.; Jonsson, M.; Klaminder, J. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. *Science* **2013**, *339*, 814–815. [CrossRef] [PubMed]
45. Klaminder, J.; Hellström, G.; Fahlman, J.; Jonsson, M.; Fick, J.; Lagesson, A.; Bergman, E.; Brodin, T. Drug-Induced Behavioral Changes: Using Laboratory Observations to Predict Field Observations. *Front. Environ. Sci.* **2016**, *4*, 1–9. [CrossRef]
46. Saaristo, M.; Lagesson, A.; Bertram, M.G.; Fick, J.; Klaminder, J.; Johnstone, C.P.; Wong, B.B.M.; Brodin, T. Behavioural effects of psychoactive pharmaceutical exposure on European perch (*Perca fluviatilis*) in a multi-stressor environment. *Sci. Total Environ.* **2019**, *655*, 1311–1320. [CrossRef] [PubMed]
47. Hellström, G.; Klaminder, J.; Finn, F.; Persson, L.; Alanärä, A.; Jonsson, M.; Fick, J.; Brodin, T. GABAergic anxiolytic drug in water increases migration behaviour in salmon. *Nat. Commun.* **2016**, *7*. [CrossRef] [PubMed]
48. Kasprzyk-Hordern, B.; Dinsdale, R.M.; Guwy, A.J. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. *Water Res.* **2009**, *43*, 363–380. [CrossRef]
49. Lajeunesse, A.; Smyth, S.A.; Barclay, K.; Sauvé, S.; Gagnon, C. Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. *Water Res.* **2012**, *46*, 5600–5612. [CrossRef]
50. Minguez, L.; Farcy, E.; Ballandonne, C.; Lepailleur, A.; Serpentini, A.; Lebel, J.M.; Bureau, R.; Halm-Lemeille, M.P. Acute toxicity of 8 antidepressants: What are their modes of action? *Chemosphere* **2014**, *108*, 314–319. [CrossRef]
51. Petrie, B.; Barden, R.; Kasprzyk-Hordern, B. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Res.* **2014**, *72*, 3–27. [CrossRef]
52. Subramanian, P.; Subbaraj, R. Effect of imipramine and amitriptyline on circadian locomotor activity rhythm in the field mouse *Mus booduga*. *Proc. Anim. Sci.* **1990**, *99*, 503–507. [CrossRef]
53. Shakunthala, V.; Najafabadi, Z.G.; Sathisha, B. Effect of Amitriptyline hydrochloride an antidepressant on courtship and reproducibility of *D. melanogaster*. *Int. J. Currrent Microbiol. Appl. Sci.* **2014**, *3*, 649–656.
54. Egan, J.; Earley, C.J.; Leonard, B.E. The effect of amitriptyline and mianserine (Org. GB94) on food motivated behaviour of rats trained in a runway: Possible correlation with biogenic amine concentration in the limbic system. *Psychopharmacology* **1979**, *61*, 143–147. [CrossRef] [PubMed]

55. Enginar, N.; Hatipoğlu, I.; Firtina, M. Evaluation of the acute effects of amitriptyline and fluoxetine on anxiety using grooming analysis algorithm in rats. *Pharmacol. Biochem. Behav.* **2008**, *89*, 450–455. [CrossRef]
56. Parra, A.; Vinader-Caerols, C.; Ferrer-Añó, A.; Urquiza, A.; Monleón, S. The effect of amitriptyline on inhibitory avoidance in mice is dose-dependent. *Psicothema* **2009**, *21*, 528–530.
57. Fong, P.P.; DiPenta, K.E.; Jonik, S.M.; Ward, C.D. Short-term exposure to tricyclic antidepressants delays righting time in marine and freshwater snails with evidence for low-dose stimulation of righting speed by imipramine. *Environ. Sci. Pollut. Res.* **2019**. [CrossRef]
58. Fong, P.P.; Molnar, N. Antidepressants cause foot detachment from substrate in five species of marine snail. *Mar. Environ. Res.* **2013**, *84*, 24–30. [CrossRef] [PubMed]
59. Fong, P.P.; Bury, T.B.; Dworkin-Brodsky, A.D.; Jasion, C.M.; Kell, R.C. The antidepressants venlafaxine ("Effexor") and fluoxetine ("Prozac") produce different effects on locomotion in two species of marine snail, the oyster drill (*Urosalpinx cinerea*) and the starsnail (*Lithopoma americanum*). *Mar. Environ. Res.* **2015**, *103*, 89–94. [CrossRef] [PubMed]
60. Bisesi, J.H.; Bridges, W.; Klaine, S.J. Reprint of: Effects of the antidepressant venlafaxine on fish brain serotonin and predation behavior. *Aquat. Toxicol.* **2014**, *151*, 88–96. [CrossRef]
61. De Oliveira, R.A.; Cunha, G.M.A.; Karla, K.D.; De Bruin, G.S.; Dos Santos-Filho, E.A.; Viana, G.S.B.; De Bruin, V.M.S. The effect of venlafaxine on behaviour, body weight and striatal monoamine levels on sleep-deprived female rats. *Pharmacol. Biochem. Behav.* **2004**, *79*, 499–506. [CrossRef]
62. McGrath, C.; Norman, T.R. The effect of venlafaxine treatment on the behavioural and neurochemical changes in the olfactory bulbectomised rat. *Psychopharmacology* **1998**, *136*, 394–401. [CrossRef] [PubMed]
63. Nowakowska, E.; Kus, K.; Chodera, A. Comparison of Behavioural Effects of Venlafaxine and Imipramine in Rats. *Arzneimittelforschung* **2012**, *53*, 237–242. [CrossRef] [PubMed]
64. Wang, C.H.; Gu, J.Y.; Zhang, X.L.; Dong, J.; Yang, J.; Zhang, Y.L.; Ning, Q.F.; Shan, X.W.; Li, Y. Venlafaxine ameliorates the depression-like behaviors and hippocampal S100B expression in a rat depression model. *Behav. Brain Funct.* **2016**, *12*, 1–10. [CrossRef] [PubMed]
65. Mesquita, S.R.; Guilhermino, L.; Guimarães, L. Biochemical and locomotor responses of *Carcinus maenas* exposed to the serotonin reuptake inhibitor fluoxetine. *Chemosphere* **2011**, *85*, 967–976. [CrossRef] [PubMed]
66. Bradley, D.J.; Forward, R.B. Phototaxis of adult brine shrimp, *Artemia salina*. *Can. J. Zool.* **1984**, *62*, 2357–2359. [CrossRef]
67. Dojmi Di Delupis, G.; Rotondo, V. Phototaxis in aquatic invertebrates: Possible use in ecotoxicity test. *Ecotoxicol. Environ. Saf.* **1988**, *16*, 189–193. [CrossRef]
68. Sumpter, J.P.; Donnachie, R.L.; Johnson, A.C. The apparently very variable potency of the anti-depressant fluoxetine. *Aquat. Toxicol.* **2014**, *151*, 57–60. [CrossRef] [PubMed]
69. Melvin, S.D.; Petit, M.A.; Duvignacq, M.C.; Sumpter, J.P. Towards improved behavioural testing in aquatic toxicology: Acclimation and observation times are important factors when designing behavioural tests with fish. *Chemosphere* **2017**, *180*, 430–436. [CrossRef]
70. Liu, L.; Davis, R.L.; Roman, G. Exploratory activity in *Drosophila* requires the kurtz nonvisual arrestin. *Genetics* **2007**, *175*, 1197–1212. [CrossRef]
71. Montgomery, K.C. The relation between exploratory behaviour and spontaneous alternation in the white rat. *J. Comp. Physiol. Psychol.* **1951**, *44*, 582–589. [CrossRef]
72. Oldham, J.; Morlock, H. The effects of open-field size on activity in the Mongolian gerbil. *Psychon. Sci.* **1970**, *20*, 290. [CrossRef]
73. Forward, R.B.; Rittschof, D. Brine shrimp larval photoresponses involved in diel vertical migration: Activation by fish mucus and modified amino sugars. *Limnol. Oceanogr.* **1999**, *44*, 1904–1916. [CrossRef]
74. Pearre, S. Problems of detection and interpretation of vertical migration. *J. Plankton Res.* **1979**, *1*, 29–44. [CrossRef]
75. Basnet, R.; Zizioli, D.; Taweedet, S.; Finazzi, D.; Memo, M. Zebrafish Larvae as a Behavioral Model in Neuropharmacology. *Biomedicines* **2019**, *7*, 23. [CrossRef] [PubMed]
76. Li, Q.; Lin, J.; Zhang, Y.; Liu, X.; Chen, X.Q.; Xu, M.Q.; He, L.; Li, S.; Guo, N. Differential behavioral responses of zebrafish larvae to yohimbine treatment. *Psychopharmacology* **2015**, *232*, 197–208. [CrossRef]
77. Van Den Bos, R.; Mes, W.; Galligani, P.; Heil, A.; Zethof, J.; Flik, G.; Gorissen, M. Further characterisation of differences between TL and AB Zebrafish (*Danio rerio*): Gene expression, physiology and behaviour at day 5 of the larval stage. *PLoS ONE* **2017**, *12*, e0175420. [CrossRef]
78. Nielsen, M.E.; Roslev, P. Behavioral responses and starvation survival of *Daphnia magna* exposed to fluoxetine and propranolol. *Chemosphere* **2018**, *211*, 978–985. [CrossRef]
79. Mulloney, B.; Acevedo, L.D.; Bradbury, A.G. Modulation of the crayfish swimmeret rhythm by octopamine and the neuropeptide proctolin. *J. Neurophysiol.* **1987**, *58*, 584–597. [CrossRef]
80. Bauknecht, P.; Jékely, G. Ancient coexistence of norepinephrine, tyramine, and octopamine signaling in bilaterians. *BMC Biol.* **2017**, *15*, 6. [CrossRef]
81. Rivetti, C.; Campos, B.; Barata, C. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. *Aquat. Toxicol.* **2016**, *170*, 289–296. [CrossRef]
82. Rivetti, C.; Campos, B.; Piña, B.; Raldúa, D.; Kato, Y.; Watanabe, H.; Barata, C. Tryptophan hydroxylase (TRH) loss of function mutations induce growth and behavioral defects in *Daphnia magna*. *Sci. Rep.* **2018**, *8*, 1–11. [CrossRef] [PubMed]

83. Bourin, M.; Hascoët, M. The mouse light-dark box test. *Eur. J. Pharmacol.* **2003**, *463*, 55–65. [CrossRef]
84. Magurran, A.E.; Girling, S.L. Predator model recognition and response habituation in shoaling minnows. *Anim. Behav.* **1986**, *34*, 510–518. [CrossRef]
85. Wong, K.; Elegante, M.; Bartels, B.; Elkhayat, S.; Tien, D.; Roy, S.; Goodspeed, J.; Suciu, C.; Tan, J.; Grimes, C.; et al. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). *Behav. Brain Res.* **2010**, *208*, 450–457. [CrossRef]

toxics

MDPI

Article

Pharmacological Modulation of Behaviour, Serotonin and Dopamine Levels in *Daphnia magna* Exposed to the Monoamine Oxidase Inhibitor Deprenyl

Marina Bellot [1], Melissa Faria [2], Cristian Gómez-Canela [1], Demetrio Raldúa [2] and Carlos Barata [2,*]

[1] Department of Analytical Chemistry and Applied (Chromatography Section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain; marina.bellot@iqs.url.edu (M.B.); cristian.gomez@iqs.url.edu (C.G.-C.)

[2] Institute for Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain; mdfqam@cid.csic.es (M.F.); drpqam@cid.csic.es (D.R.)

* Correspondence: carlos.barata@idaea.csic.es

Abstract: This study assessed the effects of the monoamine oxidase (MAO) inhibitor deprenyl in *Daphnia magna* locomotor activity. The mechanisms of action of deprenyl were also determined by studying the relationship between behaviour, MAO activity and neurotransmitter levels. Modulation of the *D. magna* monoamine system was accomplished by 24 h exposure to two model psychotropic pharmaceuticals with antagonistic and agonistic serotonin signalling properties: 10 mg/L of 4-chloro-DL-phenylalanine (PCPA) and 1 mg/L of deprenyl, respectively. Contrasting behavioural outcomes were observed for deprenyl and PCPA reflected in decreased basal locomotor activity and enhanced habituation for the former compound and delayed habituation for the latter one. Deprenyl exposure inhibited monoamine oxidase (MAO) activity and increased the concentrations of serotonin, dopamine and the dopamine metabolite 3-methoxytyramine in whole *D. magna* extracts. Our findings indicate that *D. magna* is a sensitive and useful nonvertebrate model for assessing the effects of short-term exposure to chemicals that alter monoamine signalling changes.

Keywords: *Daphnia magna*; neurotransmitter; modulation; pharmaceuticals

Citation: Bellot, M.; Faria, M.; Gómez-Canela, C.; Raldúa, D.; Barata, C. Pharmacological Modulation of Behaviour, Serotonin and Dopamine Levels in *Daphnia magna* Exposed to the Monoamine Oxidase Inhibitor Deprenyl. *Toxics* **2021**, *9*, 187. https://doi.org/10.3390/toxics9080187

Academic Editor: Ki-Tae Kim

Received: 15 July 2021
Accepted: 6 August 2021
Published: 9 August 2021

1. Introduction

Animal behaviour to environmental stimuli changes such as predation or food availability is a key fitness trait [1]. Of particular interest are behavioural responses related to predator avoidance such as sudden locomotion changes in response to visual or tactile stimuli [2,3]. Any changes in the normal behavioural conduct could compromise individual survival. Neurotransmitters modulate behavioural plasticity at the molecular level. Serotonin is one of the major neurotransmitters in the central nervous system (CNS), modulating many behaviours including perception, mood, reward, anger, aggression, appetite, memory, sexuality and attention [4]. Neurological pathologies such as schizophrenia, depression and anxiety have been related to dysfunctions in the serotonergic system. The serotonergic system is well-known in vertebrates, but little is known about many invertebrates. Tryptophan hydroxylase (TPH) is the enzyme that converts tryptophan to 5-hydroxytryptophan (5-HTP), which is the rate-limiting step of serotonin synthesis. When serotonin is released to the synaptic cleft, the serotonin transporter SERT mediates its uptake/reuptake to the serotonergic neurons, where monoamine oxidase (MAO) metabolizes serotonin to 5-hydroxy-indolecetaldehyde, which is quickly metabolized by aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of serotonin [5].

Many of the emerging contaminants present in surface waters are neuroactive substances required for highly prescribed drugs to control cardiac and neurological disorders [6]. Neuroactive compounds targeting the serotonergic system are of particular

concern due to the essential role of serotonin in both neurotransmission and neuromodulation. Thus, it is not surprising to find many investigations on the effects of selective serotonin reuptake inhibitors (SSRIs) in nontarget aquatic species like fish but also in invertebrates [7,8]. Nevertheless, information regarding other potential modes of actions, such as TPH or monoamine oxidase (MAO) inhibition, is still scarce and almost absent for many invertebrates. In this regard, the ecotoxicological model species *Daphnia magna* is a good candidate for studying the role of serotonergic signalling pathways in behaviour and its modulation by neuroactive pollutants. *D. magna* is probably the most widely use organism in aquatic toxicological evaluations. It is a well-known ecological model and has its genome fully sequenced and annotated [9]. Therefore, *D. magna* offers the opportunity to study molecular and apical effects of pharmaceuticals. Furthermore, about 80% of the molecular human drug targets are also present in the *Daphnia* genome [10]. This means that it is likely that many neuroactive pollutants affect this organism. Recently, it was shown that CRISPR/Cas9-mediated tryptophan hydroxylase (TPH) knockout clonal *D. magna* lines lack serotonin and show an abnormally high basal swimming activity and a markedly reduced habituation to repetitive light stimuli [2,11]. The selective serotonin reuptake inhibitor (SSRI) fluoxetine that is the active ingredient of Prozac increases the serotonin levels in the brain of *D. magna* and increases reproduction [12]. Conversely, chloro-DL-phenylalanine (PCPA), which is an inhibitor of TPH, decreases serotonin levels in *D. magna* [13]. Fluoxetine and PCPA, however, despite having opposite effects on serotonin levels, have similar effects on *Daphnia* cognitive behaviour, decreasing habituation to repetitive light stimuli [2]. The aim of this study was to better characterize the serotonergic system in *Daphnia*. In particular, we studied the mechanisms of action of monoamine oxidase (MAO) inhibitors, such as deprenyl, in *D. magna*, addressing its effects on the target MAO enzyme activity, neurotransmitter levels and behavioural responses. Our hypothesis is that deprenyl should increase serotonin and probably also dopamine levels in *D. magna* and have behavioural responses different from those of the drugs that depress serotonin levels, such as PCPA.

2. Materials and Methods

2.1. Experimental Animals

Five-day old juveniles from a single clone of *D. magna* (clone F) were used for exposure, behavioural and biochemical determinations assays. Further details of culture conditions are provided in the Supplementary Materials, Section S1.1.

2.2. Experimental Procedures

Deprenyl (CAS:14611-52-0) and chloro-DL-phenylalanine (PCPA; CAS: 7424-00-2) were of high-quality grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in the Milli-Q water and then diluted in ASTM hard water. *D. magna* juveniles (5-days-old) were pre-exposed to the selected compounds in ASTM hard water without food for 24 h. The selected chemicals concentrations were far below those having detrimental effects on survival or swimming (>20 mg/L; [2]). The compounds were initially screened for light stimuli motile responses using a broad concentration range ranking from 0.1 to 1000 µg/L for deprenyl and from 0.1 to 10,000 µg/L for PCPA [2]. The concentrations having the greatest effect (1 and 10 mg/L for deprenyl and PCPA, respectively) were used in the subsequent light stimuli motile response assays. For the behavioural assessment, exposures were conducted in groups of 12 individuals in 300 mL of the test medium in 500 mL glass vessels. Following 24 h of exposure, 12 animals were distributed randomly to 24-well plates (two treatments per plate). From 12 to 24 individual replicates were performed for each tested chemical. For the MAO activity and neurotransmitters assessment, exposures were carried out in groups of 20 or 5 individuals in 500 mL or 100 mL of the medium, respectively. The treatments were replicated five times. Following exposure, the individuals from each replicate (20 or 5) were pooled in an Eppendorf, the water was removed and the rest was deep-frozen in liquid N_2. The samples were stored at

−80 °C until analysis. For each behavioural assay and MAO determination, juveniles were collected from 2–4 trials of the same experimental setup conducted on different days and with different batches of animals.

2.3. Behavioural Analysis

The *Daphnia* photomotor response assay (DPRA) was performed as described in [2]. Details of the assay are provided in the Supplementary Materials, Methods, Section S1.2. The assay measured the distance moved after a sudden increase in light intensity across 30 repetitive light stimuli of 1 s followed by 4 s of darkness. Following a previous study, "enhanced photomotor response" (EPR) is defined as the area under the curve (AUC_{EPR}) for the first 10 stimuli where the response to light increases. Conversely, "habituation or non-associative learning" is defined as the area under the curve (AUCh) for the decreasing responses to stimuli [2].

To better characterize the swimming activity under darkness and upon continuous light, basal locomotor activity (BLM) and visual motor response (VMR) analyses of 5-days-old *D. magna* juveniles were also assessed using the same DanioVision system device as described below. Before the video recording, the juveniles were first acclimated for 10 min under dark conditions. Video tracking trials consisted of a 10 min cycle with a 5 min dark period followed by a 5 min light period (290 lux). The basal locomotor activity (BLM) was defined as the total distance (mm) travelled by the juveniles during the last minute of the first dark cycle. The visual motor response (VMR) was based in the hyperactivity period induced by light, which in *D. magna* increased during the first minutes and decreased afterwards.

2.4. Daphnia Monoamine Oxidase (MAO) Activity

D. magna juveniles were collected in pools of 20 individuals and homogenized in ice-cold 10 mM phosphate buffer (pH 7.6) supplemented with 1 mM EDTA using a TissueLyser® (Qiagen, Germantown, MA, USA). The volume of the buffer was adjusted to 100 juveniles/mL. Following centrifugation at 2500× *g*, 4 °C for 5 min, MAO activity was determined in the supernatant according to Faria et al. [14]. Further information is provided in the Supplementary Materials, Section S1.3.

2.5. Extraction and Analysis of Neurotransmitters

Monoaminergic neurotransmitters were extracted from pools of five juveniles according to the procedure adapted from the article by Fuertes et al. [13]. Additional details on the extraction and analysis of neurotransmitters are provided in the Supplementary Materials, Section S1.4.

2.6. Statistical Analysis

The experimental design for behavioural assay responses of controls were compared to those of treatments using either the Student's *t*-test when only controls and deprenyl treatments were used or one-way ANOVA followed by Dunnett's post-hoc test when PCPA was also considered. MAO activities and concentration of metabolites of the unexposed juveniles and the deprenyl-treated ones were compared using the Student's *t*-test. For the *Daphnia* photomotor responses, the area under the curve AUC_{EPR} and AUC_h values obtained for the individual juveniles across the repetitive light stimuli were used for statistical comparisons. The basal and visual locomotor activities of the *D. magna* juveniles monitored during 5 min of dark and 5 min of light were analysed simultaneously using repeated measures ANOVA considering the total distance moved in the last minute of darkness and in each of the five minutes of the light period as the repeated measures (hereafter referred to as the "time"). Prior to the analyses, ANOVA assumptions of data normality and variance homoscedasticity were tested. Analysis of the data was performed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Behaviour

For the *Daphnia* photomotor response assay (DPRA) to repetitive light stimuli, absolute and proportional distances moved across the tested compounds are reported in Figure 1 and the statistics are referred to the AUC values reported in the graph inlet. The statistics are depicted in Table 1. Deprenyl increased habituation significantly ($p < 0.05$) in three out of the four experiments (Figure 1A,B,D) and enhanced photomotor responses in only one of them (Figure 1D). PCPA reduced habituation in the two experiments that were performed and did not enhance photomotor responses (Figure 1C,D).

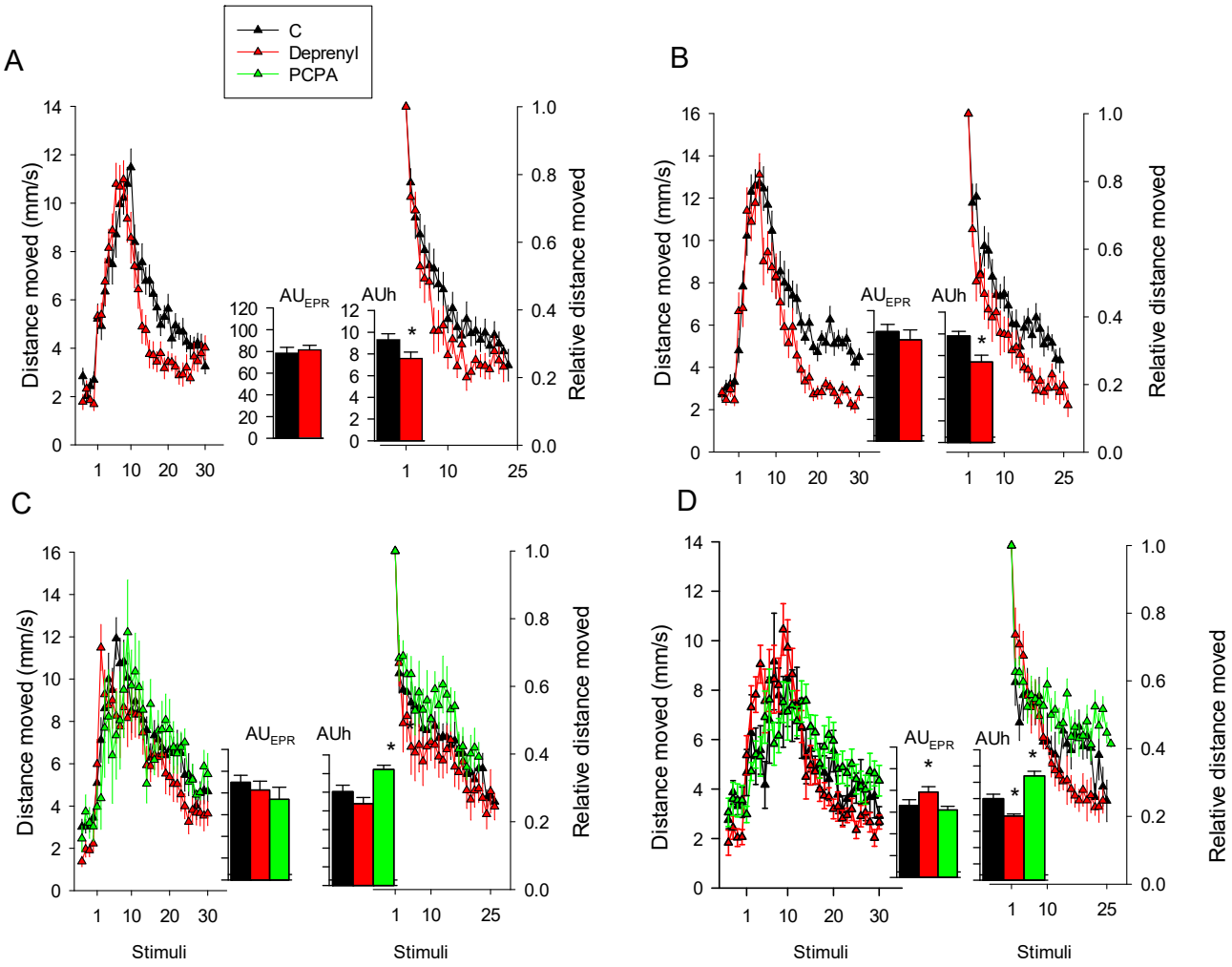

Figure 1. *Daphnia* photomotor responses to repetitive light stimuli following 24 h exposures to 1 and 10 mg/L of deprenyl and PCPA, respectively. Plots of the average distance moved ± SE (n = 12–24) against 30 tapping stimuli at 5 s ISI and corresponding bar charts (graph inlet) of the calculated area under the curve (mean ± SE) for EPR (AUC_{EPR}) and habituation AUCh phases. Within each of the four graphs, the left plots represent the full motile responses whereas the right ones show habituation responses measuring the decrease in the distance moved (proportions) relative to the maximum response to the light stimulus delivered (set to 1). Graphs (**A**,**B**) depict the data obtained across two independent experiments for deprenyl exposures, (**C**,**D**)—for deprenyl and PCPA exposures. Within the AUC bar graphs, * means significant ($p < 0.05$) treatment differeces relative to the controls following the Student's t-test or ANOVA and Dunnett's test. Axis scales for the AUC (graph inlet) are depicted in graph (**A**).

Table 1. Student's *t*-test or one-way ANOVA results testing the effects of deprenyl and PCPA on the area under the curve (AUC) values obtained from *Daphnia* photomotor responses to repetitive light stimuli. AUCEPR and AUCh are, respectively, the areas of enhanced photomotor responses during the first 10 light stimuli and during habituation afterwards. Results for the four identical experiments are reported.

		df	t,F	*P*
Experiment 1	AUCh	31	2.1	0.047
	AUCEPR	31	0.4	0.657
Experiment 2	AUCh	31	3.3	0.003
	AUCEPR	31	0.7	0.493
Experiment 3	AUCh	2,45	10.2	<0.001
	AUCEPR	2,45	0.8	0.459
Experiment 4	AUCh	2,56	10.0	<0.001
	AUCEPR	2,56	3.9	0.026

Basal locomotor activity (BLM) and visual motor response (VMR) analyses of 5-day-old *D. magna* juveniles are depicted in Figure 2. Repeated measures ANOVA denoted significant ($p < 0.05$) effects of treatment or of its interaction with time (Table 2). In all the experiments, deprenyl decreased the basal locomotor activity and in only half of them (Figure 2A,C) increased the response to light. PCPA decreased the response to light in the two experiments performed (Figure 2C,D) and increased the basal locomotor activity in one of them (Figure 2C).

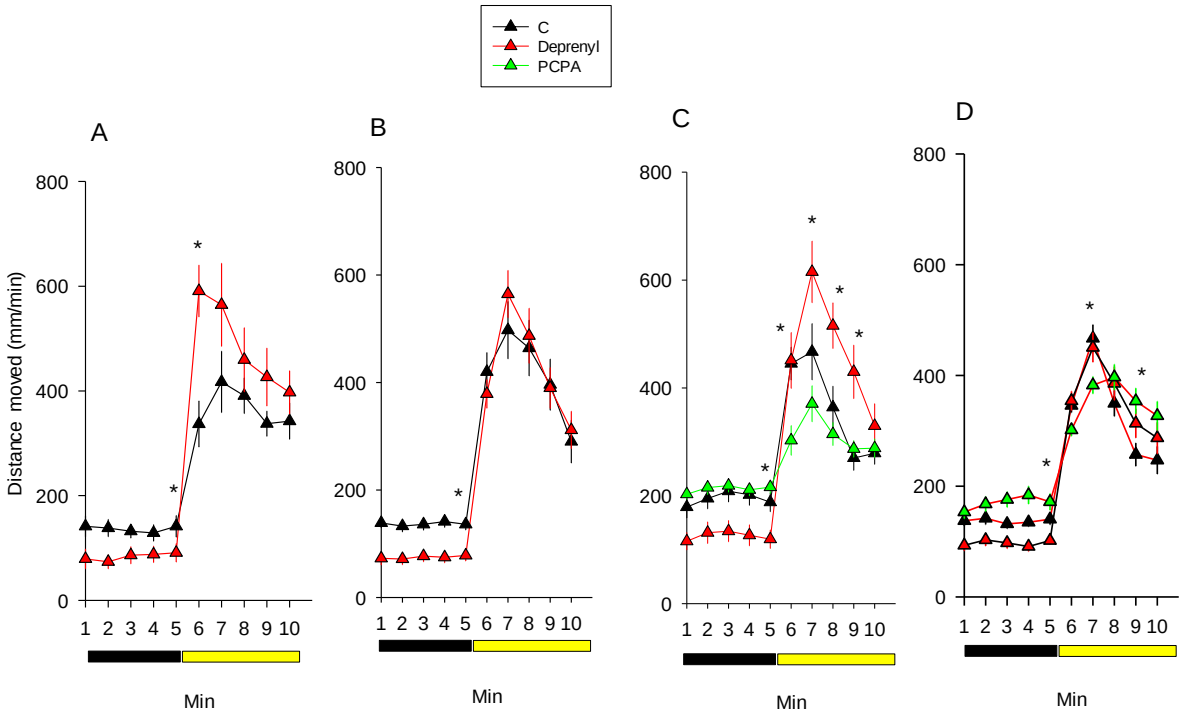

Figure 2. *Daphnia* basal locomotor activity (BLM) and visual motor responses (VMR) across 5 min in darkness and 5 min with light following 24 h exposures to 1 and 10 mg/L of deprenyl and PCPA, respectively. Plots of the average distance moved ± SE (n = 12–24) per minute across the 10 min of the monitored period are shown. Graphs (**A**,**B**) depict the data obtained across two independent experiments for deprenyl exposures and (**C**,**D**) for deprenyl and PCPA ones. Note: * during the minutes 5–10, mean significant ($p < 0.05$) treatment differences relative to the controls following the Student's *t*-test or ANOVA and Dunnett's test.

Table 2. Results of repeated measures one-way ANOVA testing for the effects of treatment, monitoring time and its interaction (Time * Treatment) on the *D. magna* visual responses across the last minute of darkness and the 5 min of light. Results for four identical experiments are reported.

		df	F	*P*
Experiment 1	Time	1,22	16.6	0.001
	Treatment	1,22	4.4	0.048
	Time * Treatment	1,22	0.0	0.923
Experiment 2	Time	1,46	12.6	0.001
	Treatment	1,46	0.2	0.688
	Time * Treatment	1,46	4.2	0.047
Experiment 3	Time	1,69	10.9	0.002
	Treatment	2,69	8.7	<0.001
	Time * Treatment	2,69	5.7	0.005
Experiment 4	Time	1,68	17.9	<0.001
	Treatment	2,68	3.9	0.026
	Time * Treatment	2,68	4.3	0.017

3.2. Biochemical and Neurotransmitter Analysis

MAO activity was significantly ($p < 0.05$) inhibited by deprenyl in the three experiments performed (Figure 3A), and deprenyl significantly ($p < 0.05$) increased serotonin (5-HT), dopamine (DA) and 3-methoxytyramine (3-MT) (Figure 3B–D). Details of the statistics and of nonsignificant metabolite values are provided in Tables S3 and S4, respectively, Supplementary Materials.

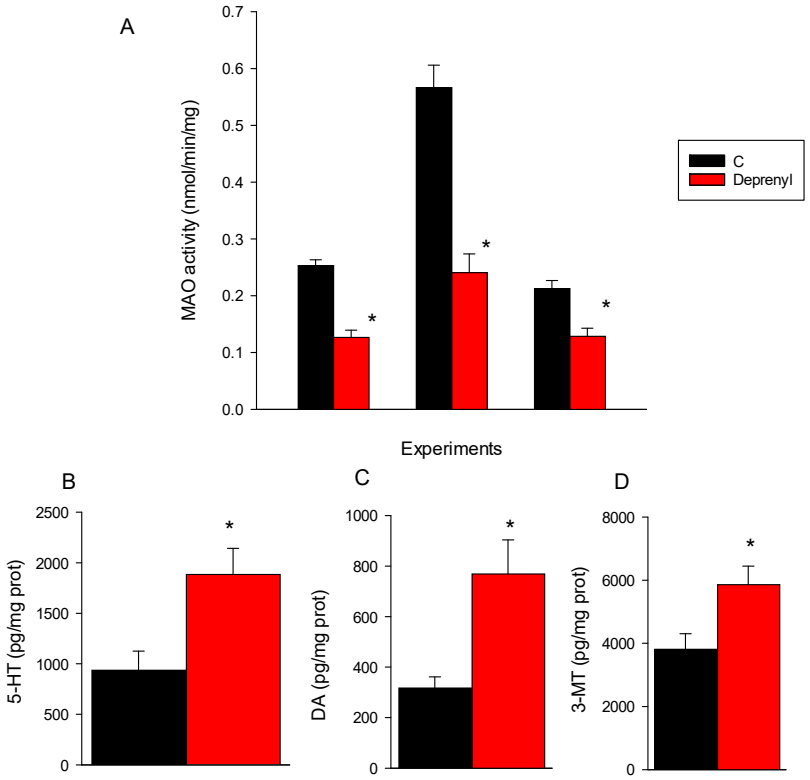

Figure 3. Effects over enzyme activity (**A**) and monoaminergic neurotransmitter levels (**B**–**D**) (means ± SE, n = 5–10) in the whole body of the *D. magna* juveniles following 24 exposures to 1 mg/L of deprenyl. For clarity, monoaminergic neurotransmitters that showed a significant change across treatments are depicted. The rest of the values are provided in Table S3, Supplementary Materials. Note: * the indicated bars mean significant ($p < 0.05$) treatment differences relative to the controls following the Student's *t*-test.

4. Discussion

In this study, we provided for the first time the results on the mode of action of deprenyl in *D. magna*. Deprenyl at 1 mg/L inhibited about 50% of the MAO activity and by doing so increased the concentrations of serotonin and dopamine by 2- and 2.4-fold, respectively. There are two types of MAO inhibitors, type A and type B. Type A inhibitors block the catabolism of noradrenaline and serotonin, and type B inhibitors block the catabolism of dopamine. Deprenyl is a MAO type B inhibitor in mammalian models; however, at high dosages, it inhibits both type A and type B MAO [15]. This means that deprenyl, apparently, has a similar mechanism of action as in vertebrates (i.e., mammals and fish) inhibiting MAO type B but also A activity and thus preventing the metabolism of serotonin and dopamine [14,16]. There is controversy on the role, if any, of the MAO types on the metabolism of monoamines in arthropods [17]. According to the previous review, alternative metabolic routes such as *N*-acetylation, γ-glutamyl conjugation, sugar conjugation, sulfation, β-alanyl conjugation are predominantly used by insects and crustaceans to metabolize monoamines. The reported evidence, however, indicates that ticks and mites have a unique MAO sensitive to deprenyl that catabolizes serotonin and dopamine [18,19]. Furthermore, in the hepatopancreas of the crab *Paralithodes camtschaticus*, there is also only one type of MAO that shows great inhibition specificity for deprenyl [20]. The *D. magna* genome contains a unique flavin-containing monoamine oxidase A gene/protein (ACC XP_032781527.1) that has 54% homology with a putative MAO protein of the shrimp *Penaeus vannamei* (XP_027230105.1), 33%—with human MAOs. These homologies seem moderate but note that the zebrafish MAO gene/protein has only 69% homology with those of humans. Thus, the results obtained in this study are in line with the previously reported studies on ticks, mites and crabs and provide evidence for the presence of a MAO-like activity in *D. magna* that is able to metabolize serotonin and dopamine.

Using similar concentrations of deprenyl (5 μM ≅ 0.94 mg/L), Faria et al. [14] reported similar background activities of MAO in zebrafish larvae, but an almost complete inhibition of MAO activity and a greater increase of serotonin than of dopamine. Phylogenetic differences between *D. magna*, fish and mammals are likely to account for the observed deprenyl specificity effect on the MAO activity and of the latter enzyme for catabolising serotonin and dopamine [21]. Deprenyl, despite decreasing the MAO activity and enhancing serotonin, did not decrease the serotonin degradation metabolite 5-hydroxyindoleacetic acid (5-HIAA). There are, however, reported discrepancies on the consequences of the MAO type A,B inhibition on serotonin degradation metabolites. The reported studies on the zebrafish exposed to deprenyl found enhanced or unchanged levels of 5-HIAA [14,22]. In rodents, MAO type A inhibition that lead to enhanced levels of serotonin did not necessarily affect its metabolite 5-HIAA [23].

Deprenyl increased, however, the concentration of the dopamine metabolite 3-methoxytyramine (3-MT) in *D. magna*, an effect previously reported for mice treated with the MAO type A inhibitor clorgyline [24].

In a previous study we found that neuroactive drugs do not always show the same target specificity in *D. magna* as in mammals [13]. In relation to this, we analysed up to 16 metabolites belonging to four neurological metabolic pathways (i.e., serotonin, catecholamine, cholinergic and GABAergic). The results obtained for deprenyl showed high specificity for its putative serotonergic and dopaminergic targets.

The study of behavioural responses indicates contrasting effects of deprenyl against compounds that are known that decrease serotonin in *Daphnia* such as PCPA [13]. Deprenyl increased habituation to repetitive light stimuli, which is a primary form of non-associative learning [25], and enhanced responses to light in 50% of the experiments performed (1 out of 4 in Figure 1; 3 out of 4 in Figure 2). Conversely, PCPA-treated organisms took longer to habituate to the repetitive light stimuli and responded to a lower extent to visual light stimuli. Interestingly, *D. magna* individuals exposed to deprenyl also had a lower basal activity, which was monitored under darkness. The behavioural features of PCPA are

consistent with the reported higher basal activity and reduced habituation of CRISPR-mediated TPH-mutated *D. magna* juveniles that lack serotonin [11]. In zebrafish larvae exposed to deprenyl, Faria et al. [14] also reported a lower basal activity, a reduced response to light or tactile stimuli and increased habituation. In fish and also in mammals, increased serotonin levels have sedative-like anxiolytic effects [14,26], whereas in *D. magna*, it is unclear. The results obtained here in *D. magna* for deprenyl apparently agree with those of zebrafish [14] since in both species it decreases the basal locomotor activity and increases habituation. However, in fish, deprenyl also decreased the response to stimuli [14], which agrees with the reported decreased anxiety-like responses in rodents [26]. In *D. magna*, deprenyl either did not affect or enhanced the response to stimuli. In relation to this, it is important to remark that, unlike in zebrafish, deprenyl increased the dopamine level to the same extent as that of serotonin. Increased dopamine levels have been associated with a hyperresponsive behaviour to mechanical stimuli in *Drosophila melanogaster* and *Caenorhabditis elegans* [27]. Of course, dopamine does not act alone in regulating behavioural responsiveness to stimuli; there are counteracting neuronal systems, such as serotonin and other monoamines [27,28]. Therefore, the observed behavioural defects in the *D. magna* exposed to deprenyl are likely to be related to the observed enhanced levels of dopamine and serotonin.

