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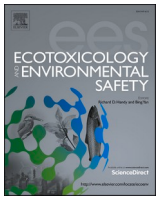

Contrasting dose response relationships of neuroactive antidepressants on the behavior of *C. elegans*

Merel A. van der Most *, Ignacio Miro Estruch, Nico W. van den Brink

Division of Toxicology, Wageningen University and Research, Wageningen 6708 WE, the Netherlands

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ABSTRACT

Antidepressant prescriptions are on a rise worldwide and this increases the concerns for the impacts of these pharmaceuticals on nontarget organisms. Antidepressants are neuroactive compounds that can affect organism's behavior. Behavior is a sensitive endpoint that may also propagate effects at a population level. Another interesting aspect of antidepressants is that they have shown to induce non-monotonic dose-response (NMDR) curves. While such NMDR relationships may have clear implications for the environmental risk, the resolution of current studies is often too coarse to be able to detect relevant NMDR. Therefore, the current study was performed into the behavioral effects (activity, feeding and chemotaxis) in *Caenorhabditis elegans* as the model organism of the selective serotonin reuptake inhibitors fluoxetine and sertraline and the acetylcholinesterase inhibiting pesticide chlorpyrifos, using a wide range of concentrations (ng/l to mg/l). In order to statistically examine the non-monotonicity, nonlinear regression models were applied to the results. The results showed a triphasic dose-response relationship for activity and chemotaxis after exposure to fluoxetine, but not to sertraline or chlorpyrifos. Effects of fluoxetine already occurred at low concentrations in the range of ng/l while sertraline only showed effects at concentrations in the µg/l range, similar to chlorpyrifos. The different responses between fluoxetine and sertraline, both SSRIs, indicate that response patterns may not always be extrapolated from chemicals with the same primary mode of action. The effects of fluoxetine at low concentrations, in a non-monotonic manner, confirm the relevance of examining such responses at low concentrations.

1. Introduction

Antidepressant prescriptions have been on a rise for decades. Concerns for the impacts of these pharmaceuticals in the environment are therefore also increasing (Ford and Fong, 2016; Silva et al., 2012). Pharmaceuticals often require metabolic stability and may therefore be quite persistent and often pass through wastewater treatment systems to end up in the environment (Rivetti et al., 2016). Antidepressants are designed to be active at low doses and often act on evolutionary conserved biochemical pathways and molecular targets, which increases the concern for effects in nontarget organisms (Ford and Fong, 2016). The neuroactive properties of antidepressant pharmaceuticals emphasize their possible effects to organisms' behavior. Behavior is generally not addressed in detail in risk assessments of chemicals, but is a non-standard endpoint that is gaining increasing attention, thanks to its relative high sensitivity compared to for example mortality and development (Anderson et al., 2004; Zhou et al., 2019). Such sensitive effects on individuals may also propagate effects at the population level (Clotfelter et al., 2004). Different scholars have therefore argued for the use of behavioral endpoints in risk assessment of chemicals (Fong et al., 2017; Steele, 2013; Weis et al., 2001).

Most antidepressants exert their effects through modifying neurotransmitter signalling pathways such as those of serotonin, dopamine, GABA and noradrenaline. These neurotransmitters have a specific, but species-dependent, role in the nervous system. Selective serotonin reuptake inhibitors (SSRIs) are one of the most commonly prescribed types of antidepressants. They block the reuptake of serotonin from the synaptic cleft so that serotonin can interact with its specific receptors longer and more intense (Guler and Ford, 2010; Silva et al., 2012). Fluoxetine and sertraline are both SSRIs that have shown to induce behavioral effects at environmentally relevant concentrations. Fluoxetine (the active ingredient of Prozac), for example, affected anti-predator behavior of

Abbreviations: AChE, Acetylcholinesterase; AIC, Akaike Information Criterion; CGC, Caenorhabditis Genetics Center; NGM, Nematode Growth Medium; NMDR, Non-monotonic dose response; LSD, least square differences; OD, optical density; SSRI, selective serotonin reuptake inhibitor.

* Corresponding author.
E-mail address: merel.vandermost@wur.nl (M.A. van der Most).

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fish, phototactic behavior of *Daphnia magna* and the ventilation rate of freshwater shrimp *Gammarus pulex* at concentrations in the range of ng/l (de Lange et al., 2009; Painter et al., 2009; Rivetti et al., 2016; Saaristo et al., 2017). Sertraline changed the amphipod *Echinogammarus marinus* velocity in a ng/l range (Bossus et al., 2014) and yellow catfish *Tachysurus fulvidraco* behavior at µg/l (Chen et al., 2021).

Interestingly, previous studies with antidepressants, including SSRIs, have shown non-monotonic dose-response (NMDR) relationships (Bossus et al., 2014; Fong et al., 2017; Ford and Fong, 2016; Guler and Ford, 2010). In a NMDR curve, the direction of the slope of the dose-response curve changes over the range of tested concentrations (Ford and Fong, 2016; Hill et al., 2018). Evaluation of NMDR relationships is relevant, because these responses could result in underestimating toxic effects at low concentrations (Hill et al., 2018). NMDR relationships have been observed for, for example, the righting time of marine snail *Ilyanassa obsolete* exposed to fluoxetine, sertraline, paroxetine and venlafaxin (all SSRIs) (Fong et al., 2017) and for phototaxis activity of the amphipod *E. marinus* after exposure to fluoxetine (Guler and Ford, 2010). A variety of theories may explain an NMDR relationship, including 1) negative feedback regulation (Fong et al., 2017), 2) receptor desensitization (Guler and Ford, 2010), 3) a plurality of molecular targets (with different receptor affinities) (Hill et al., 2018; Lagarde et al., 2015), 4) dose-dependent metabolism modulation (Hill et al., 2018; Lagarde et al., 2015) and 5) high dose acute toxicity (Fong et al., 2017; Lagarde et al., 2015). Full mechanistic understanding into these specific theories is still lacking, also for antidepressants. Furthermore, the potency of antidepressants to induce NMDR relationships appears to be variable between studies (Sumpter et al., 2014). A problem with current toxicity studies is that the range of concentrations applied in experimental studies is often limited to relatively high concentrations, but even more so the resolution in exposure concentrations is often too course to provide sufficient insights into the behavioral toxicity and possible NMDR relationships, especially at low concentrations (Sumpter et al., 2014). Therefore, it is important to perform these experiments at a wide range of concentrations. The objective if this study is to assess the potential NMDR relationships between neuroactive compounds and behavioral endpoints, using a large number of concentrations (ng/l to mg/l) of two SSRIs (fluoxetine and sertraline) and one organophosphate pesticide (chlorpyrifos) on *Caenorhabditis elegans* at multiple behavioral traits. To our knowledge, a similar study has not been conducted before. *C. elegans* was selected as a model organism because of its relatively short life cycle and ease of handling, while it still allows for the study of whole animal responses, with an intact sensory and neuromuscular system (Eom et al., 2015; Hunt, 2017).

