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Evaluating non-monotonic dose-response relationships in ecotoxicological risk assessment: A case study based on a systematic review of data on fluoxetine

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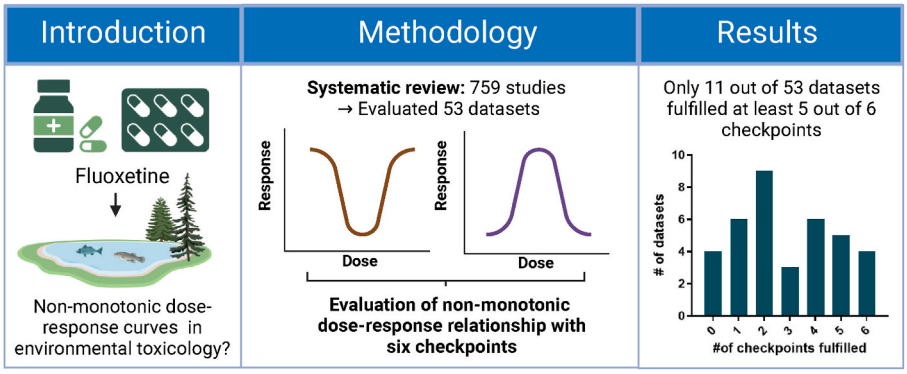
HIGHLIGHTS

- The EFSA framework proved valuable in analysing non-monotonic dose-response (NMDR) patterns in ecotoxicological studies.
- While 19 datasets met five or six checkpoints, most fluoxetine datasets lack strong evidence for NMDR relationships.
- Many NMDRs were rejected due to insufficient amount of tested concentrations or reliance on a single outlier.
- Studies focussing on mechanistic explanations for NMDRs are limited.

GRAPHICAL ABSTRACT



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ABSTRACT

The environmental presence of pharmaceuticals, including the antidepressant fluoxetine, has become a subject of concern. Numerous studies have revealed effects of fluoxetine at environmental concentrations. Some of these studies have reported non-monotonic dose-response curves (NMDRs), leading to discussion because of the inconsistent detection of subtle effects and lack of mechanistic understanding. Nevertheless, investigating NMDRs in risk assessment is important, because neglecting them could underestimate potential risks of chemicals at low levels of exposure. Identification and quantification of NMDRs in risk assessment remains challenging, particularly given the prevalence of single outliers and the lack of sound statistical analyses. In response, the European Food Safety Authority (Beausoleil et al., 2016) presented a framework delineating six checkpoints for the evaluation of NMDR datasets, offering a systematic method for their assessment. The present study applies this framework to the case study of fluoxetine, aiming to assess the weight-of-evidence for the reported NMDR relationships. Through a systematic literature search, 53 datasets were selected for analysis against the six checkpoints. The results reveal that while a minority of these datasets meet all checkpoints, a significant proportion (27%) fulfilled at least five. Notably, many studies did not meet checkpoint 3, which requires NMDRs to be based on more than a single outlier. Overall, the current study points out a number of studies with

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considerable evidence supporting the presence of NMDRs for fluoxetine, while the majority of studies lacks strong evidence. The suggested framework proved useful for analysing NMDRs in ecotoxicological studies, but it is still imperative to develop further understanding of their biological plausibility.

1. Introduction

At present, there are increasing concerns about the rising numbers of pharmaceuticals ending up in the environment (Diaz-Camal et al., 2022; Moreira et al., 2022; Singh et al., 2022). A class of pharmaceuticals that has shown increasing environmental concentrations is that of antidepressants, related to the global increase in the prevalence of depression (Gould et al., 2021; Polverino et al., 2021). Among these antidepressants is fluoxetine, the active component of Prozac. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI). SSRIs are a widely prescribed class of antidepressants that function by impeding the reuptake of serotonin in the synaptic cleft, thereby prolonging its presence and intensifying its effects (Ford and Fong, 2016; Gould et al., 2021). The primary target of the SSRIs, the SERotonin Transporter (SERT), is conserved in a wide range of species (Ford and Fong, 2016; Gould et al., 2021). Since SSRIs are designed to be biologically active, behavioural effects can also be expected in non-target organisms. Effects of fluoxetine at relatively low, environmentally relevant concentrations have indeed been observed for a number of species and endpoints. For example, changes in amphipod activity (De Lange et al., 2006) and phototaxis (Guler and Ford, 2010), mussel serotonin expression (Franzellitti et al., 2014) and nematode activity and chemotaxis (Van der Most et al., 2023) were observed upon exposure to fluoxetine in the concentration range of ng/L. Interestingly, several of these studies detecting effects at low concentrations have found non-monotonic dose-response relationships (NMDRs) (Ford and Fong, 2016; Van der Most et al., 2023).

In an NMDR, effects observed at low concentrations are no longer present at higher exposures. This is defined by a dose-response relationship where the direction of the slope of the dose-response curve changes along the range of tested concentrations (Beausoleil et al., 2016; Lagarde et al., 2015; Vandenberg, 2014) (Fig. 1). NMDRs are most frequently found for endocrine disrupting chemicals (EDCs), such as 17β-estradiol, bisphenol a, and PCBs, but also for pharmaceuticals, nutrients and vitamins (Hill et al., 2018; Lagarde et al., 2015; Varret et al., 2018). However, there is a lot of debate about the existence and relevance of NMDRs, since they are not detected in many studies, the effects are often somewhat subtle and studies often fail to provide a mechanistic underpinning. The read-across hypothesis for estimating effects in non-target organisms states that effects are only to be expected in non-target organisms when the concentrations are similar to therapeutic concentrations in a target organism (Ford and Fong, 2016; Rand-Weaver et al., 2013; Sumpter et al., 2014). The effects in the range of ng/L are sometimes difficult to reconcile with this read-across hypothesis (Rand-Weaver et al., 2013; Sumpter et al., 2014), because at those exposures internal concentrations in exposed organisms are far below therapeutic concentrations. However, critique on this hypothesis has also been raised, particularly emphasizing that SSRIs were found to have several secondary modes of action, such as effects on serotonin receptors and CYP450 enzymes, and interspecies variability in affinities for molecular targets exists, so that the read-across hypothesis might not apply (Ford and Fong, 2016; Kullyev et al., 2010; Ranganathan et al., 2001). An increasing amount of scholars consider that there is a plausible explanation for NMDRs, and they have lately been more frequently reported in literature (Beausoleil et al., 2016; Lagarde et al., 2015).

There are various mechanisms potentially explaining NMDR relationships, ranging from receptor selectivity/competition to DNA repair and dose dependent metabolism (Lagarde et al., 2015). However, the applicability of NMDRs in risk assessment is still under debate (Lagarde et al., 2015; Vandenberg, 2014). Some of the detected NMDR relationships are based on single outliers and a sound statistical analysis is often lacking, which is problematic from a regulatory perspective (Beausoleil et al., 2016; Chevillotte et al., 2017; Lagarde et al., 2015). Not taking into account NMDRs can however result in an underestimation of adverse effects of chemicals, since these non-monotonic effects are generally detected below no observed adverse effect levels (NOAELs) or in some cases even below reference doses, as shown by (Hill et al., 2018). So, when NMDRs are a likely phenomenon, it is important to take them into account in risk assessments in a regulatory framework.

In order to provide some standardisation for the detection, characterisation and interpretation of NMDRs, the European Food Safety Authority (EFSA) released a supporting publication by Beausoleil et al. (2016) reviewing non-monotonic dose-responses of substances for human risk assessment. In that review, six checkpoints were defined which can be used to evaluate NMDR datasets. However, this framework has up to this point only be applied to chemicals relevant for food safety. Pharmaceuticals and compounds related to environmental risk assessment of chemicals were not taken into account in previous analyses (Beausoleil et al., 2016), while NMDRs have also been frequently reported for those compounds, as illustrated by the example of fluoxetine. Therefore, the current study aims to take a targeted approach by applying the EFSA-framework to datasets reporting NMDRs of fluoxetine for environmentally relevant endpoints. An additional objective of the study is to check the applicability of the framework for environmental toxicological studies.