In fish and also in mammals, decreasing serotonin levels induced by PCPA cause anxiety-like behaviour such as hyperlocomotion activity and enhanced responses to stimuli [14,29]. PCPA is also known to impair learning [30]. The *D. magna* exposed to PCPA showed basal hyperactivity only in one out of the two experiments, increased the response to light in some of the trials performed, but impaired learning in all the trials (decreased habituation). This means that the *D. magna* responses to PCPA can be related to anxiogenic behaviour only in part. Nevertheless, the previous results obtained with knockout *D. magna* lacking serotonin did show higher hyperactivity, enhanced responses to light stimuli and also impaired habituation [2,11], a phenotype compatible with anxiety-like behaviour and learning impairment in rodents [30,31]. The apparently closer phenotype of genetically modified *D. magna* individuals lacking serotonin than of those enzymatically impaired by PCPA can be related to the unspecific action of the latter compound. PCPA not only reduced serotonin in *D. magna* but also decreased the levels of norepinephrine [13], which is known to modulate arousal and other types of cognitive behaviour [32].

5. Conclusions

The results reported here show that *D. magna* juveniles are sensitive to MAO inhibitors that change serotonin signalling. In addition, molecular targets of MAO modulators such as effects on the enzymatic activity and on the concentration of serotonergic and dopaminergic metabolites was also observed. The model serotonin modulator deprenyl inhibited the MAO activity and increased the serotonin, dopamine and dopamine metabolite 3-MT levels. The deprenyl-treated individuals showed consistently shorter habituation to repetitive light stimuli and reduced basal locomotor activity. Deprenyl behavioural outcomes opposed those of the PCPA drug or genetically modified individuals having reduced serotonin levels. The findings presented in this study reinforce the use of this nonvertebrate model to address behavioural and physiological roles of serotonin.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxics9080187/s1, Supplementary Methods and Supplementary Results—Tables. Table S1: List the metabolites analysed, Table S2. Quality parameters of monoamine neurotransmitters and the related metabolites, Table S3: Student's t-test results for MAO and monoaminergic neurotransmitter levels, Table S4: List of values for the nonsignificant metabolites.

Author Contributions: Conceptualization, M.B., D.R. and C.B.; Methodology, M.B. and M.F.; Validation, M.B.; Investigation, M.B.; Formal analysis, M.B. and C.B.; Writing—original draft preparation, M.B. and C.B.; Writing—review and editing, M.B., C.G.-C., D.R. and C.B.; Supervision, C.G.-C. and C.B.; Visualization, C.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by "Agencia Estatal de Investigación" from the Spanish Ministry of Science and Innovation (project PID2020-113371RB-C21), IDAEA-CSIC, Severo Ochoa Centre of Excellence (CEX2018-000794-S), which financed M.F. with Severo Ochoa funds. The work was partially supported by the Catalan Government through the network of recognized research groups (2017 SGR_902).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting the reported results will be provided upon reader's request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Burks, R.L.; Jeppesen, E.; Lodge, D.M. Littoral zone structures as Daphnia refugia against fish predators. *Limnol. Oceanogr.* **2001**, *46*, 230–237. [CrossRef]
2. Bedrossiantz, J.; Martínez-Jerónimo, F.; Bellot, M.; Raldua, D.; Gómez-Canela, C.; Barata, C. A high-throughput assay for screening environmental pollutants and drugs impairing predator avoidance in Daphnia magna. *Sci. Total Environ.* **2020**, *740*. [CrossRef]
3. Faria, M.; Prats, E.; Novoa-Luna, K.A.; Bedrossiantz, J.; Gómez-Canela, C.; Gómez-Oliván, L.M.; Raldúa, D. Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance. *Sci. Total Environ.* **2019**, *650*, 87–96. [CrossRef] [PubMed]
4. Berger, M.; Gray, J.A.; Roth, B.L. The expanded biology of serotonin. *Annu. Rev. Med.* **2009**, *60*, 355–366. [CrossRef] [PubMed]
5. O'Mahony, S.M.; Clarke, G.; Borre, Y.E.; Dinan, T.G.; Cryan, J.F. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav. Brain Res.* **2015**, *277*, 32–48. [CrossRef]
6. Busch, W.; Schmidt, S.; Kühne, R.; Schulze, T.; Krauss, M.; Altenburger, R. Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. *Environ. Toxicol. Chem.* **2016**, *35*, 1887–1899. [CrossRef]
7. Fong, P.P.; Ford, A.T. The biological effects of antidepressants on the molluscs and crustaceans: A review. *Aquat. Toxicol.* **2014**, *151*, 4–13. [CrossRef] [PubMed]
8. Brooks, B.W.; Foran, C.M.; Richards, S.M.; Weston, J.; Turner, P.K.; Stanley, J.K.; Solomon, K.R.; Slattery, M.; La Point, T.W. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* **2003**, *142*, 169–183. [CrossRef]
9. Orsini, L.; Gilbert, D.; Podicheti, R.; Jansen, M.; Brown, J.B.; Solari, O.S.; Spanier, K.I.; Colbourne, J.K.; Rush, D.; Decaestecker, E. Daphnia magna transcriptome by RNA-Seq across 12 environmental stressors. *Sci. Data* **2016**, *3*, 1–16. [CrossRef]
10. Gunnarsson, L.; Jauhiainen, A.; Kristiansson, E.; Nerman, O.; Larsson, D.G.J. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environ. Sci. Technol.* **2008**, *42*, 5807–5813. [CrossRef] [PubMed]
11. Rivetti, C.; Campos, B.; Piña, B.; Raldúa, D.; Kato, Y.; Watanabe, H.; Barata, C. Tryptophan hydroxylase (TRH) loss of function mutations induce growth and behavioral defects in Daphnia magna. *Sci. Rep.* **2018**, *8*, 11. [CrossRef] [PubMed]
12. Campos, B.; Rivetti, C.; Kress, T.; Barata, C.; Dircksen, H. Depressing Antidepressant: Fluoxetine Affects Serotonin Neurons Causing Adverse Reproductive Responses in Daphnia magna. *Environ. Sci. Technol.* **2016**, *50*. [CrossRef]
13. Fuertes, I.; Barata, C. Characterization of neurotransmitters and related metabolites in Daphnia magna juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: A targeted LC-MS/MS method. *Chemosphere* **2021**, *263*, 127814. [CrossRef] [PubMed]
14. Faria, M.; Prats, E.; Bellot, M.; Gomez-Canela, C.; Raldúa, D. Pharmacological modulation of serotonin levels in zebrafish larvae: Lessons for identifying environmental neurotoxicants targeting the serotonergic system. *Toxics* **2021**, *9*, 118. [CrossRef] [PubMed]
15. Huber, S.J.; Cummings, J.L. *Parkinson's Disease: Neurobehavioral Aspects*; Oxford University Press: Oxford, UK, 1992; ISBN 0195069692.
16. Riederer, P.; Youdim, M.B.H.; Rausch, W.D.; Birkmayer, W.; Jellinger, K.; Seemann, D. On the mode of action of L-deprenyl in the human central nervous system. *J. Neural Transm.* **1978**, *43*, 217–226. [CrossRef]
17. Sloley, B.D. Metabolism of Monoamines in Invertebrates: The Relative Importance of Monoamine Oxidase in Different Phyla. *Neurotoxicology* **2004**, *25*, 175–183. [CrossRef]
18. Kadir, H.A.; Knowles, C.O. Oxidative deamination and N-acetylation of biogenic amines by homogenates of bulb mites (Rhizoglyphus echinopus). *Comp. Biochem. Physiol. Part C Comp.* **1989**, *94*, 465–468. [CrossRef]
19. Kaufman, R.; Sloley, D. Catabolism of dopamine and 5-hydroxytryptamine by monoamine oxidase in the ixodid tick, Amblyomma hebraeum. *Insect Biochem. Mol. Biol.* **1996**, *26*, 101–109. [CrossRef]
20. Basova, N.E.; Basova, I.N.; Yagodina, O. V Monoamine Oxidase Activity in the Hepatopancreas of the Kamchatka Crab Paralithodes camtschaticus: A Substrate–Inhibitor Specificity. *J. Evol. Biochem. Physiol.* **2018**, *54*, 345–352. [CrossRef]
21. Setini, A.; Pierucci, F.; Senatori, O.; Nicotra, A. Molecular characterization of monoamine oxidase in zebrafish (Danio rerio). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2005**, *140*, 153–161. [CrossRef]

22. Sallinen, V.; Sundvik, M.; Reenilä, I.; Peitsaro, N.; Khrustalyov, D.; Anichtchik, O.; Toleikyte, G.; Kaslin, J.; Panula, P. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. *J. Neurochem.* **2009**, *109*, 403–415. [CrossRef] [PubMed]
23. Finberg, J.P.M.; Rabey, J.M. Inhibitors of MAO-A and MAO-B in psychiatry and neurology. *Front. Pharmacol.* **2016**, *7*. [CrossRef] [PubMed]
24. Kato, T.; Dong, B.; Ishii, K.; Kinemuchi, H. Brain Dialysis: In Vivo Metabolism of Dopamine and Serotonin by Monoamine Oxidase A but Not B in the Striatum of Unrestrained Rats. *J. Neurochem.* **1986**, *46*, 1277–1282. [CrossRef] [PubMed]
25. Kandel, E.R. Cellular mechanisms of learning and the biological basis of individuality. *Princ. Neural Sci.* **1991**, *3*, 1009–1031.
26. Bortolato, M.; Godar, S.C.; Davarian, S.; Chen, K.; Shih, J.C. Behavioral disinhibition and reduced anxiety-like behaviors in monoamine oxidase b-deficient mice. *Neuropsychopharmacology* **2009**, *34*, 2746–2757. [CrossRef]
27. Van Swinderen, B.; Andretic, R. Dopamine in Drosophila: Setting arousal thresholds in a miniature brain. *Proc. R. Soc. B Biol. Sci.* **2011**, *278*, 906–913. [CrossRef]
28. Fischer, A.G.; Ullsperger, M. An update on the role of serotonin and its interplay with dopamine for reward. *Front. Hum. Neurosci.* **2017**, *11*. [CrossRef]
29. Kshama, D.; Hrishikeshavan, H.J.; Shanbhogue, R.; Munonyedi, U.S. Modulation of baseline behavior in rats by putative serotonergic agents in three ethoexperimental paradigms. *Behav. Neural Biol.* **1990**, *54*, 234–253. [CrossRef]
30. Izquierdo, A.; Carlos, K.; Ostrander, S.; Rodriguez, D.; McCall-Craddolph, A.; Yagnik, G.; Zhou, F. Impaired reward learning and intact motivation after serotonin depletion in rats. *Behav. Brain Res.* **2012**, *233*, 494–499. [CrossRef]
31. Waider, J.; Popp, S.; Lange, M.D.; Kern, R.; Kolter, J.F.; Kobler, J.; Donner, N.C.; Lowe, K.R.; Malzbender, J.H.; Brazell, C.J.; et al. Genetically driven brain serotonin deficiency facilitates panic-like escape behavior in mice. *Transl. Psychiatry* **2017**, *7*, e1246. [CrossRef]
32. Mather, M.; Clewett, D.; Sakaki, M.; Harley, C.W. Norepinephrine ignites local hotspots of neuronal excitation: How arousal amplifies selectivity in perception and memory. *Behav. Brain Sci.* **2016**, *39*. [CrossRef] [PubMed]

toxics

MDPI

Article

Pharmacological Modulation of Serotonin Levels in Zebrafish Larvae: Lessons for Identifying Environmental Neurotoxicants Targeting the Serotonergic System

Melissa Faria [1,*], Eva Prats [2], Marina Bellot [3], Cristian Gomez-Canela [3] and Demetrio Raldúa [1]

[1] Institute for Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain; drpqam@cid.csic.es
[2] Research and Development Centre (CID-CSIC), Jordi Girona 18, 08034 Barcelona, Spain; eva.prats@cid.csic.es
[3] Department of Analytical Chemistry and Applied (Chromatography Section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain; marina.bellot@iqs.url.edu (M.B.); cristian.gomez@iqs.url.edu (C.G.-C.)
* Correspondence: mdfqam@cid.csic.es; Tel.: +34-93-400-61-00

Abstract: This study examines the effects of acute pharmacological modulation of the serotonergic system over zebrafish larvae's cognitive, basic, and defense locomotor behaviors, using a medium to high throughput screening assay. Furthermore, the relationship between behavior, enzyme activity related to neurotransmitter metabolism, neurotransmitter levels, and gene expression was also determined. Modulation of larvae serotonergic system was accomplished by 24 h exposure to single and opposite pharmacodynamics co-exposure to three model psychopharmaceuticals with antagonistic and agonistic serotonin signaling properties: 2.5 mM 4-Chloro-DL-phenylalanine (PCPA) and 5 µM deprenyl and 0.5 µM fluoxetine, respectively. Similar behavioral outcome was observed for deprenyl and fluoxetine, which was reflected as hypolocomotion, decrease in larvae defensive responses, and cognitive impairment. Contrarily, PCPA induced hyperlocomotion and increase in larvae escape response. Deprenyl exposure effects were more pronounced at a lower level of organization than fluoxetine, with complete inhibition of monoamine oxidase (MAO) activity, dramatic increase of 5-HT and dopamine (DA) levels, and downregulation of serotonin synthesis and transporter genes. PCPA showed mainly effects over serotonin and dopamine's main degradation metabolites. Finally, co-exposure between agonistic and antagonist serotonin signaling drugs reviled full recovery of zebrafish impaired locomotor and defense responses, 5-HT synthesis gene expression, and partial recovery of 5-HT levels. The findings of this study suggest that zebrafish larvae can be highly sensitive and a useful vertebrate model for short-term exposure to serotonin signaling changes.

Keywords: zebrafish larvae; behavior; serotonin; neurotransmitters; modulation

Citation: Faria, M.; Prats, E.; Bellot, M.; Gomez-Canela, C.; Raldúa, D. Pharmacological Modulation of Serotonin Levels in Zebrafish Larvae: Lessons for Identifying Environmental Neurotoxicants Targeting the Serotonergic System. *Toxics* **2021**, *9*, 118. https://doi.org/10.3390/toxics9060118

Academic Editor: Robyn L. Tanguay

Received: 16 April 2021
Accepted: 22 May 2021
Published: 25 May 2021

1. Introduction

Animals are able to respond to changing environmental stimuli, such as food availability or predation, through different forms of behavioral plasticity, including arousal and associative and non-associative learning [1]. Any changes in normal behavioral conduct could have dramatic consequences from individual survival to ecological disaster. Behavioral plasticity is driven at the molecular level by the action of modulatory neurotransmitters [1,2]. Serotonin (5-hydroxytryptamine) is one of the major neurotransmitters in the central nervous system (CNS), modulating behaviors like mood, sleep, aggressiveness, fear, and appetite [3]. Correlation between abnormalities in the serotonergic system and several pathologies, including affective disorders, schizophrenia, and anxiety, has been demonstrated [3].

The rate-limiting step in the synthesis of serotonin is the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by the tryptophan hydroxylase (TPH). Serotonin is then

synthesized from 5-HTP by the aromatic amino acid decarboxylase (AAAD), and is quickly transported from the cytoplasm to synaptic vesicles by the vesicular transporter SLC18A2 (VMAT2). When serotonin is released to the synaptic cleft via exocytosis, the serotonin transporter SLC6A4 (also known as SERT) is responsible for its uptake/reuptake to the serotonergic neurons. Serotonin is metabolized by MAO to 5-hydroxy-indolecetaldehyde which is quickly metabolized by an aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of serotonin [4].

Neuroactive chemicals, including both emerging and legacy pollutants, are the largest group of micropollutants present in European rivers [5]. The specific neurotoxic effect of environmental concentrations of some of these pollutants on different nontarget species, including fish, has been reported [6–8]. Considering the essential role played by serotonin in both neurotransmission and neuromodulation, neuroactive chemicals targeting serotonergic system of are of particular concern. Despite the fact that the effects of selective serotonin re-uptake inhibitors (SSRIs) in fish species have been extensively studied [9–12], information regarding other potential modes of actions, like TPH or monoamine oxidase (MAO) inhibition, is still scarce.

In this manuscript we have analyzed the basal locomotor activity, visual-motor response, and the vibrational startle response in 8 days post-fertilization (dpf) zebrafish larvae exposed during 24 h to neuroactive drugs specifically designed to increase (SERT inhibitor: fluoxetine; MAO inhibitor: deprenyl) or decrease (TPH inhibitor: chloro-DL-phenylalanine (PCPA)) serotonin levels. Moreover, changes in the profile of serotonergic and dopaminergic neurotransmitters in the heads of the exposed larvae, as well as changes in the expression of monoaminergic-related genes, have been assessed.

2. Material and Methods

2.1. Fish Husbandry and Larvae Production

All procedures regarding fish housing, larvae production, and experiments were approved by the CID-CSIC's Institutional Animal Care and Use Committees and carried out according to the institutional guidelines under a license from the local government (agreement n° 9027). For more detail please refer to Section S1.1. Fish Husbandry and Larvae Production, of the Supplementary Material.

2.2. Experimental Protocol

All chemicals used were of certified laboratory high-quality grade purchased from Sigma-Aldrich (St. Louis, MO, USA): fluoxetine (CAS:56296-78-7); chloro-DL-phenylalanine (PCPA; CAS: 7424-00-2) and deprenyl (CAS:14611-52-0). Whereas stock solutions of deprenyl and fluoxetine were prepared in DMSO and then diluted 10^{-3} in fish water for exposure solutions, chloro-DL-phenylalanine (PCPA) exposure solution was prepared directly in fish water. Previous to the experiment, the selected compounds were evaluated for toxicity, which was established either by death, gross morphology and/or swimming impairment, or clear decrease in the escape response evoked by the tapping on the plate. The highest concentration, which did not induce any of the above-mentioned criteria, was selected for this study. DMSO was added to all exposure conditions to a final concentration of 0.1%. The use of 0.1% DMSO in vehicle controls has been reported to be safe and is commonly used in the screening of zebrafish libraries of small chemicals [13,14]. For behavior assessment, exposures were conducted in 48-well microplates containing 1 mL of exposure solution and 1 larva per well. Behavior trials were directly tested without further manipulation, following 24 h of exposure (larvae from 7 to 8 dpf) (Figure 1). For MAO activity and neurotransmitters assessment, exposures were carried out in 6-well plates, where each well contained 5 larvae and 5 mL of exposure medium, and treatments placed randomly across plates. This exposure window was chosen because by 7 dpf most of the central nervous system is quite well developed and the observed effects will be mainly related with neurotoxicity instead of developmental neurotoxicity. Moreover, by using this window, any major developmental effects of the selected chemicals is

avoided, since larvae at this stage have already undergone most of their organogenesis (https://zfin.org/zf_info/zfbook/stages/, accessed on 7 May 2021), and a longer exposure was discharged to avoid having to feed larvae and therefore insert a new variable in the experiment. Exposures were performed at 28.5 °C (POL-EKO APARATURA Climatic chamber KK350, Poland) with 12L:12D photoperiod. Before sampling, larvae were euthanized by rapid chilling, transferring the larvae to ice-chilled water (2–4 °C), which is a method of euthanasia in accordance with the AVMA Guidelines on Euthanasia (https://www.avma.org/resources-tools/avma-policies/avma-guidelines-euthanasia-animals, accessed on 13 May 2021). Samples were transferred into an Eppendorf Tube and all medium water was removed. They were then immediately frozen with dry ice and then stored at −80 °C until further analysis. Larvae used for behavioral trials were then sampled for qRT-PCR analysis (n = 4/pool) while due to the large number of larvae required for neurotransmitters and MAO activity (n = 20/pool) assessment experiments were conducted separately (Figure 1). For each variable investigated, larvae were collected from 2–3 trials of the same experiment setup conducted in different days and with different batches of animals (Figure 1).

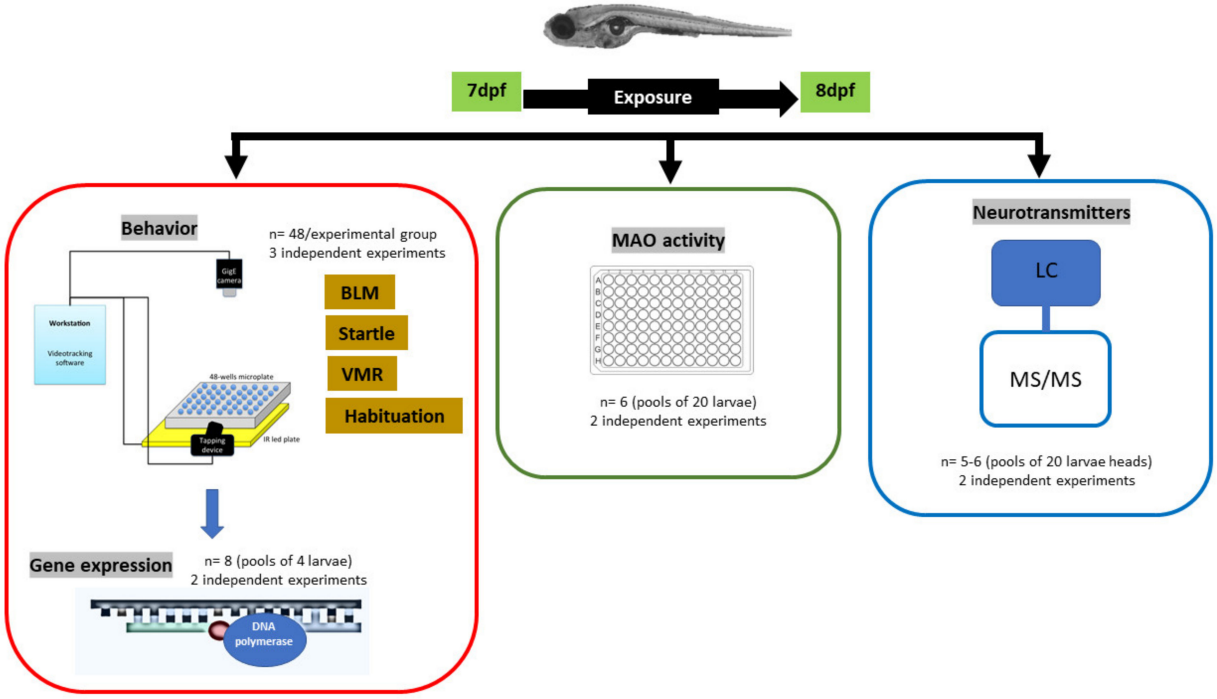

Figure 1. Diagram of the conducted study, indicating the exposure period (from 7 to 8 dpf) and the addressed variables (behavior, gene expression, MAO activity, and neurotransmitters), divided into their corresponding dataset (red, green, and blue squares). Indicated are also the number of larvae and independent experiments used for each variable.

2.3. Behavioral Analysis

Vibrational startle response assay was performed as described in [8]. The basis of this test is the escape response evoked in zebrafish larvae by a tapping stimulus. Video tracking was acquired, and EthoVision XT 9 software (Noldus, Wageningen, The Netherlands) was used to analyze the escape response. Trials were performed at 28 °C with near-infrared light. The highest intensity (intensity level: 8) was selected for the tapping stimulus and, after a 15 min acclimation period to the chamber, 50 stimulus were delivered, one every second. Videos were recorded at 30 frames per second and the vibrational startle response (VSR) for each individual larva was analyzed by measuring the distance traveled (cm) over the 1 s period following each stimulus. "Startle Response or Startle" is defined as the total distance moved (cm) in response to the first stimulus and "Habituation or non-associative

learning" as the area under the curve (AUC) of plots of distance moved relative to the response to the first stimulus [8].

Basal locomotor activity (BLM) and visual-motor response (VMR) analyses of 8 dpf zebrafish larvae were conducted with the DanioVision system associated with the Ethovision XT 11 software (Noldus, Wageningen, the Netherlands), as described by [15]. Before video recording, larvae were first acclimated for 20 min under dark conditions. Video tracking trials consisted of a 40 min cycle with a 15 min dark period followed by a 10 min light period followed by a 15 min of darkness. The BLM activity is classified as the total distance (cm) traveled by larvae during the last 10 min of the first dark cycle. The VMR is based in the hyperactivity period induced by a sudden absence of light [16], represented as the difference between total distance (cm) traveled for two minutes after and before to the beginning of the light cycle.

2.4. RNA Preparation and qRT-PCR Analysis

Analysis of larvae gene expression was conducted as previously described by Prats et al., 2017. Total RNA was extracted from 6–8 pools of 4 larvae (8 dpf), collected from two independent experiments, using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Concentration of RNA was measured in a NanoDrop™ ND-8000 spectrophotometer (260 nm) (Fisher Scientific) and its quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Values of RNA Integrity Number (RIN) ranged between 9 and 10. Following DNaseI treatment (Ambion, Austin, TX, USA), 1 μg of RNA was employed to synthesize the first strand of complementary (cDNA) using the First Strand cDNA synthesis Kit (Roche Diagnostics, Germany) and oligo(dT), according to the instructions provided by the manufacturer.

Real Time PCR was performed in a LightCycler® 480 Real-Time PCR System with SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany). Cycling parameters were 15 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 30 s at 60 °C. For each experimental condition, qPCR analyses were performed with three technical replicates for each sample. Primer sequences (Sigma-Aldrich, Steinheim, Germany) of the four selected genes (*tph1a*, *mao*, *sert,* and *vmat2*) are reported in Supplementary Table S1. Previous to any analysis, all primers were checked for their efficiency and specificity. Results were normalized using the housekeeping *ppia2* as reference gene [17] and the relative abundance of mRNA was calculated following the ΔΔCt method [18] deriving fold-change ratios from these values. The housekeeping gene remained stable across all treatments (Supplementary Table S4).

2.5. Zebrafish Monoamine-Oxidase (MAO) Activity

8 dpf zebrafish larvae were collected in pools of 20 individuals and homogenized in ice-cold 10 mM Phosphate Buffer pH 7.6 with 1 mM EDTA, to a final tissue volume concentration of 100 larvae/mL of buffer using a TissueLyser® (Qiagen, Germantown, MA, USA). After centrifuging homogenates at 2500× *g* for 5 min at 4 °C, MAO activity was immediately determined in the supernatant using the peroxidase-linked spectrophotometric assay described by Holt et al. [19] and adapted to zebrafish tissue by [20], based on the determination of the amount of H_2O_2 released during the oxidation of amines. For further information please refer to Supplementary Material Section S1.2. Zebrafish monoamine-oxidase (MAO) activity.

2.6. Extraction and Analysis of Neurotransmitters

Monoaminergic neurotransmitters were extracted from 8 pools of 20 larvae heads according to the procedure adapted from Mayol-Cabré et al. [21] based on the use of a solvent of similar polarity to that of the neurotransmitters in order to be extracted from the sample. Ultra-high-performance liquid chromatography (Acquity UPLCH-Class Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Xevo, TQS micro, Waters, Milford, MA, USA) was used to per-

form the analysis. Additional details on the extraction and analysis of neurotransmitters are provided in the Supplementary Material, Section S1.3. Monoaminergic neurotransmitters extraction and analysis.

2.7. Statistical Analysis

Analysis of data was performed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL, USA) and plotted with GraphPad Prism 8.31 for Windows (GraphPad software Inc, La Jolla, CA, USA). Data normality was assessed using Kolmogorov–Smirnov and Shapiro–Wilk tests. Multiple comparison tests were used to determine differences between normally distributed groups, while the Kruskal–Wallis test followed by Dunn's multiple comparison test against the control value was applied to test for differences between groups that did not meet parametric assumptions. One-way ANOVA followed by either Dunnett's or Tukey's test was used to compare results with those of the control group, or to determine homogenous subset groups, respectively. Scattered plots of data are presented in figures, with the median depicted as a red line. Significance was set at $p < 0.05$.

3. Results

3.1. Serotonergic Modulation—Response at the Organismal Level

A battery of behavioral tests including basal locomotor (BLM) activity, visual motor response (VMR), the acoustic/vibrational escape response (also referred as startle response) and the habituation of the startle response were used to assess the behavioral profile in zebrafish larvae exposed to prototypic compounds inhibiting MAO (deprenyl), SERT (fluoxetine), and TPH (PCPA) activities. The selected concentrations were the same as those used in a previous study [22], and similar to the latter study, no system toxicity or morphological effects were observed in larvae following exposure. A significant effect in larvae BLM was found in those exposed to deprenyl, fluoxetine, and PCPA (Figure 2A) ($H(5) = 37.310$, $p = 0.000$). Whereas, deprenyl and fluoxetine significantly decreased larvae BLM (respectively, $p = 0.000$ and $p = 0.003$), the opposite was observed for PCPA ($p = 0.027$). Furthermore, the two serotonin level enhancer drugs showed a more prominent effect than its counter drug. Curiously, complete recovery of this behavior was observed in both combined exposure conditions (Figure 2A). The same behavior was observed for the startle response of zebrafish larvae ($H(5) = 42.889$, $p = 0.000$) (Figure 2B), however, the effects in this behavior where slightly milder compared to the BLM. Full recovery of the impaired escape response was also observed when combining PCPA with either deprenyl or fluoxetine (Figure 2B). The VMR was only impaired by deprenyl and fluoxetine ($H(5) = 53.180$, $p = 0.000$), with deprenyl presenting a stronger effect over this response (Figure 2C). In spite of this, similar to the previous two behavioral responses, a full recovery was observed when in combination with PCPA. Habituation of the escape response was the only behavioral response with a more distinct outcome (Figure 2D). The non-associative learning profile was impaired by deprenyl and fluoxetine alone and in combination with PCPA ($H(5) = 37.316$, $p = 0.000$), with deprenyl, the MAO activity inhibitor, once more, presenting stronger effects than the SSRI, fluoxetine.

3.2. Serotonergic Modulation—Response at the Molecular Level

In order to better understand the changes observed in larvae behavioral outcome, following 24 h exposure to known drugs that increment and decrease serotonin levels and their combination, the neurotransmitter, biochemical and gene expression profiles were addressed.

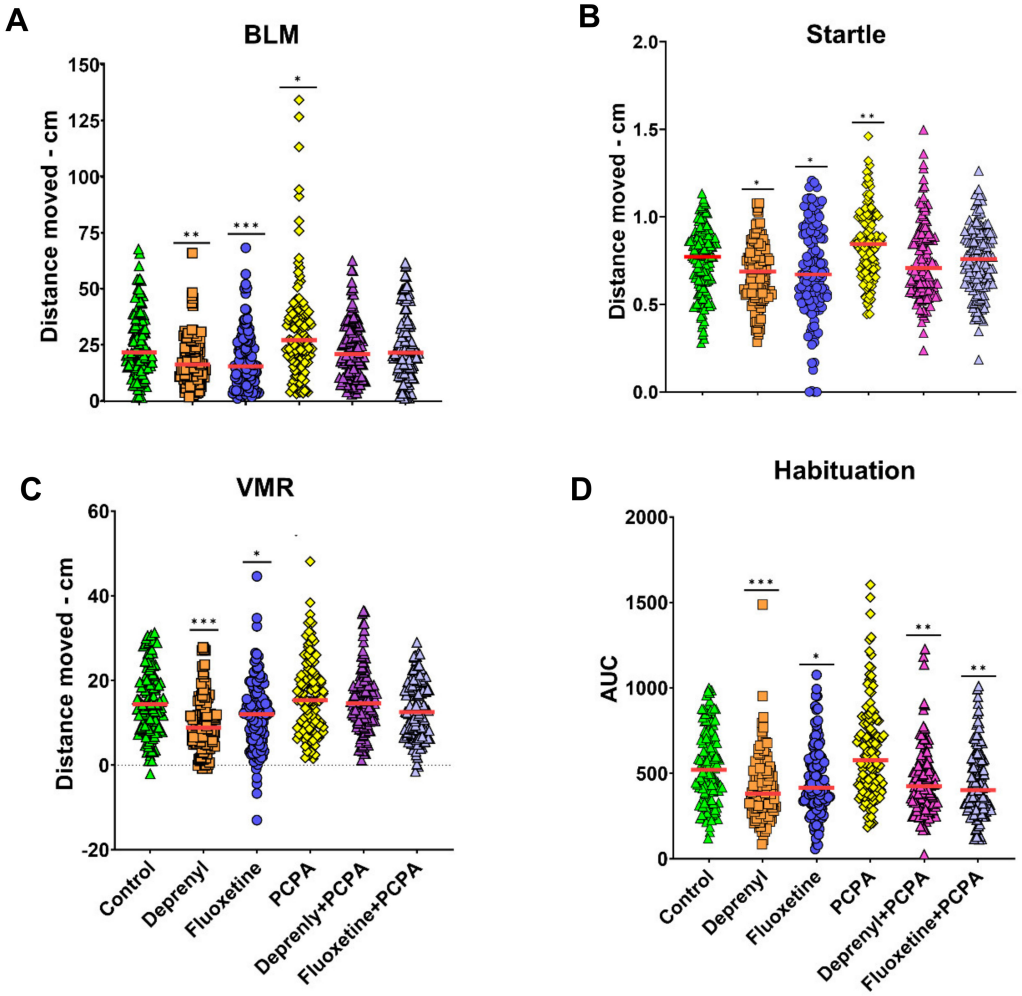

Figure 2. Behavioral changes on zebrafish 8 dpf old larvae, following 24 h waterborne exposure to 5 µM Deprenyl, 0.5 µM Fluoxetine, 2.5 mM PCPA, and the combination of 2.5 mM PCPA with either Deprenyl 5 µM or Fluoxetine 0.5 µM. (**A**) Basal locomotor (BLM) activity, represented as the total distance (cm) travelled during 10 min (n = 126–132); (**B**) acoustic/vibrational escape response (startle), represented as the total distance (cm) travelled following the delivery of a tapping stimulus (n = 127–133); (**C**) visual-motor response (VMR), representing the response of larvae due to transition of light to dark, represented as the difference of the total distance (cm) travelled by larvae during two minutes after and before the transition of light to dark (n = 121–126); (**D**) habituation of the acoustic/vibrational escape response evoked by a series of 50 tapping stimulus delivered every second represented as area under the curve (AUC) of larvae responses (n = 124–132). Data are from 3 independent experiments and are reported as scatter plots with the median (red line). Significance was set to $p < 0.05$ and can be represented as * when $p < 0.05$; ** when $p < 0.01$ and *** when $p < 0.001$, Kruskal–Wallis non-parametric test.

3.2.1. Neurotransmitter Profile

The profile of the monoaminergic neurotransmitters serotonin (5-HT) and dopamine (DA), as well as their products, norepinephrine (NE), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxytyramine (3-MT) were determined in the heads of control and exposed larvae (Figure 3). Levels of all neurochemicals with the exception of 3-MT were significantly affected by the treatments ($p < 0.05$, Supplementary Table S2—One-way ANOVA results of monoaminergic neurotransmitter levels). In a single exposure scenario of the selected drugs, deprenyl triggered a strong increase of serotonin levels ($p < 0.000$, Dunnett's test). The remaining drugs did not affect levels of this neurotransmitter. On the other hand, an important recovery of serotonin levels could be observed when deprenyl was combined with PCPA (Supplementary Table S3),

however, the observed recovery was not able to reach similar levels to those of unexposed larvae (p = 0.007, Dunnett's test) (Figure 3). Levels of the degradation product of serotonin, 5-HIAA was significantly deterred by fluoxetine (p = 0.034, Dunnett's test) and PCPA (p = 0.010, Dunnett's test), with the latter having a stronger effect over 5-HIAA levels (Figure 3, Supplementary Table S3). In addition, this same trend could be observed in larvae exposed to the combination of fluoxetine + PCPA (p = 0.022, Dunnett's test), however PCPA's strong effect seemed to have been slightly attenuated by the presence of fluoxetine (Figure 3, Supplementary Table S3). Similar to serotonin, dopamine levels were also significantly increased by deprenyl (p = 0.002, Dunnett's test), which were then fully recovered when combined with PCPA (Figure 3, Supplementary Table S3). Furthermore, levels of the MAO-mediated degradation product of dopamine, DOPAC, were found significantly higher than those of control following exposure to deprenyl and curiously also fluoxetine and PCPA (p = 0.001, p = 0.03, and p = 0.002, Dunnett's test, respectively). Furthermore, in combined exposure setups, that of deprenyl + PCPA was unable to recover DOPAC levels while full recovery could be observed for fluoxetine + PCPA (Figure 3, Supplementary Table S3). Finally, levels of norepinephrine, a neurotransmitter synthesized from dopamine through dopamine β-monooxygenase activity, was significantly decreased by PCPA (p = 0.010, Dunnett's test) (Figure 3), which then recovered when PCPA was combined with either deprenyl or fluoxetine (Supplementary Table S3).

3.2.2. Gene Expression and MAO Activity

The expression of genes involved in the 5-HT synthesis (*tph1a*), transport (*sert* and *vmat2*), and degradation (*mao*) as well as the functional activity of MAO was determined in the whole body of control and exposed larvae (Figure 4). Analysis of variance between groups for *tph1a* expression, gene encoding the rate limiting enzyme for serotonin synthesis, showed significant differences ($F_{5,42}$ = 5.458, p = 0.001), which were mainly observed due to the strong downregulation of its expression in zebrafish larvae following 24 h of exposure to 5 μM of deprenyl (p = 0.004, Dunnett's test) (Figure 4A). The remaining treatments of single exposures showed mild effects over *tph1a* expression but none were significantly different from control. On the other hand, the effect induced by deprenyl exposure was mostly recovered by combination exposure with PCPA (Figure 4A, Supplementary Table S4). The expression of the *mao* gene was significantly downregulated by most of the treatments ($F_{5,42}$ = 3.934, p = 0.005) (Figure 4B). In single exposures, the strongest effect was observed for PCPA (p = 0.001, Dunnett's test) followed by fluoxetine and deprenyl (p = 0.009 and 0.034, Dunnett's test, respectively). In combination exposures, whereas deprenyl + PCPA failed to recover expression levels (p = 0.009, Dunnett's test), fluoxetine + PCPA was able to rescue *mao* gene expression to similar levels as those expressed in control larvae. Next, to better understand MAO's potential role in the observed changes, zebrafish MAO activity was determined. Despite the observed mild effects in *mao* expression in the presence of deprenyl, MAO activity was completely abolished by deprenyl (p < 0.001, Dunnett's test) (Figure 4C, Supplementary Table S4). Furthermore, the presence of PCPA was unable to shift deprenyl's dramatic effect over MAO's activity. Curiously, the combined exposure of fluoxetine with PCPA significantly incremented the activity of this enzyme (Figure 4C, Supplementary Table S4). Finally, of the two investigated serotonin transporter genes, only *vmat2* expression was affected by the treatments ($F_{5,42}$ = 3.581, p = 0.009). Both deprenyl and PCPA significantly downregulated the expression of this gene (p = 0.026 and 0.007, Dunnett's test, respectively). Downregulated *vmat2* expression levels by PCPA were then recovered when co-exposed with fluoxetine (p > 0.05) (Figure 4D, Supplementary Table S4), while deprenyl only offered a slender recovery which was not enough to reach similar expression levels as those found in control larvae (p = 0.043) (Figure 4D).