2. Materials and methods

2.1. C. elegans maintenance and exposure scenarios of the different assays

The Bristol N2 Strain of *C. elegans* (Caenorhabditis Genetics Center (CGC), University of Minnesota, Minneapolis, USA) was used for all experiments with *E. coli* OP50 strain (also obtained from the CGC) as a food source. *C. elegans* were maintained on nematode growth medium (NGM) seeded with OP50 at 20 °C in the dark (Stiernagle, 2006). Age synchronization was performed by bleaching a *C. elegans* culture with a mixture of sodium hypochlorite (~4 %, 0.5 ml) and sodium hydroxide (1 M, 0.63 ml) and MiliQ water (1.37 ml) (Stiernagle, 2006). This process leaves only the resistant eggs, which were transferred to 50 ml M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$ to 1 litre H_2O) and allowed to hatch overnight to L1 larvae without *E. coli* (Stiernagle, 2006). The absence of a food source stalls development at the L1 stage.

For the chemotaxis, locomotion and feedings assays, L1 larvae were transferred to S medium (1 litre S Basal [5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol), H_2O to 1 litre], 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution (Stiernagle, 2006), 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$), containing *E. coli* OP50 at an optical density (at 600 nm) of 0.65–0.7 and then added to a 24-well plate (995 µl per well, ± 1 worm/µl). In the chemotaxis and locomotion assays larvae were exposed to the different compounds from L1 stage. For the assessment of activity, 96-well plates (90 µl per well, 1 worm/µl) were used and L1 larvae were allowed to develop for at least 50 h to the adult stage before the exposure (see Fig. 1) to allow for a basal measurement of activity and prevent that the activity measurements are affected by developmental toxicity (according to Wmicrotracker manufacturer's protocol). Fluoxetine hydrochloride (>99.8 %), sertraline hydrochloride (99.7 %) and chlorpyrifos-methyl (99.3 %) were purchased from Sigma Aldrich (Zwijndrecht, Netherlands). Stock solutions of the three compounds were prepared in DMSO and serial dilutions were made to obtain the required concentrations, in the range of low ng/l to high mg/l (See Table S1 for all concentrations). The final concentration of DMSO in medium was 0.5 %, as used by various former studies (Risley et al., 2016; Sofela et al., 2021; Zhou et al., 2021).

2.2. Experimental assays

2.2.1. Mortality/development

To ensure that the behavioral experiments would focus on sub-lethal concentrations, effects of the compounds on *C. elegans* mortality were tested according to Sese et al. (2009). Worms were exposed in liquid medium to different concentrations of the chemicals for 24 h (Adult-Adult) or 72 h (L1-Adult). Six replicates were included for each concentration. After exposure, around 20 worms from each well were transferred to NGM and mortality was checked by testing the response to a mechanical stimulus (Sese et al., 2009). To ensure that mortality was scored and not the lack of responsiveness, worms were washed with M9 three times and incubated further in S medium. Potential recovery after 24 h was checked. However, no worms that were classified dead responded to the mechanical stimulus after recovery, hence the mortality was verified. Possible effects on *C. elegans* development were tested by video analysis, using the EPFL Movement Tracker software (Mouchiroud et al., 2016). Video imagery was automatically analysed and the size of moving worms was quantified. Data were expressed in number of pixels. Due to mortality, size measurements for some of the highest concentrations are missing.

2.2.2. Activity assay

To measure the locomotor activity of *C. elegans*, the WMicrotracker system from InVivo Biosystems was used (PhylumTech). This instrument quantifies the joint locomotor activity of all *C. elegans* in liquid media in

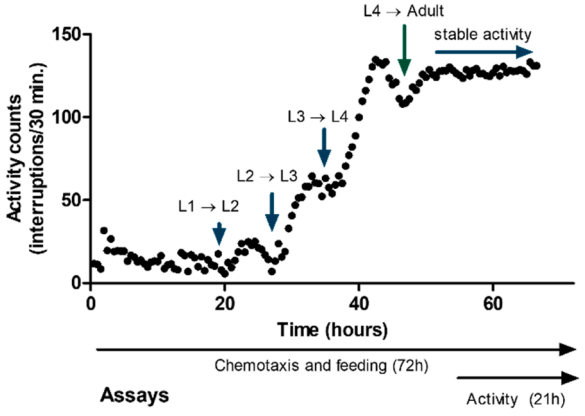

Fig. 1. Development of *C. elegans* over time expressed as activity, measured as interruption counts per 30 min with the Wmicrotracker. Chemotaxis and feeding were tested for 72 h starting exposures at L1 larvae and activity was measured for 21 h after a basal measurement of stable activity at around 50 h.

multi-well plates by measuring the number of infrared micro beam interruptions over time. Activity was expressed as the number of interruptions (count) over a timeframe of 30 min, as calculated by the Wmicrotracker software. *C. elegans* develop through different life stages and their activity increases at older life stages (Hunt et al., 2018; Fig. 1). In order to account for differences in signal due to the total number of worms between wells, a within-well basal measurement of the activity was performed after the activity had reached a plateau (~50 h), according to the manufacturer's protocol. Upon reaching this plateau in activity, worms were exposed and activity in exposure medium was measured continuously for 21 h. (Fig. 2).