2. Materials and methods

2.1. Systematic literature review: study selection and data extraction

Peer-reviewed publications on experiments testing ecotoxicological effects of fluoxetine were searched for in SCOPUS with the following

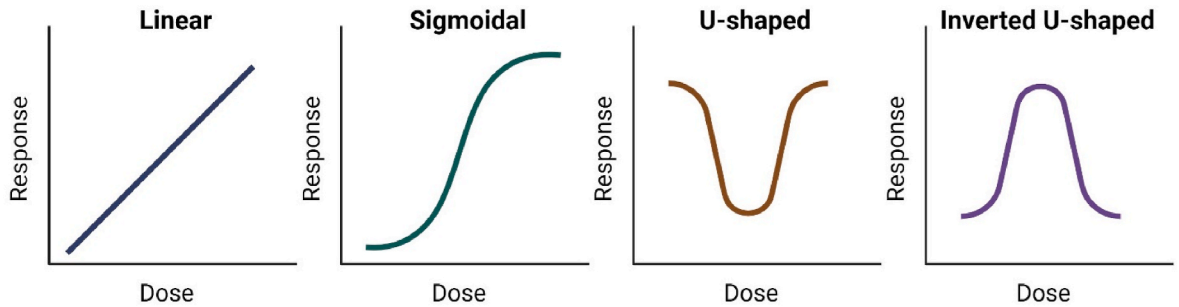

Fig. 1. Examples of monotonic (linear and sigmoidal) and non-monotonic dose-response (NMDR) curves.

search terms: (TITLE-ABS-KEY (fluoxetine) AND TITLE-ABS-KEY (ecotox* OR (environmental AND tox*) OR (aquatic AND tox*) OR (terrestrial AND tox*) OR (ecological AND risk AND assessment) OR (environmental AND impact))). The search was performed in September 2023 and 759 abstracts were obtained. Because of the width of the search terms and the number of hits, a follow-up was not deemed necessary. These abstract were further scrutinized for relevance. Abstracts were included, if they were an eco-/environmental toxicological study with original experimental toxicity data and included a dose-response relationship with exposure to fluoxetine. Supplementary Table S1 outlines reasons articles were excluded. The final study and dataset selection is summarized in Fig. 2. The supporting Excel file includes all 211 studies for which the full-text and the corresponding datasets were considered. We distinguished between studies (peer-reviewed scientific articles) and datasets (the actual dose-response curves), so one study could comprise of multiple datasets. In the end, only studies with at least 4 experimental doses of fluoxetine (additional to the reference dose) showing an NMDR were included. This criterium was based on the EFSA supporting publication (Beausoleil et al., 2016) suggesting to apply the framework to NMDR studies with at least five dose-groups (additional to the control). The relevant datasets were extracted from the selected studies using WebPlotDigitizer v4.6 (https://apps.automeris.io/wpd/), reporting the mean, the standard error or standard deviation and the sample size per dataset, which were used for further analysis. Data were transferred to a text document as is required for an input in the PROAST software (see section 2.2.2).

2.1.1. Framework for NMDRs

The framework suggested in the EFSA supporting publication (Beausoleil et al., 2016) suggests six checkpoints to apply to NMDR studies with at least five dose-groups, which can be summarized as follows:

1. Can the apparent NMDR be explained by random fluctuations around a horizontal dose-response (= no effect at all)?
2. Can the apparent NMDR be explained by random fluctuations around a monotone dose-response (MDR)?
3. Can the apparent NMDR be explained by one single potential outlying dose-group?
4. Is the effect size in one of the directions of the NMDR smaller than 5 %?
5. Is the steepness of the dose-response curve outside the range of biologically plausible/realistic dose-response shapes?
6. Does the apparent NMDR consist of more (or less) than two directions?

We applied these checkpoints to the selected datasets to assess the potential for NMDRs for fluoxetine. Answering 'no' to these checkpoints indicates a higher likelihood of NMDR and the respective checkpoints were considered as fulfilled.

2.1.2. Statistical analysis (checkpoints 1 and 2)

Checkpoints 1 and 2 focus on a statistical assessment, comparing the non-monotonic dose-response models to the null model (checkpoint 1) and the monotonic model (checkpoint 2). Dose-response analyses were performed on all selected datasets in PROAST version 70.3 (RIVM, https://www.rivm.nl/en/proast). PROAST has 56 different models, but as suggested by Beausoleil et al. (2016), models 1, 5, 31 and 33 were applied, where 1 is the null model, 5 is the monotonic model and 31 and 33 are two types of non-monotonic models (formulas in SI A2). PROAST reports log-likelihoods, AICs, fitted model parameters and confidence intervals. Statistical differences between models were determined with a critical difference in log-likelihood, based on differences in the degrees of freedom related to the amount of parameters in each model (See SI A3).

2.1.3. Checkpoints 3 to 6

Checkpoint 3, that aims at determining whether the NMDR is based on one outlier, was checked by manually drawing a monotonic line through all confidence intervals plotted by PROAST. If this monotonic model could be drawn with the exemption of only one outlier, the answer to checkpoint 3 is YES and checkpoint 3 is not fulfilled. Checkpoint 4 was determined by checking the maximum change in the response in both directions. The NMDR fitted by PROAST was used for the assessment of the effect sizes for checkpoint 4, even when the model was not deemed significant based on checkpoints 1 and/or 2. Checkpoint 5 judges if the steepness of the dose-response curve is reasonable by evaluating the steepness parameter d (reported by PROAST). A

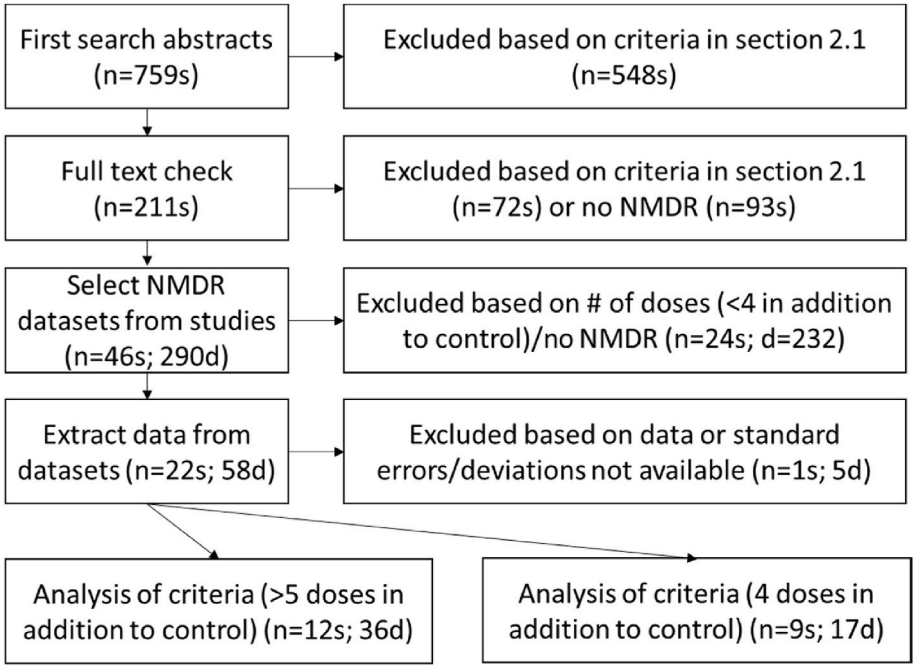

Fig. 2. Overview of the literature search for ecotoxicological studies reporting a NMDR after exposure to the SSRI fluoxetine, s = study, d = dataset.

d value between 0.25 and 4 was deemed reasonable (Beausoleil et al., 2016) and in case the value was outside of that range, checkpoint 5 was not fulfilled. In some cases of the current study however, a steep dose-response curve was calculated by PROAST, while this was not necessarily the only option based on the data. In such cases, the d was constrained to 0.25–4 and the log likelihood was compared to the log-likelihood of the fitted model without the constrained d parameter. If the log likelihood did not change considerably and the new model was also significant, checkpoint 5 was met. Checkpoint 6 evaluates the number of directions in the dose-response curve. According to Beausoleil (2016), only 2 directions are reasonable. A smooth curve was manually regressed through the confidence intervals with a minimal amount of directions needed and then the number of directions was counted. Datasets with one direction or more than two directions did not fulfil checkpoint 6.