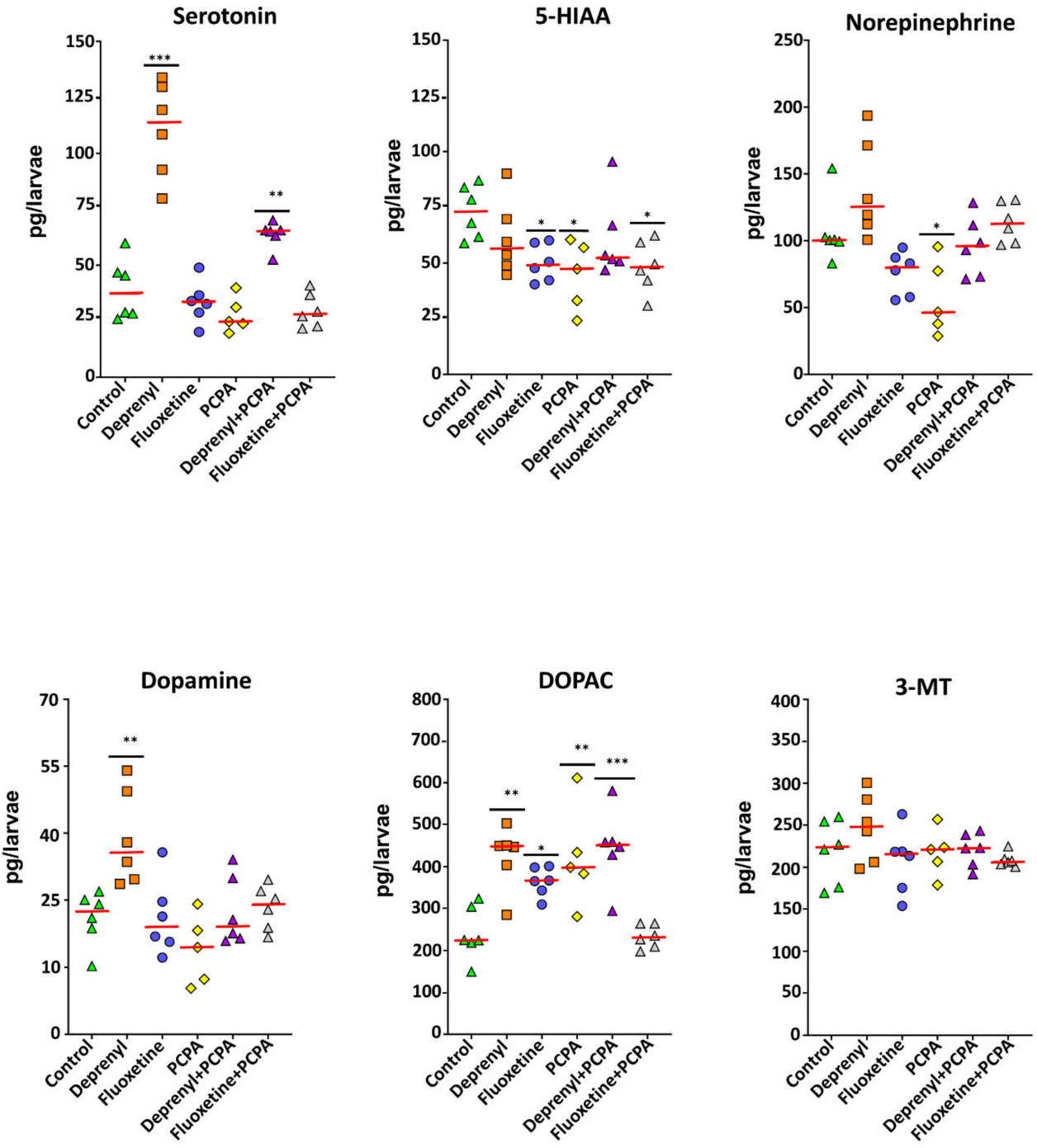

Figure 3. Monoaminergic neurotransmitter profiles of heads of zebrafish exposed to 5 μM Deprenyl, 0.5 μM Fluoxetine, 2.5 mM PCPA, and the combination of 2.5 mM PCPA with either Deprenyl 5 μM or Fluoxetine 0.5 μM. Data are reported as scatter plots with the median (red line) and n = 5–6 for all treatment groups. Significance was set to $p < 0.05$ and can be represented as * when $p < 0.05$; ** when $p < 0.01$ and *** when $p < 0.001$, one-way ANOVA with Dunnett's multiple comparison test.

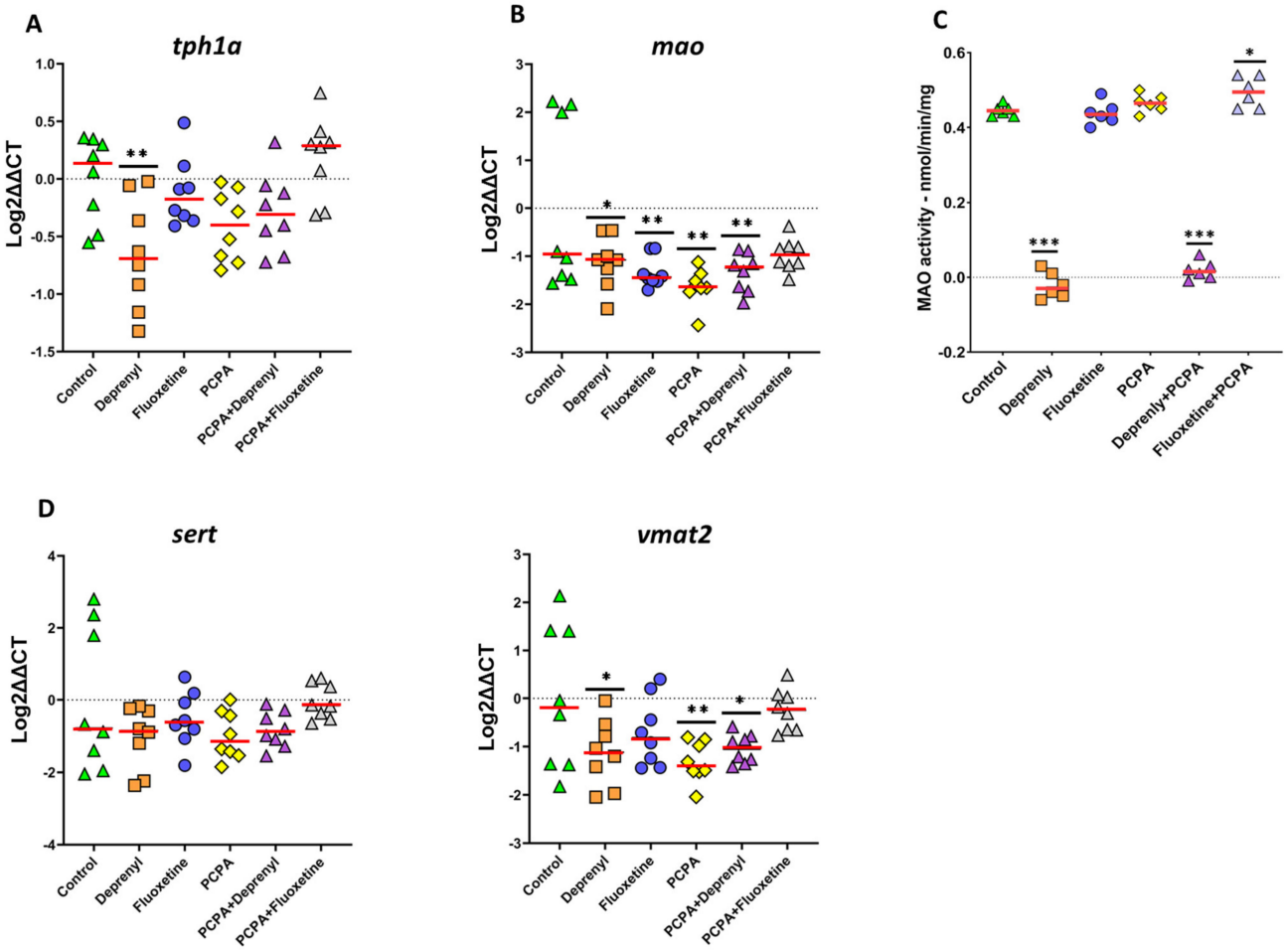

Figure 4. Effects over gene and enzyme activity levels in whole body of 8 dpf old zebrafish larvae following pharmacological modulation of the serotonergic system. (**A**) Expression of *tph1α* involved in serotonin synthesis process; (**B**,**C**) Expression of *mao* and MAO activity, respectively, involved in the degradation of serotonin; (**D**) Expression of *sert* and *vmat2* genes that respectively regulate serotonin transport from the synaptic cleft back to the presynaptic neuron and the transport of serotonin form the cell cortisol into synaptic vesicles. Data are from 2 independent experiments and are reported as scatter plots with the median (red line) and n = 6–8 for all treatment groups. Significance was set to $p < 0.05$ and can be represented as * when $p < 0.05$; ** when $p < 0.01$ and *** when $p < 0.001$, one-way ANOVA with Dunnett's multiple comparison test.

A review of the main results can be found in Supplementary Table S5.

4. Discussion

There is an increased concern for the presence of neuroactive compounds targeting the serotonergic system in many aquatic ecosystems, as changes in serotonin levels may impair many behaviors essential for population survival. The mode of action of these compounds, including both legacy and emerging pollutants, includes inhibition of serotonin synthesis, re-uptake, and degradation [23–26].

Considering that zebrafish is one of the animal models more widely used in ecotoxicology, in this study we have characterized the behavioral effect in larvae of this species of three prototypic modulators of this neurotransmitter system: deprenyl (MAOB inhibitor), fluoxetine (selective serotonin re-uptake inhibitor, SSRI), and PCPA (tryptophan hydroxylase inhibitor).

4.1. Increase of Serotonin Signaling

Serotonin plays a fundamental role in modulating multiple brain functions and motor pathways in vertebrates. The zebrafish's serotonergic system shares similarities with the

respective mammalian systems, which makes this species a feasible model for evaluating its general properties. Here, behavioral modulatory effects of deprenyl and fluoxetine were evaluated at genetic, protein, and neurochemical levels. The overall results showed that 24 h exposure to 5 μM deprenyl had a potent effect over larvae serotonergic pathways.

A total inhibition of zebrafish MAO activity has been found in larvae exposed to 5 μM deprenyl for only 24 h. As expected, fluoxetine, a chemical targeting SERT activity, did not affect the activity of this enzyme. A similar inhibition of MAO activity by deprenyl was recently reported in zebrafish larvae [27]. Furthermore, Sallinen at al. (2009) also found that exposure to deprenyl strongly decreased zebrafish MAO activity in 7 dpf larvae [26]. However, in spite of using higher deprenyl concentrations (100 μM vs. 5 μM) and longer exposure times (7 days vs. 1 day), the final effect of deprenyl MAO activity of the larvae was stronger in the present study (100% vs. 74%). The observed discrepancies may most likely be due to differences in the exposure conditions (developmental exposure vs. short term larval exposure) or in the strain of zebrafish used. Curiously, at the gene expression level, downregulation of the *mao* gene was observed for both deprenyl and fluoxetine. In contrast, no effect over zebrafish *mao* expression was detected following 79 h exposure to 0.5 μM fluoxetine [28]. However, it is worth mentioning that the exposure period in this study was between 1 to 80 h post fertilization (hpf).

The inhibition of MAO activity by deprenyl led to a significant increase in the serotonin (about 300% of the control values) and dopamine (about 150% of the control values) levels in the head of the treated larvae, as well as the downregulation of expression levels of *tph1a* and *vmat2* genes, which encode for tryptophan 5-monooxygenase the rate-limiting enzyme for serotonin synthesis and for the vesicular monoamine membrane transporter, responsible for serotonin transport from the cellular cytosol into the synaptic vesicles. These results are consistent with the fact that zebrafish MAO displays a stronger affinity for serotonin than for dopamine [20]. Furthermore, they are also within the same line as those reported by Sallinen et al. (2009), in which MAO inhibitory activity by deprenyl exposure was accompanied by a high increase in serotonin levels (up to 169% of control values after 0–5 dpf treatment with 100 μM, and up to 977% of control values after 0–7 dpf treatment with 100 μM) [26]. Interestingly, although abolition of MAO activity strongly increased serotonin, 5-HIAA levels remained unchanged in the head of deprenyl- treated larvae. This result contrasts with the dramatic decrease in the 5-HIAA levels reported in larvae treated with 100 μM deprenyl from 0–5 dpf [26]. However, different studies on the effect of MAO inhibitors performed on rodents also show increased serotonin without changes in 5-HIAA levels [29,30]. In contrast to deprenyl, fluoxetine did not affect total serotonin levels in the heads of zebrafish larvae. Acutely, SSRIs, such as fluoxetine, are designed to increase synaptic availability of serotonin by blocking the pre-synaptic serotonin transporter (SERT) and preventing its re-uptake into the pre-synaptic terminals, which does not necessarily reflect changes of total central serotonin levels [31]. However, no effect over zebrafish *sert* transcript was detected following 24 h exposure to 0.5 μM fluoxetine. Other studies, such as Airhart et al. (2007) and Cunha et al. (2018) have indeed reported a downregulating effect of fluoxetine over zebrafish *sert* transcript levels following acute exposure to 4.6 μM from 4–5 dpf or exposure to 0.5 μM from 1–80 hpf, respectively. Then again, discrepancies in exposure conditions complicate result correlations [28,32].

One of the first observations on the role of the serotonergic system in mammalian behavior concerns arousal, which usually manifests as locomotion impairment. In this study, we first studied the effect of deprenyl and fluoxetine over the motor function of the larvae, where a significant decrease in the basal locomotor activity was found, a result consistent with the decreased locomotor activity reported in larvae exposed for 2 h to 100 μM deprenyl at 7 dpf [26] and 24 h to 4.6 μM fluoxetine (4–5 dpf) [32]. The same response pattern was observed when the arousal state of larvae was addressed by triggering sensory responses following visual and vibrational stimuli. Both compounds exhibited a significant decrease in the magnitude of the escape response evoked by either stimulus. Whereas the effect on the vibrational startle was consistent with that reported in

a previous study [8], this is the first evidence using the visual-motor response paradigm. As MAOB loss of function may lead to decreased anxiety-like responses in rodents [33], the reduced response to aversive stimuli found in deprenyl-treated larvae may be considered as an anxiolytic-like effect of the hyperserotonergic phenotype. In a similar way, the prototypic SERT inhibitor fluoxetine also decreased 7 dpf zebrafish larvae escape response in the bouncing ball assay following acute exposure [34]. Non-associative learning has been studied in zebrafish larvae by monitoring the reduction in a startle response to a series of acoustic or vibrational stimuli [22,35]. Similar to this study and under the same exposure conditions, in a previous study, both compounds impaired zebrafish larvae by rapid decrease of larvae movement following consecutive tapping stimuli [22]. It has been reported that serotonergic neurons in addition to the Mauthner cells play an important role in the regulation of this form of learning in zebrafish; for example, Pantoja et al. (2016) reported decrease of total distance moved by larvae under habituation conditions following the treatment with quipazine, a serotonin receptor agonist [36].

4.2. Decrease of Serotonin Signaling

Exposure to the tryptophan hydroxylase inhibitor PCPA to reduce serotonin synthesis was used to investigate the impact of serotonin depletion in zebrafish larval locomotor behavior, escape responses, and learning. We examined the effects of serotonin reduction on the expression of mRNA transcripts, levels of neurochemicals, and enzyme activity associated with serotonin action.

As expected, PCPA did not affect zebrafish MAO activity following 24 h exposures to 2.5 mM. However, at the transcript level, *mao* expression was downregulated in exposed larvae. A possible explanation could be that underlying homeostatic mechanisms can be activated in response to changes in serotonergic signaling, such as decrease in serotonin stores. In this study, larvae exposed to 2.5 mM PCPA presented the lowest level of 5-HT across all studied compounds, with a decrease of 29% relative to control. Despite this, it was not found significantly different from larvae control levels; however, it could have been sufficient to activate adaptive mechanisms. This can be also observed in the downregulation of *vmat2* expression. The vesicular monoamine transporter type 2 (VMAT2) has an essential role in the storage and synaptic release of all monoamines, including serotonin. A two-way regulation mechanism between the activity of this monoamine transporter and levels of monoamines has already been reported [37,38]. Whereas the observed decrease of 5-HT levels was not significant from control, low levels of 5-HIAA suggests a decrease in serotonin synthesis.

Behavioral evaluation of PCPA-exposed larvae induced opposite effects of those observed for deprenyl and fluoxetine. Larvae exhibited hyperlocomotion (increased BLM), which is consistent with anxiety-like behaviors [39] and an increase in the escape behavior following a vibrational stimulus. Data about the behavioral effect of PCPA in zebrafish are scarce, and those found are mainly focused on developmental approaches, with controversial results [26,40]. On the other hand, PCPA increased rat behavioral response to turning off the electrical stimulation of the dorsal periaqueductal gray or to acoustic stimulus [41,42]. Analogous to this study, other studies have demonstrated that depleted levels of serotonin in mice have been associated to increased performance of escape-like behaviors [38,43].

4.3. Modulation of Serotoning Signaling

In order to determine if there was a direct relationship between serotonin and the behavioral effects induced by deprenyl and fluoxetine, co-exposure experiments of these chemicals with PCPA were conducted. We observed a partial but significant recovery of the normal serotonin levels in deprenyl + PCPA exposure along with full recovery of *tph1a* expression. Furthermore, total recovery of the BLM activity and VMR and vibrational startle response for all combined exposures was found, suggesting that serotonin may be a key modulator of these behaviors in zebrafish larvae. Re-establishment of the serotonin

levels and partial improvement in the locomotor activity has been previously reported in larvae co-treated with 100 μM deprenyl and 1.5 mM PCPA [26]. These results strongly suggest that serotonin is directly involved in the observed effects of deprenyl and fluoxetine on larvae behaviors. Interestingly, treatment with PCPA resulted also in a full recovery of the dopamine levels, a result consistent with the reported lack of selectivity for TPH over tyrosine hydroxylase (TH), exhibited by PCPA when this chemical is used at high concentrations [44]. Therefore, it is not possible to discard a contribution of dopamine in the observed behavioral effects.

5. Conclusions

As a final remark, our results show that zebrafish larvae can be highly sensitive to prompt serotonin signaling changes, which further reinforces the use of this model vertebrate addressing behavioral and physiological roles of serotonin. Prototypic serotonin modulator chemicals able to decline or enhance serotonin signaling lead to opposite behavioral outcomes in zebrafish larvae following 24 h of exposure. Furthermore, behaviors were then recovered in combined exposure of chemicals with opposed modes of action. In addition, modulation of the larvae serotonergic pathway was also observed at lower levels of biological origination. A review of the obtained results is available in the Supplementary Material Table S5.

The findings presented in this study can provide a useful lesson for quickly identifying the presence of serotonin modulators in the environment: (1) an environmental sample presenting decrease in all four of the studied behaviors (the observed effect will be consistent with a serotonergic-like phenotype (MAO inhibition or SSRI)); (2) analysis of levels of monoaminergic neurochemicals, especially serotonin, in larvae heads; (3) measurements of MAO activity to confirm or discharge if the chemicals' mode of action is through inhibition of serotonin metabolization or reuptake.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxics9060118/s1, Supplementary Methods and Supplementary Results—Tables and the Animal Research: Reporting of Experiments (ARRIVE) guidelines checklist declaration. Table S1: List of primers used for the qPCR. Table S2: One-way ANOVA results of monoaminergic neurotransmitter levels. Table S3: Homogeneous Subsets for neurotransmitter levels following Tukey (HDS) post hoc analysis. Table S4: Homogeneous Subsets for gene expression results following Tukey (HDS) post hoc analysis. For *ppia2* CP (crossing point) values were used and ΔΔCT values were used for the remaining genes. Table S5: Review of main observed results of this study. Arrows pointing up or down indicate significant increase or decrease of responses, respectively. Absence of responses are indicated by a hyphen. Table S6: Animal Research: Reporting of Experiments (ARRIVE) guidelines checklist (doi:10.1371/journal.pbio.3000411).

Author Contributions: M.F.; Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing—original draft, Writing—review & editing; E.P.; Investigation, Writing—original draft, Writing—review & editing, Resources, M.B.; Methodology, C.G.-C.; Supervision, D.R.; Supervision, Conceptualization, Formal analysis, Visualization, Writing—original draft, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the "Agencia Estatal de Investigación" from the Spanish Ministry of Science and Innovation and the IDAEA-CSIC, a Centre of Excellence Severo Ochoa (CEX2018-000794-S), through the SYNERGY Project "Environmental risk of complex mixtures of emerging persistent and mobile chemicals in aquatic ecosystems (MixPersiRisk)" financed with Severo Ochoa funds. The work was partially supported by the network of recognized research groups by the Catalan Government (2017 SGR_902).

Institutional Review Board Statement: Experiments were conducted at the aquatic animal care unit of the Centro de Investigación y Desarrollo—CSIC (registration n°B9900083), which hosts the IDAEA. The centre is equipped with a specific facility to house zebrafish aquariums. The proposed experimental procedures were closely reviewed and supervised by the Ethics Committee on Animal Experimentation (CEEA) of the centre and the Ethics Committee of the CSIC and approved by the competent authority (license n° 9027, approved on 6 September 2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting reported results will be provided upon reader's request.

Acknowledgments: The authors would like to thank Benjamin Piña from the IDAEA institute for his input in data analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sawin, E.R.; Ranganathan, R.; Horvitz, H.R.C. *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron* **2000**, *26*, 619–631. [CrossRef]
2. Winberg, S.; Thörnqvist, P.O. Role of brain serotonin in modulating fish behavior. *Curr. Zool.* **2016**, *62*, 317–323. [CrossRef]
3. Lillesaar, C. The serotonergic system in fish. *J. Chem. Neuroanat.* **2011**, *41*, 294–308. [CrossRef]
4. Horzmann, K.A.; Freeman, J.L. Zebrafish get connected: Investigating neurotransmission targets and alterations in chemical toxicity. *Toxics* **2016**, *4*, 19. [CrossRef]
5. Busch, W.; Schmidt, S.; Kühne, R.; Schulze, T.; Krauss, M.; Altenburger, R. Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. *Environ. Toxicol. Chem.* **2016**, *35*, 1887–1899. [CrossRef]
6. Carlson, R.W.; Bradbury, S.P.; Drummond, R.A.; Hammermeister, D.E. Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals. *Aquat. Toxicol.* **1998**, *43*, 51–68. [CrossRef]
7. Huang, Y.; Zhang, J.; Han, X.; Huang, T. The use of zebrafish (Danio rerio) behavioral responses in identifying sublethal exposures to deltamethrin. *Int. J. Environ. Res. Public Health* **2014**, *11*, 3650–3660. [CrossRef]
8. Faria, M.; Wu, X.; Luja-Mondragón, M.; Prats, E.; Gómez-Oliván, L.M.; Piña, B.; Raldúa, D. Screening anti-predator behaviour in fish larvae exposed to environmental pollutants. *Sci. Total Environ.* **2020**, *714*, 136759. [CrossRef] [PubMed]
9. Valenti, T.W.; Gould, G.G.; Berninger, J.P.; Connors, K.A.; Keele, N.B.; Prosser, K.N.; Brooks, B.W. Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRI) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. *Environ. Sci. Technol.* **2012**, *46*, 2427–2435. [CrossRef] [PubMed]
10. Nielsen, S.V.; Kellner, M.; Henriksen, P.G.; Olsén, H.; Hansen, S.H.; Baatrup, E. The psychoactive drug Escitalopram affects swimming behaviour and increases boldness in zebrafish (Danio rerio). *Ecotoxicology* **2018**, *27*, 485–497. [CrossRef]
11. Parolini, M.; Ghilardi, A.; De Felice, B.; Del Giacco, L. Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (Danio rerio). *Environ. Sci. Pollut. Res.* **2019**, *26*, 34943–34952. [CrossRef] [PubMed]
12. Simmons, D.B.D.; McCallum, E.S.; Balshine, S.; Chandramouli, B.; Cosgrove, J.; Sherry, J.P. Reduced anxiety is associated with the accumulation of six serotonin reuptake inhibitors in wastewater treatment effluent exposed goldfish Carassius auratus. *Sci. Rep.* **2017**, *7*, 1–11. [CrossRef] [PubMed]
13. Maes, J.; Verlooy, L.; Buenafe, O.E.; De Witte, P.A.M.; Esguerra, C.V.; Crawford, A.D. Evaluation of 14 Organic Solvents and Carriers for Screening Applications in Zebrafish Embryos and Larvae. *PLoS ONE* **2012**, *7*, 43850. [CrossRef] [PubMed]
14. Vliet, S.M.; Ho, T.C.; Volz, D.C. Behavioral screening of the LOPAC1280 library in zebrafish embryos. *Toxicol. Appl. Pharmacol.* **2017**, *329*, 241–248. [CrossRef] [PubMed]
15. Faria, M.; Garcia-Reyero, N.; Padrós, F.; Babin, P.J.; Sebastián, D.; Cachot, J.; Prats, E.; Arick Ii, M.; Rial, E.; Knoll-Gellida, A.; et al. Zebrafish Models for Human Acute Organophosphorus Poisoning. *Sci. Rep.* **2015**, *5*. [CrossRef]
16. Fernandes, A.M.; Fero, K.; Arrenberg, A.B.; Bergeron, S.A.; Driever, W.; Burgess, H.A. Deep brain photoreceptors control light-seeking behavior in zebrafish larvae. *Curr. Biol.* **2012**, *22*, 2042–2047. [CrossRef]
17. Prats, E.; Gómez-Canela, C.; Ben-Lulu, S.; Ziv, T.; Padrós, F.; Tornero, D.; Garcia-Reyero, N.; Tauler, R.; Admon, A.; Raldúa, D. Modelling acrylamide acute neurotoxicity in zebrafish larvae. *Sci. Rep.* **2017**, *7*, 1–12. [CrossRef]
18. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 C T Method. *Methods* **2001**, *25*, 402–408. [CrossRef]
19. Holt, A.; Sharman, D.F.; Baker, G.B.; Palcic, M.M. A Continuous Spectrophotometric Assay for Monoamine Oxidase and Related Enzymes in Tissue Homogenates oxidoreductase (deaminating) (copper-containing). *Anal. Biochem.* **1997**, *244*, 384–392. [CrossRef]
20. Anichtchik, O.; Sallinen, V.; Peitsaro, N.; Panula, P. Distinct structure and activity of monoamine oxidase in the brain of zebrafish (Danio rerio). *J. Comp. Neurol.* **2006**, *498*, 593–610. [CrossRef]
21. Mayol-Cabré, M.; Prats, E.; Raldúa, D.; Gómez-Canela, C. Characterization of monoaminergic neurochemicals in the different brain regions of adult zebrafish. *Sci. Total Environ.* **2020**, *745*, 141205. [CrossRef]
22. Faria, M.; Prats, E.; Novoa-Luna, K.A.; Bedrossiantz, J.; Gómez-Canela, C.; Gómez-Oliván, L.M.; Raldúa, D. Development of a vibrational startle response assay for screening environmental pollutants and drugs impairing predator avoidance. *Sci. Total Environ.* **2019**, *650*, 87–96. [CrossRef] [PubMed]
23. Boix, J.; Cauli, O. Alteration of serotonin system by polychlorinated biphenyls exposure. *Neurochem. Int.* **2012**, *60*, 809–816. [CrossRef] [PubMed]

24. Maximino, C.; Araujo, J.; Leão, L.K.R.; Grisolia, A.B.A.; Oliveira, K.R.M.; Lima, M.G.; Batista, E.d.J.O.; Crespo-López, M.E.; Gouveia, A.; Herculano, A.M. Possible role of serotoninergic system in the neurobehavioral impairment induced by acute methylmercury exposure in zebrafish (Danio rerio). *Neurotoxicol. Teratol.* **2011**, *33*, 727–734. [CrossRef] [PubMed]
25. Willhite, C.; Sharma, R.P. Acute dieldrin exposure in relation to brain monoamine oxidase activity and concentration of brain serotonin and 5-hydroxyindoleacetic acid. *Toxicol. Lett.* **1978**, *2*, 71–75. [CrossRef]
26. Sallinen, V.; Sundvik, M.; Reenilä, I.; Peitsaro, N.; Khrustalyov, D.; Anichtchik, O.; Toleikyte, G.; Kaslin, J.; Panula, P. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. *J. Neurochem.* **2009**, *109*, 403–415. [CrossRef]
27. Faria, M.; Prats, E.; Ramírez, J.R.R.; Bellot, M.; Bedrossiantz, J.; Pagano, M.; Valls, A.; Gomez-Canela, C.; Porta, J.M.; Mestres, J.; et al. Androgenic activation, impairment of the monoaminergic system and altered behavior in zebrafish larvae exposed to environmental concentrations of fenitrothion. *Sci. Total Environ.* **2021**, *775*, 145671. [CrossRef]
28. Cunha, V.; Rodrigues, P.; Santos, M.M.; Moradas-Ferreira, P.; Ferreira, M. Fluoxetine modulates the transcription of genes involved in serotonin, dopamine and adrenergic signalling in zebrafish embryos. *Chemosphere* **2018**, *191*, 954–961. [CrossRef]
29. McIntyre, I.M.; Norman, T.R. Serotonergic effects of isatin: An endogenous MAO inhibitor related to tribulin. *J. Neural Transm.* **1990**, *79*, 35–40. [CrossRef]
30. Finberg, J.P.M.; Rabey, J.M. Inhibitors of MAO-A and MAO-B in psychiatry and neurology. *Front. Pharmacol.* **2016**, *7*, 340. [CrossRef]
31. Norrholm, S.D.; Ouimet, C.C. Chronic fluoxetine administration to juvenile rats prevents age-associated dendritic spine proliferation in hippocampus. *Brain Res.* **2000**, *883*, 205–215. [CrossRef]
32. Airhart, M.J.; Lee, D.H.; Wilson, T.D.; Miller, B.E.; Miller, M.N.; Skalko, R.G. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). *Neurotoxicol. Teratol.* **2007**, *29*, 652–664. [CrossRef] [PubMed]
33. Bortolato, M.; Godar, S.C.; Davarian, S.; Chen, K.; Shih, J.C. Behavioral disinhibition and reduced anxiety-like behaviors in monoamine oxidase b-deficient mice. *Neuropsychopharmacology* **2009**, *34*, 2746–2757. [CrossRef] [PubMed]
34. Richendrfer, H.; Pelkowski, S.D.; Colwill, R.M.; Creton, R. On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. *Behav. Brain Res.* **2012**, *228*, 99–106. [CrossRef] [PubMed]
35. Best, J.D.; Berghmans, S.; Hunt, J.J.F.G.; Clarke, S.C.; Fleming, A.; Goldsmith, P.; Roach, A.G. Non-associative learning in larval zebrafish. *Neuropsychopharmacology* **2008**, *33*, 1206. [CrossRef] [PubMed]
36. Pantoja, C.; Hoagland, A.; Carroll, E.C.; Karalis, V.; Conner, A.; Isacoff, E.Y. Neuromodulatory Regulation of Behavioral Individuality in Zebrafish. *Neuron* **2016**, *91*, 587–601. [CrossRef]
37. Eiden, L.E.; Weihe, E. VMAT2: A dynamic regulator of brain monoaminergic neuronal function interacting with drugs of abuse. *Ann. N. Y. Acad. Sci.* **2011**, *1216*, 86–98. [CrossRef]
38. Narboux-Nême, N.; Sagné, C.; Doly, S.; Diaz, S.L.; Martin, C.B.P.; Angenard, G.; Martres, M.P.; Giros, B.; Hamon, M.; Lanfumey, L.; et al. Severe serotonin depletion after conditional deletion of the vesicular monoamine transporter 2 gene in serotonin neurons: Neural and behavioral consequences. *Neuropsychopharmacology* **2011**, *36*, 2538–2550. [CrossRef]
39. Kalueff, A.V.; Gebhardt, M.; Stewart, A.M.; Cachat, J.M.; Brimmer, M.; Chawla, J.S.; Craddock, C.; Kyzar, E.J.; Roth, A.; Landsman, S. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* **2013**, *10*, 70–86. [CrossRef] [PubMed]
40. Airhart, M.J.; Lee, D.H.; Wilson, T.D.; Miller, B.E.; Miller, M.N.; Skalko, R.G.; Monaco, P.J. Adverse effects of serotonin depletion in developing zebrafish. *Neurotoxicol. Teratol.* **2012**, *34*, 152–160. [CrossRef]
41. Carlton, P.L.; Advokat, C. Attenuated habituation due to parachlorophenylalanine. *Pharmacol. Biochem. Behav.* **1973**, *1*, 657–663. [CrossRef]
42. Sanford Kiser, R.; Lebovitz, R.M.; German, D.C. Anatomic and pharmacologic differences between two types of aversive midbrain stimulation. *Brain Res.* **1978**, *155*, 331–342. [CrossRef]
43. Soubrié, P. Reconciling the role of central serotonin neurons in human and animal behavior. *Behav. Brain Sci.* **1986**, *9*, 319–335. [CrossRef]
44. Betari, N.; Sahlholm, K.; Ishizuka, Y.; Teigen, K.; Haavik, J. Discovery and biological characterization of a novel scaffold for potent inhibitors of peripheral serotonin synthesis. *Future Med. Chem.* **2020**, 1461–1474. [CrossRef] [PubMed]

toxics

MDPI

Article

Teratogenic, Oxidative Stress and Behavioural Outcomes of Three Fungicides of Natural Origin (*Equisetum arvense*, *Mimosa tenuiflora*, Thymol) on Zebrafish (*Danio rerio*)

Raquel Vieira [1], Carlos Venâncio [1,2] and Luís Félix [1,3,4,*]

[1] Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; raquelvieira1920@gmail.com (R.V.); cvenanci@utad.pt (C.V.)
[2] Department of Animal Science, School of Agrarian and Veterinary Sciences (ECAV), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal
[3] i3S-Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal
[4] Laboratory Animal Science, IBMC-Instituto de Biologia Molecular Celular, Universidade do Porto, 4200-135 Porto, Portugal
* Correspondence: luis.felix@ibmc.up.pt

Abstract: The improper use of synthetic fungicides has raised public concerns related to environmental pollution and animal health. Over the years, plant-derived antifungals have been investigated as safer alternatives, although little scientific evidence of its neurodevelopmental effects exist. The main objective of this study was to explore the effects of three alternative natural extracts (*Equisetum arvense*, *Mimosa tenuiflora*, Thymol) with antifungal properties during the early development of zebrafish by evaluating different teratogenic, oxidative stress and behavioural outcomes. Following the determination of the 96 h-LC_{50}, exposure to sublethal concentrations showed the safety profile of both *E. arvense* and *M. tenuiflora*. However, following 96-h exposure to Thymol, increased lethality, pericardial oedema, yolk and eye deformations, and decreased body length were observed. The reduced and oxidized glutathione (GSH:GSSG) ratio was increased, and the glutathione-s-transferase activity in the group exposed to the highest Thymol concentration. Overall, these results support a more reducing environment associated with possible effects at the cellular proliferation level. In addition, the disruption of behavioural states (fear- and anxiety-like disorders) were noted, pointing to alterations in the c-Jun N-terminal kinase developmental signalling pathway, although further studies are required to explore this rationale. Notwithstanding, the results provide direct evidence of the teratogenic effects of Thymol, which might have consequences for non-target species.

Keywords: natural products; fungicides; early development; teratogenicity; zebrafish; behaviour; oxidative stress

Citation: Vieira, R.; Venâncio, C.; Félix, L. Teratogenic, Oxidative Stress and Behavioural Outcomes of Three Fungicides of Natural Origin (*Equisetum arvense*, *Mimosa tenuiflora*, Thymol) on Zebrafish (*Danio rerio*). *Toxics* **2021**, *9*, 8. https://doi.org/10.3390/toxics9010008

Received: 24 November 2020
Accepted: 4 January 2021
Published: 9 January 2021

1. Introduction

The use of agrochemicals to control plant diseases has become crucial in modern agricultural procedures, with a diverse range of commercial products being released over the last years [1]. The improper application and extensive use of these compounds have raised public concerns related to environmental pollution and animal health [2]. In fact, agrochemical residues spread in aquatic systems [2,3], compromising not only aquatic food resources but also fisheries and aquaculture. However, the effects of fungicides in non-target species have received less attention when compared to herbicides and insecticides [4]. In this context, although acceptable regulatory concentrations have been established for pesticide residues [5], local and regional studies have documented the worldwide occurrence of synthetic fungicides in surface waters in concentrations up to around 80 μg L^{-1}, which are generally higher than those observed for herbicides and insecticides (reviewed by [4]). Furthermore, these concentrations are superior to the acceptable regulatory concentrations

and above the average lowest effective concentrations in different non-target aquatic biota (reviewed by [4]).

Over the past years, phyto-fungicides (plant-based extracts or compounds) have been investigated as an alternative to synthetic fungicides and commercialised for the management of a wide range of fungal diseases in plants [6–8], and they are generally accepted as safe, easily biodegradable, environmentally friendly and with low toxicity [9,10]. However, their use is often limited due to their instability and rapid degradation, requiring higher application rates and application frequency [11]. In addition, adverse or toxic side effects for non-target species are usually reported on labels or material safety data sheets, but there is a paucity of scientific and ecotoxicological information, in particular, during neurodevelopmental periods, which are known to be critical for the population dynamics and ecosystem functioning [12]. For instance, horsetail (*Equisetum arvense*) was the first approved basic substance according to the European Regulation (EC) 1107/2009 [13], but, although not considered as a substance of concern, it has been associated with potential neurodevelopmental toxicity [14]. Likewise, "jurema preta" (*Mimosa tenuiflora*) has been described as teratogenic to higher vertebrates [15,16], and Thymol, the main monoterpene phenol isolated from plants from the *Lamiaceae* family such as *Thymus vulgaris* L. [17], has been shown to cause developmental abnormalities in chicken and zebrafish embryos [18–20] and to regulate cholinergic and antioxidant systems in cognitive dysfunctional zebrafish [21]. However, although current research shows its antifungal properties [17,22,23] and some commercial pesticide products based on natural compounds are available on the market, there is a lack of sufficient neurodevelopmental information, and further studies are needed to clarify their environmental risk to non-target organisms to define efficient and appropriate use patterns.

Among aquatic organisms, fish have been considered useful biological indicators for ecotoxicological studies [24], with the powerful and versatile teleost vertebrate zebrafish (*Danio rerio*) model being increasingly used [25,26]. In fact, its low husbandry costs, reproduction potential and embryonic external fertilization method, rapid development and transparency as well as the low ethical constrains associated with considering its embryonic stages facilitate high-throughput screenings [27,28]. Furthermore, the literature supports zebrafish embryos as more responsive to test compounds in comparison to adult fish [29,30], and they are a conservative indication of later biological changes [31]. Therefore, considering these characteristics, the present study was focused on the toxicological effects of commercially available herbal products containing *Equisetum arvense*, *Mimosa tenuiflora* and Thymol on the zebrafish embryo. In particular, the aims of this study were to evaluate the (1) morphological and physiological changes, (2) oxidative status alterations and (3) behavioural impacts induced by the test formulations during early zebrafish development.

2. Materials and Methods

2.1. Chemicals and Reagents

The commercial formulation of horsetail extract (*Equisetum arvense*) decoction (95.2% decoction of horsetail (*E. arvense* 7%), tansy herbs (*Tanaceti Hb.* conc. 2.5%), wormwood (*Artemisia absinthium* 1%) and 4.8% humus extract (15% wine marc extract)) was acquired from Aries Umweltprodukte (Horstedt, Germany). The ethanolic extract of *Mimosa tenuiflora* (Matry, 80% M. tenuiflora extract containing 1% zinc and 1% manganese) was purchased from Biagro (Valencia, Spain) and Thymol (extra pure, CAS 89-83-8) was acquired from EMD Millipore (Oeiras, Portugal). Based on the percentage of the principal component (*E. arvense*, *M. tenuiflora*, and Thymol) stock solutions of 6250, 80,000, and 500 mg L^{-1} were prepared for the *E. arvense*, *M. tenuiflora* and Thymol, respectively, and stored at 4 °C. Exposure solutions were freshly prepared in embryo water (28.0 ± 0.5 °C, 200 mg L^{-1} Instant Ocean Salt and 100 mg L^{-1} sodium bicarbonate; UV sterilized) prepared from City of Vila Real filtered tap water. Except when specified, all other chemicals were of the highest grade commercially available and obtained from standard commercial suppliers.

2.2. Animals

Adult wild-type AB strain zebrafish (*Danio rerio*) were maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Fish were maintained under standard conditions at 28.0 ± 0.5 °C with a 14 h light/10 h dark photoperiod in an open water system with continuous supply of aerated, dechlorinated, charcoal-filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5). Fish were fed with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. *nauplii* twice a day. Embryos were collected by the natural spawning method by maintaining a 2:1 male to female ratio in cages overnight. Embryos were collected within 1 h after the onset of the light cycle (at 8.00 a.m.), rinsed, and bleached with a diluted Chloramine-T solution (0.5% *w*/*v*), washed twice with embryo water and transferred into a petri dish for egg selection. Embryonic stages were denoted as hours post-fertilization (hpf) under a SMZ 445 stereomicroscope (Nikon, Japan) and 2 hpf normal fertilized embryos were used for the subsequent experiments. All animal procedures were performed in accordance with the ethical principles and other requirements on the use of laboratory animals of the EU directive (2010/63/EU) and national legislation for animal experimentation and welfare (Decreto-Lei 113/2013). In addition, two authors have a level B FELASA certification (Federation of European Laboratory Animal Science Associations) while another author has a FELASA C certification.