2.2.3. Chemotaxis assay (including movement ratio)

Chemicals can also affect the behavioral response of *C. elegans* to external stimuli, including attracting compounds. A way to determine this effect is through the chemotaxis assay (Margie et al., 2013). After 72 h exposure starting at stage L1 (Section 2.1), adult *C. elegans* were placed in the middle of an NGM filled petri dish and allowed to move for 45 min. Two quadrants of the petri dish were spiked with an attractant (0.5 % diacetyl) and two with water (MiliQ-control) (Fig. S3), according to Margie et al. (2013). After 45 min, the petri dish was moved to 4 °C to stop worm movement and the number of worms in each quadrant was counted. The Chemotaxis Index (CI) and Movement Ratio (MR) were calculated with Eqs. 1 and 2, respectively.

$$CI = \frac{Total\ number\ in\ quadrants\ "Attraction" - Total\ number\ in\ quadrants\ "Control"}{Total\ number\ of\ nematodes\ that\ moved\ out\ of\ the\ origin} \quad (1)$$

$$Movement\ ratio = \frac{Numbers\ of\ nematodes\ that\ moved\ out\ of\ the\ origin}{Total\ number\ of\ nematodes\ in\ dish} \quad (2)$$

2.2.4. Feeding assay

Feeding is another sublethal, behavioral endpoint that may be relatively sensitive to neurotoxicants (Anderson et al., 2001). One relatively simple way to quantify feeding behavior is to measure the change in optical density at 600 nm (OD600), which is a good indicator of the number of bacteria in suspension (Anderson et al., 2004). If the *C. elegans* feeding behavior is affected, this will result in reduced numbers of bacteria and therefore changes in OD600. Tests have shown that the interference of *C. elegans* in the optical density measurement is very limited (Anderson et al., 2004). After exposure in a 24-well plate, a OD600 measurement was done at 0, 24, 48 and 72 h. The total feeding was quantified by calculating the difference between the OD600 at 0 h and the other timepoints, according to Anderson et al. (2004). In this study, just the response at 48 h is displayed. Data for 24 h and 72 h can be found in Fig. S2.

2.3. Statistical analysis

Differences between treatment groups were analysed using an ANOVA, with Least Significant Differences (LSD) post hoc pairwise comparisons. A p-value < 0.05 was considered statistically significant. For mortality, a logistic regression was applied to obtain LC50 values and the corresponding confidence intervals. IBM SPSS Statistics 25 was used for the statistical analysis and data were plotted with Graphpad Prism 5.

A review by Hill et al. (2018) expresses the need to 'develop agreed upon methods, using best practices, for statistically evaluating non-monotonic relationships' (Hill et al., 2018). Therefore, nonlinear regression models by di Veroli et al. (2015) were used to analyse different types of dose-response patterns (Eqs. 4 and 5). These models adjust and multiply Hill models two or three times in order to represent biphasic and triphasic dose response curves (Fig. 3). Specific EC50s for each part of the curve are calculated. The multiplication of these models can be supported mechanistically, since it allows for interactions between different effects. For the triphasic response, for example, an initial behavioral effect can be counteracted by either a feedback mechanism or an opposing effect, and this interaction is also part of the model.

Monophasic

$$E(c) = MIN + \left(\frac{MAX(=1) - MIN}{1 + (EC50/C)^{H}}\right) \quad (3)$$

Biphasic

$$E(c) = \left(1 + \frac{Emax_1 - 1}{(EC50_1/C)^{H_1}}\right)\left(1 + \frac{Emax_2 - 1}{(EC50_2/C)^{H_2}}\right) \quad (4)$$

Triphasic

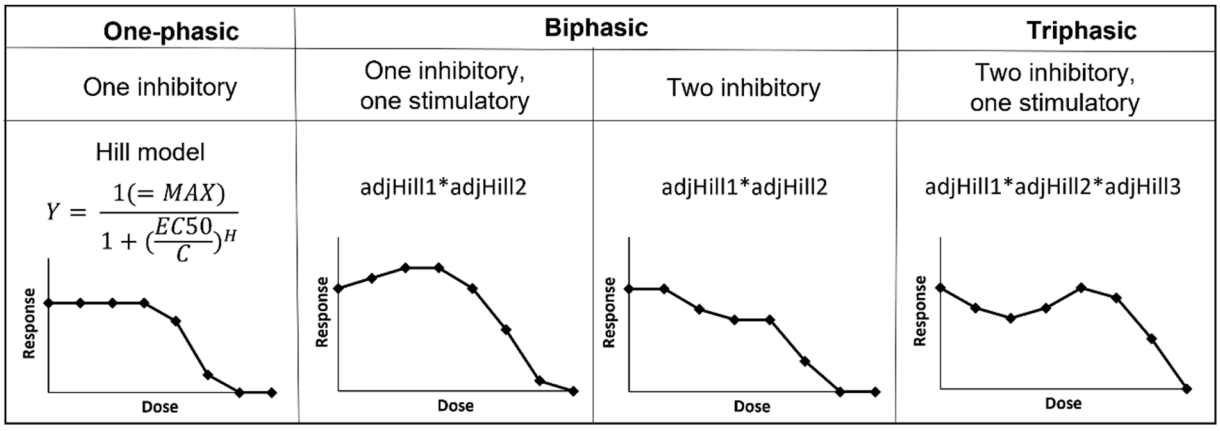

Fig. 2. Schematic representation of the three different types of nonlinear regression models that were applied to the results of the experiment, based on di Veroli et al. (2015).