3. Results

3.1. Number of included studies and how many were non-monotonic

759 studies were found based on the initial literature search (Fig. 2). Of these publications, 138 studies were selected to be relevant environmental toxicology papers studying the effect of fluoxetine based on the inclusion criteria (section 2.1) after reading the full-text (Fig. 2). Out of the 138 studies, 33% (46 studies) showed a non-monotonic pattern in at least one of the datasets (Fig. 3). According to the framework (Beausoleil et al., 2016), only studies with 5 or more doses (additional to the control) showing a NMDR curve should be included. Fig. 3 indicates that many of the studies with fluoxetine tested only 2, 3 or 4 doses in addition to the control. Only 12 studies that showed an NMDR pattern tested at least five doses (in addition to the control) and within those studies, 36 datasets gave an indication of an NMDR (Fig. 2). To extend the number of studies for inclusion, also studies and datasets with 4 doses in addition to controls were included, adding 9 studies with 17 datasets (Fig. 2). So in total, 53 datasets were analysed using the six checkpoints.

3.2. Amount of datasets and how many checkpoints they fulfil

The analysis of to what extent the 53 datasets fulfil the six checkpoints is summarized in Fig. 4. Out of the 36 datasets with 5 doses (+control), four fulfilled all of the six checkpoints, while six satisfied five checkpoints. Only one of the studies with 4 doses (+control) adhered to all checkpoints and eight fulfilled five checkpoints. Datasets fulfilling 5

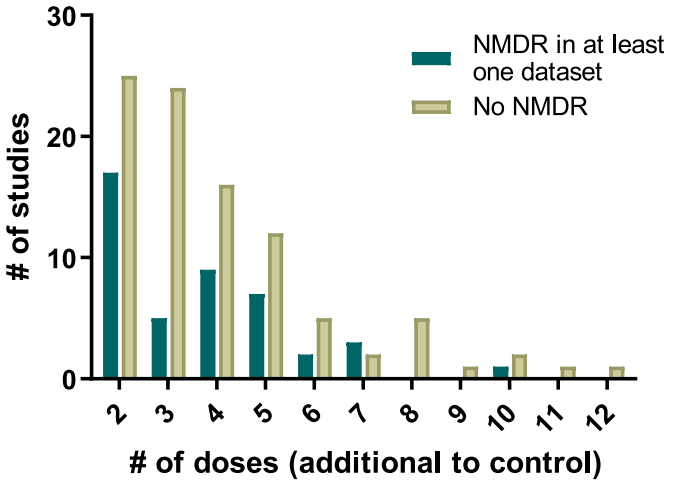

Fig. 3. The number of ecotoxicological studies testing the effect of fluoxetine, plotted against the number of doses (in addition to controls) in the experiment and the presence of an apparent NMDR in at least one dataset.

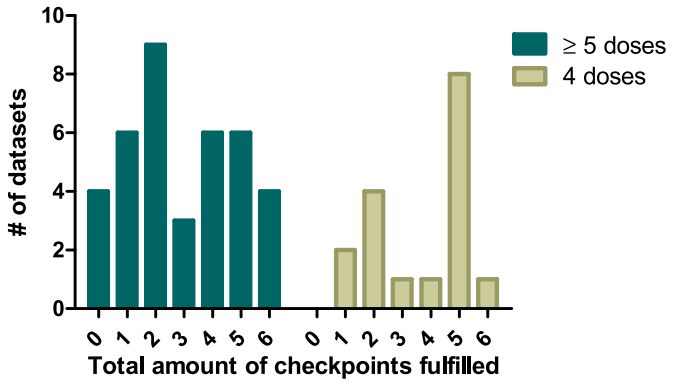

Fig. 4. A histogram plotting the number of datasets against the number of checkpoints fulfilled, for studies with 4 doses and studies with at least 5 doses (in addition to the control).

or 6 checkpoints were considered to have the more robust evidence of an NMDR, and the characteristics of these datasets are outlined in Tables 1 and 2 for further discussion.

Fig. 5 describes the percentage of datasets that fulfilled a specific checkpoint. It is clear that most of the datasets (only 12%) did not fulfil checkpoint 3 that states that an NMDR curve should not depend on just one outlier, which implies that for 88% of the studies the NMDR depends on just one outlier. Checkpoints 1 and 2 checking the significance from the null-model and a monotonic model were met by more than 50% of the studies and checkpoint 4 (stating that the effect size should be >5%) by almost all studies. Checkpoints 5 analysed the reasonability of the steepness of the dose-response curve and was only satisfied by around 40% of the datasets, but this checkpoints was only evaluated for datasets that satisfied both checkpoints 1 and 2. Therefore, the number of datasets evaluated for checkpoint 5 was only 28 out of 53 datasets and out of those 28, 21 (75%) actually fulfilled checkpoint 5.

4. Discussion

4.1. Study selection and number of datasets as outcome

The total of 759 studies initially selected based on the search terms covered a wide range of studies on fluoxetine, also including many studies without an indication of an NMDR. Contrary to former review studies focussing on non-monotonic dose-response curves (Beausoleil et al., 2016; Lagarde et al., 2015; Varret et al., 2018), our search terms did not specifically include words such as non-monotonic/hormesis/U-shaped. A significant benefit of this approach is that there was no search bias towards studies already reporting NMDR patterns. This means that also studies not specifically reporting an NMDR or hormesis were included in the analysis. Most of the identified studies (Tables 1 and 2) mention non-monotonicity somewhere in their discussion, but not in the abstract, so those studies would have been missed. Furthermore, the limited bias also allowed for an analysis of the total percentage of ecotoxicological studies with fluoxetine showing a non-monotonic dose-response curves. We could still have missed some fluoxetine studies that did not specifically mention environmental toxicology or similar terms. As indicated, around one third of the studies (46 out of 138) showed some kind of NMDR in at least one of the endpoints (Fig. 3). This in itself already indicates the importance of analysing the weight of evidence of these NMDR curves for fluoxetine. However, since many of the toxicological studies used only a few dose-groups, only 21 out of the 46 studies were selected for further analysis of the NMDR, of which only 12 studies had 5 doses or more in addition to the control.

Table 1
Studies with 5 or more doses (additional to the control) that met at least 5 of the checkpoints, cp = checkpoint.