2.3. Concentration Determination

The OECD testing guideline 236 was applied to determine the lethal concentration that causes 50% mortality (LC_{50}) with modifications. Embryos at 2 hpf were randomly distributed in 6-well culture plates (5 mL solution and 20 embryos per well) for triplicate exposure to seven test solutions (10-fold dilution from the stock solutions) for 96 h under the controlled conditions reported before. Embryo water was used as blank control and to prepare all test solutions. The exposure solutions were renewed every 24 h to keep the appropriate concentrations and water quality. The embryonic mortality was recorded daily and following correction for the percentage of mortality in the control group using Abbott's formula, and the 96 h-LC_{50} values were determined using the probit analysis. The 96 h-LC_{50} and the 95% confidence limits were calculated as 1.98 mg L^{-1} (0.50–4.13), 1.55 mg L^{-1} (0.39–3.44), and 2.35 mg L^{-1} (0.78–5.55) (Figure S1), respectively, for *E. arvense*, *M. tenuiflora*, and Thymol. Based on the calculated LC_{50}, three sub-lethal concentrations were selected for the subsequent experiments.

2.4. Embryo Toxicity

Based on the LC_{50} calculation, 0.00625—E1, 0.0625—E2 and 0.625 mg L^{-1}—E3 (about 1/300, 1/30 and 1/3 of the LC_{50}) were chosen for the *E. arvense* based formulation. For the *M. tenuiflora*, the selected concentrations were 0.008—M1, 0.08—M2 and 0.8 mg L^{-1}—M3 (around 1/200, 1/20 and 1/2 of the LC_{50}) while for Thymol the concentrations for testing were 0.01—T1, 0.1—T2 and 1 mg L^{-1}—T3 (approximately 1/200, 1/20 and 1/2 of the LC_{50}). Normally developed 3 h post fertilization (hpf) fertilized eggs were randomly placed in 6-well culture plates (50 embryos in 5 mL solution/well) and exposed to the above solutions. A blank control group (embryo water only) was also prepared and included in each plate. The plates were maintained in a constant temperature-light cycle (28 °C and 14:10 h light-dark cycle) for a period of 96 h, after which eleutheroembryo were washed twice and allowed to develop until 120 hpf (Figure 1). During the experimental period, the exposure solutions were replaced daily to maintain the appropriate concentration of the test compounds. The experiments were repeated independently five times. The zebrafish development (10 random animals removed from each group) was accompanied under a SMZ800 stereomicroscope with the cumulative mortality being assessed at 8, 24, 48, 72 and 98 hpf according to the standard guidelines [32], with dead embryos removed from the plates. Lethal parameters such as failure of somites, eye and otolith development, missing heartbeat, and nondetached tail and head, were recorded at 24, 48, 72, and 98 hpf

according to previous studies [33,34]. The spontaneous movements at 24 hpf, pigmentation formation and heart rate at 48 hpf and hatching rate at 72 hpf were evaluated as sublethal endpoints. Morphological abnormalities (body length, area of egg yolk, area of heart and eye, and head to body angle) were screened at 98 hpf in 10 randomly 3% methylcellulose-immobilized eleutheroembryo. Images of morphological defects were photographed using an inverted microscope (IX 51, Olympus, Antwerp, Belgium) and combined, merged, and processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA). Measurements were taken using the Digimizer software (version 4.1.1.0, MedCalc Software, Mariakerke, Belgium). Eleutheroembryo were further collected for subsequent biochemical analysis or washed three times with embryo medium and allowed to develop until 120 hpf for behavioural analysis. In total, five independent replicates from independent spawns were used to maximize the genetic variability of the individuals.

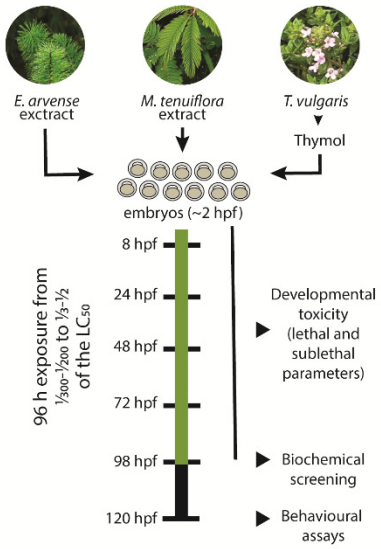

Figure 1. Schematic diagram of the zebrafish exposure to the plant-based fungicides. Collected embryos at around 2 h post-fertilization (hpf) were exposed to different concentrations of *Equisetum arvense* and *Mimosa tenuiflora* extracts and to Thymol for a period of 96 h. The selected concentrations varied from 1/300–1/200 to 1/3–1/2 of the determined LC_{50}. During the exposure period, daily lethal and sublethal parameters were assessed. After 96 h exposure, eleutheroembryo were collected for biochemical screening of different biomarkers associated with oxidative stress, energetic metabolism, and neurotransmission. At 120 hpf, the locomotor activity of the eleutheroembryo was assessed using different behavioural paradigms.

2.5. Biochemical Analysis

After exposure to the test compounds for 96 h, biochemical analysis was conducted as detailed before [35]. Around 30 randomly selected eleutheroembryo from each group were homogenized in 400 µL cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4) in a Tissuelyser II (30 Hz for 30 s, Qiagen, Hilden, Germany). Following a centrifugation at 12,000× *g* for 10 min at 4 °C in a refrigerated centrifuge (Sigma 3K30, Osterode, Germany), supernatant protein concentration was determined using the Bradford method at 595 nm with bovine serum albumin (BSA) as a standard. The overall reactive oxygen species (ROS) generation was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) at 485 nm (excitation) and 530 nm (emission). Changes in oxidative stress indicators, such as the activity of superoxide dismutase (Cu/Zn-SOD) and catalase (CAT), were evaluated at 560 nm and at 240 nm, respectively. The activity of glutathione peroxidase (GPx) and glutathione-s-transferase (GST) were measured at 340 nm. The reduced (GSH) and oxidized glutathione (GSSG) were derivatized with ortho-phthalaldehyde (OPA) and measured at 320 nm and 420 nm for excitation and emission wavelengths, respectively. The ratio between

glutathione (GSH:GSSG) was used to describe the redox ratio (oxidative stress index, OSI). The content of malondialdehyde (MDA) was estimated by the quantification of the MDA-TBA adducts at 530 nm with a correction for non-specific adducts at 600 nm. The lactate dehydrogenase (LDH) was assayed at 340 nm and the acetylcholinesterase (AChE) activity at 405 nm. All samples (10 µL) were tested in duplicate and measured against a reagent blank at 30 °C using a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) or a Varian Cary Eclipse (Varian, Australia) spectrofluorometer, equipped with a microplate reader. To integrate all the biomarker responses into a general "stress index", the integrated biomarker response index version 2 (IBRv2) was calculated according to a previous method [36] representing the stress level at each tested concentration, based on the principle of reference deviation. Overall, data were normalized to control values and log-transformed (Yi) to diminish variability, and the overall mean (µ) and standard deviation (s) calculated. Data were further standardized as $Zi = (Yi - \mu)/s$. The difference between Zi and Z0 (control) was then calculated to determine A values and the IBRv2 was calculated by summing the absolute values of A.

2.6. Locomotor Behaviour Analysis

The zebrafish eleutheroembryo locomotor behaviour (exploratory open field test), the patterns of avoidance (in response to a bouncing ball stimulus) and anxiety-like behaviours (in the visual motor response test) were analysed 24 h after the end of the exposure, at 120 hpf, in a climatized dark room as previously described [34,35,37]. Briefly, 6-well agarose-coated plates containing 1 randomly picked eleutheroembryo per well (5 per group) were placed above a 15.6" laptop LCD screen (1366 × 768 pixels resolution) showing a white Microsoft PowerPoint (Microsoft Corp., Washington, DC, USA) presentation. A 14.2 megapixels Sony Nex-5 digital camera was used to record the exploratory behaviour (mean speed, total distance moved, percentage of time spent in each zone, mean distance to centre zone (5 mm radius circle) of the well, mean absolute turn angle, and percentage of time active) of the eleutheroembryo during 10 min after a period of acclimation (5 min). After the analysis of exploratory behaviour, the avoidance response was measured by the eleutheroembryo's ability to respond to a visual stimulus (a red bouncing ball present at the upper half of the well and moving from left to right) provided by the presentation in the Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA) during alternating periods (10 min). In addition, the anxiety-like behaviour of eleutheroembryo was monitored in duplicate conditions of continuous visible light (10 min) and dark (10 min) using an infrared-capable camera (GENIUSPY, GS-NQ140CML) with a 3.6 mm lenses using the same plate configuration. The TheRealFishTracker software was used to video-track individuals and eleutheroembryo exhibiting obvious malformations in the exposure, and control groups were excluded to avoid the interference of morphological effects.

2.7. Statistical Analysis

The statistical analyses were performed on the averaged values from each independent exposure using the GraphPad Prism software (Prism 8). The LC_{50} values were calculated using a variable slope model. The normality of data was controlled using Shapiro Wilk's test, and the homoscedasticity was checked with Brown-Forsythe's test. When data followed the normal distribution, differences among groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test and data expressed as mean ± standard deviation (SD). When data followed a non-normal distribution, the data treatment was performed using the non-parametric Kruskal-Wallis analysis of variance followed by Dunn's test with a Bonferroni correction for multiple comparisons and data expressed as medians and interquartile range (25th; 75th percentiles). The student's *t* test was used to evaluate differences for the aversive behavioural responses. A $p < 0.05$ was considered to be a statistically significant difference.

3. Results

3.1. Teratogenic Effects of Phyto-Fungicide Formulations

The effects on the embryo development were evaluated from ~2 hpf and for a period of 96 h with different parameters being evaluated at specific time-points (Table 1). At 24 hpf, no significant changes were observed in the development of the tail, head and somites following exposure to any of the phyto-fungicides (Supplementary Table S1). Similarly, the spontaneous movements were not affected by *E. arvense* ($X^2(3) = 7.859$, $p = 0.050$) or *M. tenuiflora* ($X^2(3) = 1.037$, $p = 0.792$), nor by Thymol ($X^2(3) = 4.907$, $p = 0.179$) (Table 1). At 48 hpf, the eyes, otoliths, pigmentation, and blood circulation were visible in all treated embryos (Table S1), and no changes were depicted in the heart rate of the individuals for *E. arvense* ($F(3,16) = 3.065$, $p = 0.058$) or *M. tenuiflora* ($F(3,16) = 0.649$, $p = 0.595$), nor by Thymol ($F(3,16) = 2.357$, $p = 0.110$) (Table 1). At 72 hpf, and despite slight variations in the Thymol-exposed individuals ($X^2(3) = 2.159$, $p = 0.540$, Table S1), no significant changes were apparent for the oedema presence. At this time-point, as shown in Table 1, the hatching rate did not differ among *E. arvense* ($X^2(3) = 2.201$, $p = 0.532$), *M. tenuiflora* ($F(3,15) = 1.039$, $p = 0.404$) or Thymol ($F(3,16) = 0.145$, $p = 0.931$) treated embryos.

At 98 hpf, embryo development in the control groups was as expected with around 80% of the animals showing a normal development with mortalities of about 10%, and 6 to 10% malformed individuals (Figure 2A) without significant changes after 96 h exposure to *E. arvense* or to *M. tenuiflora* ($p > 0.05$). However, after 96 h exposure to Thymol ($X^2(3) = 12.46$, $p = 0.006$), the cumulative mortality increased significantly after exposure to T3 ($p = 0.004$) in relation to the control group, while no significant differences were verified between the other groups. Similarly, at this time point, malformed eleutheroembryos ($X^2(3) = 9.827$, $p = 0.020$) were noticed in T3 group ($p = 0.029$), showing a higher percentage in relation to the control group (Figure 2A,B). The quantitative analysis (Table S1) showed that the most evident malformations were related to the yolk ($X^2(3) = 11.81$, $p = 0.008$), pericardial ($F(3,15) = 3.516$, $p = 0.041$), and eye ($X^2(3) = 9.377$, $p = 0.025$) areas and to the overall body length of the eleutheroembryo ($F(3,15) = 6.231$, $p = 0.006$). In this regard, exposure for 96 h to Thymol caused a decreased yolk ($p = 0.034$ between T3 and the control group and $p = 0.017$ between T3 and T1), an increased pericardial ($p = 0.042$ between T3 and the control group), and a decreased eye ($p = 0.039$ between T3 and the control group). In addition, exposure to T2 and T3 caused a significant reduction on the body length of 98 h eleutheroembryos in relation to the control group ($p = 0.026$ and $p = 0.018$, respectively).

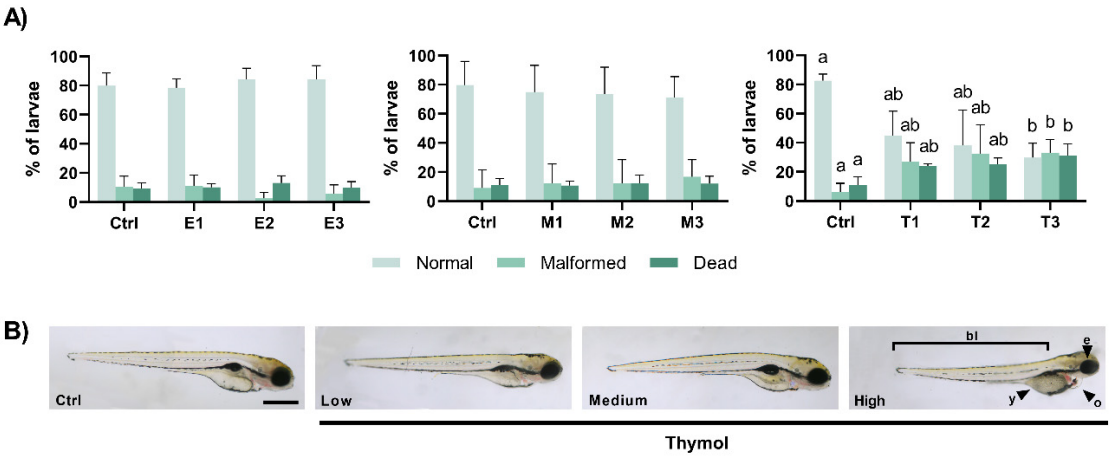

Figure 2. (**A**) Percentages of normal, malformed and dead eleutheroembryo at the end of the exposure period (at 98 hpf). Values are presented as mean ± SD of five replicates per treatment ($n = 10$ random embryos per replicate). Different lowercase letters represent statistical differences among treatment groups (one-way ANOVA, $p < 0.05$). (**B**) Representative views of the malformations observed in eleutheroembryo exposed to Thymol. Malformations were observed after exposure to the highest concentration of Thymol (T3) namely as abnormal eye (e), yolk (y), and pericardiac oedema (o) and by the decreased body length (bl). The scale bar represents 500 μm.

Table 1. Effects of the different exposures on spontaneous movements, heart rate and hatching rate in zebrafish embryos. Morphometric parameters were recorded in zebrafish eleutheroembryo at 98 hpf.

Phyto-Fungicide	Group	24 hpf	48 hpf	72 hpf	98 hpf			
		Spontaneous Movements (units/min)	**Heart Rate (beats/min)**	**Hatching Rate (%)**	**Body Length (mm)**	**Yolk Area (mm^2)**	**Heart Area (mm^2)**	**Eye Area (mm^2)**
E. arvense	Ctrl	2.0 (2.0–2.5)	123.6 ± 1.82	70.0 (65.5–71.5)	3.75 (3.71–3.77)	0.21 (0.20–0.21)	0.034 ± 0.001	0.082 (0.082–0.085)
	E1	2.0 (2.0–3.0)	124.2 ± 3.42	70.0 (66.0–71.0)	3.74 (3.72–3.78)	0.21 (0.21–0.22)	0.033 ± 0.003	0.083 (0.081–0.084)
	E2	1.0 (1.0–2.0)	117.0 ± 3.39	68.0 (63.5–71.5)	3.53 (3.49–3.65)	0.22 (0.22–0.22)	0.035 ± 0.003	0.075 (0.074–0.079)
	E3	2.0 (2.0–2.5)	121.2 ± 6.57	68.0 (61.5–68.5)	3.72 (3.65–3.80)	0.20 (0.20–0.21)	0.032 ± 0.004	0.081 (0.079–0.085)
M. tenuiflora	Ctrl	2.0 (0.5–2.0)	113.2 ± 7.19	62.5 ± 7.7	3.56 ± 0.20	0.22 (0.21–0.23)	0.038 ± 0.005	0.081 ± 0.015
	M1	1.0 (1.0–1.5)	115.0 ± 9.82	65.6 ± 5.3	3.48 ± 0.26	0.24 (0.23–0.24)	0.035 ± 0.006	0.086 ± 0.015
	M2	2.0 (1.0–2.0)	120.0 ± 5.92	56.4 ± 13	3.65 ± 0.14	0.24 (0.22–0.25)	0.039 ± 0.005	0.088 ± 0.010
	M3	1.0 (0.5–2.5)	118.8 ± 11.4	54.0 ± 16	3.60 ± 0.15	0.25 (0.22–0.25)	0.038 ± 0.005	0.087 ± 0.011
Thymol	Ctrl	2.0 (0.5–2.0)	144.2 ± 21.7	60.8 ± 7.6	3.47 ± 0.10 ^{a}	0.21 (0.20–0.22) ^{a}	0.031 ± 0.001 ^{a}	0.079 (0.078–0.082) ^{a}
	T1	0.0 (0.0–2.5)	159.6 ± 5.32	58.6 ± 3.1	3.44 ± 0.09 ^{a,b}	0.21 (0.20–0.23) ^{a}	0.037 ± 0.002 ^{ab}	0.072 (0.061–0.079) ^{a,b}
	T2	1.0 (0.0–1.0)	165.6 ± 5.68	59.4 ± 5.4	3.32 ± 0.05 ^{b}	0.23 (0.22–0.24) ^{a,b}	0.037 ± 0.006 ^{a,b}	0.070 (0.063–0.073) ^{a,b}
	T3	2.0 (1.5–2.0)	145.4 ± 20.5	59.0 ± 5.2	3.30 ± 0.04 ^{b}	0.25 (0.24–0.28) ^{b}	0.038 ± 0.003 ^{b}	0.067 (0.062–0.073) ^{b}

Parametric data is presented as mean and standard deviation while non-parametric data presented as median and interquartile range of five independent replicates. Statistical analysis was performed using the one-way ANOVA followed by Turkey's test or by Kruskal–Wallis test followed by Dunn's test ($p < 0.05$). Different lowercase letters represent statistical differences among groups for each analysed parameter.

3.2. Biochemical Markers Affected by the Phyto-Fungicide Exposure

The biochemical changes induced by the exposure to these phyto-fungicides were evaluated at the end of the exposure by some ROS-mediated and related parameters, which were normalised to control values and are summarized in Figure 3 (original data is shown in Supplementary Tables S2–S4). After exposure to E1, an elevated AChE activity ($F(3,16) = 3.526$, $p = 0.039$) was detected in relation to the control group ($p = 0.041$). No other difference was noted after exposure to *E. arvense*. Similarly, no biochemical changes were observed in zebrafish following exposure to *M. tenuiflora*. However, when embryos were exposed to Thymol, an increase in the GSH:GSSG ratio ($F(3,16) = 11.21$, $p < 0.001$) was observed for T1 ($p < 0.001$), T2 ($p < 0.001$) and T3 ($p = 0.025$) in relation to the control group. Exposure to T3 also resulted in an increased activity of GST activity ($X^2(3) = 12.60$, $p = 0.006$) in relation to the control group ($p = 0.003$). No other change was perceived. The star plot representations for each compound (Figure 3B) shows how each individual biomarker contributed to the IBRv2 index obtained for each compound. Overall, a negative relationship between the IBRv2 values for *E. arvense* and *M. tenuiflora* was obtained with the lowest concentrations showing higher values in relation to the lowest concentrations which may be associated to the individual changes observed. On the other hand, the IBRv2 index was similar in the Thymol exposed groups although changes were observed in the individual biomarkers.

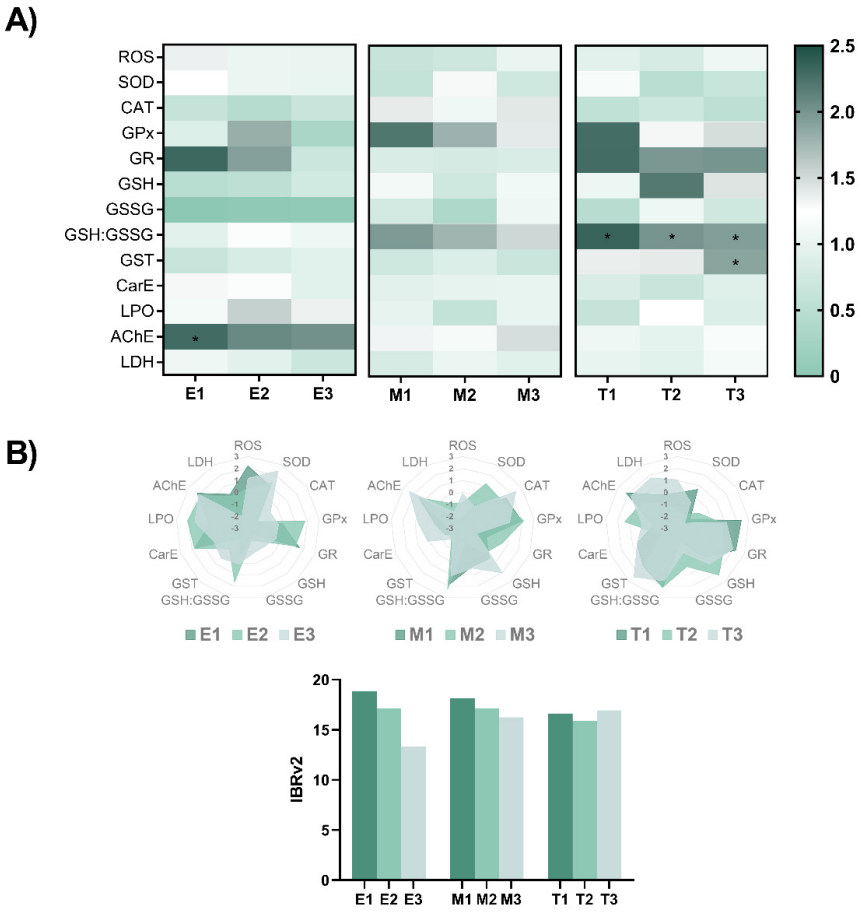

Figure 3. (**A**) Heatmap of biochemical parameters measured in zebrafish eleutheroembryo at the end of the exposure to the different phyto-fungicide. Data from at least five independent samples ($n = 100$ individuals per replicate). The data used for the evaluation of the biochemical parameters were normalised to the control group value. Parametric data is expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. The * indicate significant differences relative to the control group ($p < 0.05$). (**B**) Star plots of A values obtained and IBRv2 value for biomarker responses of zebrafish embryos exposed for 96-h to the different plant-based fungicides.

3.3. Behavioural Responses Induced by the Different Formulations

The behavioural responses evaluated at 120 hpf after the 96-h exposure to the phyto-fungicides are shown in Figure 4. Regarding the exploratory behaviours, no significant changes were observed following exposure (Figure 4A and Supplementary Tables S5–S7). Concerning the ability to escape the red bouncing ball (aversive stimulus, Figure 4B), the individuals exposed to *E. arvense* and *M. tenuiflora* showed their ability to escape from the stimulus by remaining for more time in the area without the stimulus ($p < 0.05$). However, after exposure to Thymol, eleutheroembryo from the T2 and T3 group showed a reduced ability to escape the aversive stimulus ($p = 0.453$ and $p = 0.765$, respectively), spending the same amount of time in both halves of the well. The individuals were also tested for anxiety-like behavioural changes using the light/dark test and the results are shown in Figure 4C. In comparison to the control group, no significant changes were perceived after exposure to *E. arvense* and *M. tenuiflora* regardless of the lightning conditions (for *E. arvense*: 10 min: $F(3,16) = 3.325$, $p = 0.050$; 20 min: $F(3,16) = 8.286$, $p = 0.002$ with significant differences between E1 and E3 ($p = 0.002$) and between E2 and E3 ($p = 0.014$); 30 min: $F(3,15) = 1.370$, $p = 0.290$ and 40 min: $F(3,16) = 0.578$, $p = 0.638$ and for *M. tenuiflora*: 10 min: $F(3,14) = 3.469$, $p = 0.045$ with significant differences between M1 and M3 ($p = 0.030$); 20 min: $F(3,16) = 0.736$, $p = 0.546$; 30 min: $F(3,15) = 2.129$, $p = 0.155$ and 40 min: $F(3,16) = 0.510$, $p = 0.682$). Thymol exposure during initial zebrafish development induced no changes on the first light period ($F(3,16) = 0.458$, $p = 0.715$), but exposure to T3 induced hyperactivity in relation to the control group ($p = 0.047$) in the first dark period ($F(3,16) = 2.919$, $p = 0.023$). However, these differences disappeared in the second light ($F(3,14) = 1.602$, $p = 0.234$) and dark ($F(3,16) = 3.223$, $p = 0.061$) periods.

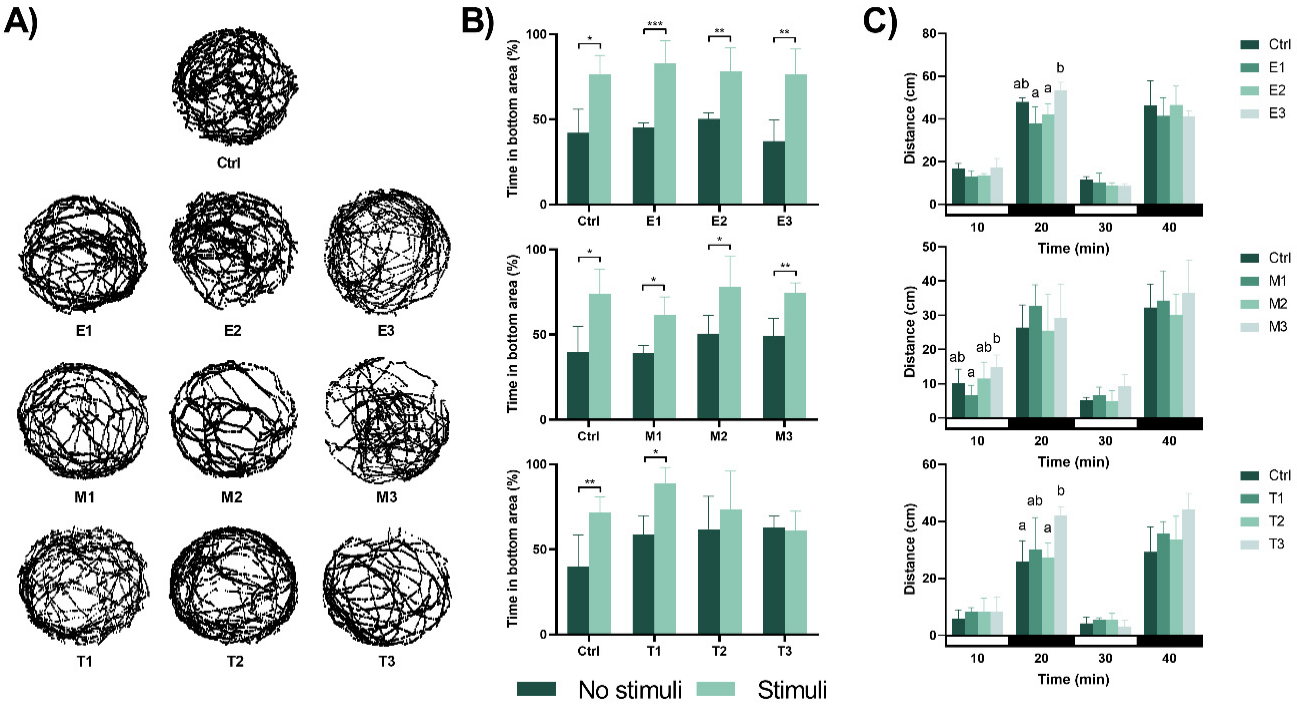

Figure 4. Effects of the different concentrations of the phyto-fungicide on zebrafish eleutheroembryo motor behaviour. (**A**) Representative swimming tracks of untreated and treated zebrafish eleutheroembryo at 120 hpf. No significant changes were observed between the different treatments and the control group during the 10 min recording period. (**B**) Avoidance behaviour of the zebrafish eleutheroembryo in the presence of an aversive stimulus. Data is expressed as mean ± SD from five independent replicates (5 eleutheroembryo assayed for each treatment). Statistical analysis was performed using *t*-test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (**C**) Distance moved during the visual motor response test. Data represent the mean distance moved during 10 min assay and is expressed as mean ± SD from five independent replicates (5 eleutheroembryo assayed for each treatment). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

4. Discussion

The worldwide occurrence of synthetic fungicides in aquatic environments has led to the investigation of different plant-based extracts or compounds as safer alternatives with low toxicity. However, adverse or toxic side effects have been reported in higher vertebrates [14–16,18]. Furthermore, there is a lack of ecotoxicological information about these products, which can have environmental implications. In this study, the toxicological effects of commercially available herbal products containing *E. arvense*, *M. tenuiflora*, and Thymol were tested using zebrafish embryos. The results showed the safety of *E. arvense* and *M. tenuiflora* with no lethality, and only a slight increase of AChE activity was observed following exposure to the lowest concentration of *E. arvense*, which may have no biological significance as no other association with both biochemical and behavioural markers could be made despite the IBRv2 index suggesting higher stress levels in this concentration. On the other hand, after exposure to Thymol, mortality and malformed development were observed. In addition, changes in the glutathione ratio and the disruption of behavioural responses were perceived, although no changes in the IBRv2 index were depicted.

Results from the current study highlighted a higher toxicity of Thymol to zebrafish in early life stages in comparison to the remaining test compounds. While the calculated LC_{50} values are in line with those observed for both synthetic [35,38] and other natural compounds in this species (reviewed in [39]), no studies have been found in the literature regarding the embryo toxicological effects of *E. arvense* and *M. tenuiflora* in aquatic species, although a previous study has shown a higher EC_{50} for *E. arvense* extract in another aquatic model (*Daphnia* sp., 50–100 mg L^{-1} [40]). Additionally, a previous study has shown that Thymol exhibits a lower toxicity (3× higher LC_{50} of 42.35 μM~6.36 mg L^{-1}) [19] in comparison to the current study, which could be explained by different species' sensitivities among different laboratories. Notwithstanding, craniofacial and skeletal deformities were similarly observed at higher concentrations (50 μM~7.5 mg L^{-1} [19] and 40 mg L^{-1} [20]), further supporting the teratogenic potential of Thymol to zebrafish embryos, even at lower concentrations. During embryogenesis, cells acquire distinct functions and specific positions, giving rise to a functional, complex, and multicellular organism through a set of early molecular and cellular mechanisms [41]. The modulation of these signalling pathways is required for the early patterning decisions, and previous studies have shown that changes in these signalling pathways result in defective development [42,43]. Although information is limited, Thymol is known to play multiple modulatory roles. For instance, it has been shown to down-regulate PI3K/Akt and ERK pathways [44], which are the key mechanisms involved in cell growth, proliferation, differentiation and survival [45,46]. However, the complex interplay between these pathways, the knockdown of PI3K/Akt signalling genes have been associated with embryonic lethality (reviewed and summarised by [47]), which may justify the observed effects. In addition, the negative regulation of PI3K/Akt signalling by the overexpression of its inhibitor (PTEN) has been shown to impair cell movements during gastrulation, resulting in developmental defects, including heart oedema, small or missing eyes and short tail [48], as observed after Thymol exposure. Overall, these studies demonstrate that the PI3K/Akt signalling pathway may play an integral role in the teratogenicity of Thymol, although the underlying mechanism remains to be defined, requiring further insights.

Notwithstanding, previous studies have shown that the inhibition of PI3K/Akt signalling hinders the activation of Nrf2 [49], the master regulator of the anti-oxidative response. Changes in the Nrf2-mediated antioxidant response have been previously described in Thymol-induced malformations of zebrafish embryos [19]. This is a crucial antioxidant signalling molecule for developmental processes [50], and deficiencies in its levels have been shown to induce embryonic lethality and severe oxidative stress in mice [51]. Collectively, data gathered from previous studies point to the Nrf2-antioxidant signalling pathway, and its activation by oxidative stress plays a pivotal role in the teratogenesis of Thymol. Oxidative stress results from an imbalance between the production and accumulation of oxygen reactive species (ROS), which can impair embryonic develop-

ment [52]. Among the different oxidative stress-related parameters, glutathione-associated assays are often the primary choice [53], with the calculation of the redox status (GSH:GSSG ratio) being traditionally reported as a biomarker of oxidative stress [53]. Although the dynamics of glutathione during early development are yet to be understood, changes in its levels are associated with developmental effects [54]. Yet, in the current study, no changes were observed for GSH and GSSG levels following exposure to Thymol. However, a significant increase in the GSH:GSSG ratio was observed. The interplay between glutathione expression and changes in the redox state is important for the correct development of the organism [55]. A higher GSH:GSSG ratio has been described to occur in situations in which the redox environment is more reducing, preventing oxidative modifications [56] and being associated with cell proliferation [54]. The proper coordination of cell proliferation is critical for the correct embryogenesis [57], and Nrf2 has been considered to control proliferation and differentiation by maintaining the redox state [58]. In addition, although not observed in this study, Thymol has been shown to increase GSH levels [59,60], which can affect cell proliferation in different ways, such as the regulation of c-Jun N-terminal kinase (JNK) and P38- mitogen-activated protein kinase (MAPK) pathways [61], modulation of cellular redox environment [62] and/or by affecting cytokine levels [63]. However, the relation between these effects and the modifications that may originate from the observed zebrafish eleutheroembryo malformations is not clear, and further research on this topic will be needed.

Nevertheless, glutathione is also involved in the cellular detoxification system, as it is used to conjugate a wide variety of exogenous compounds. In this context, phase II conjugation often involves glutathione-s-transferase (GST)-catalysed conjugation of GSH [64]. In the current study, GST activity increased following exposure to the highest concentration of Thymol. Although not described in aquatic species, Thymol has been shown to elevate GST activity in other non-target species [65–68], associated with a response to increased oxidative damage caused by reactive species. Yet, the increase in oxidative damage was not observed in the current study following Thymol exposure, as seen by the different oxidative-related parameters. Thus, other mechanisms might be involved. In view of this, GSTs are also implicated as modulators of cell proliferation and cell death by controlling the activity of members of the MAPK pathways, particularly by inhibiting JNK [69]. The inhibition of JNK has been shown to cause embryonic growth retardation, malformations and death of zebrafish [70,71], as observed in the current study. In accordance with this, Thymol has been suggested to modulate the in vitro expression of JNK [72,73] in a concentration dependent manner. Therefore, and although no in vivo information could be found in the literature, further studies are needed to elucidate the molecular mechanism involved in Thymol teratogenic effects.

Thymol exposure resulted in the disruption of behavioural responses in zebrafish eleutheroembryo, as observed by the lack of response to a threatening moving object and the increased distance moved in the dark period, which are associated with fear- and anxiety-like behaviours, respectively [74]. These emotional responses involve profound changes and specified activity patterns in the zebrafish brain [75]. Although a recent study has shown Thymol to improve the cholinergic nervous system and antioxidative stress in a cognitive dysfunction model [21], no supporting behavioural information could be found for zebrafish embryo. Yet, Thymol has been shown to affect the behaviour (depression- and antidepressant-like) of mice [76,77]. The modulation of emotional states in zebrafish are controlled by the habenula [78], an evolutionarily conserved structure of the vertebrate brain. The disruption of this structure was found to increase fear [79] and contribute to anxiety disorders [80], as observed following exposure to Thymol. The correct function and development of habenular circuits in zebrafish has a strong association with the correct embryo neurogenesis [81]. This is dependent upon complex intrinsic and extrinsic signalling factors interactions [82], with neurogenesis impairment being associated with various brain disorders. In accordance, altered neurogenesis has been previously described by changes in JNK signalling [83,84], and different behavioural phenotypes have been

described in JNK-knockdown animal models [85]. Additionally, JNK has been shown as a dominant controller of behavioural moods [86]. Therefore, understanding the specific function of this signalling pathways in the Thymol-induced teratogenic effects will provide potentially important insights into the molecular mechanisms underlying the observed teratogenic effects.

5. Conclusions

In conclusion, the present study demonstrates the safety profile of both *E. arvense* and *M. tenuiflora* at sublethal concentrations during the early development of zebrafish. Yet, the data obtained further support the teratogenic potential of Thymol during early developmental stages as shown by the increased lethality and malformations. In addition, oxidative changes were observed, suggesting a change in the oxidative environment, which may be associated with effects at the proliferation level. While further studies are required to validate this hypothesis, the disruption of behavioural states further suggests alterations on the early embryonic signalling patterns. Taken together, the results obtained improve the risk assessment of these compounds, raising questions about the potential non-safe use of Thymol, which might have direct ecotoxicological consequences in non-target species.

Supplementary Materials: The following are available online at https://www.mdpi.com/2305-6304/9/1/8/s1, Table S1: Sublethal effects of the different exposures on the development of zebrafish embryos and eleutheroembryo, Table S2: Biochemical parameters evaluated at 98 hpf in zebrafish embryos exposed to *Equisetum arvense* extract, Table S3: Biochemical parameters evaluated at 98 hpf in zebrafish embryos exposed to *Mimosa tenuiflora* extract, Table S4: Biochemical parameters evaluated at 98 hpf in zebrafish embryos exposed to Thymol, Table S5 Exploratory behaviour of 120 hpf eleutheroembryo exposed to *Equisetum arvense* extract, Table S6: Exploratory behaviour of 120 hpf eleutheroembryo exposed to *Mimosa tenuiflora* extract, Table S7: Exploratory behaviour of 120 hpf eleutheroembryo exposed to Thymol.

Author Contributions: Conceptualization, C.V. and L.F.; methodology, C.V. and L.F.; investigation, R.V.; resources, C.V. and L.F.; data curation, R.V.; writing—original draft preparation, R.V.; writing—review and editing, C.V. and L.F.; supervision, C.V. and L.F.; funding acquisition, C.V. and L.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by European Investment Funds by FEDER/COMPETE/POCI–Operational Competitiveness and Internationalization Programme, under the project POCI-01–0145-FEDER-006958 and National Funds by FCT—"Fundação para a Ciência e a Tecnologia", under the project UIBD/04033/2020 and under the PhD grant number SFRH/BD/144904/2019.

Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the use of early-life stages of zebrafish which do not fall within the regulations of the European Directive 2010/63/EU on the protection of animals in research.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analysed during this study are included in this published article (and its supplementary information files).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Jeschke, P. Progress of modern agricultural chemistry and future prospects. *Pest Manag. Sci.* **2016**, *72*, 433–455. [CrossRef]
2. Carvalho, F.P. Pesticides, environment, and food safety. *Food Energy Secur.* **2017**, *6*, 48–60. [CrossRef]
3. Gonzalez-Rey, M.; Tapie, N.; Le Menach, K.; Devier, M.H.; Budzinski, H.; Bebianno, M.J. Occurrence of pharmaceutical compounds and pesticides in aquatic systems. *Mar. Pollut. Bull.* **2015**, *96*, 384–400. [CrossRef] [PubMed]
4. Zubrod, J.P.; Bundschuh, M.; Arts, G.; Bruhl, C.A.; Imfeld, G.; Knabel, A.; Payraudeau, S.; Rasmussen, J.J.; Rohr, J.; Scharmuller, A.; et al. Fungicides: An overlooked pesticide class? *Environ. Sci. Technol.* **2019**, *53*, 3347–3365. [CrossRef] [PubMed]
5. Li, Z.; Jennings, A. Worldwide regulations of standard values of pesticides for human health risk control: A review. *Int. J. Environ. Res. Public Health* **2017**, *14*, 826. [CrossRef] [PubMed]

6. Abbey, J.A.; Percival, D.; Abbey, L.; Asiedu, S.K.; Prithiviraj, B.; Schilder, A. Biofungicides as alternative to synthetic fungicide control of grey mould (*Botrytis cinerea*)—Prospects and challenges. *Biocontrol Sci. Technol.* **2019**, *29*, 241–262. [CrossRef]
7. Lorsbach, B.A.; Sparks, T.C.; Cicchillo, R.M.; Garizi, N.V.; Hahn, D.R.; Meyer, K.G. Natural products: A strategic lead generation approach in crop protection discovery. *Pest Manag. Sci.* **2019**, *75*, 2301–2309. [CrossRef] [PubMed]
8. Palou, L.; Ali, A.; Fallik, E.; Romanazzi, G. GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. *Postharvest Biol. Technol.* **2016**, *122*, 41–52. [CrossRef]
9. Marrone, P.G. Pesticidal natural products—Status and future potential. *Pest Manag. Sci.* **2019**, *75*, 2325–2340. [CrossRef]
10. Pavela, R.; Benelli, G. Essential oils as ecofriendly biopesticides? Challenges and constraints. *Trends Plant Sci.* **2016**, *21*, 1000–1007. [CrossRef]
11. Damalas, C.A.; Koutroubas, S.D. Botanical pesticides for eco-friendly pest management: Drawbacks and limitations. In *Pesticides in Crop Production: Physiological and Biochemical Action*; Wiley: Chichester, UK, 2020; pp. 181–193.
12. Vagner, M.; Zambonino-Infante, J.L.; Mazurais, D. Fish facing global change: Are early stages the lifeline? *Mar. Environ. Res.* **2019**, *147*, 159–178. [CrossRef] [PubMed]
13. Marchand, P.A. Basic substances under EU pesticide regulation: An opportunity for organic production? *Org. Farming* **2017**, *3*, 16–19. [CrossRef]
14. Ortega Garcia, J.A.; Angulo, M.G.; Sobrino-Najul, E.J.; Soldin, O.P.; Mira, A.P.; Martinez-Salcedo, E.; Claudio, L. Prenatal exposure of a girl with autism spectrum disorder to 'horsetail' (*Equisetum arvense*) herbal remedy and alcohol: A case report. *J. Med. Case Rep.* **2011**, *5*, 129. [CrossRef] [PubMed]
15. Medeiros, R.M.; de Figueiredo, A.P.; Benicio, T.M.; Dantas, F.P.; Riet-Correa, F. Teratogenicity of *Mimosa tenuiflora* seeds to pregnant rats. *Toxicon* **2008**, *51*, 316–319. [CrossRef]
16. Pimentel, L.A.; Correa, F.R.; Gardner, D.; Panter, K.E.; Dantas, A.F.; Medeiros, R.M.; Mota, R.A.; Araujo, J.A. *Mimosa tenuiflora* as a cause of malformations in ruminants in the northeastern Brazilian semiarid rangelands. *Vet. Pathol.* **2007**, *44*, 928–931. [CrossRef]
17. Marchese, A.; Orhan, I.E.; Daglia, M.; Barbieri, R.; Di Lorenzo, A.; Nabavi, S.F.; Gortzi, O.; Izadi, M.; Nabavi, S.M. Antibacterial and antifungal activities of thymol: A brief review of the literature. *Food Chem.* **2016**, *210*, 402–414. [CrossRef]
18. Verrett, M.J.; Scott, W.F.; Reynaldo, E.F.; Alterman, E.K.; Thomas, C.A. Toxicity and teratogenicity of food additive chemicals in the developing chicken embryo. *Toxicol. Appl. Pharmacol.* **1980**, *56*, 265–273. [CrossRef]
19. Krishnan, M.; Kim, D.K.; Gie Kim, S.; Kang, S.C. Thymol exposure mediates pro-oxidant shift by regulating Nrf2 and apoptotic events in zebrafish (*Danio rerio*) embryos. *Environ. Toxicol. Pharmacol.* **2019**, *65*, 1–8. [CrossRef]
20. He, T.; Li, X.; Wang, X.; Xu, X.; Yan, X.; Li, X.; Sun, S.; Dong, Y.; Ren, X.; Liu, X.; et al. Chemical composition and anti-oxidant potential on essential oils of *Thymus quinquecostatus* Celak. from Loess Plateau in China, regulating Nrf2/Keap1 signaling pathway in zebrafish. *Sci. Rep.* **2020**, *10*, 11280. [CrossRef]
21. Capatina, L.; Todirascu-Ciornea, E.; Napoli, E.M.; Ruberto, G.; Hritcu, L.; Dumitru, G. *Thymus vulgaris* essential oil protects zebrafish against cognitive dysfunction by regulating cholinergic and antioxidants systems. *Antioxidants* **2020**, *9*, 1083. [CrossRef]
22. Garcia, D.; Ramos, A.J.; Sanchis, V.; Marín, S. *Equisetum arvense* hydro-alcoholic extract: Phenolic composition and antifungal and antimycotoxigenic effect against *Aspergillus flavus* and *Fusarium verticillioides* in stored maize. *J. Sci. Food Agric.* **2013**, *93*, 2248–2253. [CrossRef] [PubMed]
23. Llorens, E.; Mateu, M.; González-Hernández, A.I.; Agustí-Brisach, C.; García-Agustín, P.; Lapeña, L.; Vicedo, B. Extract of *Mimosa tenuiflora* and *Quercus robur* as potential eco-friendly management tool against Sclerotinia sclerotiorum in *Lactuca sativa* enhancing the natural plant defences. *Eur. J. Plant Pathol.* **2019**, *153*, 1105–1118. [CrossRef]
24. Hinton, D.E.; Kullman, S.W.; Hardman, R.C.; Volz, D.C.; Chen, P.J.; Carney, M.; Bencic, D.C. Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? *Mar. Pollut. Bull.* **2005**, *51*, 635–648. [CrossRef] [PubMed]
25. Bambino, K.; Chu, J. Zebrafish in toxicology and environmental health. *Curr. Top. Dev. Biol.* **2017**, *124*, 331–367. [CrossRef]
26. Magyary, I. Recent advances and future trends in zebrafish bioassays for aquatic ecotoxicology. *Ecocycles* **2018**, *4*, 12–18. [CrossRef]
27. Scholz, S.; Fischer, S.; Gundel, U.; Kuster, E.; Luckenbach, T.; Voelker, D. The zebrafish embryo model in environmental risk assessment—Applications beyond acute toxicity testing. *Environ. Sci. Pollut. Res. Int.* **2008**, *15*, 394–404. [CrossRef]
28. Bugel, S.M.; Tanguay, R.L.; Planchart, A. Zebrafish: A marvel of high-throughput biology for 21(st) century toxicology. *Curr. Environ. Health Rep.* **2014**, *1*, 341–352. [CrossRef]
29. Domingues, I.; Oliveira, R.; Lourenco, J.; Grisolia, C.K.; Mendo, S.; Soares, A.M. Biomarkers as a tool to assess effects of chromium (VI): Comparison of responses in zebrafish early life stages and adults. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2010**, *152*, 338–345. [CrossRef]
30. Sisman, T. Early life stage and genetic toxicity of stannous chloride on zebrafish embryos and adults: Toxic effects of tin on zebrafish. *Environ. Toxicol.* **2011**, *26*, 240–249. [CrossRef]
31. Knobel, M.; Busser, F.J.; Rico-Rico, A.; Kramer, N.I.; Hermens, J.L.; Hafner, C.; Tanneberger, K.; Schirmer, K.; Scholz, S. Predicting adult fish acute lethality with the zebrafish embryo: Relevance of test duration, endpoints, compound properties, and exposure concentration analysis. *Environ. Sci. Technol.* **2012**, *46*, 9690–9700. [CrossRef]
32. OECD Guidelines. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. In *OECD Guidelines for the Testing of Chemicals, Section 2*; OECD: Paris, France, 2013; pp. 1–22. [CrossRef]

33. Felix, L.M.; Serafim, C.; Valentim, A.M.; Antunes, L.M.; Campos, S.; Matos, M.; Coimbra, A.M. Embryonic stage-dependent teratogenicity of ketamine in zebrafish (*Danio rerio*). *Chem. Res. Toxicol.* **2016**, *29*, 1298–1309. [CrossRef] [PubMed]
34. Felix, L.M.; Serafim, C.; Martins, M.J.; Valentim, A.M.; Antunes, L.M.; Matos, M.; Coimbra, A.M. Morphological and behavioral responses of zebrafish after 24 h of ketamine embryonic exposure. *Toxicol. Appl. Pharmacol.* **2017**, *321*, 27–36. [CrossRef] [PubMed]
35. Vieira, R.; Venancio, C.A.S.; Felix, L.M. Toxic effects of a mancozeb-containing commercial formulation at environmental relevant concentrations on zebrafish embryonic development. *Environ. Sci. Pollut. Res. Int.* **2020**, *27*, 21174–21187. [CrossRef] [PubMed]
36. Sanchez, W.; Burgeot, T.; Porcher, J.M. A novel "Integrated Biomarker Response" calculation based on reference deviation concept. *Environ. Sci. Pollut. Res. Int.* **2013**, *20*, 2721–2725. [CrossRef] [PubMed]
37. Felix, L.M.; Antunes, L.M.; Coimbra, A.M.; Valentim, A.M. Behavioral alterations of zebrafish larvae after early embryonic exposure to ketamine. *Psychopharmacology* **2017**, *234*, 549–558. [CrossRef]
38. Andrade, T.S.; Henriques, J.F.; Almeida, A.R.; Machado, A.L.; Koba, O.; Giang, P.T.; Soares, A.; Domingues, I. Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos. *Aquat. Toxicol.* **2016**, *170*, 390–399. [CrossRef]
39. Chahardehi, A.M.; Arsad, H.; Lim, V. Zebrafish as a successful animal model for screening toxicity of medicinal plants. *Plants* **2020**, *9*, 1345. [CrossRef]
40. Andreu, V.; Levert, A.; Amiot, A.; Cousin, A.; Aveline, N.; Bertrand, C. Chemical composition and antifungal activity of plant extracts traditionally used in organic and biodynamic farming. *Environ. Sci. Pollut. Res. Int.* **2018**, *25*, 29971–29982. [CrossRef]
41. Schier, A.F. Axis formation and patterning in zebrafish. *Curr. Opin. Genet. Dev.* **2001**, *11*, 393–404. [CrossRef]
42. Salazar, V.S.; Gamer, L.W.; Rosen, V. BMP signalling in skeletal development, disease and repair. *Nat. Rev. Endocrinol.* **2016**, *12*, 203–221. [CrossRef]
43. Reynolds, K.; Kumari, P.; Sepulveda Rincon, L.; Gu, R.; Ji, Y.; Kumar, S.; Zhou, C.J. Wnt signaling in orofacial clefts: Crosstalk, pathogenesis and models. *Dis. Model. Mech.* **2019**, *12*, dmm037051. [CrossRef] [PubMed]
44. Lv, R.; Chen, Z.Z. Thymol inhibits cell migration and invasion by downregulating the activation of PI3K/AKT and ERK pathways in human colon cancer cells. *Trop. J. Pharm. Res.* **2017**, *16*, 2895–2901. [CrossRef]
45. Akieda, Y.; Ogamino, S.; Furuie, H.; Ishitani, S.; Akiyoshi, R.; Nogami, J.; Masuda, T.; Shimizu, N.; Ohkawa, Y.; Ishitani, T. Cell competition corrects noisy Wnt morphogen gradients to achieve robust patterning in the zebrafish embryo. *Nat. Commun.* **2019**, *10*, 4710. [CrossRef] [PubMed]
46. Hikasa, H.; Sokol, S.Y. Wnt signaling in vertebrate axis specification. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a007955. [CrossRef]
47. Yu, J.S.; Cui, W. Proliferation, survival and metabolism: The role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development* **2016**, *143*, 3050–3060. [CrossRef]
48. Finkielsztein, A.; Kelly, G.M. Altering PI3K-Akt signalling in zebrafish embryos affects PTEN phosphorylation and gastrulation. *Biol. Cell* **2009**, *101*, 661–678. [CrossRef]
49. Koundouros, N.; Poulogiannis, G. Phosphoinositide 3-Kinase/Akt signaling and redox metabolism in cancer. *Front. Oncol.* **2018**, *8*, 160. [CrossRef]
50. Hahn, M.E.; Timme-Laragy, A.R.; Karchner, S.I.; Stegeman, J.J. Nrf2 and Nrf2-related proteins in development and developmental toxicity: Insights from studies in zebrafish (*Danio rerio*). *Free Radic. Biol. Med.* **2015**, *88*, 275–289. [CrossRef]
51. Leung, L.; Kwong, M.; Hou, S.; Lee, C.; Chan, J.Y. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. *J. Biol. Chem.* **2003**, *278*, 48021–48029. [CrossRef]
52. Dennery, P.A. Oxidative stress in development: Nature or nurture? *Free Radic. Biol. Med.* **2010**, *49*, 1147–1151. [CrossRef]
53. Massarsky, A.; Kozal, J.S.; Di Giulio, R.T. Glutathione and zebrafish: Old assays to address a current issue. *Chemosphere* **2017**, *168*, 707–715. [CrossRef] [PubMed]
54. Timme-Laragy, A.R.; Goldstone, J.V.; Imhoff, B.R.; Stegeman, J.J.; Hahn, M.E.; Hansen, J.M. Glutathione redox dynamics and expression of glutathione-related genes in the developing embryo. *Free Radic. Biol. Med.* **2013**, *65*, 89–101. [CrossRef] [PubMed]
55. Tierbach, A.; Groh, K.J.; Schonenberger, R.; Schirmer, K.; Suter, M.J. Glutathione S-Transferase protein expression in different life stages of zebrafish (*Danio rerio*). *Toxicol. Sci.* **2018**, *162*, 702–712. [CrossRef] [PubMed]
56. Schafer, F.Q.; Buettner, G.R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **2001**, *30*, 1191–1212. [CrossRef]
57. Liu, Y.; Sepich, D.S.; Solnica-Krezel, L. Stat3/Cdc25a-dependent cell proliferation promotes embryonic axis extension during zebrafish gastrulation. *PLoS Genet.* **2017**, *13*, e1006564. [CrossRef]
58. Murakami, S.; Motohashi, H. Roles of Nrf2 in cell proliferation and differentiation. *Free Radic. Biol. Med.* **2015**, *88*, 168–178. [CrossRef]
59. Ozkan, A.; Erdogan, A. A comparative study of the antioxidant/prooxidant effects of carvacrol and thymol at various concentrations on membrane and DNA of parental and drug resistant H1299 cells. *Nat. Prod. Commun.* **2012**, *7*, 1557–1560. [CrossRef]
60. Guvenc, M.; Cellat, M.; Gokcek, I.; Yavas, I.; Yurdagul Ozsoy, S. Effects of thymol and carvacrol on sperm quality and oxidant/antioxidant balance in rats. *Arch. Physiol. Biochem.* **2019**, *125*, 396–403. [CrossRef]
61. Kandil, S.; Brennan, L.; McBean, G.J. Glutathione depletion causes a JNK and p38MAPK-mediated increase in expression of cystathionine-gamma-lyase and upregulation of the transsulfuration pathway in C6 glioma cells. *Neurochem. Int.* **2010**, *56*, 611–619. [CrossRef]

62. Day, R.M.; Suzuki, Y.J. Cell proliferation, reactive oxygen and cellular glutathione. *Dose Response* **2006**, *3*, 425–442. [CrossRef]
63. Obrador, E.; Navarro, J.; Mompo, J.; Asensi, M.; Pellicer, J.A.; Estrela, J.M. Glutathione and the rate of cellular proliferation determine tumour cell sensitivity to tumour necrosis factor in vivo. *Biochem. J.* **1997**, *325 Pt 1*, 183–189. [CrossRef]
64. Glisic, B.; Mihaljevic, I.; Popovic, M.; Zaja, R.; Loncar, J.; Fent, K.; Kovacevic, R.; Smital, T. Characterization of glutathione-S-transferases in zebrafish (*Danio rerio*). *Aquat. Toxicol.* **2015**, *158*, 50–62. [CrossRef] [PubMed]
65. Glavan, G.; Novak, S.; Bozic, J.; Jemec Kokalj, A. Comparison of sublethal effects of natural acaricides carvacrol and thymol on honeybees. *Pestic. Biochem. Physiol.* **2020**, *166*, 104567. [CrossRef] [PubMed]
66. Sasaki, K.; Wada, K.; Tanaka, Y.; Yoshimura, T.; Matuoka, K.; Anno, T. Thyme (*Thymus vulgaris* L.) leaves and its constituents increase the activities of xenobiotic-metabolizing enzymes in mouse liver. *J. Med. Food* **2005**, *8*, 184–189. [CrossRef] [PubMed]
67. Kumrungsee, N.; Pluempanupat, W.; Koul, O.; Bullangpoti, V. Toxicity of essential oil compounds against diamondback moth, *Plutella xylostella*, and their impact on detoxification enzyme activities. *J. Pest Sci.* **2014**, *87*, 721–729. [CrossRef]
68. Shahriari, M.; Zibaee, A.; Sahebzadeh, N.; Shamakhi, L. Effects of alpha-pinene, trans-anethole, and thymol as the essential oil constituents on antioxidant system and acetylcholine esterase of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *Pestic. Biochem. Physiol.* **2018**, *150*, 40–47. [CrossRef] [PubMed]
69. Laborde, E. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. *Cell Death Differ.* **2010**, *17*, 1373–1380. [CrossRef] [PubMed]
70. Xiao, Y.; Zhou, Y.; Xiong, Z.; Zou, L.; Jiang, M.; Luo, Z.; Wen, S.; Liu, W.; Liu, S.; Li, W. Involvement of JNK in the embryonic development and organogenesis in zebrafish. *Mar. Biotechnol. (NY)* **2013**, *15*, 716–725. [CrossRef]
71. Valesio, E.G.; Zhang, H.; Zhang, C. Exposure to the JNK inhibitor SP600125 (anthrapyrazolone) during early zebrafish development results in morphological defects. *J. Appl. Toxicol.* **2013**, *33*, 32–40. [CrossRef]
72. Liang, D.; Li, F.; Fu, Y.; Cao, Y.; Song, X.; Wang, T.; Wang, W.; Guo, M.; Zhou, E.; Li, D.; et al. Thymol inhibits LPS-stimulated inflammatory response via down-regulation of NF-kappaB and MAPK signaling pathways in mouse mammary epithelial cells. *Inflammation* **2014**, *37*, 214–222. [CrossRef]
73. Gholijani, N.; Gharagozloo, M.; Farjadian, S.; Amirghofran, Z. Modulatory effects of thymol and carvacrol on inflammatory transcription factors in lipopolysaccharide-treated macrophages. *J. Immunotoxicol.* **2016**, *13*, 157–164. [CrossRef] [PubMed]
74. Colwill, R.M.; Creton, R. Imaging escape and avoidance behavior in zebrafish larvae. *Rev. Neurosci.* **2011**, *22*, 63–73. [CrossRef] [PubMed]
75. Jesuthasan, S. Fear, anxiety, and control in the zebrafish. *Dev. Neurobiol.* **2012**, *72*, 395–403. [CrossRef] [PubMed]
76. Capibaribe, V.C.C.; Vasconcelos Mallmann, A.S.; Lopes, I.S.; Oliveira, I.C.M.; de Oliveira, N.F.; Chaves, R.C.; Fernandes, M.L.; de Araujo, M.A.; da Silva, D.M.A.; Valentim, J.T.; et al. Thymol reverses depression-like behaviour and upregulates hippocampal BDNF levels in chronic corticosterone-induced depression model in female mice. *J. Pharm. Pharmacol.* **2019**, *71*, 1774–1783. [CrossRef]
77. Deng, X.Y.; Li, H.Y.; Chen, J.J.; Li, R.P.; Qu, R.; Fu, Q.; Ma, S.P. Thymol produces an antidepressant-like effect in a chronic unpredictable mild stress model of depression in mice. *Behav. Brain Res.* **2015**, *291*, 12–19. [CrossRef]
78. Okamoto, H.; Agetsuma, M.; Aizawa, H. Genetic dissection of the zebrafish habenula, a possible switching board for selection of behavioral strategy to cope with fear and anxiety. *Dev. Neurobiol.* **2012**, *72*, 386–394. [CrossRef]
79. Agetsuma, M.; Aizawa, H.; Aoki, T.; Nakayama, R.; Takahoko, M.; Goto, M.; Sassa, T.; Amo, R.; Shiraki, T.; Kawakami, K.; et al. The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nat. Neurosci.* **2010**, *13*, 1354–1356. [CrossRef]
80. Lee, A.; Mathuru, A.S.; Teh, C.; Kibat, C.; Korzh, V.; Penney, T.B.; Jesuthasan, S. The habenula prevents helpless behavior in larval zebrafish. *Curr. Biol.* **2010**, *20*, 2211–2216. [CrossRef]
81. Fore, S.; Acuña-Hinrichsen, F.; Mutlu, K.A.; Bartoszek, E.M.; Serneels, B.; Faturos, N.G.; Chau, K.T.P.; Cosacak, M.I.; Verdugo, C.D.; Palumbo, F.; et al. Functional properties of habenular neurons are determined by developmental stage and sequential neurogenesis. *Sci. Adv.* **2020**, *6*, eaaz3173. [CrossRef]
82. Schmidt, R.; Strahle, U.; Scholpp, S. Neurogenesis in zebrafish—From embryo to adult. *Neural Dev.* **2013**, *8*, 3. [CrossRef]
83. Amura, C.R.; Marek, L.; Winn, R.A.; Heasley, L.E. Inhibited neurogenesis in JNK1-deficient embryonic stem cells. *Mol. Cell. Biol.* **2005**, *25*, 10791–10802. [CrossRef] [PubMed]
84. Castro-Torres, R.D.; Landa, J.; Rabaza, M.; Busquets, O.; Olloquequi, J.; Ettcheto, M.; Beas-Zarate, C.; Folch, J.; Camins, A.; Auladell, C.; et al. JNK Isoforms Are Involved in the Control of Adult Hippocampal Neurogenesis in Mice, Both in Physiological Conditions and in an Experimental Model of Temporal Lobe Epilepsy. *Mol. Neurobiol.* **2019**, *56*, 5856–5865. [CrossRef] [PubMed]
85. Coffey, E.T. Nuclear and cytosolic JNK signalling in neurons. *Nat. Rev. Neurosci.* **2014**, *15*, 285–299. [CrossRef] [PubMed]
86. Hollos, P.; Marchisella, F.; Coffey, E.T. JNK Regulation of depression and anxiety. *Brain Plast.* **2018**, *3*, 145–155. [CrossRef] [PubMed]

toxics

MDPI

Article

Assessing Combined Effects for Mixtures of Similar and Dissimilar Acting Neuroactive Substances on Zebrafish Embryo Movement

Afolarin O. Ogungbemi [1,2,*], Riccardo Massei [3], Rolf Altenburger [1], Stefan Scholz [1] and Eberhard Küster [1]

1 Department of Bioanalytical Ecotoxicology, UFZ—Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany; rolf.altenburger@ufz.de (R.A.); stefan.scholz@ufz.de (S.S.); eberhard.kuester@ufz.de (E.K.)
2 Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany
3 Department of Effect-Directed Analysis, UFZ—Helmholtz Centre for Environmental Research, Permoserstraße 15, 04318 Leipzig, Germany; riccardo.massei@ufz.de
* Correspondence: afolarin.ogungbemi@ufz.de

Abstract: Risk assessment of chemicals is usually conducted for individual chemicals whereas mixtures of chemicals occur in the environment. Considering that neuroactive chemicals are a group of contaminants that dominate the environment, it is then imperative to understand the combined effects of mixtures. The commonly used models to predict mixture effects, namely concentration addition (CA) and independent action (IA), are thought to be suitable for mixtures of similarly or dissimilarly acting components, respectively. For mixture toxicity prediction, one important challenge is to clarify whether to group neuroactive substances based on similar mechanisms of action, e.g., same molecular target or rather similar toxicological response, e.g., hyper- or hypoactivity (effect direction). We addressed this by using the spontaneous tail coiling (STC) of zebrafish embryos, which represents the earliest observable motor activity in the developing neural network, as a model to elucidate the link between the mechanism of action and toxicological response. Our objective was to answer the following two questions: (1) Can the mixture models CA or IA be used to predict combined effects for neuroactive chemical mixtures when the components share a similar mode of action (i.e., hyper- or hypoactivity) but show different mechanism of action? (2) Will a mixture of chemicals where the components show opposing effect directions result in an antagonistic combined effect? Results indicate that mixture toxicity of chemicals such as propafenone and abamectin as well as chlorpyrifos and hexaconazole that are known to show different mechanisms of action but similar effect directions were predictable using CA and IA models. This could be interpreted with the convergence of effects on the neural level leading to either a collective activation or inhibition of synapses. We also found antagonistic effects for mixtures containing substances with opposing effect direction. Finally, we discuss how the STC may be used to amend risk assessment.

Keywords: mixture toxicity; neurotoxicity; antagonism; organophosphate; acetylcholinesterase inhibitors; GABA; behavior; risk assessment; spontaneous movement activity

Citation: Ogungbemi, A.O.; Massei, R.; Altenburger, R.; Scholz, S.; Küster, E. Assessing Combined Effects for Mixtures of Similar and Dissimilar Acting Neuroactive Substances on Zebrafish Embryo Movement. *Toxics* **2021**, *9*, 104. https://doi.org/10.3390/toxics9050104

Academic Editors: Demetrio Raldúa, Carlos Barata and Melissa Faria

Received: 29 March 2021
Accepted: 28 April 2021
Published: 6 May 2021

1. Introduction

Chemicals typically occur as mixtures in the environment and hence, organisms are exposed to a combination of these chemicals. However, prospective risk assessment is conducted for single chemicals and may not account for combined effects [1]. Since it is practically impossible to test all the possible combinations of chemical exposure, modeling of mixture toxicity allows one to at least predict an expected effect of several chemicals from their individual effects.

Two common mixture toxicity models are concentration addition (CA) and independent action (IA). CA is based on the notion that mixture toxicity can be predicted by the

addition of the fractions of exposure and effect concentrations for the mixture components. In addition, the single components of the mixture should cause a similar effect or target a similar receptor in the organism [2]. On the other hand, IA may be applied when compounds are acting independently [3] which has been interpreted as acting on different target sites in the organism [4]. Both models have been found to be reasonably predictive in several studies exposing unicellular organisms to bioactive compounds with known mechanisms of action [5–7]. Nevertheless, these models cannot predict the interaction of chemicals at the physical, toxicokinetic or toxicodynamic level [8]. In this case, CA and IA models may be used to evaluate observations as antagonistic (less effect than predicted) or synergistic (higher effect than predicted) and to quantify such deviations.

Neuroactive chemicals are often found in insecticidal and pharmaceutical products in which they represent active ingredients designed to interact with specific targets and receptors of the nervous system. Busch et al. [9] found that neuroactive substances are the largest group (13%) of chemicals detected in European surface waters. Despite neuroactive substances being often detected in the environment, only a few studies have explored how neuroactive substances act in mixtures to induce combined neurotoxicity (e.g., Corbel et al. [10]; Yang et al. [11]) and how to use the mode of action knowledge to group them for mixture effect prediction using CA and IA models.

Zebrafish embryos are considered as an alternative model to animal testing since they are considered to feel less pain or distress [12]. Due to behavioral patterns already established in embryonic stages, embryos are also frequently used as a model for neurotoxicity assessment. Several behavioral test methods have been developed such as spontaneous tail coiling (STC), photomotor response (PMR) and locomotor response (LMR) (reviewed in Ogungbemi et al. [13]). Despite the potential of non-lethal endpoints such as behavior for ecotoxicology research, the applicability of CA and IA models to such endpoints for mixture effect prediction is not well studied. Hence, it is valuable to investigate the applicability of CA and IA models for such experimental systems to predict and understand how mixtures of neuroactive substances may act in the environment. To implement mixture models, bioassays capable of quantitatively detecting impact on the nervous system are required. In this study we explored the spontaneous tail coiling (STC) of zebrafish embryos, one frequently used assay for assessing neuroactivity. STC represents the earliest motor activity observed in developing zebrafish embryos. It is the result of the innervation of the muscles by the primary motor neurons and can be observed beginning at 17 hours post-fertilization (hpf) [14,15]. Measurement of the STC frequency has been proposed as an indicator of adverse effects on the function and development of the nervous system which could lead to population and ecosystem effects [13,16]. Consequently, the STC has been used to study the effects of diverse neuroactive chemicals [17–20]. Until now the STC has not been used as a test method to measure mixture neurotoxicity based on a chemical's mode or mechanism of action. In this study, we define the mechanism of action as the interaction of neuroactive chemicals with specific molecular targets such as acetylcholinesterase (AChE) and gamma aminobutyric acid (GABA) activated ion channels. On the other hand, mode of action is defined here as the series of key events (including the mechanism of action) in the nervous system leading to a measurable toxicological response such as hyper- or hypoactivity behavior phenotypes (referred to as effect direction onwards). Hypoactivity refers to a decrease in the STC frequency, while hyperactivity refers to the increase with respect to the level in non-exposed embryos.

The STC test has been shown to discriminate movement activity changes due to exposure to chemicals with different modes of action causing either hyper- or hypoactivity but not those with different mechanisms of action [13,17]. Based on previous results in Ogungbemi et al. [13,17], we postulate the STC neuroactivity hypothesis which states that a neuroactive substance will induce increased STC (hyperactivity) in zebrafish embryos if its mechanism of action directly or indirectly leads to activation of the neuronal synapse and vice versa for hypoactivity. For example, different mechanisms of action such as AChE inhibition and GABA antagonism may both enhance neuronal activation potential in the

neuromuscular synapses by inducing the inflow of sodium ions and blocking the inflow of chloride ions respectively [21]. Both mechanisms are expected to cause hyperactivity response regardless of the different target receptors. Similarly, compounds activating GABA receptors or blocking sodium channels may cause hypoactivity by enhancing the inhibitory synapses [22].

Based on such prior knowledge about the link between the mechanism of action and toxicological response, we defined two levels of similarity for our mixture toxicity expectation: (1) The mixture components are known to have similar target receptors or mechanism of action and (2) they show similar toxicological response (i.e., effect direction: hyper- or hypoactivity) in the STC test. Therefore, we selected mixture components based on the above factors. Compounds expected to induce hyperactivity were chlorpyrifos, chlorpyrifos-oxon and hexaconazole while abamectin, carbamazepine and propafenone are anticipated to induce hypoactivity in the STC test.

The link between effect direction and mechanism of action has been shown for single substances. In contrast, it is still open if this also works for mixture components with similar or dissimilar mechanisms of action. Therefore, the goal of the present study is to address the following questions: (1) Can the additivity models CA or IA be used to predict combined effects for neuroactive chemical mixtures when the components share a similar mode of action (hyper- or hypoativity) but show different mechanism of action? (2) Will a mixture of chemicals where the components show opposing effect direction result in an antagonistic combined effect? CA or IA cannot be used to predict the opposing effects and therefore we define antagonistic effect in this case as a counteracting effect and not a lower effect than predicted by CA or IA. We demonstrate that mixtures of neuroactive substances with different mechanisms of action follow the additivity concept and we propose ways to use the STC test in risk assessment.

2. Materials and Methods

2.1. Test Organism

Zebrafish embryos were raised from an in-house hybrid strain (OBI-WIK strain, F3 generation). The adults were cultured under 14 h light/10 h dark photoperiod in 120 L aquaria (tap water, 26.5 $\pm$ 1 °C). Adult fish were fed twice a day either with commercial dry food flakes or *Artemia* sp. and physicochemical parameters of the aquaria water were frequently measured (pH 7–8; water hardness 2–3 mmol/L, conductivity 540–560 µS/cm, nitrate < 2.5 mg/L, nitrite < 0.025 mg/L, ammonia < 0.6 mg/L, oxygen saturation 87–91%). Spawning was initiated by inserting spawning trays 4–6 h before the end of the light cycle prior to the spawning day. Eggs were collected and cleaned 1 h after the onset of light. Fertilized embryos were selected according to Kimmel et al. [23] with a microscope and embryos between the 16th and 128th cell stage were used to start the exposure.

2.2. Chemicals

Chlorpyrifos (99.9%, CASRN 2921882), hexaconazole (CASRN 79983-71-4), abamectin (100%, CASRN 71751412) and propafenone-hydrochloride (CASRN 34183-22-7) were purchased from Sigma-Aldrich. Carbamazepine (99%, CASRN 298464) was purchased from Acros Organic^{TM} and chlorpyrifos-oxon (97.9%, CASRN 5598152) from Dr. Ehrenstorfer GmbH. Stock solutions were prepared in 100% dimethyl-sulfoxide (DMSO) and diluted in ISO water as specified in ISO 7346-3 (1996) (80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl). The properties, effect concentrations and model parameters for single substances used in mixture modeling are given in Table 1.

Table 1. Properties and effects of single substances in the spontaneous tail coiling (STC) test.

Substance	Chemical Class	Mechanism of Action [a]	Expected Activity, i.e., Effect Direction	STC EC_{50} (μmol/L) [b]	Slope of crc [b]
Chlorpyrifos	Organophosphate	Acetylcholinesterase inhibitor *	Hyperactivity	1.85 (1.95)	1.30
Chlorpyrifos-oxon	Organophosphate	Acetylcholinesterase inhibitor *	Hyperactivity	0.32 (0.44)	1
Hexaconazole	Triconazole	Ergosterol biosynthesis inhibitor *	Hyperactivity	4.03 (3.63)	1.80
Abamectin	Avermectin	Activation of GABA-gated chloride channel [$]	Hypoactivity	0.06 (0.09)	1.70
Carbamazepine	Dibenzazepine	Sodium channel blocker [#]	Hypoactivity	271	2.28
Propafenone	Aromatic Ketone	Sodium channel blocker [#]	Hypoactivity	32 (46)	1.94

[a] Mechanism of action was obtained from different sources including [#] http://drugbank.com * pesticide properties database (https://sitem.herts.ac.uk/aeru/ppdb/index.htm) and [$] Sánchez-Bayo, (2012) [24]; [b] Data obtained from Ogungbemi et al., (2020), the minimum and maximum of the concentration–response curves (crc) were set to 0 and 100, respectively. Values in parenthesis were obtained from independent experiments and were used for the mixture modelling.

2.3. Mixture Testing in the STC Test

Several mixtures were designed to investigate the appropriate classification for similar and dissimilar neuroactive substances which is suitable for mixture effect prediction using CA or IA models. Mixture components were selected according to their mechanism of action and effect direction (hyper- or hypoactivity) as follows (Figure 1 and Table 2): Mixture A—compounds with the same mechanism of action and same effect direction; Mixture B—compounds with different mechanism of action but same effect direction; Mixture C—compounds in A and B; Mixture D—compounds with a different mechanism of action and different effect direction. Mixtures A and B are binary while C and D are ternary. The exposure concentrations of the mixtures given in Table 2 are based on mixture ratios of the single substances calculated as molar fraction of their effect concentrations (EC_{50}). The EC_{50} concentration was selected to ensure that all components in the mixture contribute to the effect. Mixture D was particularly designed to understand if and how dissimilar compounds with different mechanisms of action and opposing effect direction would interact in the STC test. Although components of mixture D are equitoxic (in terms of EC_{50} ratio), the mixture was designed to reflect an unequitoxic scenario with respect to effect direction (0.33 hypoactivity: 0.66 hyperactivity).

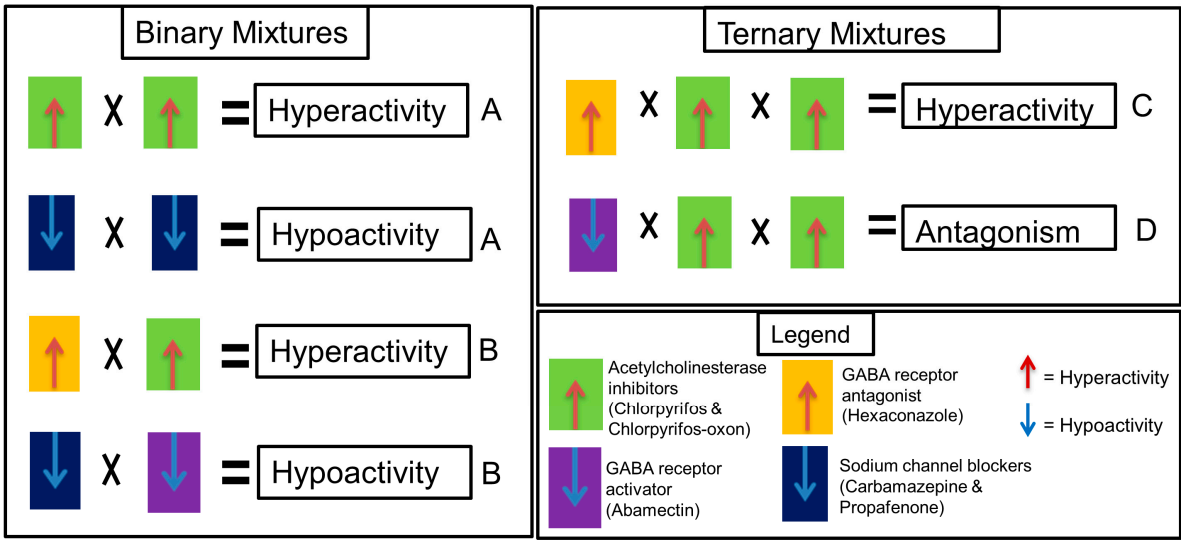

Figure 1. Mixture design scheme representing the hypotheses of this study. The letters A, B, C and D represent the mixture design according to Table 2. Each equation scheme for mixtures A, B and C represents a hypothesis whether concentration addition (CA) or independent action (IA) models could predict the hyper- or hypoactivity effects expected for mixtures with similar and dissimilar mechanisms of action. Equation for mixture D represents an antagonistic effect hypothesis.

Table 2. Summary of the mixture design, observed toxicity and predicted toxicity.

Mixture	Substances	Observed Activity	Mixture Ratio [a]	Exposure Concentration (µmol/L) [b]	Predicted EC_{50} (µmol/L)		Observed EC_{50} (µmol/L)
					CA	IA	
Mixture A	Chlorpyrifos and chlorpyrifos-oxon	Hyperactivity	0.816:0.184	0, 0.25, 0.5, 1, 2, 4 0, 0.1, 0.3, 0.9, 2.7, 5 0, 0.313, 0.625, 1.25, 2.5, 5	1.19	1.16	1.25
	Carbamazepine and propafenone	Hypoactivity	0.86:0.14	0, 40, 80, 160, 320 0, 78, 125, 200, 320	159	207	132
Mixture B	Hexaconazole and chlorpyrifos	Hyperactivity	0.65:0.35	0, 0.94, 1.87, 3.75, 7.5, 15 0, 0.75, 1.5, 3, 5.73, 12 0, 0.625, 1.25, 2.5, 5, 10 0, 0.625, 1.25, 2.5, 5, 10	2.79	3.69	2.79
	Abamectin and propafenone	Hypoactivity	0.002:0.998	0, 2.8, 5.6, 11.3, 22.5, 45 0, 4.38, 8.75, 17.5, 35, 70	23	27.6	17.4
Mixture C	Chlorpyrifos, hexaconazole and chlorpyrifos-oxon	Hyperactivity	0.603:0.324 :0.073	0, 0.75, 1.5, 3, 6, 12 0, 0.33, 1, 3, 9	2	2.19	1.95
Mixture D	Chlorpyrifos, hexaconazole and abamectin	Hyper and Hypoactivity	0.34:0.64 :0.02	0, 1.25, 2.5, 5 0, 1, 2, 4	- *	-	-
Simulation of Hyperactive Mixture A	Chlorpyrifos-oxon, (chlorpyrifos and hexaconazole)	Hyperactivity	0.184:(0.286 :0.53)	0, 0.313, 0.625, 1.25, 2.5, 5 0, 0.1, 0.3, 0.9, 2.7	-	-	-
Simulation of Hyperactive Mixture B	Hexaconazole, (chlorpyrifos and chlorpyrifos-oxon)	Hperactivity	0.65:(0.286 :0.064)	0, 0.625, 1.25, 2.5, 5, 10 0, 0.33, 1, 3, 9	-	-	-

* no mixture and toxicity predictions; [a] Mixture ratios are calculated as molar fraction of the total concentration. The ratio in the mixture is defined by the ratio of EC_{50}s. [b] The given exposure concentrations refer to the exposure range of independent experiments. In subsequent experiments, often different ranges were used to promote a better description of concentration–response curves. All concentration ranges were combined for concentration–response modelling.