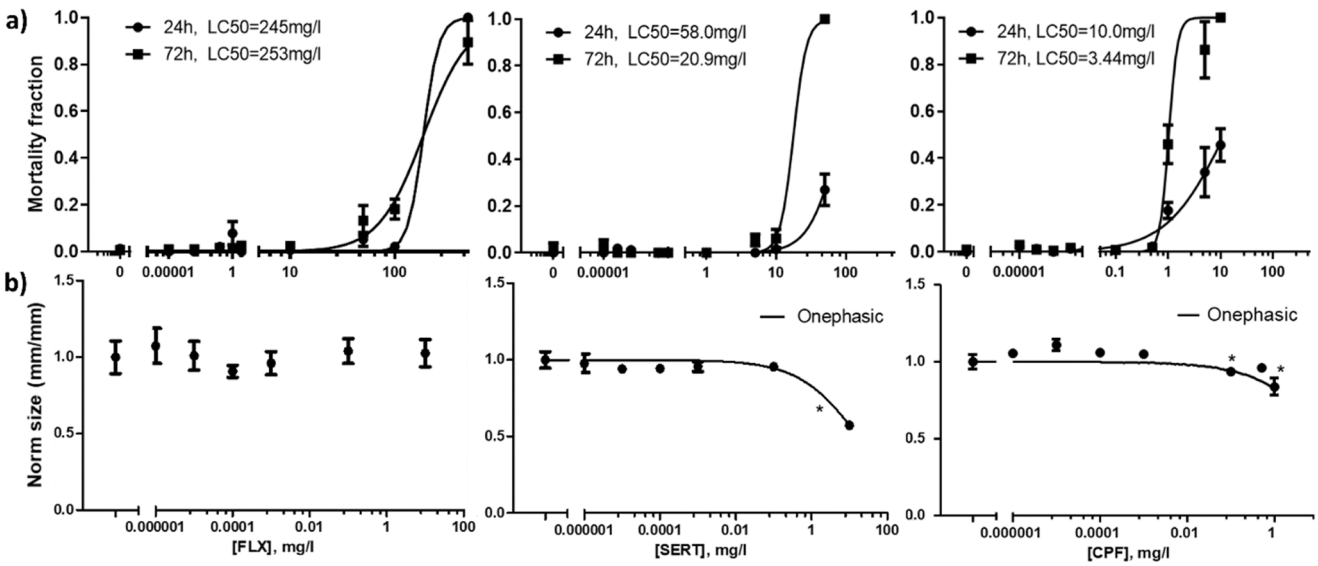

Fig. 3. a) Mortality of *C. elegans* after 24 or 72 h exposure to fluoxetine (FLX), sertraline (SERT) or chlorpyrifos (CPF) and b) Relative size of *C. elegans* after 72 h exposure from L1 larvae to adult stage to fluoxetine (FLX), sertraline (SERT) and chlorpyrifos (CPF). Data were obtained through automated video analysis and normalized to the control. Bars represent the SEM and * indicate statistical significance ($p < 0.05$ with ANOVA LSD pairwise comparisons).

$$E(c) = \left(1 + \frac{Emax_1 - 1}{(EC50_1/C)^{H_1}}\right)\left(1 + \frac{Emax_2 - 1}{(EC50_2/C)^{H_2}}\right)\left(1 + \frac{Emax_3 - 1}{(EC50_3/C)^{H_3}}\right) \quad (5)$$

where E(c) = effect at concentration C, MIN = lower plateau of the response, MAX = upper plateau of the response, Emax = maximum effect/virtual asymptote (1 = no effect, 0 = 100 % effect), EC50 = C at which E is 50 % of Emax/MAX and H = Hill constant and subscripts (1,2 and 3) represent different parts of the curve.

Data were normalized to the average response of the non-exposed control. For the Monophasic Hill model, Eq. (3) was used and the maximum value (MAX) was set at 1 (the average of the control) to minimize the risk that the non-monotonic pattern will not be recognized. In case the MAX is not specified, it may be calculated as the combined average of the effects at the control and low concentration, resulting in a risk of smearing subtle effects at low concentrations into the average. For the biphasic and triphasic model, this MAX= 1 is already included in the equation so that was not changed (see Eqs. 4 and 5). Furthermore, for assays where the maximum effect goes to 0 (all except for feeding where there was a background), the MIN (monophasic) and Emax (bi- and triphasic) were set at 0. The best performing model was determined based on the corrected Akaike information criterion (AICc) calculation (Eq. 6) and the model with the lowest AICc weight was chosen (Eq. 7) (Akaike, 1973; Newland, 2019). AICc was preferred over AIC, because of the relatively low sample size.

$$AICc = n\ln\left(\frac{SSerror}{n}\right) + 2K + \frac{2K(K+1)}{(n-K-1)}, \quad (6)$$

where K = # parameters + 1, N = # of observations, SSerror = residual sum of squares.

$$AICweight = \exp(-0.5 * \Delta AICc) \Big/ \sum \exp(-0.5 * \Delta AICc) \quad (7)$$

3. Results and discussion

3.1. Effects on mortality and development

The mortality experiments indicate that chlorpyrifos was most toxic, followed by sertraline and fluoxetine (Fig. 3a). The corresponding LC50s (+ 95 % CI) are 10.0 (8.51–12.1) mg/l, 58.0 (52.6–65.9) mg/l and 245 (184−371) mg/l for 24 h, and 3.44 (2.75–4.53) mg/l, 20.9 (15.0–40.5) mg/l and 253 (203−332) mg/l for 72 h for respectively chlorpyrifos, sertraline and fluoxetine. In the behavioral experiments maximum concentrations tested were up to respectively 1, 50 and 100 mg/l, thus including some concentrations that may affect mortality, but mainly representing sublethal concentrations. Despite the similar modes of action, sertraline appeared more toxic than fluoxetine, as indicated in earlier studies (Johnson et al., 2007). *C. elegans* development (represented by size) was affected at 100 mg/l fluoxetine, ≥ 10 mg/l sertraline and ≥ 0.1 mg/l chlorpyrifos (Fig. 3b). These developmental effects can result in altered behavior and were therefore taken into account in the analysis of behavioral responses.