Date	Authors	Species	Endpoint	# of doses	Tested dose range	Lowest sign. effect conc.	Shape	# of cps fulfilled (cp not fulfilled)
2021	Al Shuraiqi et al.	Zebrafish	Swimming speed	7	5–5000 ng/l	5 ng/l	U	6
2016	Rivetti et al.	*Daphnia magna*	phototactic index	7	0.1 mg/l - 100 μg/l	1 ng/l	U	6
2023	van der Most et al.	*C. elegans*	Chemotaxis index	7	1 ng/l - 100 mg/l	1 ng/l	U	6
2014	Franzellitti et al.	Mussels	pka activity mantle/ gonads	5	0.03 ng/l - 300 ng/l	0.03 ng/l	U	5 (3)
			5-HT mRNA expression			0.03 ng/l	∩	5 (3)
			5-HT mRNA expression			0.03 ng/l	∩	5 (3)
			ABCB mRNA expression			0.3 ng/l	U	6
2015	Maranho et al.	Amphipod	DBF	5	0.01–100 ng/g	1 ng/g	U	5 (3)
			GST			0.1 ng/g	U	5 (3)
			EROD			0.1 ng/g	U	5 (3)

Table 2
Studies with 4 doses (additional to the control) that met at least 5 of the checkpoints, cp = checkpoint.

Date	Authors	Species	Endpoint	# of doses	Tested dose range	Lowest sign. effect conc.	Shape	# of cps fulfilled (cp not fulfilled
2014	Bossus et al.	Amphipod	Gene expression - NEUC	4	0.001–1 μg/l	1 ng/l	U	5 (3)
2019	de Farias et al.	Zebrafish (embryos)	Swimming time	4	0.88–500 μg/l	0.88 μg/l	U	5 (3)
2019	Duarte et al.	Common goby	EROD	4	0.1–100 μg/l	0.5 μg/l	∩	5 (3)
			GST			0.5 μg/l	∩	5 (3)
2004	Foran et al.	Japanese Medaka	Plasma estradiol females	4	0.1–5 μg/l	0.1 μg/l	∩	5 (3)
2015	Oliveira et al.	Springtail	AChE activity	4	0.04–40 mg/kg	0.4 mg/kg	U	5 (3)
2010	Guler and Ford	Amphipod	phototaxis index trial 2	4	0.01–10 μg/l	0.01 μg/l	∩	5 (3)
			geotaxis index trial 1			–	∩	5 (3)
			geotaxis index trial 2			0.1 μg/l	∩	6

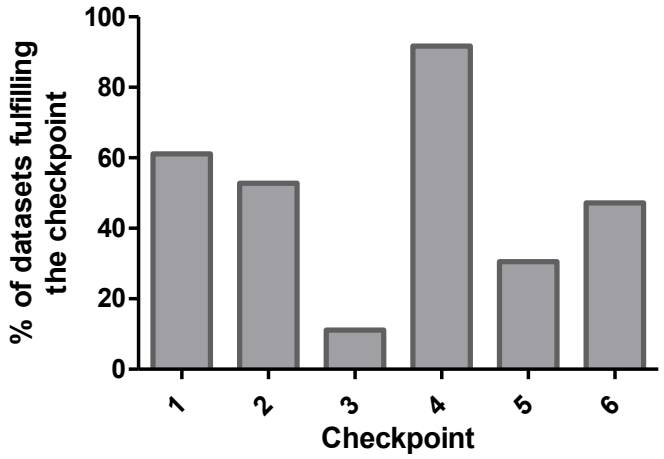

Fig. 5. The percentage of datasets (out of 53) fulfilling each specific checkpoint.

4.2. Weight of evidence of reported NMDR curves for fluoxetine based on assessment of the checkpoints

Among the 53 datasets scrutinized, only 19 datasets met a minimum of five checkpoints, as illustrated in Fig. 3. Tables 1 and 2 provide additional details on these datasets. Among datasets including at least 5 doses, only 11% (4 out of 36) satisfied all six checkpoints, while 28% (10 out of 36) fulfilled at least five of the checkpoints. In a comparable analysis focussing solely on chemicals relevant for food safety (Beausoleil et al., 2016; Varret et al., 2018), the corresponding percentages were 6% and 20%, respectively. The total number of datasets included in this previous and the current study are too small to perform in-depth statistics, but the results indicate quite similar percentages, slightly higher for the current study. Notably, checkpoint 3, stating the NMDR should not solely rely on a single outlier, was only satisfied by 12% of the datasets reporting NMDRs (Fig. 4). This implies that for 88% of the studies reporting an NMDR the non-monotonous behaviour depends on just one outlier, which illustrates the need for more in-depth analysis of such studies and reports on the occurrence of NMDR. Some previous studies have argued that if the NMDR is based on one outlier, this outlier could be regarded as an experimental artifact and, consequently, disregarded. However, outliers are not necessarily artefacts. If the study design of such studies would have included more concentrations in similar ranges as the 'outlier' group, this could address this limitation and might result in satisfying checkpoint 3. Therefore, study design is essential for the analysis of the evidence for the NMDR.

Even though the analysis excluded many NMDR datasets, a substantial number of datasets satisfying at least five checkpoints remained, suggesting the potential occurrence of NMDR effects following exposure to fluoxetine. However, to confirm this, an analysis of the biological plausibility with a mechanistic explanation becomes pertinent, as discussed in the next section. Moreover, Beausoleil et al. (2016) state that 'convincing evidence of NMDR always requires at least one other independent study, performed in another laboratory and examining the same substance-endpoint combination, which reproduces the non-monotonicity.' This is not yet the case for the studies we selected and we therefore recommend a repetition of those studies by different independent laboratories, and applaud journals to publish such results.

4.3. Mechanistic explanations and biological plausibility

Of the 11 studies reporting the 21 datasets in Tables 1 and 2, only seven provide a clear mechanistic explanation of the NMDR. Some propose the potential involvement of the desensitization of G-protein

coupled receptors (GPCR) (Bossus et al., 2014; Guler and Ford, 2010; Rivetti et al., 2016; Van der Most et al., 2023). GPCR desensitization is a process where receptors become less responsive to stimulation after prolonged or repeated exposure to a ligand, such as serotonin. This type of negative feedback prevents overstimulation, and can, for example, also lead to a decrease in serotonin release (Guler and Ford, 2010). The desensitization is more pronounced and rapid at higher concentrations of the ligand, which could explain NMDR by reducing the sensitivity of the system with increasing dose. Foran et al. (2004) do not specifically mention GPCR desensitization, but emphasize the potential of regulatory biofeedback mechanisms to mitigate the increase in serotonin levels. Other ways of biofeedback are also mentioned, such as an increase in serotonin receptor expression to replace old receptors (Franzellitti et al., 2014). The finite amount of endogenous serotonin present could also lead to a saturation of the effect (Bossus et al., 2014; Guler and Ford, 2010). Furthermore, serotonin and fluoxetine are both known to have multiple molecular targets and if these have different sensitivities or affinities and result in opposing effects, this could also result in an NMDR (Van der Most et al., 2023).

Duarte et al. (2019) observed NMDR patterns for assays of the detoxification enzymes CYP450 (EROD activity) and GST, examining the metabolic activity in fish. They found an inverted U-shaped dose-response relationship to the exposure of the common goby (*Pomatoschistus microps*) to fluoxetine, with an increase in EROD and GST activity at lower concentrations, but a return to baseline levels at the higher concentration. The hypothesized mechanisms behind this NMDR are different from the proposed modes of action for the NMDR for other types of endpoints. The authors suggest the possibility of down-regulating genes associated with detoxification pathways or direct enzyme inhibition by fluoxetine and its metabolites (Duarte et al., 2019). Since EROD and GST activity are different endpoints than the behavioural and neurotoxic endpoints tested by the other studies, the mechanistic explanation for the NMDR is also different. Interestingly, Maranho et al. (2015) performed similar assays, but with amphipods instead of fish, and actually observed the opposite result: a U-shaped instead of an inverted U-shaped dose-response curve for EROD, GST and DBF (biomarker for CYP3A4) activities in amphipods with increasing concentration of fluoxetine. However, the authors acknowledge that the physiological mechanism underlying this observation is still unknown (Maranho et al., 2015). None of the studies from Tables 1 and 2 actually performed follow-up experiments to elucidate the suggested mechanisms for non-monotonicity of the observed dose-response curves.