To test if the simple case assumption of CA, i.e., substances are a dilution of each other and an equitoxic concentration of one can replace another [5], holds true for combined neurotoxicity effects in the STC test, we performed dilution experiments with the ternary mixture to simulate the hyperactivity mixtures A and B (chlorpyrifos and chlorpyrifos-oxon as well as chlorpyrifos and hexaconazole respectively). A portion of chlorpyrifos was replaced with an EC_{50} equitoxic portion of hexaconazole in mixture A and chlorpyrifos-oxon in mixture B (Table 2).

The detailed procedures for STC testing have been previously reported in detail [25]. Briefly, twenty fertilized embryos were exposed in 20 mL of the mixture solution prepared from DMSO stock solution (0.1% maximum concentration) of the components, within a 60 mm glass crystallization dish covered with a watchmaker glass. Two replicates per concentration and at least 2 independent experiments were conducted. The exposed embryos were incubated at 28 °C under 14 h light/10 h dark photoperiod for 21 ± 1 h. On the next day, at 24 hpf, exposed embryos were removed from the incubator and allowed to acclimatize to room temperature for at least 30 min. Videos of normally developed embryos (without any obvious malformation) were recorded for 60 s. Collected videos were analyzed for STC counts per minute (STC frequency) by means of a workflow using the KNIME® Analytical Platform [25,26].

2.4. Mixture Modeling

Mixture toxicity modeling was performed to investigate the capacity of concentration addition (CA) and independent action (IA) models to predict the combined effect of similar and dissimilar neuroactive substances. Effect data for the single substances used for mixture modelling were obtained from a previous study [17]. The CA mixture modeling is based on the effect concentration of the individual chemicals and it considers chemicals in a mixture to be a dilution of each other [5]. It is used to predict the mixture toxicity of chemicals with a similar mechanism of action.

$$ECx_{Mix} = \sum_{i=1}^{n} \frac{P_i^{-1}}{ECx_i} \quad (1)$$

Equation (1) shows the mathematical representation of the CA model where ECx_{Mix} is the total concentration of the mixture provoking x effect (i.e., 50% effect), P_i is the fraction of component i which represents the concentration of component i in the mixture, ECx_i is the concentration of component i provoking x effect, when applied singly.

The IA mixture modeling is based on the effect induced by individual chemicals in a mixture. It is usually applied to predict the mixture toxicity of chemicals with the dissimilar mechanism of action.

$$EC_{Mix} = 1 - \prod_{i=1}^{n}(1 - EC_i) \quad (2)$$

Equation (2) shows the mathematical representation of the IA model where EC_{Mix} is the total effect of the mixture and EC_i is the effect of component i in the mixture when applied singly. Mixture toxicity modeling was performed using an in-house excel sheet and the mixtox package in R [27].

2.5. Concentration–Response Modeling

Data from the mixture experiment were obtained as STC count per minute (STC frequency). The mean STC frequency was estimated for the exposed 20 embryos. The absolute STC frequency varied between the independent experiments. To combine results from independent experiments, mean percentage change in STC frequency with respect to unexposed embryos was estimated for independent experiments. Concentration–response

modeling of the percentage change in STC frequency was performed using the 4-parameter logistic function (LL.4) of the drc package in R [28].

$$y = c + \frac{(d - c)}{1 + \left(\frac{x}{e}\right)^b} \quad (3)$$

Equation (3) shows the concentration (x) response (y) model where b is the slope; c and d are the minimum and maximum STC response set to 0 and 100, respectively; and e is the inflection point, e.g., the EC_{50}.

In cases of hyperactivity, the maximum effect of STC frequency was different for the three tested hyperactive chemicals—chlorpyrifos, chlorpyrifos-oxon and hexaconazole (see Figure 2). Mixture prediction using different maximal of the percentage STC effect would have been based on a non-equitoxic mixture ratio of EC_{50}, EC_{41} and EC_{24} for hexaconazole, chlorpyrifos and chlorpyrifos-oxon respectively. To equalize the mixture ratio and maximum effect, the percentage STC change (obtained by normalizing to control) was standardized by dividing with the maximum percentage effect for each chemical to obtain a standardized percentage hyperactivity effect leading to 100% maximum effect for all hyperactive chemicals (Figure 2). This allowed us to obtain a similar half-maximum effect (EC_{50}) for the 3 chemicals. Skipping this hyperactivity standardization step would have led to the unpredictability of mixture effects higher than that of the chemical with the least maximal effect. Scholze et al. [29] used the toxic unit extrapolation approach to equalize and extend the dose–response curves for partial agonists. However, the observed hyperactivity effect in this study is usually followed by hypoactivity (possibly due to paralysis) at higher concentrations and this could indicate a saturated hyperactive effect. This appears not to support partial agonism but rather, the differential maximal effect of the 3 chemicals could be an indication of different mechanisms of hyperactive action. A partial agonist is expected to act as an antagonist in the presence of a full agonist [30] but this was not observed in the present study. Consequently, we consider the standardized percentage hyperactivity effect to be more representative of the observations and for mixture modeling in this study. The effect concentration causing a 50% increase or decrease of the STC was estimated from the concentration–response curve and the confidence interval was estimated as 2 times the standard error.

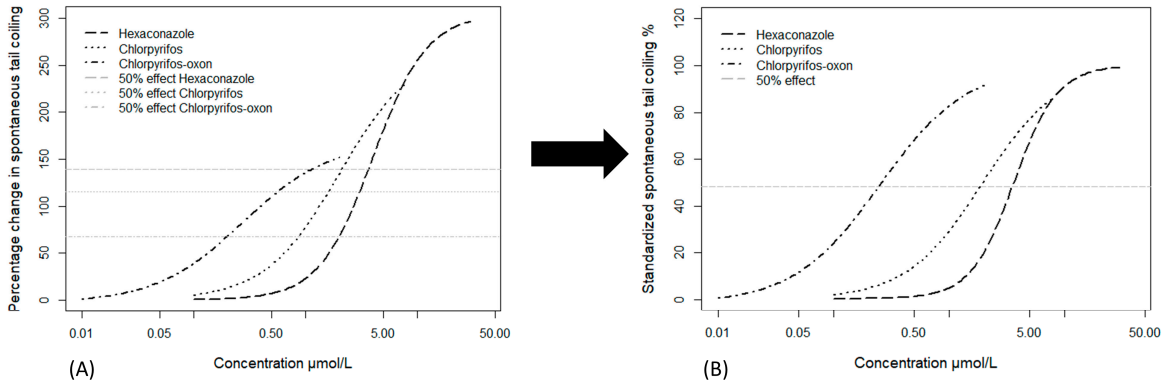

Figure 2. Visual representation of the data transformation for hyperactivity-inducing chemicals: (**A**) Concentration response curves showing different maximal for the hyperactivity inducing substances. The horizontal lines show EC_{50}, EC_{41} and EC_{24} which corresponds to the 50% effect for hexaconazole, chlorpyrifos and chlorpyrifos-oxon respectively; (**B**) Standardized concentration–response curves for the hyperactivity substances. The horizontal line shows the same 50% effect for the 3 substances after standardization. Data taken from Ogungbemi et al. (2020) [17].

2.6. Measurement of the Exposure Concentrations

Measurement of exposure concentrations was conducted to verify that test compounds were present in adequate concentrations in the test. Chemical measurement was

performed only for one independent experiment of the binary mixtures since the same relation of measured and nominal concentrations were expected for other independent experiments and also for the ternary mixture. For quantifying chlorpyrifos/chlorpyrifos-oxon and chlorpyrifos/hexaconazole mixtures, chemical analyses were conducted using an HPLC system (Merck-LaChrom) with diode array (model L7450) detector. One mL of the exposure solution for each concentration of the respective mixtures was sampled and 30 µL was injected directly. A reversed-phase column (Lichrospher 60 Reverse Phase (RP) select B, Merck, C-8), with a particle size of 5 µm was used. The column temperature was set to 40 °C and the flow rate was adjusted to 0.5 mL/min. Different mobile phase ratios of AcN:water was used for chlorpyrifos/chlorpyrifos-oxon (57:43%, elution time of 15 min) and chlorpyrifos/hexaconazole (65:35%, elution time of 12 min). The substances were detected at an absorbance of 207 nm. For quantifying carbamazepine/propafenone and abamectin/propafenone mixtures, chemical analyses were performed on a linear ion trap/Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Scientific, Waltham, MA, USA). Samples were diluted 100 (carbamazepine/propafenone) and 10 (abamectin/propafenone) times with ISO water before injection. An Agilent 1200 series HPLC system with a Kinetex C18 column (100 × 3 mm, 2.6 µm particle size, Phenomenex) was used for chromatographic separation after injection of 10 µL of sample. We used 0.1% formic acid and methanol containing 0.1% formic acid as mobile phases at a column temperature of 40 °C and a flow rate of 0.4 mL/min. The analysis was conducted in full scan mode with a mass range of m/z 100–1000 in negative and positive mode ESI with a nominal resolving power of 100,000 (referenced to m/z 400). For peak integration, compound calibration, and compound quantification, the software program TraceFinder 3.2 (Thermo Scientific, Waltham, MA, USA) was used.

3. Results

3.1. Chemical Analysis

Results of the chemical analysis are shown in Table 3. Measured concentrations were close to the nominal concentration, typically with a maximum deviation of about 20% for the highest tested concentrations for propafenone (+37 in Hypoactive Mixture A and −3% in Hypoactive Mixture B), carbamazepine (−8.8%), chlorpyrifos (−20 and −20% in both mixtures), chlorpyrifos-oxon (+19%) and hexaconazole (+15%). Measured concentrations of abamectin were below the detection limit (MDL) in all measurements. Reasons might be due to losses or rather adsorption to the test vessels because of its high lipophilicity ($\log D_{pH7.4(ACD/Labs)}$ of 5.85). It is important to note that chlorpyrifos concentrations in DMSO stock solutions declined by 25–40% after 2 months of storage. However, this reduction in concentration did not lead to a significant difference in the STC effect (Data not shown). Therefore, we used the nominal concentrations for further mixture toxicity evaluations based on the assumption that a 20% difference between nominal and measured concentrations will not cause a significant change in the observed effect.

Table 3. Measured concentrations of single substances in each mixture in micromole/liter. Values in round brackets are the percentage change of the measured concentrations with respect to the nominal concentrations while values in squared brackets are nominal concentrations that are below detection limit.

Hyperactive Mixture A		Hypoactive Mixture A		Hyperactive Mixture B		Hypoactive Mixture B	
Chlorpyrifos	**Chlorpyrifos-Oxon**	**Carbamazepine**	**Propafenone**	**Chlorpyrifos**	**Hexaconazole**	**Abamectin**	**Propafenone**
<MDL [0.25]	<MDL [0.05]	92.2 (+36)	22.1 (+120)	<MDL [0.2]	0.4 (−4)	<MDL [0.009]	6.0 (+37)
0.2 (−59)	<MDL [0.1]	128.0 (+20)	33.1 (+89)	0.2 (−50)	0.8 (+5)	<MDL [0.018]	11.4 (+31)
0.7 (−32)	0.5 (+109)	190.8 (+11)	47.7 (+70)	0.6 (−37)	1.8 (+10)	<MDL [0.035]	20.2 (+15)
1.8 (−12)	0.6 (+39)	250.7 (−8.8)	61.3 (+37)	1.4 (−23)	3.6 (+10)	<MDL [0.07]	31.4 (−10)
3.2 (−20)	1.1 (+19)			2.8 (−20)	7.5 (+15)	<MDL [0.14]	68.0 (−3)

MDL = Method detection limit. Chlorpyrifos MDL = 0.1 µM, Chlorpyrifos-oxon MDL = 0.1 µM, Hexaconazole MDL = 0.3 µM, Carbamazepine MDL = 0.0045 µM, Propafenone MDL = 0.0034 µM, Abamectin MDL = 0.0005 µM.

3.2. Description of Mixture Effect in Comparison to CA and IA Models

The effects of single substances used in the mixture testing have already been described in Ogungbemi et al. [17] and are summarized in Table 1. The mixture effects exceeded those of the single substances for all mixtures. Concentration–response curves for the observed and predicted mixture effects, as well as those for the single substances, are shown in Figure 3. Observed and predicted EC_{50} values are also shown in Table 2.

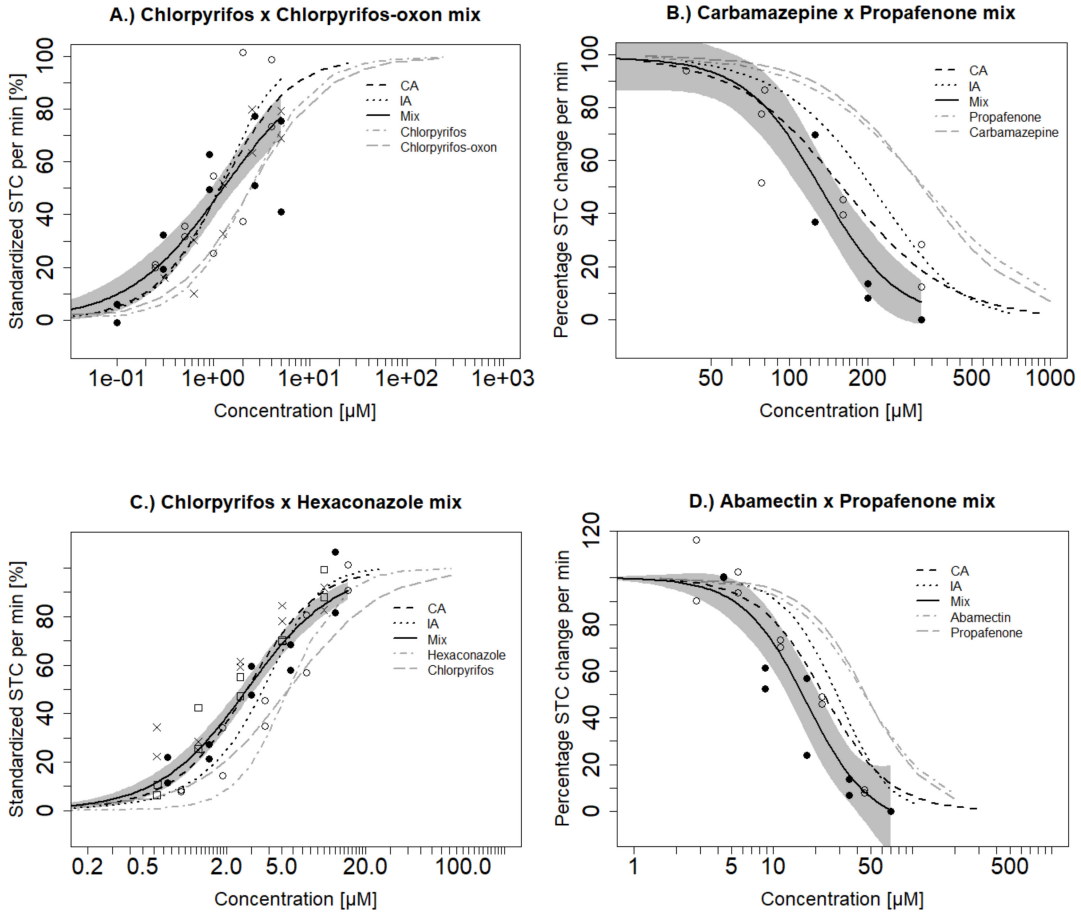

Figure 3. Comparison of observed (Mix) versus predicted effects of binary mixtures based on the concentration addition (CA) and independent action (IA) models in the STC. Furthermore, mixture effects are compared to single substances effects: (**A**) Hyperactivity Mixture A; (**B**) Hypoactivity Mixture A; (**C**) Hyperactivity Mixture B; (**D**) Hypoactivity Mixture B. Grey shaded areas represent the confidence interval of the fitted mixture model for the observed effect. Different symbols represent the observed mean of the STC effect for 20 embryos exposed in independent mixture experiments.

Hyperactive Mixture A (chlorpyrifos and chlorpyrifos-oxon) (see Section 2.3 or Table 2 for the definition of the mixture name) induced hyperactivity with an EC_{50} of 1.25 µM. The CA and IA models were similar and they both predicted the EC_{50} of the mixture (Table 2). The prediction curves were within the confidence boundary of the tested mixture at low and mid concentrations but both models slightly deviated and overestimated the effect at higher concentrations (Figure 3A). The Hypoactive Mixture A (carbamazepine and propafenone) caused hypoactivity with an EC_{50} of 132 µM. Both CA and IA (EC_{50} of 159 µM and 207µM, respectively) underestimated the mixture effect. Nevertheless, CA was predictive at low and medium-high concentrations (50–150 µM) while IA was less predictive and slightly underestimated the hypoactivity effects except at the lowest concentration range up to 100 µM (Figure 3B). Overall the estimation difference was always below a factor of 2 for CA and IA.

Hyperactive Mixture B (chlorpyrifos and hexaconazole) showed hyperactivity with an EC_{50} of 2.79 µM (Table 2). CA could predict the exact observed EC_{50} of the mixture but IA slightly underestimated the mixture effect [EC_{50} = 3.69 µM] (Figure 3C). Hypoactive Mixture B (abamectin and propafenone) showed hypoactivity with an EC_{50} of 17.4 µM. Both CA and IA slightly underestimated the mixture toxicity with EC_{50} values of 23 and 27.6 µM respectively. CA aligned with the confidence boundary of the observed mixture effect while IA deviated from the observed concentration–response curve (Figure 3D). Further, we tested a ternary mixture (Mixture C comprising of chlorpyrifos, chlorpyrifos-oxon and hexaconazole). Both CA and IA models showed similar predictions and were predictive of the observed mixture effect (Figure 4). In general, we observe a trend where CA and IA could very well predict mixture hyperactivity effects but to a slightly lesser extent for the hypoactivity effects—though these differences were minor.

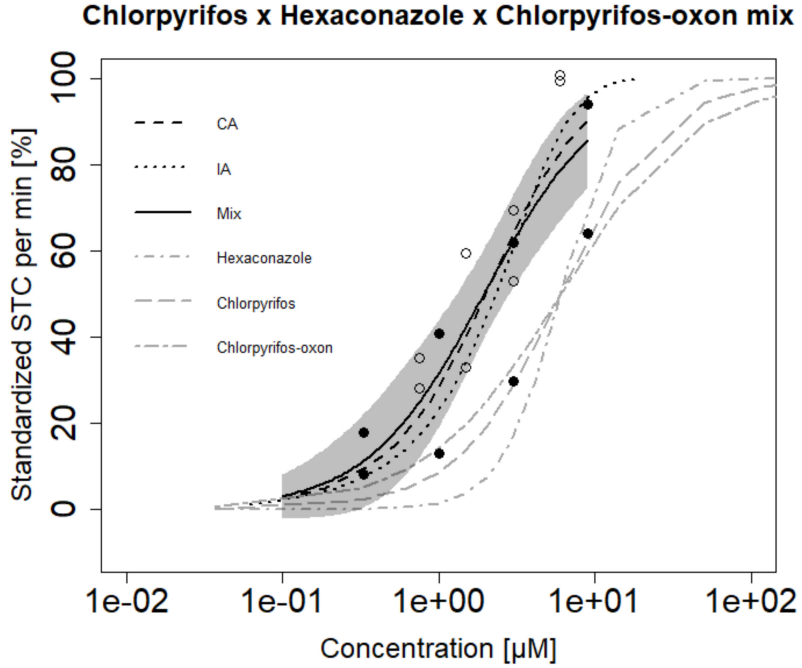

Figure 4. Comparison of observed (Mix) versus predicted effects of a ternary mixture based on the concentration addition (CA) and independent action (IA) models for mixture C. Furthermore, mixture effects are compared to single substances effects: Grey shaded areas represent the confidence interval of the fitted mixture model for the observed effect. Different symbols represent observed mean of STC effect for 20 embryos exposed in independent mixture experiments.

Further, we investigated the CA assumption that substances are dilutions of each other. Results show that substituting portions of chlorpyrifos in the Hyperactivity Mixtures A and B with hexaconazole and chlorpyrifos-oxon respectively, induced similar concentration–response curves as the non-substituted mixture (Figure 5A,B). The mixture of chlorpyrifos-oxon and (chlorpyrifos + hexaconazole) showed an EC_{50} of 1.77 µM which was higher than that of chlorpyrifos-oxon and chlorpyrifos mixture by only a factor of 1.4. An EC_{50} of 2.13 µM was estimated for hexaconazole and (chlorpyrifos + chlorpyrifos-oxon) which was lower than the hexaconazole and chlorpyrifos mix by only a factor of 1.3.

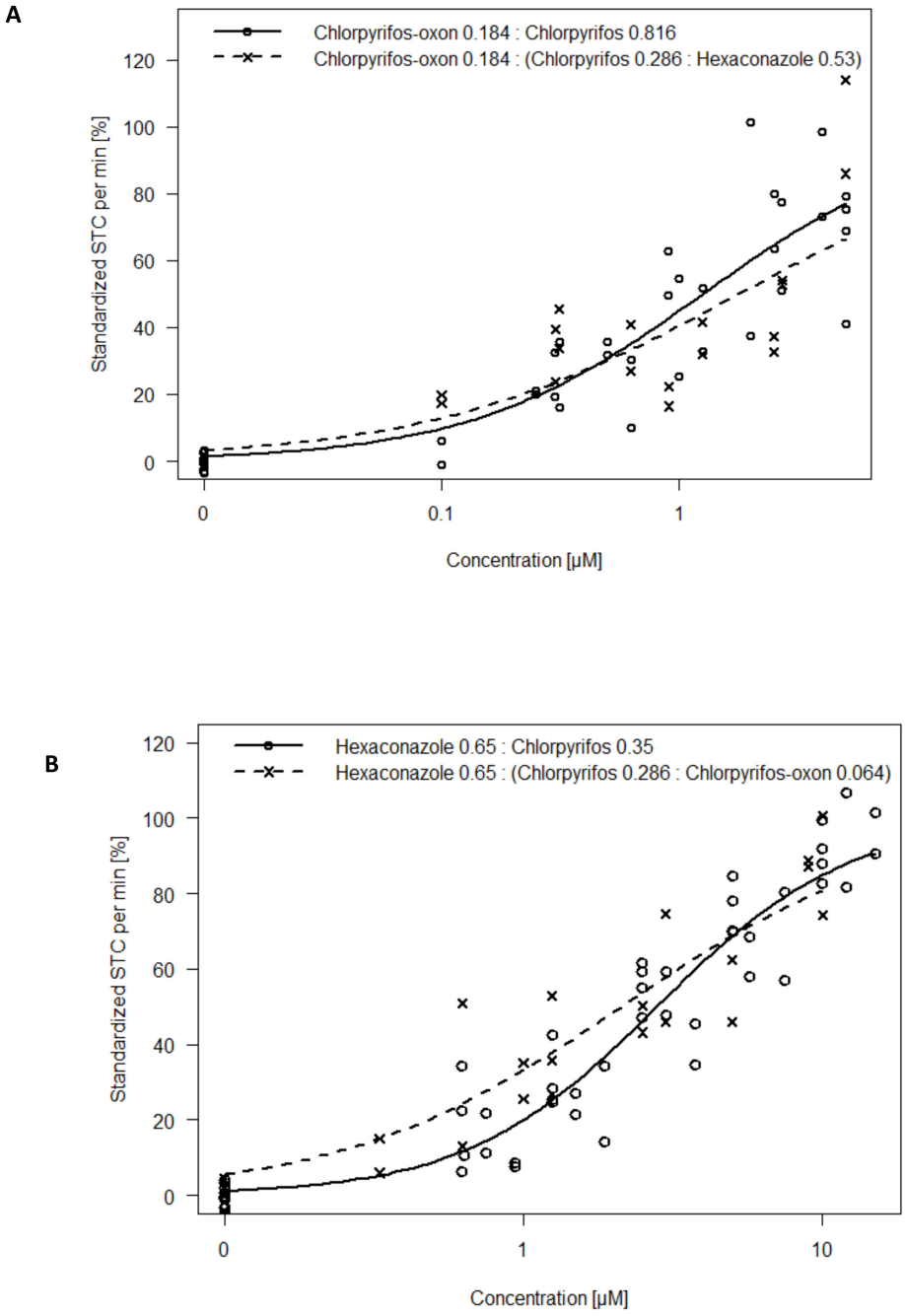

Figure 5. A ternary mixture is used to simulate a binary mixture by replacing a portion of one of the binary components with an equitoxic proportion of another substance: (**A**) Concentration–response curves for Hyperactive Mixture A containing chlorpyrifos-oxon and chlorpyrifos. Portions of chlorpyrifos were replaced with hexaconazole; (**B**) Concentration–response curves for Hyperactive Mixture B containing hexaconazole and chlorpyrifos. Portions of chlorpyrifos were replaced with chlorpyrifos-oxon.

3.3. Antagonistic Mixture Effects in the STC Test

Exposure of substances inducing opposing effect direction may induce antagonistic effects. Therefore, we exposed a ternary mixture of dissimilar substances (Mixture D) with different mechanisms of action and opposing effect directions (i.e., hyper- and hypoactivity). Mixtures were designed to reflect an unequitoxic scenario (0.33 hypoactivity: 0.66 hyperactivity; with respect to the corresponding EC_{50} values) by mixing the hypoactivity causing abamectin with two hyperactivity causing substances (chlorpyrifos

and hexaconazole). The result shows that the antagonistic effect of abamectin significantly decreased the hyperactivity effect expected from hexaconazole and chlorpyrifos (Hyperactive Mixture B). Furthermore, hypoactivity effect relative to control was observed at mid-high concentration of the mixture (Figure 6).

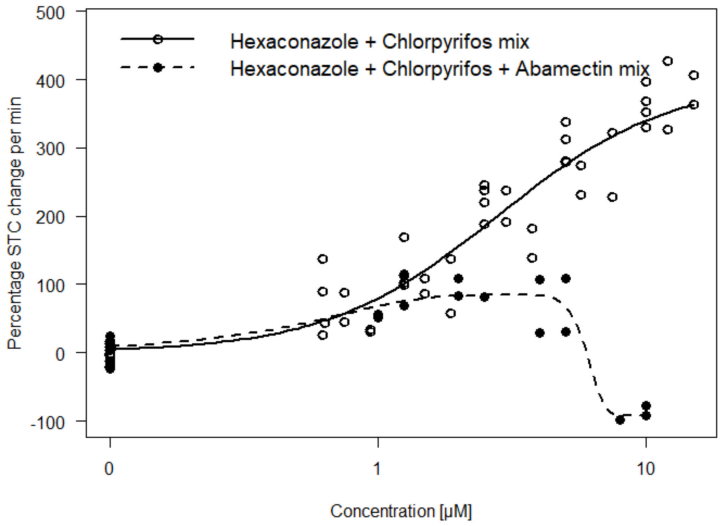

Figure 6. Comparison of concentration–response curves for hexaconazole and chlorpyrifos (Hyperactive Mixture B) with or without the addition of abamectin. Addition of abamectin decreases the hyperactivity effect (i.e., indicating an antagonistic effect) observed for the mixture without abamectin. A gaussian function was fitted to the data to model the biphasic effect of the mixture with abamectin.

4. Discussion

In order to evaluate the mixture toxicity of neuroactive compounds, two main challenges have to be considered regarding the application of prediction models: (1) Neuroactive chemicals in mixtures interact with different biochemical targets. To capture the effects of such a mixture, a possibility is to measure the effects at converging key events. (2) Mixtures may comprise of neuroactive chemicals with opposing effects. Consequently, we explored (1) whether mixture effects of neuroactive substances with similar effect directions (whether hyper- or hypoactivity) but different mechanisms of action would be additive and if concentration addition (CA) or independent action (IA) models can predict such mixture effect and (2) if mixtures of neuroactive substances with different mechanisms/modes of action and opposing effect direction would induce observable antagonistic effects. In order to address these challenges, we used an established behavior test, the spontaneous tail coiling (STC) of zebrafish embryos. It is responsive to diverse mechanisms of actions that finally translate to increased or reduced frequency of spontaneous movements as a result of either activation or inhibition of the neuronal synapse leading to hyper- or hypoactivity respectively (STC neuroactivity hypothesis). Accordingly, we hypothesized that neuroactive chemicals inducing the same response (either hyper- or hypoactivity) in the STC test can be predicted from CA or IA models. In contrast, compounds with modes of action with opposing effects would result in antagonistic effects if compared to individual compounds.

4.1. Mixture Components with Different Mechanisms of Action but Similar Effect Direction Can Act in an Additive Way

The first goal of the present study was focused on addressing the question—"Can additivity be assumed for a mixture of substances with the same mode of action (e.g., antiandrogenic) but not the same mechanism of action (e.g., receptor-blocking and inhibition of androgen production)?" which was posed in Kortenkamp et al. [31]. Based on theory, the CA model is adequate to predict mixture toxicity of similarly acting components (i.e., similar mechanisms of action) while IA is assumed to hold for dissimilarly acting

chemicals. However, CA may also be applied to predict the effect of chemicals showing similar toxicological responses (i.e., hyper- or hypoactivity) or modes of action [32]. We hypothesized that irrespective of the mechanism of action, compounds inducing the same toxicological response (whether hyper- or hypoactivity) would also lead to an additive response in the STC. This allows defining the similarity/dissimilarity of mixture components based on the combined knowledge of both the mechanism of action and toxicological response. Results from the current study indicate that mixture toxicity of chemicals such as propafenone and abamectin as well as chlorpyrifos and hexaconazole that are known to induce different mechanisms of action but similar effect directions were predictable using CA and IA models. (Figure 3C,D). Predictions of the IA model were very close to those of CA and this is not surprising for a binary mixture considering that the differences between the models increase with more mixture components [33]. However, there was also no difference in the prediction of CA and IA for the ternary Mixture C (Figure 4). CA and IA models could also predict the combined effect of pyrethroids and organophosphates in a D. magna immobility assay [34]. The predictability of the mixture models for differing neuro-mechanisms as observed in zebrafish embryos and daphnids may not be applicable in other test systems or endpoints with different levels of complexity or specificity [35]. For instance, CA and IA are expected to give different predictions for simpler but specific neuro-endpoints such as neural electric signal which may not reflect an integrated output as the STC but this remains to be investigated. Therefore, it is dependent on the mechanistic understanding of the test endpoint if neuroactive substances acting on different targets in the nervous system should be considered as similarly or dissimilarly acting components [34]. This also indicates that the assessment of similarity/dissimilarity of mixture components should go beyond knowledge of molecular targets and should consider other factors such as toxicological response and secondary mode of action [36].

4.2. Mechanistic Understanding of the Predictability Power of CA and IA

The STC is presumed to be generated by depolarizations which trigger action potentials in the synapses of the primary motor neurons [37]. Consequently, it is not farfetched to consider different target interactions or mechanisms of action as similarly acting in so far as they result in the same key event (activation or inhibition of neuronal synapses) and same toxicological response (hyper- or hypoactivity). In this case, we may consider neuroactivity via the STC endpoint to be an integrated effect on neuronal synapses and CA might be more appropriate to predict mixture effects of chemicals in the STC. We showed in the present study the capacity of CA to predict mixture B (substances with different mechanisms of action but similar effect direction). This is consistent with previous studies on nervous system-related endpoints. For example, Wolansky et al. [38] found that CA was a good predictor of the mixture neurotoxicity of different pyrethroids on the motor activity of rats and Gonçalves et al. [39] reported that CA was adequate to predict the mixture effect of PAHs on fish behavior.

Based on the confidence interval of the experimental mixture, the IA model was slightly less predictive (a factor of about 1.6% deviation) for hypoactivity effects (Figure 2B,D). This could be due to unspecific effects such as axonal deformation and malformations which might contribute additional effect to the primary hypoactivity of the embryo [17]. Such additional effects would likely be captured as an integrative hypoactivity effect in the CA model. Further, the accuracy of the IA model in complex organisms such as zebrafish embryos has been questioned due to converging signaling pathways and inter-dependent subsystems [31,35,40]. For instance, Corbel et al. [10] found that carbamate and pyrethroid had a converging effect on acetylcholine concentration in the synapse even though they have different mechanisms of action. Estrogen receptor activation was also seen as an integrated effect of different cascading steroidal receptor signaling [29]. In addition, we could simulate concentration additive mixtures by replacing a portion of the mixture component with another similar acting substance (similar effect direction but different mechanism of action) (Figure 4A,B). This adds credence to the CA assumption that components can be described as a dilution of

each other in the STC test. However, the results of mixture assessment with STC do not allow to favor one of the models as the differences between CA and IA were quite small.

Mixture toxicity prediction using CA and IA models assumes that the mixture components do not interact to affect the uptake, distribution, metabolism and elimination of each other [8,41]. Mixture interaction of neuroactive substances may occur via the biotransformation pathways due to the reduced activation or competition for biotransformation sites [42]. Organophosphates were found to be a major synergistic group due to their ability to inhibit esterases which are responsible for phase 2 biotransformation of chemicals [43]. However, we did not observe synergistic interaction of a mixture of chlorpyrifos and its oxon metabolite in the present study and this could be due to potential limited biotransformation capacity of early stages of the zebrafish embryo [44] or the sensitivity of our test system. Other mixture neurotoxicity studies have shown interaction effects. For example, a mixture of chlorpyrifos and nickel on zebrafish embryos was found to be antagonistic [45] and the mixture of atrazine and chlorpyrifos was assessed as synergistic [46]. However, 120 and 96 hpf embryos, which should have higher rates for biotransformation into the active oxon metabolite, were used in these studies.

4.3. Mixture Components with Different Mechanisms of Action and Opposing Effect Direction Are Antagonistic

We investigated the STC outcome for mixtures comprising of different mechanisms of action as well as opposing effect directions (Mixture D). The results show that mixtures with both hyper- and hypoactivity-inducing components will lead to antagonistic interaction (Figure 6). Our results corroborate the recommendation of a chemical grouping for mixture analysis based on common adverse outcomes (hyper- and hypo-activity in this case) with less emphasis on the similarity of the mechanism of action [31]. Information on common adverse outcomes such as hyper- and hypoactivity will be useful to qualitatively predict mixture outcomes of multi-component/complex mixtures as well as to understand deviations from additivity. For instance, the antagonistic effects of abamectin on the hyperactivity level of the mixture of chlorpyrifos and hexaconazole (Figure 6) would have been unexplainable if only a mechanism of action-based classification was used. This particularly applies to endpoints with opposing effect directions such as locomotor activity or even gene response. For such endpoints, chemicals that primarily induce hyperactivity at low concentrations may cause hypoactivity at higher concentrations due to seizures and paralysis [13]. The use of chemicals inducing such biphasic activity as a component in a mixture without considering the primary effect direction could lead to misinterpretation of its impact on the combined effect. This biphasic activity was also observed for Mixture D in the current study and could be due to the relatively higher counteractive potency of abamectin (EC_{50} of 0.06 μM) induced at high mixture concentrations in comparison to the hyperactivity effect of chlorpyrifos and hexaconazole with much higher EC_{50}s (Figure 6).

Hyper- and hypoactivity response could also be used as an effect-based strategy for bio-monitoring of complex environmental mixtures which can facilitate the identification of chemicals inducing mixture neurotoxicity that would not have been detected with analytical chemical measurements [47,48]. However, equitoxic ratio of substances with opposing effect direction could lead to normalization or mitigation of the expected individual effects or mixture effects approaching control level. This counteracting effect could be a huge challenge for diagnostic risk assessment. Therefore, effect evaluation with STC as converging key event of a complex environmental mixture may only indicate an effect size related to the amount of neuroactive components if they show effect in the same direction (i.e., hyper- or hypoactivity). With opposing effects in the STC, effect evaluation may not relate to the cumulative exposure levels. However, this may present a better evaluation of the exposure level regarding the relevant biological effects and potential hazards. Nevertheless, a solution could be to spike environmental mixtures with a positive control such that deviations from the known effect size of the positive control could be an indication of the inherent effect of the mixture. In prospective mixture evaluation, one solution could be to employ a non-equitoxic mixture ratio design (e.g., 25% compound A

and 75% compound B or vice versa) for opposing acting substances such that the strength of the counteracting effect is weakened. This non-equitoxic design was useful to evaluate Mixture D in the current study. However, this approach may lead to hidden effects and could give a false perspective of effect assessment. Regardless, it is necessary to elaborate on when effect normalization is an acceptable ecological risk.

5. Conclusions

We found that mixtures of neuroactive substances with different mechanisms of action but similar effect direction are additive and could be predicted using CA or IA models. Convergence and integration of effects in the nervous system provides a mechanistic understanding to support similarity classification of neuroactive compounds not only based on mechanisms of action but also considering the toxicological response or effect direction (whether hyper- or hypoactivity). Consequently, we recommend considering toxicological response or effect direction as an additional grouping factor when applying CA and IA models. On the other hand, mixtures of substances with different mechanisms of action and opposing effect direction are antagonistic. Being able to detect neurotoxicity within an environmental sample (complex mixture) is relevant since neuroactive chemicals are usually dominating concentrations of contaminants in the environment and may be major drivers of mixture toxicity. Since established effect-based tools may overlook or may not capture neurotoxicity, in this study, we propose a way to use the STC test for risk assessment despite counteracting effects which could complicate proper evaluation.

Author Contributions: Conceptualization, A.O.O., R.M., R.A., S.S. and E.K.; methodology, A.O.O., R.M., S.S. and E.K.; formal analysis, A.O.O., R.A. and E.K.; investigation, A.O.O.; writing—original draft preparation, A.O.O.; writing—review and editing, A.O.O., R.M., R.A., S.S. and E.K.; visualization, A.O.O.; supervision, E.K. and S.S.; project administration, E.K.; funding acquisition, E.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the German Federal Ministry of Education and Research (BMBF) within the Neurobox Project (FKZ 02WRS1419F). In addition parts of the study were co-financed by the UFZ integrated project Exposome.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.4640059.