3.2. Effects on behavioral traits

Fig. 4 displays the results of the behavioral experiments on activity, feeding, chemotaxis and the related movement ratio. Table 1 provides the corresponding statistical outcomes of the nonlinear regression models (more details in Table S2). *C. elegans* behavior was clearly affected by all chemicals, also at sublethal concentrations. The results obtained confirm the higher sensitivity of behavior compared to more traditional endpoints such as mortality and development (Anderson et al., 2004). Only for chlorpyrifos, behavior was affected in a similar concentration range as mortality and development. The lowest EC50s (Table 1) were as low as 1 ng/l for the triphasic dose-response models, orders of magnitudes lower than the LC50s in Fig. 3a. Also for mono and bi-phasic responses, some of the EC50 values were significantly lower than the related LC50s, such as for the activity response to sertraline (EC50 0.02 or 5.7 mg/l (Fig. 4b) compared to LC50 58 mg/l (Fig. 3a)) and the feeding responses to fluoxetine (EC50s 0.89 ng/l and 7.0 mg/l (Fig. 4c) compared to LC50 ~250 mg/l (Fig. 3a)) and sertraline (EC50 0.39 μg/l (Fig. 4c) compared to LC50 of 21–58 mg/l (Fig. 3a)). The high EC50s were often comparable to the LC50s, hence the higher EC50 levels could be related to different mechanisms than the lower EC50 levels within the same experiment. The nature of the responses differs between the compounds. Fluoxetine exposure produced a triphasic NMDR pattern for both activity and chemotaxis (Fig. 4), with effects at low concentrations that disappeared at intermediate concentrations and reappeared at high concentrations. For chemotaxis (Fig. 4d), these low concentration effects were statistically significant at 1, 10 and 100 ng/l. The movement ratio (Fig. 4e) was only affected at 10 and 100 mg/l, so the effect at low concentrations did not appear to just be caused by a lack

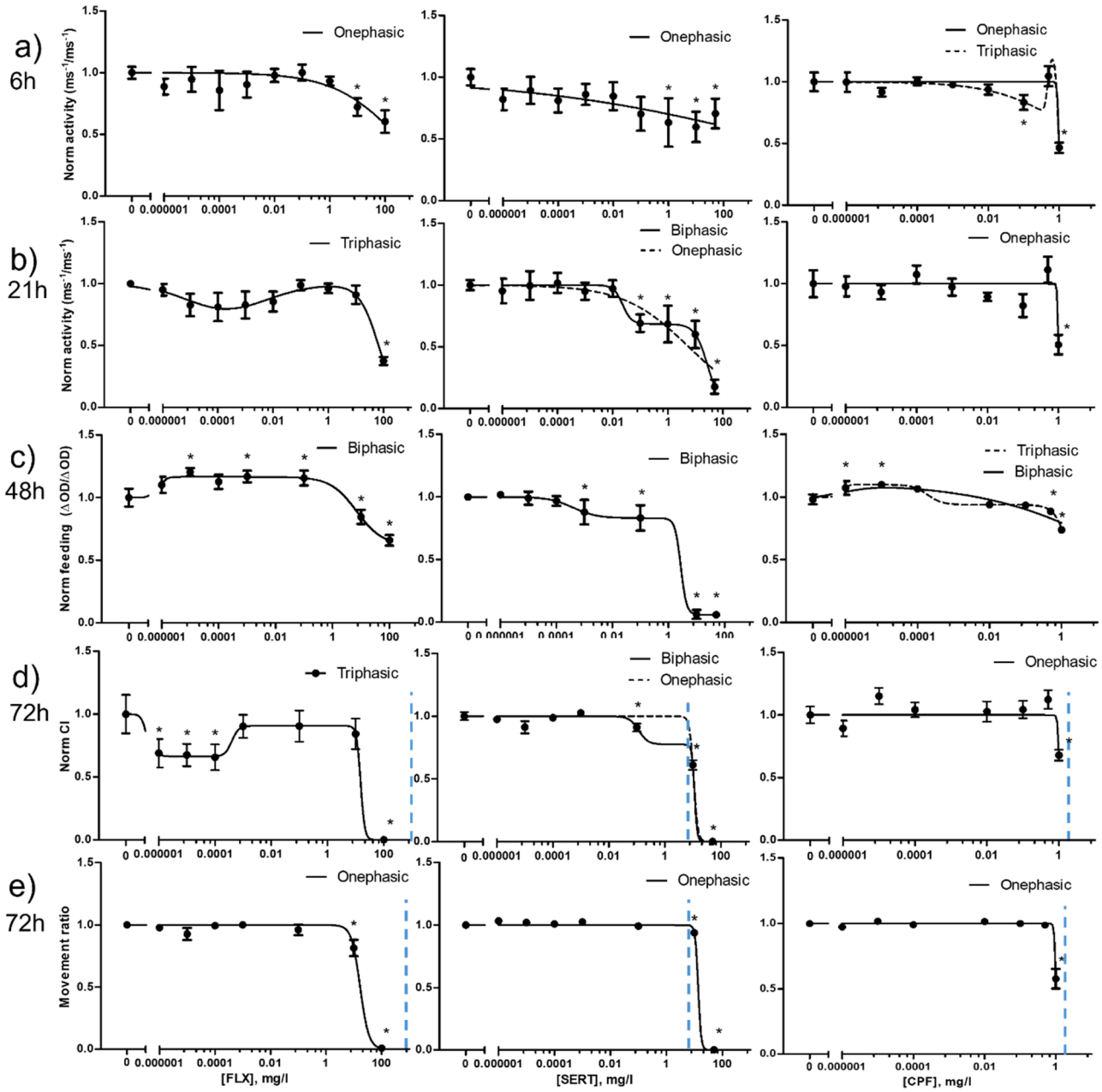

Fig. 4. Behavioral changes of *C. elegans* after exposure to either fluoxetine (FLX), sertraline (SERT) or chlorpyrifos (CPF) in a and b) activity (after 6 h (a) or 21 h (b)), c) feeding (48 h exposure), d) chemotaxis index (72 h exposure) and e) movement ratio (72 h exposure). Data were normalized to the control. Bars represent the SEM and * indicate statistical significance ($p < 0.05$ with ANOVA LSD pairwise comparisons). Dashed vertical line in Figs. d and e represents the LC50 for similar exposure time (72 h L1-A): 253, 20.9 and 3.44 mg/l for respectively fluoxetine, sertraline and chlorpyrifos.

of movement, but might also be related to a particular chemotactic behavior, e.g. sensing. For activity (Fig. 4a/b), the low concentration responses were not significantly different from the control, but the AICc analysis still favored a triphasic model after 21 h (Table 1). The concentrations at which an NMDR response was detected, differed between chemotaxis and activity. This could be explained by different exposure times (21 h versus 72 h) or a varying sensitivity between the two assays. Small increases in feeding were also observed after fluoxetine exposure (Fig. 4c).