Several studies have focussed on specific elements of the aforementioned theorized mechanisms. For instance, receptor internalization, a process that often follows receptor desensitization, was observed in human cells when exposed to exogenous serotonin (Bohn and Schmid, 2010). It is essential to note that this was specific to external serotonin exposure rather than fluoxetine exposure, but similar effects could be expected, since fluoxetine exposure results in an increase of serotonin in the synaptic cleft. Another study, by Winder et al. (2009), looked at the effects of fluoxetine on serotonin levels in sheepshead minnow *Cyprinodon variegatus*. They investigated the ratio between 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, and serotonin (5-HT). The 5-HIAA/5-HT ratio was increased for higher concentrations of fluoxetine and the researchers suggested that this was linked to an elevated biotransformation of serotonin at the higher dose levels. This would confirm the negative feedback theory concerning serotonin levels. Nevertheless, the potential influence of opposing molecular targets in these processes cannot be disregarded. For instance, in the nematode *C. elegans,* two serotonin producing neurons were found to exhibit opposing effects on the nematode's locomotion (Churgin et al., 2017). Through one neuronal pathway serotonin encourages exploration (roaming), while via another neuronal pathway, it facilitates a sedentary state (dwelling). Hence, serotonin can both enhance and decrease locomotion in *C. elegans,* and if this is also the case for other species, it could also be a potential explanation for an NMDR provided these receptors are activated at different concentrations. These examples indicate that different mechanisms could be involved in the occurrence of NMDRs, and might operate simultaneously. However, a specific study validating this for fluoxetine exposure over a range of concentrations is lacking. Furthermore, the studies did not measure internal concentrations or discuss the read-across hypothesis, a commonly mentioned criticism on the presence of NDMRs.

4.4. Studies not detecting NMDRs

Up to this point, we have focussed on the studies which reported NMDR curves for which we evaluated the weight of evidence using the six checkpoints. However, as indicated, two third (92 out of 138) of the environmental toxicology studies with fluoxetine did not find an NMDR relationship in any of their studied endpoints. To gain further insights into these studies, this paragraph further discusses the 17 studies that used at least six experimental concentrations in addition to the control (see SI Excel 5). Of these 17 studies, 3 look at cell viability (Caminada et al., 2006; Fernández et al., 2013; Schnell et al., 2009), 3 focus on algae or phytoplankton (community) growth or photosynthesis (Backhaus et al., 2011; Bi et al., 2018; Grzesiuk et al., 2016), and 5 examined endpoints such as mortality, development, and teratogenic effects (Di Poi et al., 2014; Orozco-Hernández et al., 2022; Richards and Cole, 2006; Schweizer et al., 2022; Van de Maele et al., 2021). The experimental design of those studies was thus less aimed at measuring endpoints directly relating to the serotonin nervous system, which might be a reason for the absence of NMDRs. However, 5 of the 17 studies actually examined behavioural endpoints (Atzei et al., 2021; Fong and Molnar, 2008; Margiotta-Casaluci et al., 2014; Varano et al., 2017; Zindler et al., 2020). An investigation of zebrafish visual motor response detected a LOEC for fluoxetine of around 90 ng/L (Zindler et al., 2020) and freshwater bivalve spawning was affected at 3 μg/L, reporting a PEC/PNEC of 14.2 (Fong and Molnar, 2008). So, while these studies on fluoxetine did not detect an NMDR, they did find behavioural effects at low concentrations.

However, the three other studies detected effects only at higher concentrations. The reported EC50 for chronic effects of fluoxetine on *D. magna* reproduction was much higher (0.23 mg/L) (Varano et al., 2017). It is worthy to note that the authors did not examine doses lower than 0.05 mg/L, so the range of tested concentrations is limited. The effect of fluoxetine on zebrafish embryos was tested through the light-dark transition test at a wide range of concentrations (0.00001–10 μM) and a BMC5 of 0.05–0.2 mg/L was detected (Atzei et al., 2021). A study using the fathead minnow tank diving test also examined a wide concentration range (0.1–64 μg/L) and reported monotonic behaviour with a LOEC of 38 μg/L (Margiotta-Casaluci et al., 2014). Interestingly, the latter study also measured bioaccumulation and compared fish internal concentrations to Human Therapeutic Plasma Concentrations (H_TPCs). The results obtained showed that only internal concentrations above the H_TPCs induced anxiolytic response, which validates the species read-across hypothesis (Margiotta-Casaluci et al., 2014; Rand-Weaver et al., 2013). As mentioned previously, the effects at low nominal concentrations in the range of ng/L are sometimes difficult to reconcile with the read-across hypothesis, so it is relevant that Margiotta-Casaluci et al. (2014) validated this hypothesis for read-across from one species to another to obtain insight in adverse effects of fluoxetine. However, bioaccumulation can still vary between species and that SSRIs are also known to have multiple molecular targets, and the presence, role, and affinity of the molecular targets can be species-specific may hamper such read-across (Ford and Fong, 2016). Furthermore, the therapeutic effects of fluoxetine are hypothesized to be related to long-term neuroadaptive changes, such as changes in neuroplasticity, serotonin release and receptor gene expression (Harmer et al., 2017). These neuroadaptive changes could also vary between species. Therefore, these other modes of actions, neuroadaptive changes, and species-specific bioaccumulation and target-affinities should first be

established to further validate the read-across from one species to another for fluoxetine.

The analysis in this section points out that the presence or absence of NMDRs was not consistent among studies. Two of these studies tested behavioural endpoints with a wide range of doses and did not detect NMDRs. This inconsistency emphasizes the importance of replicating previous studies, whether they are reporting NMDRs or not, in order to gain further insights into these contradictions. The type of endpoint measured or species studied appeared to be relevant for the detection of NMDRs. However, it is also important to note that endpoints such as behaviour have in some cases shown higher variability in their outcomes and are context-dependent, making it difficult to detects subtle effects (Ågerstrand et al., 2020; Butcher et al., 1979; Ford et al., 2021; Jirkof et al., 2020; Pacholski et al., 2017). Therefore, standardization of behavioural endpoints and characterization of intra- and interlaboratory variability as well as of species variability is essential in improving the reliability of studies reporting the presence but also of studies reporting the absence of NMDRs (Ågerstrand et al., 2020; Ford et al., 2021). Especially if an NMDR is dependent on a single outlier, the results should be interpreted with care, and studies should be repeated.

4.5. The translation of NMDR effects to population level responses and biodiversity impacts

For the datasets meeting at least five of the checkpoints, the concentrations at which significant effects occur are low and environmentally relevant, often within the nanograms per liter range (Tables 1 and 2). This implies that these responses may have implications in the environment and should therefore be taken into account in environmental risk assessment. However, it is important to highlight that non-monotonic dose-responses were mostly detected at the molecular level, encompassing gene expression and enzyme activities (as delineated in Tables 1 and 2). Additionally, certain NMDR curves were reported for behavioural effects such as alterations in photo/geo/chemotaxis and swimming speed/distance (Tables 1 and 2). Behaviour is known to be a subtle endpoint, often characterized by high variability, which makes the detection of NMDRs even more challenging. Moreover, how these molecular, cellular and organismal responses translate into responses on a population level or even impacts on biodiversity (as shown in Fig. 6), remains to be determined (Committee et al., 2021). None of the studies we identified found such NMDRs for population level responses. This translational aspect to adverse effects is relevant for emphasizing the importance of these NMDRs for environmental risk assessment.