Acknowledgments: Firstly, we would like to acknowledge the high-spirited patronage by the late Tamara Grummt throughout the whole project! We also appreciate Gianina Jakobs and Franz Gierstorf for the discussions during the design of the experiments and for supporting the experimental work respectively. We highly acknowledge the help by Nicole Schweiger for fish culture and embryo production.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Faust, M.; Backhaus, T.; Altenburger, R.; Dulio, V.; van Gils, J.; Ginebreda, A.; Kortenkamp, A.; Munthe, J.; Posthuma, L.; Slobodnik, J.; et al. Prioritisation of water pollutants: The EU Project Solutions proposes a methodological framework for the integration of mixture risk assessments into prioritisation procedures under the European Water Framework Directive. *Environ. Sci. Eur.* **2019**, *31*. [CrossRef]
2. Belden, J.B.; Gilliom, R.J.; Lydy, M.J. How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integr. Environ. Assess. Manag.* **2007**, *3*, 364–372. [CrossRef] [PubMed]
3. Bliss, C.I. The Toxicity of Poisons Applied Jointly. *Ann. Appl. Biol.* **1939**, *26*, 585–615. [CrossRef]
4. Hewlett, P.S.; Plackett, R.L. A Unified Theory for Quantal Responses to Mixtures of Drugs: Non-Interactive Action. *Biometrics* **1959**, *15*, 591–610. [CrossRef]
5. Altenburger, R.; Backhaus, T.; Boedeker, W.; Faust, M.; Scholze, M.; Grimme, L.H. Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting chemicals. *Environ. Toxicol. Chem.* **2000**, *19*, 2341–2347. [CrossRef]

6. Backhaus, T.; Altenburger, R.; Boedeker, W.; Faust, M.; Scholze, M.; Grimme, L.H. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. *Environ. Toxicol. Chem.* **2000**, *19*, 2348–2356. [CrossRef]
7. Faust, M.; Altenburger, R.; Backhaus, T.; Blanck, H.; Boedeker, W.; Gramatica, P.; Hamer, V.; Scholze, M.; Vighi, M.; Grimme, L.H. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquat. Toxicol.* **2001**, *56*, 13–32. [CrossRef]
8. Cedergreen, N.; Svendsen, C.; Backhaus, T. Toxicity Prediction of Chemical Mixtures. *Encycl. Environ. Manag.* **2013**, 2572–2581. [CrossRef]
9. Busch, W.; Schmidt, S.; Kühne, R.; Schulze, T.; Krauss, M.; Altenburger, R. Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. *Environ. Toxicol. Chem.* **2016**, *35*, 1887–1899. [CrossRef]
10. Corbel, V.; Stankiewicz, M.; Bonnet, J.; Grolleau, F.; Hougard, J.M.; Lapied, B. Synergism between insecticides permethrin and propoxur occurs through activation of presynaptic muscarinic negative feedback of acetylcholine release in the insect central nervous system. *Neurotoxicology* **2006**, *27*, 508–519. [CrossRef]
11. Yang, Y.; Ma, H.; Zhou, J.; Liu, J.; Liu, W. Joint toxicity of permethrin and cypermethrin at sublethal concentrations to the embryo-larval zebrafish. *Chemosphere* **2014**, *96*, 146–154. [CrossRef]
12. Strähle, U.; Scholz, S.; Geisler, R.; Greiner, P.; Hollert, H.; Rastegar, S.; Schumacher, A.; Selderslaghs, I.; Weiss, C.; Witters, H.; et al. Zebrafish embryos as an alternative to animal experiments-A commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod. Toxicol.* **2012**, *33*, 128–132. [CrossRef]
13. Ogungbemi, A.; Leuthold, D.; Scholz, S.; Küster, E. Hypo- or hyperactivity of zebrafish embryos provoked by neuroactive substances: A review on how experimental parameters impact the predictability of behavior changes. *Environ. Sci. Eur.* **2019**. [CrossRef]
14. Saint-Amant, L.; Drapeau, P. Time course of the development of motor behaviors in the zebrafish embryo. *J. Neurobiol.* **1998**, *37*, 622–632. [CrossRef]
15. Kimmel, C.B.; Patterson, J.; Kimmel, R.O. The development and behavioral characteristics of the startle response in the zebra fish. *Dev. Psychobiol.* **1974**, *7*, 47–60. [CrossRef]
16. Selderslaghs, I.W.T.; Hooyberghs, J.; De Coen, W.; Witters, H.E. Locomotor activity in zebrafish embryos: A new method to assess developmental neurotoxicity. *Neurotoxicol. Teratol.* **2010**, *32*, 460–471. [CrossRef]
17. Ogungbemi, A.O.; Teixido, E.; Massei, R.; Scholz, S.; Küster, E. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. *Neurotoxicol. Teratol.* **2020**, *81*, 106918. [CrossRef]
18. Zhang, K.; Liang, J.; Brun, N.R.; Zhao, Y.; Werdich, A.A. Rapid Zebrafish Behavioral Profiling Assay Accelerates the Identification of Environmental Neurodevelopmental Toxicants. *Environ. Sci. Technol.* **2021**, *55*, 1919–1929. [CrossRef]
19. Vliet, S.M.; Ho, T.C.; Volz, D.C. Behavioral screening of the LOPAC1280 library in zebrafish embryos. *Toxicol. Appl. Pharmacol.* **2017**, *329*, 241–248. [CrossRef]
20. De Oliveira, A.A.S.; Brigante, T.A.V.; Oliveira, D.P. Tail coiling assay in zebrafish (Danio rerio) embryos: Stage of development, promising positive control candidates, and selection of an appropriate organic solvent for screening of developmental neurotoxicity (DNT). *Water* **2021**, *13*, 119. [CrossRef]
21. Casida, J.E.; Durkin, K.A. Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **2013**, *58*, 99–117. [CrossRef] [PubMed]
22. Söderpalm, B. Anticonvulsants: Aspects of their mechanisms of action. *Eur. J. Pain* **2002**, *6*, 3–9. [CrossRef]
23. Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B.; Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **1995**, *203*, 253–310. [CrossRef]
24. Sánchez-Bayo, F. Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. *J. Environ. Anal. Toxicol.* **2012**, *s4*. [CrossRef]
25. Ogungbemi, A.O.; Teixido, E.; Massei, R.; Scholz, S.; Küster, E. Automated measurement of the spontaneous tail coiling of zebrafish embryos as a sensitive behavior endpoint using a workflow in KNIME. *MethodsX* **2021**, *8*, 101330. [CrossRef]
26. Teixido, E.; Klüver, N.; Ogungbemi, A.O.; Küster, E.; Scholz, S. Evaluation of Neurotoxic effects in zebrafish embryos by automatic measurement of early motor behaviors. In *Experimental Neurotoxicology Methods*; Neuromethods; Springer: Berlin/Heidelberg, Germany, 2021.
27. Zhu, X.W.; Chen, J.Y. Mixtox: An r package for mixture toxicity assessment. *R J.* **2016**, *8*, 422–434. [CrossRef]
28. Ritz, C.; Streibig, J.C. Bioassay analysis using R. *J. Stat. Softw.* **2005**, *12*. [CrossRef]
29. Scholze, M.; Silva, E.; Kortenkamp, A. Extending the applicability of the dose addition model to the assessment of chemical mixtures of partial agonists by using a novel toxic unit extrapolation method. *PLoS ONE* **2014**, *9*. [CrossRef]
30. Jackson, A. Partial Agonist. In *Encyclopedia of Psychopharmacology*; Stolerman, I.P., Ed.; Springer: Berlin/Heidelberg, Germany, 2010; pp. 959–960. ISBN 978-3-540-68706-1.
31. Kortenkamp, A.; Backhaus, T.; Faust, M. State of the Art Report on Mixture Toxicity. Final Report Contract N. 2009, pp. 1–391. Available online: http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf (accessed on 6 December 2017).
32. Cleuvers, M. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicol. Lett.* **2003**, *142*, 185–194. [CrossRef]

33. Drescher, K.; Boedeker, W. Assessment of the Combined Effects of Substances: The Relationship between Concentration Addition and Independent Action. *Biometrics* **1995**, *51*, 716–730. [CrossRef]
34. Rose, S.; Altenburger, R.; Sturm, A. Mixture toxicity effects of sea louse control agents in Daphnia magna. *Chemosphere* **2016**, *144*, 599–606. [CrossRef]
35. Jakobs, G.; Krüger, J.; Schüttler, A.; Altenburger, R.; Busch, W. Mixture toxicity analysis in zebrafish embryo: A time and concentration resolved study on mixture effect predictivity. *Environ. Sci. Eur.* **2020**, *32*, 143. [CrossRef]
36. Cedergreen, N.; Christensen, A.M.; Kamper, A.; Kudsk, P.; Mathiassen, S.K.; Streibig, J.C.; Sørensen, H. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environ. Toxicol. Chem.* **2008**, *27*, 1621–1632. [CrossRef]
37. Drapeau, P.; Saint-Amant, L.; Buss, R.R.; Chong, M.; McDearmid, J.R.; Brustein, E. Development of the locomotor network in zebrafish. *Prog. Neurobiol.* **2002**, *68*, 85–111. [CrossRef]
38. Wolansky, M.J.; Gennings, C.; DeVito, M.J.; Crofton, K.M. Evidence for dose-additive effects of pyrethroids on motor activity in rats. *Environ. Health Perspect.* **2009**, *117*, 1563–1570. [CrossRef]
39. Gonçalves, R.; Scholze, M.; Ferreira, A.M.; Martins, M.; Correia, A.D. The joint effect of polycyclic aromatic hydrocarbons on fish behavior. *Environ. Res.* **2008**, *108*, 205–213. [CrossRef]
40. Schmidt, S.; Busch, W.; Altenburger, R.; Küster, E. Mixture toxicity of water contaminants-effect analysis using the zebrafish embryo assay (Danio rerio). *Chemosphere* **2016**, *152*, 503–512. [CrossRef]
41. Altenburger, R.; Nendza, M.; Schüürmann, G. Mixture toxicity and its modeling by quantitative structure-activity relationships. *Environ. Toxicol. Chem.* **2003**, *22*, 1900–1915. [CrossRef]
42. Corbett, J.R. *The Biochemical Mode of Action of Pesticides*; Academic Press: Cambridge, MA, USA, 1974; 330p. [CrossRef]
43. Cedergreen, N. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. *PLoS ONE* **2014**, *9*. [CrossRef]
44. Kühnert, A.; Vogs, C.; Aulhorn, S.; Altenburger, R.; Küster, E.; Busch, W.; Kühnert, A.; Vogs, C.; Altenburger, R.; Hollert, H.; et al. Biotransformation in the zebrafish embryo –temporal gene transcription changes of cytochrome P450 enzymes and internal exposure dynamics of the AhR binding xenobiotic benz[a]anthracene. *Environ. Pollut.* **2017**, *230*, 1–11. [CrossRef]
45. Kienle, C.; Köhler, H.R.; Gerhardt, A. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 1740–1747. [CrossRef]
46. Pérez, J.; Domingues, I.; Monteiro, M.; Soares, A.M.V.M.; Loureiro, S. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. *Environ. Sci. Pollut. Res.* **2013**, *20*, 4671–4680. [CrossRef] [PubMed]
47. Bal-Price, A.; Crofton, K.M.; Leist, M.; Allen, S.; Arand, M.; Buetler, T.; Delrue, N.; FitzGerald, R.E.; Hartung, T.; Heinonen, T.; et al. International STakeholder NETwork (ISTNET): Creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch. Toxicol.* **2015**, *89*, 269–287. [CrossRef] [PubMed]
48. De Baat, M.L.; Kraak, M.H.S.; Van der Oost, R.; De Voogt, P.; Verdonschot, P.F.M. Effect-based nationwide surface water quality assessment to identify ecotoxicological risks. *Water Res.* **2019**, *159*, 434–443. [CrossRef] [PubMed]

toxics

MDPI

Article

Environmentally Relevant Mixture of Pesticides Affect Mobility and DNA Integrity of Early Life Stages of Rainbow Trout (*Oncorhynchus mykiss*)

Shannon Weeks Santos [1], Jérôme Cachot [1,*], Bettie Cormier [1], Nicolas Mazzella [2], Pierre-Yves Gourves [1], Christelle Clérandeau [1], Bénédicte Morin [1] and Patrice Gonzalez [1]

[1] CNRS, EPOC, EPHE, University of Bordeaux, UMR 5805, 33600 Bordeaux, France; shannon.wks@gmail.com (S.W.S.); bettie.cormier@u-bordeaux.fr (B.C.); pierre-yves.gourves@u-bordeaux.fr (P.-Y.G.); christelle.clerandeau@u-bordeaux.fr (C.C.); benedicte.morin@u-bordeaux.fr (B.M.); patrice.gonzalez@u-bordeaux.fr (P.G.)

[2] National Research Institute of Science and Technology for Environment and Agriculture, 33612 Aix-en-Provence, France; nicolas.mazzella@inrae.fr

* Correspondence: jerome.cachot@u-bordeaux.fr

Abstract: The aim of this study was to analyze the impact of three concentrations of a pesticide mixture on the first development stages of rainbow trout (*Oncorhynchus mykiss*). The mixture was made up of three commonly used pesticides in viticulture: glyphosate (GLY), chlorpyrifos (CPF) and copper sulfate (Cu). Eyed stage embryos were exposed for 3 weeks to three concentrations of the pesticide mixture. Lethal and sub-lethal effects were assessed through a number of phenotypic and molecular endpoints including survival, hatching delay, hatching success, biometry, swimming activity, DNA damage (Comet assay), lipid peroxidation (TBARS), protein carbonyl content and gene expression. Ten target genes involved in antioxidant defenses, DNA repair, mitochondrial metabolism and apoptosis were analyzed using real-time RT-qPCR. No significant increase of mortality, half-hatch, growth defects, TBARS and protein carbonyl contents were observed whatever the pesticide mixture concentration. In contrast, DNA damage and swimming activity were significantly more elevated at the highest pesticide mixture concentration. Gene transcription was up-regulated for genes involved in detoxification (*gst* and *mt1*), DNA repair (*ogg1*), mitochondrial metabolism (*cox1* and 12S), and cholinergic system (*ache*). This study highlighted the induction of adaptive molecular and behavioral responses of rainbow trout larvae when exposed to environmentally realistic concentrations of a mixture of pesticides.

Keywords: copper; glyphosate; chlorpyrifos; early life stages; rainbow trout; swimming behavior; DNA damage; development

Citation: Weeks Santos, S.; Cachot, J.; Cormier, B.; Mazzella, N.; Gourves, P.-Y.; Clérandeau, C.; Morin, B.; Gonzalez, P. Environmentally Relevant Mixture of Pesticides Affect Mobility and DNA Integrity of Early Life Stages of Rainbow Trout (*Oncorhynchus mykiss*). *Toxics* **2021**, *9*, 174. https://doi.org/10.3390/toxics9080174

Academic Editor: Markus Brinkmann

Received: 24 June 2021
Accepted: 20 July 2021
Published: 22 July 2021

1. Introduction

Increased concern about chemical contaminants in natural environments has led to a growth in research aimed at predicting the impacts of these contaminants on ecosystems.

Pesticides are the only kind of chemicals that are purposely re-leased into the environment [1]. Currently, more than 400 chemical compounds are used to treat crops against pests and weeds [2]. Global pesticide usage is calculated at 4.6 million tons per year [1,3]. It has been estimated that 98% of pesticides applied to agricultural crops do not reach their intended target, and could affect terrestrial and aquatic ecosystems. Studies have shown that 40% of worldwide land mass poses a risk in terms of pesticide runoff into rivers and streams [4]. While most ecotoxicological studies focus on individual pesticides, several reports have also confirmed that pesticides are usually found in complex mixtures at low concentrations [2,5], and aquatic organisms are directly exposed to them [6–8]. Exposure to pesticide mixtures may trigger additive effects when molecules have similar modes of

action and affect the same molecular target, or by independent action when they have dissimilar modes of action affecting the same or different molecular targets, and therefore synergism or antagonism may occur [9].

Because of its anti-microbial and anti-fungal properties, copper (Cu), also listed as a priority substance in the water Framework Directive (WFD) [10], is widely used to protect vineyards from fungal diseases. Given its intensive use for both conventional and organic agriculture, Cu is a widely present pollutant in the environment, and transfer from soils to aquatic ecosystems is likely to occur [11,12]. While it has an essential role in numerous cellular processes, excess Cu generates toxicity by producing reactive oxygen species (ROS) which may cause damages to lipids, proteins and nucleic acids, leading to cell death [13,14]. In a previous study, first stages of rainbow trout were found to be very sensitive to Cu [15]. Indeed, after a 3-week exposure to environmental concentrations of Cu (2 and 20 μg/L), inhibitory effect on hatching and significant induction of malformations were observed. In addition, several genes were down-regulated in Cu-exposed rainbow trout, especially those involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle regulation (*p53*).

Glyphosate (GLY) is a broad-spectrum, non-selective and systemic herbicide used in numerous phytosanitary products. It acts as an inhibitor of the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimic acid pathway that is common in all plants. Because of its extensive use, especially in genetically modified crops [16], high concentrations (ranging from 7.5 to 700 μg/L) have been found in different streams and lakes near agricultural basins [17–19]. Several reports have highlighted the sub-lethal effects of GLY on fish in particular on DNA integrity [20–22], acetylcholinesterase (AChE) inhibition [23], swimming behavior alterations [24,25], and antioxidant enzyme activities [26,27]. We reported that, following a 3-week embryo-larval exposure to glyphosate (0.1 and 1 mg/L), rainbow trout larvae exhibited reduced head size when compared to control larvae. Additionally, swimming behavior was also affected and exposed larvae had increased mobility compared to non-exposed larvae [28].

Chlorpyrifos (CPF) is an organophosphorus insecticide (OP) which has been widely used in the past by both industrial and private users. However, because of its high toxicity for humans and animals, the use of the product for domestic purposes has been restricted (US EPA), and there is a declining trend in its consumption [3]. Because it is on the list of priority substances for the WFD, its presence in surface and groundwater is closely monitored. While CPF has been detected predominantly in sediments from cultivated areas, low concentrations have also been observed in stream water, where maximum concentrations ranged between 0.06 and 0.45 μg/L in the USA and Argentina, respectively [29,30]. As an OP, CPF affects the nervous system by inhibiting the AChE enzyme [31], disrupting the transmission of nerve impulses through synaptic terminals and impacting essential functions such as respiration and swimming behavior of fish [32,33]. In a recent study, low concentrations of CPF (0.3 and 3 μg/L) did not affect rainbow trout embryonic and larval viabilities. However, sub-lethal effects were observed on mobility of larvae, which was reduced for those exposed to 3 μg/L of CPF compared to control conditions. Low concentrations of CPF also down-regulated genes involved in steroid hormone pathways such as *er-b* and *cyp19a1* [34].

These three compounds (Cu, GLY and CPF), which have differing modes of action and functions, may be applied to crops simultaneously. Given the ease with which they are transported into water bodies through runoff, spray drift, or groundwater, it could be valuable to consider the toxicity of their combined effects, using realistic environmental concentrations, in aquatic organisms. To date, data on the effects of pesticide mixtures remain scarce, particularly relating to the early life stages of fish. To assess the sublethal effects of environmental concentrations of a pesticide mixture, a 3-week exposure was performed on rainbow trout embryos and several developmental and behavioral endpoints were recorded. The endpoints studied included survival, hatching delay, hatching success, morphological anomalies, swimming behavior, genotoxicity (measured by the comet assay), lipid peroxidation and protein carbonyl content, and gene transcription levels.

The expression levels of ten genes were selected according to their biological functions in antioxidant defenses (*cat*, *sod*), detoxification (*mt1*, *gst*), mitochondrial metabolism (*cox1*, 12S), cholinergic system (*ache*), DNA repair (*ogg1*, *rad*) or apoptosis (*bax*).

2. Materials and Methods

2.1. Test Chemicals

Chlorpyrifos-ethyl (CAS No. 2921-88-2) was purchased from ChemService (Merseyside, UK). Copper sulfate ($CuSO_4 \cdot H_2O$, CAS No. 7758-99-8, 99.99%) was purchased from Sigma Aldrich (Lyon, France). Glyphosate was purchase as a commercial formulation (available on the market) Roundup®GT Max. The active substance of Roundup®GT Max is 480 g/L of glyphosate acid, which is equivalent to 588 g/L of potassium salt of glyphosate.

2.2. Exposure System

Eyed-stage rainbow trout embryos (*Oncorhynchus mykiss*) were purchased from INRAe-PEIMA (Sizun, FR). Embryos were exposed for 3 weeks from stage 240 DD (Degree Days) to larvae stage 500 DD at 12 ± 0.5 °C. Stock solutions of copper (0.67, 2 and 20 mg/L) and glyphosate (33.3, 100 and 1000 mg/L) were prepared using osmosis water. Stock solutions of chlorpyrifos-ethyl (33.3, 100 and 1000 mg/L) were prepared using DMSO (dimethyl sulfoxide) as a solvent. Each condition (control-solvent mixture conditions) contained 0.0003% DMSO.

Prior embryonic exposure, experimental apparatus units and tanks (1 L in polyethylene terephthalate) were saturated with chlorpyrifos diluted in distilled water for 2 weeks with the studied concentrations for each condition, to ensure saturation of aquaria and avoid dramatic decrease of this compound during exposure. Test solutions were pre-pared by dilution of the stock solutions in a 5 L tank of spring water (dechlorinated) from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, 7.5 pH, <1.2 mg/L Cl^-), oxygenated and renewed every two days. The experimental conditions were designed as follows: condition A (solvent control), condition B (0.1 µg/L of CPF + 0.67 µg/L of Cu + 33.3 µg/L of GLY), condition C (0.3 µg/L of CPF + 2 µg/L of Cu + 100 µg/L of GLY) and condition D (3 µg/L of CPF, 20 µg/L of Cu + 1000 µg/L of GLY). Conditions C (3-fold condition B) and D (10-fold condition C), which agree with the medium and highest concentrations in this study, correspond to the lowest and highest concentrations previously studied [15,28]. Each studied condition consisted of three replicates with 75 embryos in one L aquaria. A peristaltic pump (ISMATEC, ISM942) allowed the maintenance of a continuous flow rate (9 mL/min) of contaminated water from tanks into the incubation aquaria. Dissolved oxygen was measured each day with a fiber-optic mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany) and data was recorded with OxyView v6.02 software (PreSens Precision Sensor).

2.3. Chemical Analysis of Pollutants in Water

Water samples were collected at T_0 (at the moment of exposure), T_{24} and T_{48} (before water was renewed). Water samples were analyzed to determine Cu concentrations. Water samples of 40 mL for each condition were acidified with 5% of nitric acid (Nitric acid 65%, Fluka). Copper concentrations in water of condition "20 µg/L" and fish samples were analyzed by inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent Technologies), whereas copper concentrations in water of controls and "2 µg/L" conditions were analyzed using an atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, CA, USA). Detection limit (DL) for ICP-OES was 2.26 µg/L Cu^{2+} and for atomic absorption was 0.5 µg/L Cu^{2+}. Glyphosate concentration and its main metabolite amino-methyl-phosphonic acid (AMPA) in water samples were analyzed using the method described by [35]. These samples were analyzed using an HPLC-ESI MS (Dionex Ultimate 3000, Thermo Fisher scientific—API 2000 ABSciex). Concentrations were determined with a calibration curve from 1 to 10 µg L^{-1}. To ensure accuracy of each analysis, isotope-labeled surrogates was quantified, derivatization blanks

were analyzed and quality controls were performed every 10 samples during analysis. Finally, data processing was performed with Analyst V1.6.2. CPF concentrations in water samples were measured by chromato-graphic methods using the Trace GC Ultra Gas Chromatograph (Thermo Fisher Scientific) equipped with an AS-3000 Autosampler (Thermo Fisher Scientific) and coupled to a TSQ Quantum GC Triple Quadrupole (Thermo Fisher Scientific). The limit of quantification was 100 ng/L. The control of the device and the data processing were carried out by the XCalibur software.

2.4. Embryo-Toxicity Assay

Embryonic and larval viability was recorded daily, and dead embryos were removed. Embryonic and larval viability denote the number of living individuals compared to the total number of embryos at the start of the experiment or total number of hatching larvae. Hatching rate is calculated by dividing the number of hatched embryos by the total number of embryos at the beginning of the experiment. Duration of development expressed in degree-days (DD) is the duration of embryonic development from fertilization to hatching. At the end of the experiment, 15 larvae per replicate (n = 45) were placed individually in a Petri dish with ice water and a few drops of carbonated water to sedate them. Photos of each larva were taken with stereomicroscope (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG, Schlieren, Switzerland). From the photos, total body length, head length and yolk sac area were measured for each larva. Larvae were also observed for the presence of developmental anomalies including edemas, yolk-sac absorption, spinal malformations, craniofacial anomalies, and presence of hemorrhages [15,36].

2.5. Swimming Behavior Analyses

Swimming behavior analysis was carried out on 10 larvae per replicate (n = 30) at the end of the 3-week exposure. For 30 min, larvae were acclimated in the dark in 6-well microplates containing 3 mL of exposure solution at 12.0 ± 0.5 °C. After acclimation, the microplates were placed in the recording chamber (Daniovision Image Analysis System with Ethovision software version 12.0 Noldus) coupled to a thermoregulatory system set at 12 ± 0.5 °C (Pilot one®, Huber). The DanioVision recording chamber includes a white light that can mimic a day/night cycle. Trout larvae were subjected to a light stress with a cycle duration of 30 min including 10 min dark, 10 min light and 10 min dark. An infrared camera in the recording chamber recorded the motion of each larva in response to light stimulation. The Ethovision software recorded the larval velocity every 30 s. Average velocity, total distance swam and time of mobility were determined for each light and dark phase.

2.6. Comet Assay with FPG Enzyme

Comet assay was performed on blood cells sampled by decapitation of 6 larvae per replicate (n = 18) using a heparinized pipette. Before decapitation, larvae were sedated using iced water and a few drops of carbonated water. Samples were immediately frozen in liquid nitrogen in microtubes with 200 µL of cryo-conservation solution (250 mM sucrose, 40 mM citrate trisodique, 5% DMSO, pH adjusted to 7.6 with nitric acid 1 M) until analysis. To improve the sensibility of the comet as-say, slices were incubated with form-amido-pyrimidine glycosylase (Fpg) enzyme for 30 min at 37 °C, as described by [37]. The comet assay was assessed following the protocol of [36] and [15]. After 20 min of DNA unwind in alkaline solution, electrophoresis was performed with a voltage of 25 V and 300 mA for 20 min. After nuclei separation, slides were stained with 20 µg/mL of ethidium bromide solution, and comet lecture was carried out using an epifluorescence microscope (Olympus BX51) at ×20 equipped with an Olympus U-RFL-T reflected fluorescence system lamp. 100 nuclei per slide were quantified using the Comet Assay IV software (Instrument Perspective LtD). Results are expressed as a percentage of DNA tail.

2.7. Biochemical Analysis

2.7.1. Preparation of Supernatant

Pools of 2 larvae (Three pools per replicate, n = 9) (approximately 250 mg for one pool) were homogenized in 250 µL of phosphate buffer (0.1 M; pH 7.5; 4 °C) using an UltraTurrax® tissue homogenizer with a potter at 9000 g and 4 °C. The supernatant S9 fraction was obtained after centrifugation at 9000× g for 25 min at 4 °C. Each S9 fraction was split up into three tubes for total protein, TBARS and carbonyl protein analysis.

2.7.2. Total Protein

The protein content was determined according to the method described by [38] on the S9 fraction. Measurements were performed using bovine serum albumin as standard, and absorbance was recorded at 750 nm using a spectrophotometer microplate reader (Synergy HT, BioTek).

2.7.3. Lipid Peroxidation (TBARS)

Lipid peroxidation was performed as reported by [39], adjusted for a microplate reader. A volume of 500 µL of a solution containing 20% of butylated hydroxytoluene (BHT) and 20% of trichloroacetic acid (TCA) was added to 500 µL of S9 fraction. The mixture was centrifuged at 9000× g for 10 min. Then, 600 µL of the supernatant was added to 120 µL of HCl and 480 µL of TRISbase (25 mM)-TBA (thio-barbituric acid—100 mM) and heated at 80 °C for 15 min. Afterwards, samples were cooled in iced water and mixed. The absorbance of the mixtures was measured using a UV-spectrophotometer (Synergy HT, BioTek) at 530 nm. Results were expressed as nmol of thio-barbituric acid reactive substance (TBARS) equivalents per mg of protein.

2.7.4. Protein Carbonyl Assay

Protein carbonyl content was performed using the procedure of [40]. S9 fraction (500 µL) was added to 50 µL of a solution of streptomycin sulfate (11%)-phosphate buffer (100 mM pH 7.4), mixed and incubated for 15 min at room temperature. Mixtures were centrifuged at 6000× g for 10 min and then split up into two tubes. The first one was used as a control and contained 200 µL of supernatant and 800 µL of HCl (2.5 M), and the other was used as a sample, where 200 µL of supernatant was added to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 Mm), and then left incubated for 1 h. Proteins were precipitated with 20% TCA (trichloroacetic acid), and the formed pellets were washed 3 times by resuspension with 1 mL of ethanol-ethyl acetate (v:v). Pellets were solubilized with 500 µL of 6 M guanidine hydrochloride and centrifuged at 10,000× g for 10 min. The measure of carbonyl content was performed with a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results are expressed as nanomoles of DNPH incorporated/mg of protein, using the molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

2.8. Gene Expression

At the end of the exposure, 6 larvae per replicate (n = 18) were sampled and kept individually in RNA later buffer (Qiagen). Samples were stored at −80 °C.

2.8.1. RNA Extraction

Total RNAs were extracted in whole larvae using the kit SV Total RNA Isolation system" (Promega) following the indications of the provider. Larvae were mixed and homogenized with the MP fastprep®-24 (Biorad, 6 m/s, 40 s) using ceramic beads (MP Biomedicals, Lysing Matrix D bulk).

2.8.2. Retro-Transcription of Total RNA into cDNA

The reverse transcription of total purified RNA was performed using the kit "GoScript Reverse Transcription System" (Promega). A mixture of 1 µL of oligo dT (1 µM), 1 µL of hexanucleotides (1 µM) and 10 µL of total purified RNA (1 µg) were mixed and heated for

5 min at 70 °C followed by 5 min at 4 °C with a thermocycler (Eppendorf Mastercycler) to allow primer annealing. Afterwards, 1 µL of dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of MgCl2 (25 mM), 1 µL of reverse transcriptase (1 U/µL) and 0.5 µL of RNAsine were added. Reverse transcription was then performed at 42 °C for 1 h. The cDNA samples were kept at −20 °C until analysis by quantitative real-time PCR.

2.8.3. Quantitative Real-Time PCR

The studied primers were designed using Primer3plus soft-ware (Table 1). Each primer-pair was tested and showed an efficiency greater than 95%. Real-time qPCR was performed using GoTaq® qPCR Master Mix kit (Promega). Each reaction mixture was made up of 1 µL of cDNA sample, 2 µL of specific primer pair mix (200 µM each) and 17 µL of a mix consisted of Nuclease-Free Water and GoTaq® qPCR Master containing SyberGreen fluorescent dye. Real-time PCR reactions were carried out in a Mx3000P® qPCR system (Stratagene), and the amplification program was one cycle at 95 °C for 10 min, then 45 amplification cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The specificity of the amplifications was checked using the dissociation curve of the PCR products. The dissociation curve was acquired by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C.

Table 1. Accession number and specific primer pairs for the *Oncorhynchus mykiss* used in our study.

Gene	Accession Number	Primer (5′–3′)
rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA [a] TTATGTCCGTCCCTCTGGGT [b]
cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG [a] GTCCAGGATGGGAAGTTGC [b]
sod Cu/Zn	NM_001124329.1	TGATTGGGGAGATCTCGGGT [a] CGGGTCCAGTGAGAGTCAAC [b]
gst	BT073173.1	ATTTTGGGACGGGCTGACA [a] CCTGGTGCTCTGCTCCAGT [b]
cox1	KP013084.1	TCGTTTGAGCCGTGCTAGTT [a] CTTCTGGGTGGCCGAAGAAT [b]
12S	KY798500.1	GCGCCAGCTTAAAACCCAAA [a] GCCCATTTCTTCCCACCTCA [b]
ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT [a] GTAAGGACCCCATGGCTGTC [b]
rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT [a] GTATTTGAGGGTGGCAGCCT [b]
bax	BT074328.1	CAGAAAACCCAGGGAGGCAT [a] AGAACACATCCTGGGCACAG [b]
mt1	M18104.1	GTGGATCCTGCAAGTGCTCA [a] GTAATGCACCAGGCCTCACT [b]
ache	XM_021577686	AGGAGGGTTCTACAGCGGAT [a] TATCCTGGACCCACTGGAGG [b]

[a] Forward primer [b] Reverse primer.

For each gene, the cycle thresholds (Ct) were obtained from the software MxPro™ qPCR. Two reference genes (*rpl7* and *ef1α*) were used, and the level of gene transcription was normalized with the mean of Ct value of reference genes according to the method of $2^{\Delta\Delta Ct}$ [41]. Induction (>2) or repression (<0.5) factors were determined by the ratio of transcription levels of each condition and the control. The list of studied genes and primer pairs are presented in Table 1.

2.9. Statistics

Each condition was carried out in 3 independent replicates. Results are presented as mean ± SD (standard deviation). Normality of data distribution was verified by the Shapiro-Wilk test ($p < 0.01$), and the homogeneity of variances by the Levene test ($p < 0.05$). When data followed a normal distribution, a one-way ANOVA analysis was used ($p < 0.05$)

followed by the Tukey post-hoc test. If normality was not met, the non-parametric test of Kruskal-Wallis ($p < 0.05$) was used. All statistical analysis was performed using R software.

3. Results

3.1. Condition of Exposure

Concentrations of CPF (chlorpyrifos), GLY (glyphosate) and Cu were analyzed at each water change at T_0, T_{24}, and T_{48} in the different treatments to estimate the losses of the compound (Table 2). To analyze the complexes between Cu-GLY in solution, GLY was treated with a metal complex EDTA (ethylene diamine tetra-acetic acid), and Cu was acidified with HNO_3. In all conditions, the measured concentrations of CPF were inferior to the nominal concentrations at T_0. For the condition B, all CPF concentrations were below the detection limit. For the two other conditions C and D, CPF concentration strongly declined after a few hours due likely to compound sorption to exposure unit. In the case of GLY, measure concentration was superior to the nominal concentrations at T_0, and slight decreases were observed at T_{24} and T_{48}, but always higher than the nominal concentration. For Cu, the measured concentration was below the quantification limit for the B condition but was very close to the targeted concentrations for conditions C and D at T_{24}. In contrast to the two other compounds, Cu concentration increased over time.

Table 2. Nominal and measured concentrations of chlorpyrifos (CPF), copper (Cu) and glyphosate (GLY) for each condition. Concentrations of samples were analyzed at T_0, T_{24} and T_{48}.

	CPF (µg/L) [1]		**Cu^{2+} (µg/L)** [2]		**GLY (µg/L)** [3]		
	Nominal Concentration	**Measured Concentration**	**Nominal Concentration**	**Measured Concentration**	**Nominal Concentration**	**Measured Concentration**	
				Acidified (HNO_3)		No EDTA	EDTA
Condition B	0.1	T_0 < 0.04	0.67	T_0 < 1.1	33.3	T_0 63.3	64.7
		T_{24} < 0.04		T_{24} < 1.1		T_{24} 67.5	49.2
		T_{48} < 0.04		T_{48} < 1.1		T_{48} 55.4	42.2
Condition C	0.3	T_0 0.18	2.0	T_0 2.03	100	T_0 160.0	175.8
		T_{24} < 0.04		T_{24} 4.16		T_{24} 140.0	168.0
		T_{48} < 0.04		T_{48} 4.81		T_{48} 154.0	137.25
Condition D	3.0	T_0 1.63	20.0	T_0 19.63	1000	T_0 1345.0	1785.0
		T_{24} 0.1		T_{24} 26.32		T_{24} 1645.0	2022.5
		T_{48} 0.05		T_{48} 26.65		T_{48} 1775.0	1790.0

[1] Limit of quantification: 0.13 µg/L, limit of detection: 0.04 µg/L. [2] Limit of quantification: 1.1 µg/L, limit of detection: 0.1 µg/L. [3] Limit of quantification: 30, 600 and 6000 ng/L.

3.2. Embryonic and Larval Survival

Dissolved oxygen varied from 84% to 94.6% throughout the embryonic and larval exposure. After 3 weeks exposure to the mixture of pesticides, no significant mortality was observed in embryos and larvae in any of the conditions studied (Table 3). Embryonic and larval survival reached more than 95% in all conditions. Hatchability was high, and more than 90% of embryos succeeded in hatching. No significant differences between conditions were observed for the duration of embryonic development.

3.3. Phenotypic Effects

Biometric measurements included total size, head size and yolk sac area. No significant alterations were observed in biometrics for larvae exposed to mixtures of CPF, Cu and GLY compared to non-exposed larvae (Table 3). Similarly, no significant increases of developmental anomalies were noted on larvae at the end of the three weeks exposure. Percentages of abnormal larvae were 15.0 ± 2.8, 23.7 ± 3.6, 15.0 ± 7.8 and 22.8 ± 11.3% for control and conditions B, C and D, respectively (Table 3).

3.4. Swimming Behavior

Average velocity (mm/s) and mobility (s) of rainbow trout larvae subjected to light stress are presented in Figure 1. Average velocity did not show any significant difference

between conditions, and only a slight increase between conditions A and C (p-value = 0.052) was observed at the basal period (dark 1, Figure 1A). On the other hand, larvae exposed to condition C displayed a significant increase in their mobility compared to control larvae ($p < 0.05$) in both dark periods (dark 1 and 2, Figure 1B). In contrast, no significant differences were observed when larvae were subjected to light stress, due to their high variability.

Table 3. Viability and developmental anomalies of early life stages of rainbow trout exposed to a mixture of three pesticides, copper, glyphosate and chlorpyrifos. Values represent Mean ± SD. No significant differences were observed, n = 3, ANOVA.

	Control A	Condition B	Condition C	Condition D
Acute toxicity				
Embryo viability (%)	98.7 ± 2.3	96.8 ± 3.4	96.4 ± 1.5	94.2 ± 4.1
Larval viability (%)	97.7 ± 2.1	94.5 ± 4.8	96.0 ± 3.2	95.2 ± 4.9
Cumulative viability (%)	96.4 ± 0.8	91.7 ± 7.3	92.6 ± 3.1	89.5 ± 3.5
Hatching rate (%)	95.6 ± 2.0	92.0 ± 4.6	92.0 ± 4.8	90.2 ± 4.1
Sub-lethal toxicity				
Duration of development (DD)	301.1 ± 2.2	294.9 ± 12.2	305.7 ± 1.1	309.9 ± 8.3
Total length (mm)	19.5 ± 0.3	19.6 ± 0.2	19.8 ± 0.2	19.1 ± 0.2
Head length (mm)	4.5 ± 0.0	4.5 ± 0.1	4.6 ± 0.0	4.3 ± 0.2
Ratio of head/body length (%)	23.2 ± 0.1	23.1 ± 0.2	23.1 ± 0.1	22.4 ± 0.7
Area of yolk sac (mm^2)	10.8 ± 0.2	10.1 ± 0.4	10.4 ± 0.5	10.5 ± 0.1
Abnormalities (%)				
Total	15.0 ± 2.8	23.7 ± 3.6	15.0 ± 7.8	22.8 ± 11.3
Oedemas	7.5 ± 8.4	12.7 ± 6.2	9.3 ± 5.6	11.9 ± 6.5
Spinal	7.5 ± 8.4	16.6 ± 11.2	12.9 ± 11.3	16.5 ± 14.8
Craniofacial	1.8 ± 3.2	5.4 ± 5.3	11.3 ± 4.5	14.0 ± 4.5
Haemorrhages	7.6 ± 6.6	0.0 ± 0.0	9.3 ± 5.6	11.9 ± 6.5

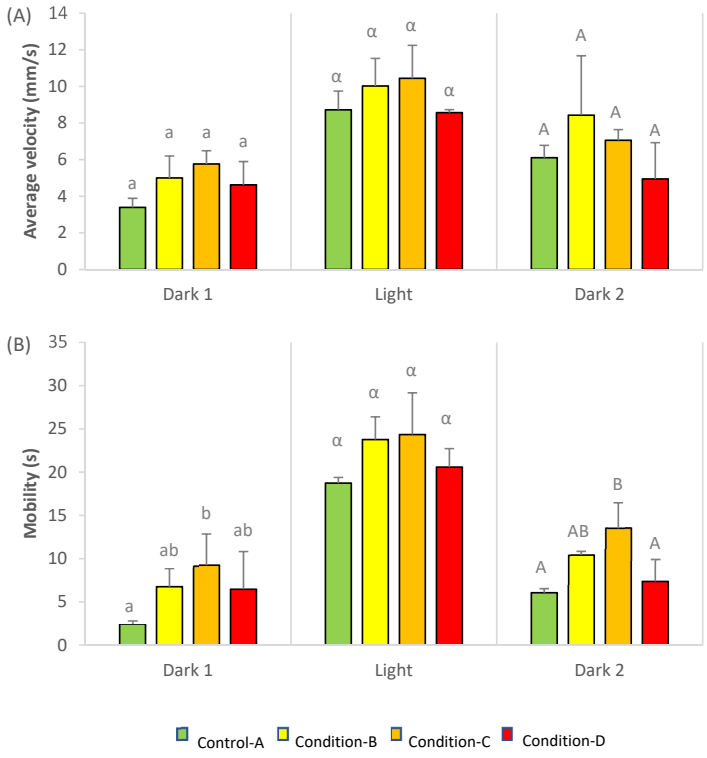

Figure 1. Velocity average (mm/s) (**A**) and cumulative time of mobility (s) (**B**) of rainbow trout larvae following exposure to a mixture of three pesticides, copper, glyphosate and chlorpyrifos. Different letters at the top of the bars indicate significant differences between conditions. Values represent Mean ± SD (n = 3, ANOVA, $p < 0.05$).