Sertraline, even though it has a similar primary mode of action as fluoxetine, mainly showed mono- or biphasic dose response relationships. The biphasic pattern is likely explained by two different effects; 1) a behavioral effect through the blocking of the serotonin reuptake transporter at lower concentrations, and 2) an effect on survival/development (Fig. 3) at higher concentrations that also affected behavior. Feeding appeared to be most sensitive to sertraline exposure with significant effects at 0.001 mg/l. For chlorpyrifos, most effects occur at high concentrations, close to the lethal concentrations, but a small but significant increase in feeding was observed at lower concentrations (Fig. 4c).

Serotonin is responsible for the slowing response of *C. elegans* when encountering food (Ranganathan et al., 2001; Sawin et al., 2000), explaining the reduction in activity upon exposure to both SSRIs. Exposure to SSRIs increased velocity/activity in amphipods *E. Marinus* (Bossus et al., 2014; de Lange et al., 2009) and the crab *Carcinus maenas* (Mesquita et al., 2011). The increase instead of decrease in activity for those studies indicate that the effect may be species specific, depending on the role of serotonin in the nervous system, or on the experimental

Table 1
Modelling outcomes - applying monophasic, biphasic and triphasic nonlinear regression (Eqs. 3–5) models to the dose-response data of three behavioral endpoints of C. elegans (activity, chemotaxis and feeding) exposed to fluoxetine, sertraline and chlorpyrifos. AICc = corrected Aikaike Information Criterion, weight corresponding to AICc value, EC50 = half maximum effective concentration. All EC50 values are in mg/l. Light blue color indicates AICc weight > 0.1, dark blue color indicates AICc weight > 0.8.

		Activity 6h			Activity 21h			Chemotaxis			Feeding (48h)		
		Fluoxetine	Sertraline	Chlorpyrifos	Fluoxetine	Sertraline	Chlorpyrifos	Fluoxetine	Sertraline	Chlorpyrifos	Fluoxetine	Sertraline	Chlorpyrifos
onephasic	AICc	-183.30	-157.38	-233.17	-190.03	-181.58	-192.51	-103.63	-236.23	-164.32	-161.34	-249.33	-230.47
	weight	0.95	0.97	0.68	0.04	0.32	0.88	0.01	0.39	0.90	0.00	0.00	0.01
	R2	0.19	0.13	0.53	0.32	0.57	0.34	0.30	0.94	0.31	0.35	0.97	0.56
	EC50	249.97	7906.23	0.99	65.84	5.71	1.00	14.02	10.78	1.04	10.57	0.38	3.32
biphasic	AICc	-177.35	-150.48	-229.97	-188.55	-183.10	-187.08	-108.19	-237.10	-159.85	-185.81	-267.38	-236.39
	weight	0.05	0.03	0.14	0.02	0.67	0.06	0.06	0.60	0.10	1.00	1.00	0.10
	R2	0.21	0.14	0.56	0.38	0.63	0.36	0.46	0.95	0.35	0.67	0.98	0.67
	EC50	1.00E-06	9.30E-02	1.02E-03	1.00E-06	2.38E-02	1.01E-03	5.00E-07	1.17E-01	2.90E-02	8.92E-07	3.90E-04	1.00E-06
	EC502	150.00	50.06	1.00	60.00	28.37	1.01	15.00	11.49	1.02	7.02	2.74	180.00
	type	x	x	2 inhib	x	2 inhib	x	x	2 inhib	x	1 stim, 1 inhib	2 inhib	1 stim, 1 inhib
triphasic	AICc	-172.61	-144.63	-230.52	-196.30	-174.93	-187.30	-113.56	-229.19	-152.06	-173.30	-255.50	-240.68
	weight	0.00	0.00	0.18	0.94	0.01	0.06	0.93	0.01	0.00	0.00	0.00	0.89
	R2	0.25	0.17	0.62	0.52	0.63	0.44	0.59	0.95	0.37	0.66	0.98	0.75
	EC50	3.10E-07	3.04E-07	1.49	8.58E-06	8.44E-02	7.62E-03	5.85E-07	1.85E-03	4.74E-02	0.10	8.46E-04	8.55E-07
	EC502	2.00E-04	9.00E-02	0.48	1.84E-03	5.59E-04	0.52	3.97E-04	4.66E-02	2.97E-02	9.00E-07	2.58E-02	1.88E-04
	EC503	245.00	228.09	0.96	66.04	46.93	0.93	15.10	11.14	1.03	8.00	2.10	1.91