4.6. Suitability of the framework for ecotoxicological studies

The results of the present study reveal that the framework comprising six checkpoints was straightforward to apply and instrumental in assessing the likelihood of NMDR curves for the fluoxetine datasets. Nonetheless, working with this framework did raise some questions regarding certain checkpoints that may require further consideration. Checkpoint 3, that states that the presence of an NMDR should not be based on one single outlier, also takes the control into account as a potential outlier. However, when a control is well characterized, within normal physiological boundaries and has shown limited variability in previous independent experiments, it could potentially be deemed trustworthy and may not necessarily be considered an outlier. This is especially important for ecotoxicological studies, where it is often not feasible to test many replicates and concentrations in vivo. The validity of the control response may be verified with other studies or experiments, which may provide further justification. If the control would not be considered an outlier, three times more studies would fulfil checkpoint 3 (SI A4), increasing the likelihood of NMDRs.

Furthermore, the usefulness of checkpoint 4, only including studies with an effect size greater than 5%, can be questioned. Ecotoxicological in vivo endpoints often display a relatively high variation compared to in vitro endpoints. This also results in a higher standard deviation and an effect size of 5% is therefore not likely to be reported as a significant effect. Hence, in case in environmental studies effects are significant, the study most likely meets checkpoint 4.

Finally, checkpoint 6 asserts that an NMDR curve should only have two directions, as three directions are deemed biologically implausible. However, it is worth noting that three-directional curves have also been reported (Hill et al., 2018; Van der Most et al., 2023). At high concentrations, the feedbacks could be insufficient to counteract the effects, or alternative mechanisms, such as mortality, could induce a third phase in the dose-response curve's slope trajectory. A few of the datasets from the 53 analysed datasets have shown these kind of three-directional NMDR curves (as outlined in SI A5, Fig. S2 and Table S3). However, none of the dose-response models in PROAST have the capability to analyse three directions. To address this, we excluded the final doses of datasets that produced the third direction, thus generating two-directional dose-response datasets. Nevertheless, none of these adjusted datasets fulfilled at least five of the checkpoints, so this was not deemed relevant for this analysis (SI A5). Three-directional dose-response models are also available and could be used for future analysis (Di Veroli et al., 2015; Van der Most et al., 2023).

4.7. Future evaluation of non-monotonic dose-response curves

In summary, the framework suggested by Beausoleil et al. (2016) was demonstrated to be useful in assessing NMDR curves in ecotoxicological studies. Applying the six checkpoints through statistical analysis in PROAST provided a straightforward method for evaluating the NMDRs. While 19 datasets fulfilled at least five of the checkpoints, the majority of studies lacked sufficient evidence for NMDRs. Many initially identified NMDR curves were rejected due to factors such as a low number of doses, insignificantly improved models compared to null or monotonic models, or reliance on a single outlier. While the selected studies offered insights into the occurrence of NMDRs, they did not provide clear mechanistic explanations underlying the non-monotonic responses. As discussed, the applicability of the read-across hypothesis for fluoxetine also has to be further validated. We therefore suggest future studies should focus on elucidating the mechanisms behind fluoxetine-induced NMDR curves, and on determining the role of secondary modes of action, and species-specific bioaccumulation, target-affinities, and neuro-adaptive changes. Additionally, we recommend replicating some of the studies listed in Tables 1 and 2 in different laboratories to verify the reproducibility of the results. As indicated, NMDRs have been reported

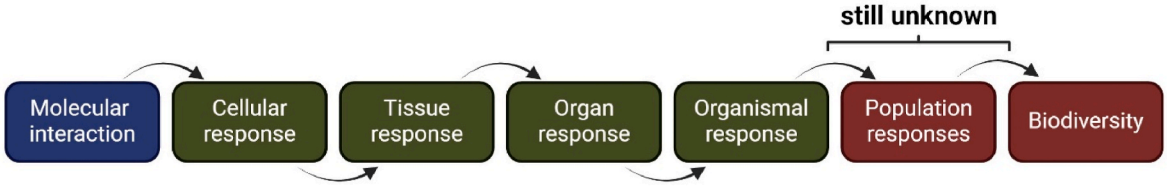

Fig. 6. Responses among different levels of biological organisation. How non-monotonic organismal responses translate to population level and biodiversity impacts remains to be established.

for a multitude of EDCs, pharmaceuticals, nutrients, and vitamins (Hill et al., 2018; Lagarde et al., 2015; Varret et al., 2018). The framework used in the present review to evaluate NMDRs has been applied before by EFSA to evaluate for example bisphenol A, chlorpyrifos, and PCBs (Beausoleil et al., 2016). The present study was a case-study applying this targeted approach for a compound relevant for environmental risk assessment, fluoxetine. The results obtained indicate that a similar approach could be used to test the weight of evidence of the reported NMDRs for other (groups of) compounds relevant for environmental risk assessment.

Future studies should be designed to also include low-dose levels, to be able to detect NMDR relationships at environmentally relevant concentrations. The absence of low-dose testing may lead to overlooking NMDRs and severely underestimating environmental risks. However, conducting experiments with extensive replicates at various concentrations in vivo is not always feasible, which may hamper applying this framework for environmental risk assessment of chemicals. New approach methodologies (NAMs) could offer a solution (Committee et al., 2021). NAMs devoid animal testing, while using a wide range of technologies, methodologies and approaches, such as in silico, in vitro and ex vivo approaches (Stucki et al., 2022). These approaches could both help in elucidating the mechanisms behind non-monotonic responses and could also allow for high-throughput screening to test for the potential of NMDRs and the use of an adequate number of concentrations tested in addition to the control. However, in vivo experiments are still relevant in confirming these responses at a whole organismal level and to confirm the transfer to population level responses. To conclude, the current evaluation of the existing datasets on ecotoxicological effects of fluoxetine points out a few studies with considerable evidence, but also shows that many studies fail to provide substantial proof, due to a limited amount of tested concentrations or the reliance on a single outlier. Consequently, the recommendations outlined for future studies hold considerable importance.

CRediT authorship contribution statement

Merel A. van der Most: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Ivonne M.C.M. Rietjens:** Writing – review & editing, Conceptualization. **Nico W. van den Brink:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Excel Supplementary Information for all analysed studies and datasets

Ågerstrand, M., Arnold, K., Balshine, S., Brodin, T., W Brooks, B., Maack, G., S McCallum, E., Pyle, G., Saaristo, M., Ford, A.T., 2020. Emerging investigator series: use of behavioural endpoints in the regulation of chemicals. Environ. Sci. J. Integr. Environ. Res.: Process. Impacts 22 (1), 49–65. https://doi.org/10.1039/C9EM00463G.

Atzei, A., Jense, I., Zwart, E.P., Legradi, J., Venhuis, B.J., van der Ven, L.T.M., Heusinkveld, H.J., Hessel, E.V.S., 2021. Developmental neurotoxicity of environmentally relevant pharmaceuticals and mixtures thereof in a zebrafish embryo behavioural test. Int. J. Environ. Res. Publ. Health 18 (13). https://doi.org/10.3390/ijerph18136717. Article 13.

Backhaus, T., Porsbring, T., Arrhenius, Å., Brosche, S., Johansson, P., Blanck, H., 2011. Single-substance and mixture toxicity of five pharmaceuticals and personal care products to marine periphyton communities. Environ. Toxicol. Chem. 30 (9), 2030–2040. https://doi.org/10.1002/etc.586.

Beausoleil, C., Beronius, A., Bodin, L., Bokkers, B.g. h., Boon, P.e., Burger, M., Cao, Y., De Wit, L., Fischer, A., Hanberg, A., Leander, K., Litens-Karlsson, S., Rousselle, C., Slob, W., Varret, C., Wolterink, G., Zilliacus, J., 2016. Review of non-monotonic dose-responses of substances for human risk assessment. EFSA Supporting Publ. 13 (5), 1027E. https://doi.org/10.2903/sp.efsa.2016.EN-1027.