3.5. DNA Damage

Results of comet assay from blood cells are presented in Figure 2 with and without treatment by Fpg enzyme prior the alkaline unwinding of DNA. Fpg treatment was used to increase the sensitivity of the test. No significant differences in DNA damage were detected between conditions with the classical comet assay and tail DNA intensity ranging from 3.6 to 6.4%. When nuclei were treated with Fpg enzyme, a three to four times increase of DNA damage was observed in comparison to results without Fpg treatment for the same exposure condition. In addition, DNA damage was significantly increased in larvae from control (16.2 ± 3.0%) to condition D (23.3 ± 1.5%).

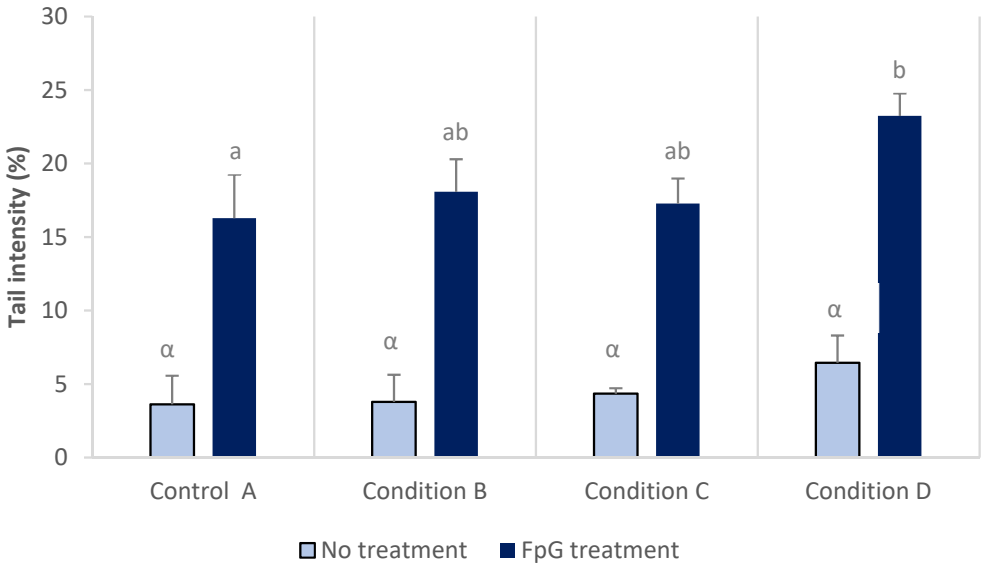

Figure 2. DNA damage in blood cells from rainbow trout larvae after exposure to a mixture of three pesticides (copper, glyphosate and chlorpyrifos), with- and without treatment with the Fpg enzyme. Different letters at the top of the bars indicate significant differences between conditions. Absence of letters indicates no significant differences (Mean ± SD, $n = 3$, ANOVA, $p < 0.05$).

3.6. Lipid Peroxidation and Protein Carbonyls

A significant reduction in TBARS levels was observed on larvae from condition D when compared to control (Figure S1A). However, no significant alterations in protein carbonyl contents were observed in larvae from all conditions when compared to non-exposed larvae (Figure S1B).

3.7. Gene Expression

Gene expression levels from whole larvae of rainbow trout revealed several up-regulated genes following a three weeks exposure to pesticide mixture (Table 4). Mixtures of GLY, Cu and CPF resulted in a significant induction of genes involved in detoxification (*gst* and *mt1*), DNA repair (*ogg1*), mitochondrial metabolism (*cox1* and 12S) and cholinergic system (*ache*). High variability in gene transcription was observed for genes involved in oxidative stress (*cat*, *sod*) and no significant differences were observed, nor was the gene involved in apoptosis (*bax*) regulated. Strong genetic induction for 5 out of 10 genes was observed in larvae from condition D, indicating the likelihood that a higher concentration in the mixtures can lead to more severe toxic effects.

Table 4. Transcription levels of whole larvae of rainbow trout after 3 weeks of exposure to a mixture of copper, glyphosate and chlorpyrifos. Data was expressed as induction (above 2) or repression (below 0.5) factors compared to the control condition. Asterisks (*) refer to significant differences compared to control condition (n = 3, ANOVA, $p < 0.05$). /: identical to control.

Gene	Condition B	Condition C	Condition D
cat	/	/	/
sod	/	/	/
gst	2.5 *	/	/
cox1	5.3 *	3.8 *	23.8 *
12s	4.9 *	5.2 *	32.4 *
ogg1	4.4 *	6.3 *	34.8 *
rad51	/	/	/
bax	/	/	/
mt1	9.4 *	/	12.7 *
ache	4.5 *	7.0 *	36.7 *

4. Discussion

Numerous reports have shown that most aquatic ecosystems located close to farmland are contaminated with diverse kinds of pesticide, at different concentrations, which can have an impact on aquatic biota [6,9,42]. Commercial pesticide formulations including copper (Cu), chlorpyrifos (CPF) and glyphosate (GLY) are widely used for crop treatments, and are frequently detected in wetlands and streams, usually transported by runoff [11,17,19,43,44]. The interaction between these molecules can lead to changes in their overall toxicity as a function of the synergistic and/or antagonistic effects [5]. Each studied compound has a different mode of action, and its interaction may imply an induction or an inhibition of a specific metabolic pathway [5].

Our results show no significant mortality of embryos and larvae of rainbow trout exposed to low, moderate and highly concentrated mixtures of Cu, CPF and GLY. In previous studies performed with individual pesticides, no mortality was observed in early life stages of rainbow trout exposed to GLY [28] and CPF [34]. However, significant embryonic mortality (about 10%) and a high frequency of half-hatched embryos (25%) have previously been observed for embryos exposed to 20 µg/L of Cu [15]. In this study, the addition of CPF and GLY seems to inhibit the toxicity of Cu for embryos and larvae of rainbow trout. This result could be explained by possible interactions between compounds. Indeed, GLY is known to be a strong chelator of heavy metals such as Cu [45,46]. The functional groups of glyphosate (amine, carboxylate and phosphate) can react with metal ions to form complexes, resulting in a decreased bio-availability of Cu [47,48]. Our findings are consistent with similar studies with freshwater cladocera (*Ceriodaphnia dubia*) and earthworms (*Eisenia fetida*). Indeed, neonates of *C. dubia* were exposed to seven heavy metals, both alone and in binary mixtures with Roundup®, and most of the metals displayed less than additive toxicity [48]. In the same study, LC50-48h for *C. dubia* was estimated at 10 µg/L of Cu^{2+}, but in the presence of GLY mortality was reduced to 95%. GLY could also affect the bioavailability of metals: for instance, it favors uptake of Hg and decreases uptake of Ag [48]. Zhou [47] tested the interactions between Cu and GLY on the toxicity of earthworm (*Eisenia fetida*). Acute toxicity of Cu for *E. fetida* was calculated at 0.11 mg/L (LC50-48h), but when it was combined with GLY at concentrations of 0, 2 and 10 mg/L, worm mortalities declined from 57%, to 3% and 0%, respectively. The authors observed that the free Cu^{2+} was reduced with the presence of GLY, preventing the accumulation of Cu in worms.

For the swimming behavior of rainbow trout larvae exposed to single pesticides, we observed that GLY (100 µg/L) induced hyperactivity of larvae, increasing their velocity and mobility under light stimulation [28]. Similar observations were obtained by [24,49,50] in different fish species. On the other hand, larvae exposed to CPF (3 µg/L) were significantly less mobile than non-exposed larvae [34]. Comparable results were observed by [51]

and [52], usually related to a decreased of acetylcholinesterase (AChE) activity [53–55]. In this study, rainbow trout larvae exposed to the mixture of pesticides at low and medium concentrations were significantly more mobile than control larvae. Mobility of larvae exposed to high concentrations of pesticide did not significantly differ from controls, and no differences were observed after light stimulation. Bonifacio [56] exposed adult females of ten spotted livebearer fish (*Cnesterodon decemmaculatus*) for 6 weeks at concentrations of CPF (0.1 to 1 μg/L) and GLY (0.2 to 2 mg/L). They observed that fish exposed only to CPF had reduced swimming activity, but no differences were observed for fish exposed to GLY and binary mixtures. They hypothesized that GLY could decrease the effect of CPF on swimming activity. In another study, the addition of Cu also diminished the impact of CPF on swimming behavior of adult zebrafish (*Danio rerio*) exposed to a mixture of Cu (6.3 to 40 μg/L) and CPF (35 to 220 μg/L) for 24 h [57]. Indeed, decreased swimming activity was observed in zebrafish exposed to the highest concentration of CPF, suggesting that the addition of Cu may block the biochemical and neurological impacts of CPF or modify its uptake by the neuron. Responses in terms of both hyperactivity and hypoactivity were observed on females of *Jenynsia multidentata* exposed for 24 and 96 h to a binary mixture of CPF (0.4 and 4 μg/L) and cypermethrin (0.04 and 0.4 μg/L) [53]. Low mixture concentrations significantly increased their swimming activity in the upper area of the aquaria, and higher mixture concentrations reduced their swimming activity and caused them to favor the bottom of the tank [53]. Alterations of normal behavior may also depend on the ratio and concentrations of pollutants that are present in a mixture. For example, Kienle [51] exposed zebrafish embryos for 11 days to mixtures of CPF, at 0.25 and 1 mg/L, and nickel chloride ($NiCl_2$), at 7.5 and 15 mg/L. When fish were exposed to individual compounds, opposing behaviors were observed, i.e., CPF increased their swimming activity while nickel decreased it. Interestingly, when nickel was combined with low concentrations of CPF, the swimming activity of larvae also decreased; but, combined with higher concentrations of CPF, larvae increased their activity. Kienle [51] argued that both compounds have different modes of action, and in a mixture they may act independently of each other. We can therefore suppose that the hyperactivity observed in our larvae may be an effect caused mostly by the dominance of GLY, in accordance with our previous results [28]. However, since there was an absence of significant response to light change for larvae exposed to mixtures of pesticides, we can assume that the presence of Cu and CPF antagonized or reduced the effects of GLY on larvae. Furthermore, numerous studies have documented the relationship between AChE activity and behavioral changes in fish [54,58,59]. In our work, we observed that ache gene expression was up-regulated in all conditions compared to control, suggesting that alterations observed on behavior of larvae could be related to this induction. Indeed, several pesticides, such as chlorpyrifos and glyphosate, have the capacity to inhibit the AChE activity [23,60]. This inhibition may cause acetylcholine accumulation in synapses of cholinergic neurons, leading to deficiency of important functions such as swimming and feeding [61,62].

When embryos of rainbow trout were exposed separately to Cu, GLY and CPF, no significant increase in DNA damage on blood cells was observed after 3-week exposure compared to non-exposed larvae [15,28,34]. Blood cells from larvae exposed individually to GLY and CPF were also treated with the Fpg enzyme (forma-mido-pyrimidine DNA glycosylase), but no significant changes were observed. Fpg is a DNA-based excision repair enzyme that makes it possible to detect and remove lesions related to basic sites (apuric or apyrimidic), alkylation and oxidative damage (8-oxoguanine) induced by Reactive Oxygen Species (ROS) [37]. In this study, the addition of Fpg enzyme reveals additional DNA damage, in particular for larvae exposed to the highest mixture concentration (condition D). DNA damage has previously been detected by the comet assay in various aquatic organisms exposed individually to Cu, GLY and CPF using environmental concentrations [20,21,63,64]. Few studies have examined the genotoxicity effects of mixtures of pesticides on fish. DNA damage was only observed in hemocytes of the freshwater clam *Corbicula fluminea* exposed to a herbicide mixture of Roundup® (2 and 10 mg/L) and

atrazine (2 and 10 µg/L) for 96 h, but no genotoxic effects were observed when clams were exposed to the herbicides separately using the same concentrations [65]. In another study, a mixture of endo-sulfan and CPF (0.94 to 1.88 µg/L) also caused significant DNA damage to erythrocytes of fingerlings of Nile tilapia *Oreochromis niloticus*, after a 70-days exposure [66]. The genotoxicity of mixtures of metals has also been studied using the micronucleus assay on erythrocytes of fish, *Synodontis clarias* and *Tilapia nitolica*, exposed to Cu and zinc (Zn) [67]. In this case, micronuclei frequency was significantly increased when fish were exposed to binary mixtures, compared with exposure to individual metals. No significant protein carbonyl content was observed in exposed larvae when compared to control group. However, TBARS levels from larvae exposed to condition D were significantly lower than control ones. Exposure to individual compounds showed a significant reduction in TBARS levels in larvae exposed to 0.1 mg/L of GLY [28], while Cu and CPF alone did not modify TBARS [15,34]. As we hypothesized previously, the absence of increased TBARS levels and protein carbonyls may be the result of an efficient anti-oxidant system, serving to defend against oxidative stress as observed by [27,68,69] in piava (*Leporinus obtusidens*), freshwater catfish (*Channa punctatus*) and *Anguilla anguilla*, respectively.

With regard to gene expression levels, 3-week exposure to all conditions was mainly associated with the up-regulation in the genes investigated, with similar patterns observed in each of the studied conditions. The exposure to the mixture of the three compounds led to the up-regulation of genes involved notably in detoxification, mitochondrial metabolism and DNA repair. Cu, GLY and CPF are well known to produce individually reactive oxygen species (ROS), and the enzymes catalase (CAT) and superoxide dismutases (SOD) protect the cells from oxidative damage caused by ROS. In our study, no significant regulation of cat and cytoplasmic *sodCu/Zn* genes were observed on exposed larvae because of their elevated variability. However, many studies have shown evidence of the capability of these compounds to induce antioxidant gene expression [62,70–72]. ROS neutralization could also be completed by mitochondrial *sodMn* or *gpx* (Glutathione peroxidase) via glutathione oxidation, but these two genes were not investigated in our study. Genes involved in detoxification, *gst* and *mt1*, were induced, especially in larvae exposed to the lowest and the highest mixture concentrations. Expression of *gst* (glutathione s-transferase) is usually up-regulated when fish are exposed to xenobiotics. In our study, *gst* expression was significantly increased in larvae exposed to the lowest mixture concentration (condition B), and this could indicate that larvae were able to implement defense mechanisms against low concentrations of pesticides. GST proteins play an important role in detoxifying xenobiotic compounds by catalyzing the conjugation of reduced glutathione (GSH) on primary metabolites from phase I metabolism [73]. *Mt1* gene encodes the metallothionein proteins, usually induced by metal exposure, such as to copper, but it has also an antioxidant role [74]. Metallothionein proteins have the capacity to bind xenobiotic heavy metals through their thiol groups, providing protection against metal toxicity and oxidative stress [74]. In a previous study using Cu [15], rainbow trout larvae showed a down-regulation of *mt1*, *mt2* and *gst* genes after 3-weeks exposure. However, no significant changes of *gst* gene expression were observed on larvae exposed individually to GLY and CPF [28,34]. Therefore, we can presume that the induction of these genes is the consequence of an additive effect over these functions on larvae exposed to the mixture of pesticides. In our study, a significant induction of *cox1* (cytochrome c-oxidase subunit 1) and 12S gene expression was observed in all conditions compared to control larvae. These up-regulations were even stronger in larvae exposed to the highest mixture concentrations (condition D). An induction of these genes could reveal an increased mitochondrial number per cell and a possible mitochondrial electron transport chain disruption from the lowest mixture concentration tested. The mitochondria is involved in the production of energy for cellular metabolism by synthesizing ATP (adenosine triphosphate). Since the mitochondria is a major source of ROS (superoxide and hydrogen peroxide) through the disruption of the electron-transport chain [75], the up-regulation of *cox1* could denote an increased demand for energy to fight the effects of toxicants. Furthermore, the putative increase

of mitochondria could also stimulate ROS production [75,76]. The observed induction of *cox1* and *12S* genes could mean a high-energy demand, in the form of ATP, to defend cells against ROS production or to repair cellular damage by pesticides. This demand of energy may even be required for functions that we have not considered in this study. We also observed, in all conditions, an induction of DNA repair gene expression, ogg1, which is responsible for the removal of 8-oxoguanine as a result of ROS production. The over-expression of *ogg1* (X34.8) on larvae exposed to the highest mixture concentration is probably related to the significant induction of DNA damage that we observed on blood cells after treatment with Fpg enzyme. Consequently, DNA lesions observed in blood cells were likely to be related to oxidative damage. The up-regulation of DNA repair (*ogg1*) and detoxification (*gst* and *mt1*) gene expression may indicate that defense mechanisms were effectively implemented to avoid the effects of ROS production in cells and DNA. This could relate to high demand for energy, with up-regulation observed for 12S and *cox1* gene. However, to validate this relationship, future research could focus on the expression of the mitochondrial superoxide dismutase gene (*sodMn*), which transforms the superoxide anion into hydrogen peroxide, and the expression of glutathione peroxidase (*gpx*), which detoxifies the hydrogen peroxide into water.

It was not clear whether the observed gene regulation in larvae was a consequence of a toxic effect or an adaptation effect. However, since no significant toxic effects were observed on exposed larvae (e.g., mortality, lipid/protein oxidation), we considered that the significant gene up-regulation was mostly an adaptive effect triggered to deal with toxic exposure. At higher mixture concentrations, energy demand increased and the DNA repair, mt1 and ache gene expression greatly increase. This indicates a defense mechanisms induction to prevent toxic effects resulting from increased exposure to chemicals. When comparing the pattern of gene expression from the present work with previous work done with individual exposure to Cu, GLY and CPF [15,28,34], we could consider that the joint action of the three pollutants had an additive or a synergistic effects since the observed effects of the mixture were stronger than the isolated substances.

5. Conclusions

Sub-chronic exposure to a mixture of copper, glyphosate and chlorpyrifos on rainbow trout embryos can induce hyperactivity and DNA damage at the higher tested concentrations. In addition, several genes were up-regulated, especially those involved in detoxification, mitochondrial metabolism, cholinergic system and DNA repair. The up-regulation of *gst*, *ogg1*, *mt1*, *cox1* and *12S* gene expression suggests that exposure to the mixture of the three pesticides at realistic environmental concentrations promotes cellular defense mechanisms, indicating the induction of adaptive responses which could limit the occurrence of more severe effects. Our results provide new information about the spectrum of effects and the mechanisms involved in cellular response of rainbow trout embryos exposed to an environmentally relevant mixture of pesticides.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxics9080174/s1, Figure S1: Content of lipid peroxidation expressed as nanomoles of TBARS/mg of protein (A), and content of protein carbonyl expressed as nanomoles of carbonyl/mg of protein (B) in whole body of rainbow trout larvae exposed to a mixture of thee pesticides, copper, glyphosate and chlorpyrifos.

Author Contributions: Conceptualization, J.C. and P.G.; methodology, N.M., P.-Y.G., C.C., B.M.; validation, J.C., N.M., P.-Y.G., B.M., P.G.; formal analysis, S.W.S., B.C., N.M., P.-Y.G.; investigation, S.W.S. and B.C.; resources, C.C.; data curation, S.W.S., J.C., N.M., P.-Y.G., B.M., P.G.; writing—original draft preparation, S.W.S.; writing—review and editing, J.C. and P.G.; visualization, S.W.S., J.C. and P.G.; supervision, J.C. and P.G.; project administration, J.C.; funding acquisition, J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant from SENACYT (Secretaría Nacional de Ciencia y Tecnología e Innovación) as a part of a PhD program.

Institutional Review Board Statement: Not applicable since this study involved experiments on yolk sac stage of rainbow trout.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data can be requested from the corresponding author.

Acknowledgments: The authors would like to thank the INRAE PEIMA at Sizun for suppling rainbow trout embryos and, Misters Lionel Gordon and Laurent Labbe for their technical advice on the culture of embryos and larvae. This work benefitted from the French GDR "Aquatic Ecotoxicology" framework which aims at fostering stimulating scientific discussions and collaborations for more integrative approaches. Authors would like to thank James Emery for providing English proofreading services.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sánchez-Bayo, F. Chapter 4: Impacts of agricultural pesticides on terrestrial ecosystems. *Ecolofical Impacts Toxic Chem.* **2011**, 63–87. [CrossRef]
2. Stone, W.W.; Gilliom, R.J.; Ryberg, K.R. Pesticides in U.S. streams and rivers: Occurrence and trends during 1992–2011. *Environ. Sci. Technol.* **2014**, *48*, 11025–11030. [CrossRef]
3. Zhang, W.; Jiang, F.; Ou, J. Global pesticide consumption and pollution: With China as a focus. *Proc. Int. Acad. Ecol. Environ. Sci.* **2011**, *1*, 125–144. [CrossRef]
4. Ippolito, A.; Kattwinkel, M.; Rasmussen, J.J.; Schäfer, R.B.; Fornaroli, R.; Liess, M. Modeling global distribution of agricultural insecticides in surface waters. *Environ. Pollut.* **2015**, *198*, 54–60. [CrossRef]
5. Krishnan, K.; Brodeur, J. Toxic interactions among environmental pollutants: Corroborating laboratory observations with human experience. *Environ. Health Perspect* **1994**, *102*, 11–17. [CrossRef]
6. Relyea, R.A. A cocktail of contaminants: How mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* **2009**, *159*, 363–376. [CrossRef]
7. Relyea, R.; Hoverman, J. Assessing the ecology in ecotoxicology: A review and synthesis in freshwater systems. *Ecol. Lett.* **2006**, *9*, 1157–1171. [CrossRef]
8. Schäfer, R.B.; Von Der Ohe, P.C.; Rasmussen, J.; Ke, B.J.; Beketov, M.A.; Schulz, R.; Liess, M. Thresholds for the effects of Pesticides on Invertebrate Communities and Leaf Breakdown in Stream Ecosystems. *Environ. Sci. Technol.* **2012**, *46*, 5134–5142. [CrossRef]
9. Beyer, J.; Petersen, K.; Song, Y.; Ruus, A.; Grung, M.; Bakke, T.; Tollefsen, K.E. Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. *Mar. Environ. Res.* **2014**, *96*, 81–91. [CrossRef]
10. WFD Water Framework Directive. Available online: http://ec.europa.eu/environment/water/water-framework/index_en.html (accessed on 23 October 2000).
11. Devez, A.; Gomez, E.; Gilbin, R.; Elbaz-Poulichet, F.; Persin, F.; Andrieux, P.; Casellas, C. Assessment of copper bioavailability and toxicity in vineyard runoff waters by DPASV and algal bioassay. *Sci. Total Environ.* **2005**, *348*, 82–92. [CrossRef]
12. Ruyters, S.; Salaets, P.; Oorts, K.; Smolders, E. Copper toxicity in soils under established vineyards in Europe: A survey. *Sci. Total Environ.* **2013**, *443*, 470–477. [CrossRef]
13. Manzl, C.; Enrich, J.; Ebner, H.; Dallinger, R.; Krumschnabel, G. Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. *Toxicology* **2004**, *196*, 57–64. [CrossRef]
14. Prousek, J. Fenton chemistry in biology and medicine. *Pure Appl. Chem.* **2007**, *79*, 2325–2338. [CrossRef]
15. Weeks Santos, S.; Cachot, J.; Gourves, P.Y.; Clérandeau, C.; Morin, B.; Gonzalez, P. Sub-lethal effects of waterborne copper in early developmental stages of rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicol. Environ. Saf.* **2019**, *170*, 778–788. [CrossRef]
16. Van Bruggen, A.H.C.; He, M.M.; Shin, K.; Mai, V.; Jeong, K.C.; Finckh, M.R.; Morris, J.G. Environmental and health effects of the herbicide glyphosate. *Sci. Total Environ.* **2018**, *616–617*, 255–268. [CrossRef]
17. Aparicio, V.C.; De Gerónimo, E.; Marino, D.; Primost, J.; Carriquiriborde, P.; Costa, J.L. Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. *Chemosphere* **2013**, *93*, 1866–1873. [CrossRef]
18. Peruzzo, P.J.; Porta, A.A.; Ronco, A.E. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environ. Pollut.* **2008**, *156*, 61–66. [CrossRef]
19. Coupe, R.H.; Kalkhoff, S.J.; Capel, P.D.; Gregoire, C. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. *Pest. Manag. Sci.* **2012**, *68*, 16–30. [CrossRef]
20. Çavaş, T.; Könen, S. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis* **2007**, *22*, 263–268. [CrossRef]
21. Guilherme, S.; Gaivão, I.; Santos, M.A.; Pacheco, M. European eel (*Anguilla anguilla*) genotoxic and pro-oxidant responses following short-term exposure to Roundup®—A glyphosate-based herbicide. *Mutagenesis* **2010**, *25*, 523–530. [CrossRef]

22. Guilherme, S.; Santos, M.A.; Barroso, C.; Gaivão, I.; Pacheco, M. Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): Considerations on chemical interactions and DNA damaging mechanisms. *Ecotoxicology* **2012**, *21*, 1381–1390. [CrossRef]
23. Salbego, J.; Pretto, A.; Gioda, C.R.; De Menezes, C.C.; Lazzari, R.; Radünz Neto, J.; Baldisserotto, B.; Loro, V.L. Herbicide formulation with glyphosate affects growth, acetylcholinesterase activity, and metabolic and hematological parameters in Piava (*Leporinus obtusidens*). *Arch. Environ. Contam. Toxicol.* **2010**, *58*, 740–745. [CrossRef]
24. Sinhorin, V.D.G.; Sinhorin, A.P.; Teixeira, J.M.S.; Miléski, K.M.L.; Hansen, P.C.; Moeller, P.R.; Moreira, P.S.A.; Baviera, A.M.; Loro, V.L. Metabolic and Behavior Changes in Surubim Acutely Exposed to a Glyphosate-Based Herbicide. *Arch. Environ. Contam Toxicol.* **2014**, 659–667. [CrossRef]
25. Bridi, D.; Altenhofen, S.; Gonzalez, J.B.; Reolon, G.K.; Bonan, C.D. Glyphosate and Roundup® alter morphology and behavior in zebrafish. *Toxicology* **2017**, *392*, 32–39. [CrossRef]
26. de Moura, F.R.; Brentegani, K.R.; Gemelli, A.; Sinhorin, A.P.; Sinhorin, V.D.G. Oxidative stress in the hybrid fish jundiara (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) exposed to Roundup Original®. *Chemosphere* **2017**, *185*, 445–451. [CrossRef]
27. Glusczak, L.; Loro, V.L.; Pretto, A.; Moraes, B.S.; Raabe, A.; Duarte, M.F.; Da Fonseca, M.B.; De Menezes, C.C.; De Sousa Valladão, D.M. Acute exposure to glyphosate herbicide affects oxidative parameters in piava (*Leporinus obtusidens*). *Arch. Environ. Contam. Toxicol.* **2011**, *61*, 624–630. [CrossRef]
28. Weeks Santos, S.; Gonzalez, P.; Cormier, B.; Mazzella, N.; Bonnaud, B.; Morin, S.; Clérandeau, C.; Morin, B.; Cachot, J. A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, *Oncorhynchus mykiss*. *Aquat. Toxicol.* **2019**, *216*, 105291. [CrossRef]
29. Marino, D.; Ronco, A. Cypermethrin and Chlorpyrifos Concentration Levels in Surface Water Bodies of the Pampa Ondulada, Argentina. *Bull. Environ. Contam. Toxicol.* **2005**, *75*, 820–826. [CrossRef]
30. Jergentz, S.; Mugni, H.; Bonetto, C.; Schulz, R. Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. *Chemosphere* **2005**, *61*, 817–826. [CrossRef]
31. Barron, M.G.; Plakas, S.M.; Wilga, P.C.; Ball, T. Absorption, tissue distribution and metabolism of chlorpyrifos in channel catfish following waterborne exposure. *Environ. Toxicol. Chem.* **1993**, *12*, 1469–1476. [CrossRef]
32. Fulton, M.H.; Key, P.B. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ. Toxicol. Chem.* **2001**, *20*, 37–45. [CrossRef]
33. Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol.* **2003**, *13*, 57–149. [CrossRef]
34. Weeks Santos, S.; Gonzalez, P.; Cormier, B.; Mazzella, N.; Moreira, A.; Clérandeau, C.; Morin, B.; Cachot, J. Sub-lethal exposure to environmental concentrations of chlorpyrifos affect swimming activity of rainbow trout larvae. *Environ. Toxicol. Chem. Rev.* **2021**, In Press.
35. Fauvelle, V.; Nhu-Trang, T.T.; Feret, T.; Madarassou, K.; Randon, J.; Mazzella, N. Evaluation of Titanium Dioxide as a Binding Phase for the Passive Sampling of Glyphosate and Aminomethyl Phosphonic Acid in an Aquatic Environment. *Anal. Chem.* **2015**, *87*, 6004–6009. [CrossRef]
36. Le Bihanic, F.; Morin, B.; Cousin, X.; Le Menach, K.; Budzinski, H.; Cachot, J. Developmental toxicity of PAH mixtures in fish early life stages. Part I: Adverse effects in rainbow trout. *Environ. Sci. Pollut. Res.* **2014**, *21*, 13720–13731. [CrossRef]
37. Kienzler, A.; Tronchère, X.; Devaux, A.; Bony, S. Assessment of RTG-W1, RTL-W1, and PLHC-1 fish cell lines for genotoxicity testing of environmental pollutants by means of a Fpg-modified comet assay. *Toxicol. Vitr.* **2012**, *26*, 500–510. [CrossRef]
38. Lowry, O.; Rosebrough, N.; Farr, L.; Randall, R. Protein measurement with the folin phenol reagent. *Readings* **1951**, *193*, 265–275. [CrossRef]
39. Buege, J.A.; Aust, S.D. Microsomal Lipid Peroxidation. *Methods Enzymol.* **1978**, *52*, 302–310. [CrossRef] [PubMed]
40. Augustyniak, E.; Adam, A.; Wojdyla, K.; Rogowska-Wrzesinska, A.; Willetts, R.; Korkmaz, A.; Atalay, M.; Weber, D.; Grune, T.; Borsa, C.; et al. Validation of protein carbonyl measurement: A multi-centre study. *Redox Biol.* **2015**, *4*, 149–157. [CrossRef]
41. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
42. Vidal-Dorsch, D.E.; Bay, S.M.; Mays, M.A.; Greenstein, D.J.; Young, D.; Wolf, J.; Pham, D.; Loguinov, A.V.; Vulpe, C. Using Gene Expression to Assess the Status of Fish from Anthropogenically Influenced Estuarine Wetlands. *Environ. Sci. Technol.* **2011**, *46*, 69–77. [CrossRef]
43. Davis, A.P.; Shokouhian, M.; Ni, S. Loading estimates of lead, copper, cadmium, and zinc in urban runoff from specific sources. *Chemosphere* **2001**, *44*, 997–1009. [CrossRef]
44. Phillips, P.J.; Bode, R.W. Pesticides in surface water runoff in south-eastern New York State, USA: Seasonal and stormflow effects on concentrations. *Pest. Manag. Sci.* **2004**, *60*, 531–543. [CrossRef]
45. Subramaniam, V.; Hoggard, P.E. Metal Complexes of Glyphosate. *J. Agric. Food Chem.* **1988**, *36*, 1326–1329. [CrossRef]
46. Glass, R.L. Metal Complex Formation by Glyphosate. *J. Agric. Food Chem.* **1984**, *32*, 1249–1253. [CrossRef]
47. Zhou, C.F.; Wang, Y.J.; Yu, Y.C.; Sun, R.J.; Zhu, X.D.; Zhang, H.L.; Zhou, D.M. Does glyphosate impact on Cu uptake by, and toxicity to, the earthworm Eisenia fetida. *Ecotoxicology* **2012**, *21*, 2297–2305. [CrossRef]
48. Tsui, M.T.K.; Wang, W.X.; Chu, L.M. Influence of glyphosate and its formulation (Roundup®) on the toxicity and bioavailability of metals to *Ceriodaphnia dubia*. *Environ. Pollut.* **2005**, *138*, 59–68. [CrossRef] [PubMed]

49. Ayoola, S.O. Toxicity of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*) juvenile. *Afr. J. Agric. Res.* **2008**, *3*, 825–834.
50. Zhang, S.; Xu, J.; Kuang, X.; Li, S.; Li, X.; Chen, D.; Zhao, X.; Feng, X. Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*). *Chemosphere* **2017**, *181*, 270–280. [CrossRef]
51. Kienle, C.; Köhler, H.R.; Gerhardt, A. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 1740–1747. [CrossRef]
52. Richendrfer, H.; Pelkowski, S.D.; Colwill, R.M.; Créton, R. Developmental sub-chronic exposure to chlorpyrifos reduces anxiety-related behavior in zebrafish larvae. *Neurotoxicol. Teratol.* **2012**, *34*, 458–465. [CrossRef]
53. Bonansea, R.I.; Wunderlin, D.A.; Amé, M.V. Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures. *Ecotoxicol. Environ. Saf.* **2016**, *129*, 311–319. [CrossRef] [PubMed]
54. Sandahl, J.F.; Baldwin, D.H.; Jenkins, J.J.; Scholz, N.L. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. *Environ. Toxicol. Chem.* **2005**, *24*, 136–145. [CrossRef] [PubMed]
55. Yen, J.; Donerly, S.; Levin, E.D.; Linney, E.A. Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. *Neurotoxicol. Teratol.* **2011**, *33*, 735–741. [CrossRef]
56. Bonifacio, A.F.; Cazenave, J.; Bacchetta, C.; Ballesteros, M.L.; Bistoni, M.D.L.A.; Amé, M.V.; Bertrand, L.; Hued, A.C. Alterations in the general condition, biochemical parameters and locomotor activity in *Cnesterodon decemmaculatus* exposed to commercial formulations of chlorpyrifos, glyphosate and their mixtures. *Ecol. Indic.* **2016**, *67*, 88–97. [CrossRef]
57. Tilton, F.A.; Tilton, S.C.; Bammler, T.K.; Beyer, R.P.; Stapleton, P.L.; Scholz, N.L.; Gallagher, E.P. Transcriptional impact of organophosphate and metal mixtures on olfaction: Copper dominates the chlorpyrifos-induced response in adult zebrafish. *Aquat. Toxicol.* **2011**, *102*, 205–215. [CrossRef]
58. Faria, M.; Garcia-Reyero, N.; Padrós, F.; Babin, P.J.; Sebastián, D.; Cachot, J.; Prats, E.; Arick, M.; Rial, E.; Knoll-Gellida, A.; et al. Zebrafish Models for Human Acute Organophosphorus Poisoning. *Sci. Rep.* **2015**, *5*, 15591. [CrossRef]
59. Kavitha, P.; Venkateswara Rao, J. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, Gambusia affinis. *Environ. Toxicol. Pharmacol.* **2008**, *26*, 192–198. [CrossRef] [PubMed]
60. Rodríguez-Fuentes, G.; Rubio-Escalante, F.J.; Noreña-Barroso, E.; Escalante-Herrera, K.S.; Schlenk, D. Impacts of oxidative stress on acetylcholinesterase transcription, and activity in embryos of zebra fish (*Danio rerio*) following Chlorpyrifos exposure. *Comp. Biochem. Physiol.* **2015**, *172–173*, 19–25. [CrossRef]
61. Glusczak, L.; Dos Santos Miron, D.; Moraes Silveira, B.; Rodrigues Simões, R.; Chitolina Schetinger, M.R.; Morsch, V.M.; Loro, V.L. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comp. Biochem. Physiol. Toxicol. Pharmacol.* **2007**, *146*, 519–524. [CrossRef]
62. Topal, A.; Atamanalp, M.; Uçar, A.; Oruç, E.; Kocaman, E.M.; Sulukan, E.; Akdemir, F.; Beydemir, Ş.; Kilinç, N.; Erdoğan, O. Effects of glyphosate on juvenile rainbow trout (*Oncorhynchus mykiss*): Transcriptional and enzymatic analyses of antioxidant defence system, histopathological liver damage and swimming performance. *Ecotoxicol. Environ. Saf.* **2015**, *111*, 206–214. [CrossRef]
63. Guecheva, T.; Henriques, J.A.P.; Erdtmann, B. Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay). *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2001**, *497*, 19–27. [CrossRef]
64. Ali, D.; Nagpure, N.S.; Kumar, S.; Kumar, R.; Kushwaha, B.; Lakra, W.S. Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food Chem. Toxicol.* **2009**, *47*, 650–656. [CrossRef]
65. dos Santos, K.C.; Martinez, C.B.R. Genotoxic and biochemical effects of atrazine and Roundup®, alone and in combination, on the Asian clam *Corbicula fluminea*. *Ecotoxicol. Environ. Saf.* **2014**, *100*, 7–14. [CrossRef]
66. Ambreen, F.; Javed, M. Pesticide mixture induced DNA damage in peripheral blood erythrocytes of freshwater fish, *Oreochromis niloticus*. *Pak. J. Zool.* **2018**, *50*, 339–346. [CrossRef]
67. Obiakor, M.O.; Okonkwo, J.C.; Ezeonyejiaku, C.D.; Ezenwelu, C.O. Genotoxicology: Single and Joint Action of Copper and Zinc to *Synodontis clarias* and Tilapia nilotica. *J. Appl. Sci. Environ. Manag.* **2010**, *14*, 59–64. [CrossRef]
68. Ahmad, I.; Hamid, T.; Fatima, M.; Chand, H.S.; Jain, S.K.; Athar, M.; Raisuddin, S. Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. *Biochim. Biophys.* **2000**, *1523*, 37–48. [CrossRef]
69. Marques, A.; Guilherme, S.; Gaivão, I.; Santos, M.A.; Pacheco, M. Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods—Insights into the mechanisms of genotoxicity and DNA repair. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* **2014**, *166*, 126–133. [CrossRef] [PubMed]
70. Woo, S.; Yum, S.; Park, H.S.; Lee, T.K.; Ryu, J.C. Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oryzias javanicus*). *Comp. Biochem. Physiol. Toxicol. Pharmacol.* **2009**, *149*, 289–299. [CrossRef] [PubMed]
71. Casanova, F.M.; Honda, R.T.; Ferreira-nozawa, M.S. Effects of Copper and Cadmium Exposure on mRNA Expression of Catalase, Glutamine Synthetase, Cytochrome P450 and Heat Shock Protein 70 in Tambaqui Fish (*Colossoma macropomum*). *Gene Expr. Genet. Genom.* **2013**, *6*, 1. [CrossRef]
72. Benedetto, A.; Brizio, P.; Squadrone, S.; Scanzio, T.; Righetti, M.; Gasco, L.; Prearo, M.; Abete, M.C. Oxidative stress related to chlorpyrifos exposure in rainbow trout: Acute and medium term effects on genetic biomarkers. *Pestic. Biochem. Physiol.* **2016**, *129*, 63–69. [CrossRef] [PubMed]
73. Mukhopadhyay, D.; Chattopadhyay, A. Induction of oxidative stress and related transcriptional effects of sodium fluoride in female zebrafish liver. *Bull. Environ. Contam. Toxicol.* **2014**, *93*, 64–70. [CrossRef] [PubMed]

74. Ruttkay-Nedecky, B.; Nejdl, L.; Gumulec, J.; Zitka, O.; Masarik, M.; Eckschlager, T.; Stiborova, M.; Adam, V.; Kizek, R. The role of metallothionein in oxidative stress. *Int. J. Mol. Sci.* **2013**, *14*, 6044–6066. [CrossRef]
75. Chen, Q.; Vazquez, E.J.; Moghaddas, S.; Hoppel, C.L.; Lesnefsky, E.J. Production of reactive oxygen species by mitochondria: Central role of complex III. *J. Biol. Chem.* **2003**, *278*, 36027–36031. [CrossRef] [PubMed]
76. Achard-Joris, M.; Gonzalez, P.; Marie, V.; Baudrimont, M.; Bourdineaud, J.P. Cytochrome c oxydase subunit I gene is up-regulated by cadmium in freshwater and marine bivalves. *BioMetals* **2006**, *19*, 237–244. [CrossRef]

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Toxics Editorial Office
E-mail: toxics@mdpi.com
www.mdpi.com/journal/toxics

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-0365-4259-1