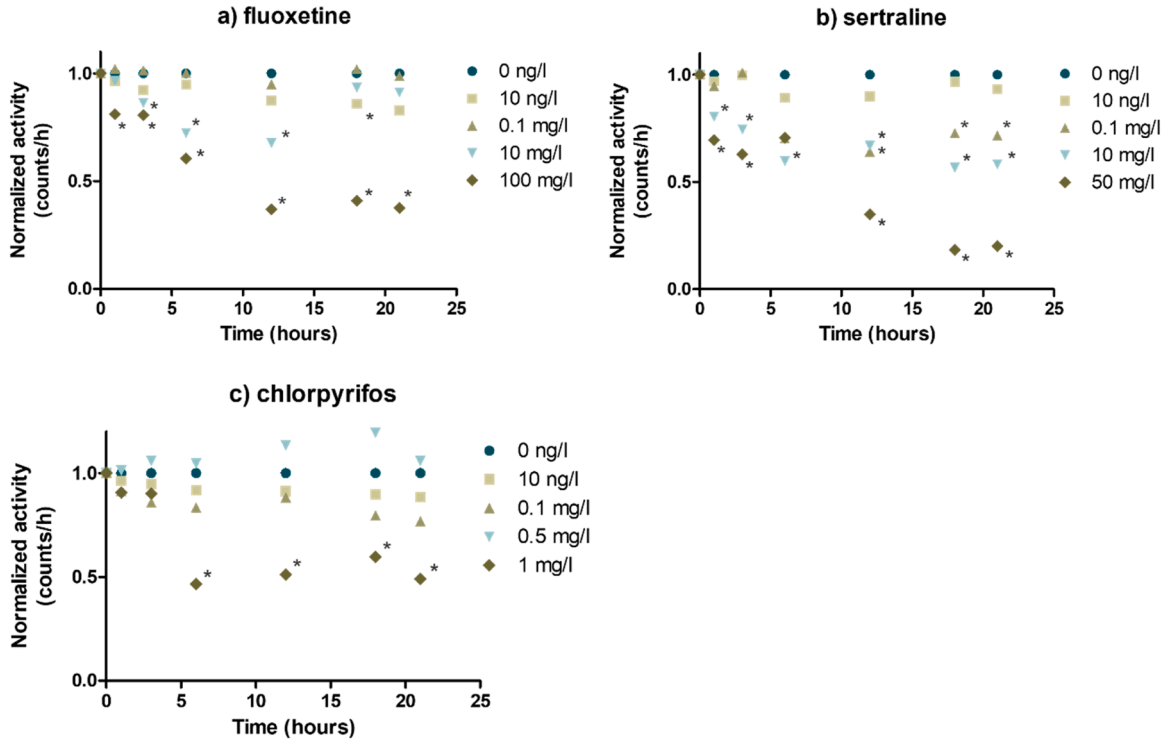

Fig. 5. *C. elegans* activity after exposure to fluoxetine (a), sertraline (b) and chlorpyrifos (c), continuously measured with the Wmicrotracker (Phylumtech). * indicate statistical significance ($p < 0.05$ with ANOVA LSD pairwise comparisons relative to the control at the same timepoint). Time points were selected to accurately show developments over time.

conditions. Interestingly, the decrease in activity at low concentrations of fluoxetine was associated with an increase in feeding. This increased feeding can be linked to the food-specific slowing response, but serotonin itself also stimulates pharyngeal pumping (Chase and Koelle, 2007). Sertraline, on the other hand, did not enhance feeding, again raising the question how the modes of action of fluoxetine and sertraline compare. Serotonin also plays a particularly important role in modulating the exploratory behavior of *C. elegans* when introduced to new environments and situations, as it is responsible for chemosensory and olfactory learning and sensitivity (Chao et al., 2004)(Chao et al., 2004). Effects of SSRIs on chemotaxis were therefore to be expected. Other studies also found effects of fluoxetine by increasing photo and geotactic behavior in amphipods *E. marinus* (Guler and Ford, 2010) and intensifying phototactic behavior in *Daphnia magna* (Rivetti et al., 2016). The direction of the effect was again opposite to our results, further confirming the species and experiment specificity. Locomotory changes linked to inactivation of AChE are often caused by paralysis through hypertension (Kumar et al., 2018). This explains the effects of chlorpyrifos on activity only close to the lethal concentrations. There is no clear role of ACh in chemotaxis behavior (Jorgensen et al., 2007) and our results also show an effect only at the highest concentrations, likely related to a limited movement as the movement ratio was clearly

affected here. ACh stimulates pharyngeal pumping (Jorgensen, Kaplan, and Rand, 2007), which was observed for the feeding response of chlorpyrifos.

3.3. Time dependent effects on activity and NMDR theories

Because the Wmicrotracker tracked activity over a full 21 h exposure period, analysis of changes in responses over time was possible. Upon exposure to all chemicals, activity decreased over the first 5–10 h, after which it either stabilized or recovered (Fig. 5). For 10 mg/l fluoxetine, the activity clearly recovered over time, while for 100 mg/l, it did not after 21 h (Fig. 5a). The same absence of recovery was also seen for 0.1 mg/l & 10 mg/l sertraline (Figs. 5b) and 1 mg/l chlorpyrifos (Fig. 5c). For 50 mg/l sertraline, the activity continued to decrease over time reaching nearly 0 which is likely linked to mortality (Fig. 3). The stabilization or even reduction in the response over time, may be explained by feedback mechanisms and receptor-ligand kinetics. There is only a limited amount of endogenous serotonin produced and the accumulation of serotonin in the synaptic cleft due to reduced reuptake is therefore finite, leading to a stabilization of the response over time (de Lange et al., 2009; Guler and Ford, 2010). Pre-synaptic serotonin auto receptors can also be activated by an increased amount of serotonin in the synaptic cleft and inhibit the further release of serotonin (Fong et al., 2017; Guler and Ford, 2010). Another feedback mechanism specific to *C. elegans* is the presence of serotonin absorbing neurons that can act as temporal–spatial regulators of extrasynaptic serotonin (Jafari et al., 2011). Furthermore, receptors for neurotransmitters can be desensitized due to ligand induction at relatively high concentrations of neurotransmitters over a prolonged period of time (Guler and Ford, 2010; Rivetti et al., 2016). Such receptors then become unresponsive and the (behavioral) effect may be reduced.

Interestingly, these time dynamics are also helpful in explaining the NMDR pattern, that was observed for both activity and the chemotaxis index after exposure to fluoxetine. It is known that receptors will reach desensitization earlier for high concentrations than for lower concentrations of chemicals and responses at higher concentrations are therefore counteracted earlier (Bossus et al., 2014). The recovery at 10 mg/l of FLX may explain the lack of effects after prolonged exposure, as seen for both activity and the chemotaxis index after exposure to fluoxetine. The effects at lower concentrations (e.g. 10 ng/l) on the other hand, actually seem to increase over time (Fig. 5a), with a significant decrease in activity only after 18 h. This lack of recovery or negative feedbacks visible at lower concentrations could explain the NMDR relationship at 21 h, but more research is needed to confirm the underlying molecular mechanisms. One interesting observation is that 0.1 mg/l fluoxetine does not appear to produce an effect at any of the time points, which challenges this hypothesis. More research into serotonin signalling and the actual levels of serotonin in different types of neurons will help to further elucidate these mechanisms. Two other theories for NMDR as discussed in the introduction are dose-dependent metabolism modulation and a plurality of molecular targets with different receptor affinities. Dose-dependent metabolism modulation is often applicable when the metabolite is more or less toxic than the parent compound. In the case of fluoxetine, however, the metabolite norfluoxetine was found to be equipotent or only slightly more toxic (Andrés-Costa et al., 2017; Nałecz-Jawecki, 2007). The theory of different molecular targets may still be applicable, since *C. elegans* serotonergic neurons have different primary receptors, which may also translate into opposing effects (Churgin et al., 2017). However, this still does not explain the differences in dose-response relationship between fluoxetine and sertraline.