Bi, R., Zeng, X., Mu, L., Hou, L., Liu, W., Li, P., Chen, H., Li, D., Bouchez, A., Tang, J., Xie, L., 2018. Sensitivities of seven algal species to triclosan, fluoxetine and their mixtures. Sci. Rep. 8 (1), 15361 https://doi.org/10.1038/s41598-018-33785-1.

Bohn, L.M., Schmid, C.L., 2010. Serotonin receptor signaling and regulation via β-arrestins. Crit. Rev. Biochem. Mol. Biol. 45 (6), 555–566. https://doi.org/10.3109/10409238.2010.516741.

Bossus, M.C., Guler, Y.Z., Short, S.J., Morrison, E.R., Ford, A.T., 2014. Behavioural and transcriptional changes in the amphipod Echinogammarus marinus exposed to two antidepressants, fluoxetine and sertraline. Aquat. Toxicol. 151, 46–56. https://doi.org/10.1016/j.aquatox.2013.11.025.

Butcher, R.E., Hoar, R.M., Nolan, G.A., Vorhees, C.V., 1979. Interlaboratory comparison of behavioral testing. J. Assoc. Off. Anal. Chem. 62 (4), 840–843. https://doi.org/10.1093/jaoac/62.4.840.

Caminada, D., Escher, C., Fent, K., 2006. Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish cell lines. Aquat. Toxicol. 79 (2), 114–123. https://doi.org/10.1016/j.aquatox.2006.05.010.

Chevillotte, G., Bernard, A., Varret, C., Ballet, P., Bodin, L., Roudot, A.-C., 2017. Probabilistic assessment method of the non-monotonic dose-responses-Part II: robustness assessment. Food Chem. Toxicol. 110, 214–228. https://doi.org/10.1016/j.fct.2017.10.030.

Churgin, M.A., McCloskey, R.J., Peters, E., Fang-Yen, C., 2017. Antagonistic serotonergic and octopaminergic neural circuits mediate food-dependent locomotory behavior in Caenorhabditis elegans. J. Neurosci. 37 (33), 7811–7823. https://doi.org/10.1523/JNEUROSCI.2636-16.2017.

Committee, E.S., More, S., Benford, D., Hougaard Bennekou, S., Bampidis, V., Bragard, C., Halldorsson, T., Hernandez-Jerez, A., Koutsoumanis, K., Lambré, C., 2021. Opinion on the impact of non-monotonic dose responses on EFSA' s human health risk assessments. EFSA J. 19 (10), e06877.

De Lange, H.J., Noordoven, W., Murk, A.J., Lürling, M., Peeters, E.T.H.M., 2006. Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat. Toxicol. 78 (3), 209–216. https://doi.org/10.1016/j.aquatox.2006.03.002. Scopus.

Di Poi, C., Evariste, L., Serpentini, A., Halm-Lemeille, M.P., Lebel, J.M., Costil, K., 2014. Toxicity of five antidepressant drugs on embryo–larval development and metamorphosis success in the Pacific oyster, Crassostrea gigas. Environ. Sci. Pollut. Control Ser. 21 (23), 13302–13314. https://doi.org/10.1007/s11356-013-2211-y.

Di Veroli, G.Y., Fornari, C., Goldlust, I., Mills, G., Koh, S.B., Bramhall, J.L., Richards, F. M., Jodrell, D.I., 2015. An automated fitting procedure and software for dose-response curves with multiphasic features. Sci. Rep. 5 (1) https://doi.org/10.1038/srep14701. Article 1.

Diaz-Camal, N., Cardoso-Vera, J.D., Islas-Flores, H., Gómez-Oliván, L.M., Mejía-García, A., 2022. Consumption and ocurrence of antidepressants (SSRIs) in pre- and post-COVID-19 pandemic, their environmental impact and innovative removal methods: a review. Sci. Total Environ. 829, 154656 https://doi.org/10.1016/j.scitotenv.2022.154656.

Duarte, I.A., Pais, M.P., Reis-Santos, P., Cabral, H.N., Fonseca, V.F., 2019. Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine. Mar. Environ. Res. 147, 24–31. https://doi.org/10.1016/j.marenvres.2019.04.002.

Fernández, C., Carbonell, G., Babín, M., 2013. Effects of individual and a mixture of pharmaceuticals and personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2). J. Appl. Toxicol. 33 (11), 1203–1212. https://doi.org/10.1002/jat.2752.

Fong, P.P., Molnar, N., 2008. Norfluoxetine induces spawning and parturition in estuarine and freshwater bivalves. Bull. Environ. Contam. Toxicol. 81 (6), 535–538. https://doi.org/10.1007/s00128-008-9558-7. Scopus.

Ford, A.T., Ågerstrand, M., Brooks, B.W., Allen, J., Bertram, M.G., Brodin, T., Dang, Z., Duquesne, S., Sahm, R., Hoffmann, F., Hollert, H., Jacob, S., Klüver, N., Lazorchak, J. M., Ledesma, M., Melvin, S.D., Mohr, S., Padilla, S., Pyle, G.G., et al., 2021. The role of behavioral ecotoxicology in environmental protection. Environ. Sci. Technol. 55 (9), 5620–5628. https://doi.org/10.1021/acs.est.0c06493.

Ford, A.T., Fong, P., 2016. The effects of antidepressants appear to be rapid and at environmentally relevant concentrations. Environ. Toxicol. Chem. 35 https://doi.org/10.1002/etc.3087 null.

Franzellitti, S., Buratti, S., Capolupo, M., Du, B., Haddad, S.P., Chambliss, C.K., Brooks, B. W., Fabbri, E., 2014. An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels. Aquat. Toxicol. 151, 14–26. https://doi.org/10.1016/j.aquatox.2013.11.016.

Gould, S.L., Winter, M.J., Norton, W.H.J., Tyler, C.R., 2021. The potential for adverse effects in fish exposed to antidepressants in the aquatic environment. Environ. Sci. Technol. 55 (24), 16299–16312. https://doi.org/10.1021/acs.est.1c04724.

Grzesiuk, M., Wacker, A., Spijkerman, E., 2016. Photosynthetic sensitivity of phytoplankton to commonly used pharmaceuticals and its dependence on cellular phosphorus status. Ecotoxicology 25 (4), 697–707. https://doi.org/10.1007/s10646-016-1628-8.

Guler, Y., Ford, A.T., 2010. Anti-depressants make amphipods see the light. Aquat. Toxicol. 99 (3), 397–404. https://doi.org/10.1016/j.aquatox.2010.05.019.

Harmer, C.J., Duman, R.S., Cowen, P.J., 2017. How do antidepressants work? New perspectives for refining future treatment approaches. Lancet Psychiatr. 4 (5), 409–418. https://doi.org/10.1016/S2215-0366(17)30015-9.

Hill, C.E., Myers, J.P., Vandenberg, L.N., 2018. Nonmonotonic dose–response curves occur in dose ranges that are relevant to regulatory decision-making. Dose Response 16 (3), 1559325818798282. https://doi.org/10.1177/1559325818798282.

Jirkof, P., Abdelrahman, A., Bleich, A., Durst, M., Keubler, L., Potschka, H., Struve, B., Talbot, S.R., Vollmar, B., Zechner, D., Häger, C., 2020. A safe bet? Inter-laboratory variability in behaviour-based severity assessment. Lab. Anim 54 (1), 73–82. https://doi.org/10.1177/0023677219881481.

Kullyev, A., Dempsey, C.M., Miller, S., Kuan, C.-J., Hapiak, V.M., Komuniecki, R.W., Griffin, C.T., Sze, J.Y., 2010. A genetic survey of fluoxetine action on synaptic transmission in Caenorhabditis elegans. Genetics 186 (3), 929–941. https://doi.org/10.1534/genetics.110.118877.

Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., Rousselle, C., 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. Environ. Health 14 (1), 13. https://doi.org/10.1186/1476-069X-14-13.

Maranho, L.A., Moreira, L.B., Baena-Nogueras, R.M., Lara-Martín, P.A., DelValls, T.A., Martín-Díaz, M.L., 2015. A candidate short-term toxicity test using Ampelisca brevicornis to assess sublethal responses to pharmaceuticals bound to marine sediments. Arch. Environ. Contam. Toxicol. 68 (2), 237–258. https://doi.org/10.1007/s00244-014-0080-0. Scopus.

Margiotta-Casaluci, L., Owen, S.F., Cumming, R.I., Polo, A. de, Winter, M.J., Panter, G. H., Rand-Weaver, M., Sumpter, J.P., 2014. Quantitative cross-species extrapolation between humans and fish: the case of the anti-depressant fluoxetine. PLoS One 9 (10), e110467. https://doi.org/10.1371/journal.pone.0110467.

Moreira, D.G., Aires, A., de Lourdes Pereira, M., Oliveira, M., 2022. Levels and effects of antidepressant drugs to aquatic organisms. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 256, 109322 https://doi.org/10.1016/j.cbpc.2022.109322.

Orozco-Hernández, J.M., Gómez-Oliván, L.M., Elizalde-Velázquez, G.A., Heredia-García, G., Cardoso-Vera, J.D., Dublán-García, O., Islas-Flores, H., SanJuan-Reyes, N., Galar-Martínez, M., 2022. Effects of oxidative stress induced by environmental relevant concentrations of fluoxetine on the embryonic development on *Danio rerio*. Sci. Total Environ. 807, 151048 https://doi.org/10.1016/j.scitotenv.2021.151048.

Pacholski, L., Chapman, P., Hood, A., Peters, M., 2017. A cautionary note: Ceriodaphnia dubia inter-laboratory test variability. Bull. Environ. Contam. Toxicol. 98 (1), 53–57. https://doi.org/10.1007/s00128-016-1946-9.

Polverino, G., Martin, J.M., Bertram, M.G., Soman, V.R., Tan, H., Brand, J.A., Mason, R. T., Wong, B.B.M., 2021. Psychoactive pollution suppresses individual differences in fish behaviour. Proc. Biol. Sci. 288 (1944), 20202294 https://doi.org/10.1098/rspb.2020.2294.

Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G.H., Owen, S.F., Sumpter, J. P., 2013. The read-across hypothesis and environmental risk assessment of pharmaceuticals. Environ. Sci. Technol. 47 (20), 11384–11395. https://doi.org/10.1021/es402065a.

Ranganathan, R., Sawin, E.R., Trent, C., Horvitz, H.R., 2001. Mutations in the Caenorhabditis elegans serotonin reuptake transporter MOD-5 reveal serotonin-dependent and -independent activities of fluoxetine. J. Neurosci. 21 (16), 5871–5884. https://doi.org/10.1523/JNEUROSCI.21-16-05871.2001.

Richards, S.M., Cole, S.E., 2006. A toxicity and hazard assessment of fourteen pharmaceuticals to Xenopus laevis larvae. Ecotoxicology 15 (8), 647–656. https://doi.org/10.1007/s10646-006-0102-4.

Rivetti, C., Campos, B., Barata, C., 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in Daphnia magna. Aquat. Toxicol. 170, 289–296. https://doi.org/10.1016/j.aquatox.2015.07.019.

Schnell, S., Bols, N.C., Barata, C., Porte, C., 2009. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. Aquat. Toxicol. 93 (4), 244–252. https://doi.org/10.1016/j.aquatox.2009.05.007.

Schweizer, M., von der Ohe, P.C., Gräff, T., Kühnen, U., Hebel, J., Heid, C., Kundy, L., Kuttler, J., Moroff, F.-M., Schlösinger, A.-F., Schulze-Berge, P., Triebskorn, R., Panagopoulou, E., Damalas, D.E., Thomaidis, N.S., Köhler, H.-R., 2022. Heart rate as an early warning parameter and proxy for subsequent mortality in *Danio rerio* embryos exposed to ionisable substances. Sci. Total Environ. 818, 151744 https://doi.org/10.1016/j.scitotenv.2021.151744.

Singh, A., Saidulu, D., Gupta, A.K., Kubsad, V., 2022. Occurrence and fate of antidepressants in the aquatic environment: insights into toxicological effects on the aquatic life, analytical methods, and removal techniques. J. Environ. Chem. Eng. 10 (6), 109012 https://doi.org/10.1016/j.jece.2022.109012.

Stucki, A.O., Barton-Maclaren, T.S., Bhuller, Y., Henriquez, J.E., Henry, T.R., Hirn, C., Miller-Holt, J., Nagy, E.G., Perron, M.M., Ratzlaff, D.E., Stedeford, T.J., Clippinger, A.J., 2022. Use of new approach methodologies (NAMs) to meet regulatory requirements for the assessment of industrial chemicals and pesticides for effects on human health. Front. Toxicol. 4. https://www.frontiersin.org/articles/10.3389/ftox.2022.964553.

Sumpter, J., Donnachie, R.L., Johnson, A., 2014. The apparently very variable potency of the anti-depressant fluoxetine. Aquat. Toxicol. 151, 57–60. https://doi.org/10.1016/j.aquatox.2013.12.010.

Van de Maele, M., Janssens, L., Stoks, R., 2021. Evolution of tolerance to chlorpyrifos causes cross-tolerance to another organophosphate and a carbamate, but reduces tolerance to a neonicotinoid and a pharmaceutical. Aquat. Toxicol. 240, 105980 https://doi.org/10.1016/j.aquatox.2021.105980.

Van der Most, M.A., Miro Estruch, I., Van den Brink, N.W., 2023. Contrasting dose response relationships of neuroactive antidepressants on the behavior of C. elegans. Ecotoxicol. Environ. Saf. 250, 114493 https://doi.org/10.1016/j.ecoenv.2022.114493.

Vandenberg, L.N., 2014. Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol a as a case study. dose-response Dose Response 12 (2), 13–20. https://doi.org/10.2203/dose-response.13-020.Vandenberg. Vandenberg.

Varano, V., Fabbri, E., Pasteris, A., 2017. Assessing the environmental hazard of individual and combined pharmaceuticals: acute and chronic toxicity of fluoxetine and propranolol in the crustacean Daphnia magna. Ecotoxicology 26 (6), 711–728. https://doi.org/10.1007/s10646-017-1803-6.

Varret, C., Beronius, A., Bodin, L., Bokkers, B.G.H., Boon, P.E., Burger, M., De Wit-Bos, L., Fischer, A., Hanberg, A., Litens-Karlsson, S., Slob, W., Wolterink, G., Zilliacus, J., Beausoleil, C., Rousselle, C., 2018. Evaluating the evidence for non-monotonic dose-response relationships: a systematic literature review and (re-)analysis of in vivo toxicity data in the area of food safety. Toxicol. Appl. Pharmacol. 339, 10–23. https://doi.org/10.1016/j.taap.2017.11.018.

Zindler, F., Stoll, S., Baumann, L., Knoll, S., Huhn, C., Braunbeck, T., 2020. Do environmentally relevant concentrations of fluoxetine and citalopram impair stress-related behavior in zebrafish (Danio rerio) embryos? Chemosphere 261, 127753. https://doi.org/10.1016/j.chemosphere.2020.127753.