3.4. Differences between compounds

The study outcomes showed clear differences between fluoxetine, sertraline and chlorpyrifos. Since chlorpyrifos has a different mode of action than sertraline and fluoxetine, differences were to be expected. The apparent dissimilarities between fluoxetine and sertraline, on the other hand, raise some questions. Both compounds are known SSRIs, and although they may have varying potencies, the way that they induce effects was expected to be similar. These differences in NMDR patterns has also been observed in former studies, where predator avoidance and escape velocity in fathead minnow *P. promelas* showed an NMDR pattern for fluoxetine, but was monotonic for sertraline (Painter et al., 2009; Valenti et al., 2012). Other studies, however, report non-monotonic dose-responses in behavior for both fluoxetine and sertraline in amphipods *E. marinus* and marine snails *I. obsoleta* (Bossus et al., 2014; Fong et al., 2017). The study by Bossus et al. (2014) simultaneously tested transcriptional changes and locomotion velocity in response to light in the amphipod *E. marinus,* exposed to fluoxetine and sertraline. Even though the behavioral response was non-monotonic for both sertraline and fluoxetine, they found a downregulation in genes involved in phototransduction in a non-monotonic manner only for fluoxetine (Bossus et al., 2014).

A possible explanation for the differences between these compounds may be the presence of dissimilar secondary modes of action, e.g. molecular/biochemical specific target sites, different from the principle mode of action which is through the reuptake transporter MOD-5 (Kullyev et al., 2010; Ranganathan et al., 2001). Fluoxetine, for example, was found to directly bind to the serotonin receptor SER-7 in *C. elegans* resulting in similar behavioral effects (Kullyev et al., 2010), which has not been explored for sertraline. Ford and Fong (2016) pointed out that other possible molecular targets of fluoxetine may include 'dopamine reuptake transporters, 5-HT2 receptors, sigma receptors, muscarinic cholinergic receptors and cytochrome P450′s′. This demands further studies, such as radioligand binding studies as recommended by Bidel et al. (2016) and Fong et al. (2017). Slight differences between the effects of sertraline and fluoxetine on rat behavior have been observed, also indicating that they may have different, additional mechanisms of action (Sokolowski and Seiden, 1999; Stanford et al., 2002), other than the serotonin transporter but potentially involving the pre-synaptic serotonin autoreceptor and binding to other receptors (Sokolowski and Seiden, 1999). Another possible difference between sertraline and fluoxetine is that fluoxetine has an active metabolite, norfluoxetine, which is similarly or even slightly more active, while sertraline's active metabolite (desmethylsertraline) has a much lower potency. Furthermore, fluoxetine is also a more potent CYP inhibitor and can even inhibit its own metabolism. This raises the question how internal kinetics of fluoxetine and norfluoxetine develop over time, compared to sertraline and desmethylsertraline.

3.5. Statistical evaluation of the NMDR relationships

Combining the nonlinear regression models with the AICc helped in the development of 'agreed upon methods, using best practices, for how non-monotonic relationships should be evaluated statistically', as called for in a recent review (Hill et al., 2018). This, however, could only be established by the use of a large number of (low) concentration treatments along wide exposure gradients. The nonlinear regression models in the current study show chemical specific patterns across concentrations.

The nonlinear regression models allowed for the analysis of complex NMDR patterns (di Veroli et al., 2015). The complexity of these models has strengths and weaknesses. Analysing NMDR curves this way allows for the calculation of EC50s for different parts of the dose response curve and by modelling NMDR patterns, they can be statistically compared. Furthermore, because the nonlinear regression models are based on the Hill model, they also have a mechanistic underpinning. On the other hand, complex models with many parameters pose a risk of overfitting the data. The AIC and especially the AICc, however, is meant to prefer the more simple models and penalize the use of many explanatory variables. Although the AIC comparison is based on weights and not on a clear significance value, the ease of using this criterium and the penalty

for model complexity still indicate the great usefulness of this method.

4. Conclusions and potential implications for risk assessment

This study examined effects of fluoxetine, sertraline and chlorpyrifos on *C. elegans* behavior. Effects at low concentrations were found and signify the relevance of including behavior as a sensitive endpoint. Three different endpoints and a range of concentrations were used, which allowed for the application of nonlinear regression models to statistically test NMDR relationships. Clear triphasic NMDR relationships were found for fluoxetine activity and chemotaxis with effects at environmentally relevant concentrations. Fluoxetine has been detected in surface waters and wastewater effluents at concentrations in the range of ng/l to ug/l, similar to the relevant effect concentrations in this study (Brooks et al., 2003; Silva et al., 2012). The non-monotonic dose response relationship found for fluoxetine indicates a risk for neglecting effects at low concentrations when the resolution of the used concentrations in toxicity testing is limited (Hill et al., 2018). Differences in the non-monotonic responses between endpoints have been detected before and non-monotonicity therefore seems endpoint dependent (Ford et al., 2018). The inclusion of time dynamics helped in understanding some of the possible feedback mechanisms related to NMDR relationships, but more data are needed to confirm the related theories. The discussion points out many uncertainties related to the (alternative) modes of action of fluoxetine and sertraline that require further investigation. An important area for future research is a further mechanistic comparison between fluoxetine and sertraline, for both toxicokinetics and toxicodynamics. Furthermore, future studies should focus on elucidating the mechanisms behind antidepressant induced non-monotonicity, for example through examining opposing molecular targets, dose-dep endent changes in metabolism and changes in serotonin levels for different exposure concentrations of fluoxetine and sertraline. The detected dissimilarities between fluoxetine and sertraline also raise uncertainties on how to perform read across information between chemicals with supposed similar modes of action. The use of *C. elegans* as a model organism allowed for the study of these sensitive behavioral effects.

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CRediT authorship contribution statement

Conceptualization: **M.M., N.B.**, Methodology: **M.M., I.M.E.**, Investigation: **M.M., I.M.E.**, Formal analysis: **M.M.**, Writing - original draft: **M.M.**, Writing - review & editing: **N.B, I.M.E**.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114493.

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