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Effects of antidepressant exposure on aquatic communities assessed by a combination of morphological identification, functional measurements, environmental DNA metabarcoding and bioassays

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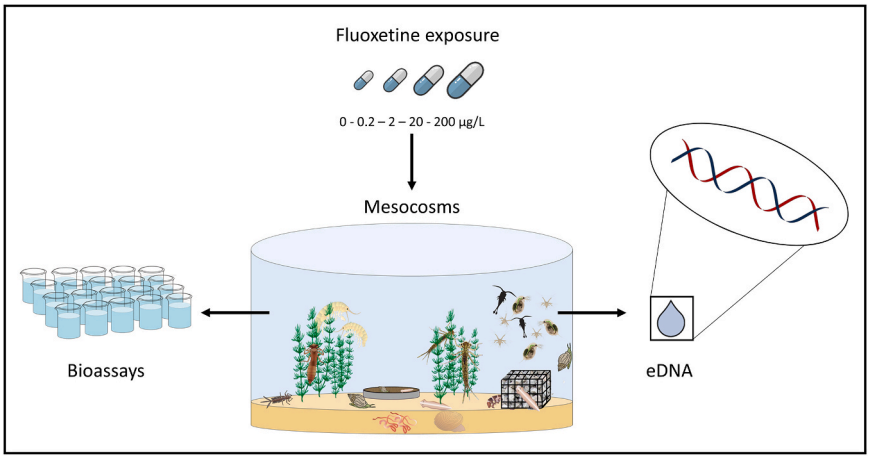
HIGHLIGHTS

- We found effects of the antidepressant fluoxetine on aquatic communities.
- The combination of morphological identification and non-traditional assessment tools provided complementary information.
- Environmentally realistic fluoxetine concentrations can affect aquatic ecosystems.
- Behaviour is the most sensitive endpoint.
- Some studies reported nonmonotonic responses when exposed to fluoxetine.

GRAPHICAL ABSTRACT



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ABSTRACT

The antidepressant fluoxetine is frequently detected in aquatic ecosystems, yet the effects on aquatic communities and ecosystems are still largely unknown. Therefore the aim of this study is to assess the effects of the long-term application of fluoxetine on key components of aquatic ecosystems including macroinvertebrate-, zooplankton-, phytoplankton- and microbial communities and organic matter decomposition by using traditional

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Communities
eDNA
Bioassays
Ecosystem functioning

and non-traditional assessment methods. For this, we exposed 18 outdoor mesocosms (water volume of 1530 L and 10 cm of sediment) to five different concentrations of fluoxetine (0.2, 2, 20 and 200 μg/L) for eight weeks, followed by an eight-week recovery period. We quantified population and community effects by morphological identification, environmental DNA metabarcoding, *in vitro* and *in vivo* bioassays and measured organic matter decomposition as a measure of ecosystem functioning. We found effects of fluoxetine on bacterial, algal, zooplankton and macroinvertebrate communities and decomposition rates, mainly for the highest (200 μg/L) treatment. Treatment-related decreases in abundances were found for damselfly larvae (NOEC of 0.2 μg/L) and Sphaeriidae bivalves (NOEC of 20 μg/L), whereas *Asellus aquaticus* increased in abundance (NOEC <0.2 μg/L). Fluoxetine decreased photosynthetic activity and primary production of the suspended algae community. eDNA assessment provided additional insights by revealing that the algae belonging to the class Cryptophyceae and certain cyanobacteria taxa were the most negatively responding taxa to fluoxetine. Our results, together with results of others, suggest that fluoxetine can alter community structure and ecosystem functioning and that some impacts of fluoxetine on certain taxa can already be observed at environmentally realistic concentrations.

1. Introduction

Aquatic communities are threatened by a wide range of chemical stressors, including pharmaceuticals which are identified as contaminants of emerging concern (Boxall et al., 2012). Pharmaceuticals are ubiquitous in aquatic ecosystems worldwide and antidepressants make up one group of pharmaceuticals that have frequently been detected in aquatic systems (Santos et al., 2010; Wilkinson et al., 2022). Among the most commonly used antidepressants globally are selective serotonin reuptake inhibitors (SSRIs) which inhibit the reuptake of the neurotransmitter serotonin in humans. The SSRI fluoxetine is under the trade name Prozac® frequently described to humans and concentrations have been reported up to 0.35 μg/L in surface waters whereas in wastewater effluent fluoxetine concentrations can be as high as 3.5 μg/L (Salahinejad et al., 2022).

Recent studies have shown that fluoxetine can affect aquatic organisms. Fish are the most well-studied group of organisms with regard to the effects of fluoxetine, and studies revealed changes in behavior, reproduction and enzyme levels (see Correia et al. (2022), Gould et al. (2021) and Salahinejad et al. (2022) for reviews). For invertebrate species, behavioral studies have shown effects of fluoxetine on feeding and reproduction in bivalves at concentrations of 0.02 μg/L and higher (Lazzara et al., 2012; Hazelton et al., 2013) and on the swimming behavior of amphipods at 0.1 μg/L (De Lange et al., 2006; Guler and Ford, 2010; Bossus et al., 2014) and decapods at 25,000 μg/L (Hamilton et al., 2016). Although the majority of studies focused on behavioral endpoints, it is not only behavior that is found to be affected by fluoxetine. Lazzara et al. (2012) reported that fluoxetine induced spawning at concentrations as low as 0.02 μg/L in zebra mussels. Next to that, a decrease in reproductive output in number of egg masses was found for freshwater snails (*Physa acuta*) after three years of exposure to a mean 0.03 μg/L fluoxetine concentration (Henry et al., 2022). Pery et al. (2008) found effects of fluoxetine on the life cycle of *Daphnia magna* and the snail *Potamopyrgus antipodarum* at a concentration of about 10 μg/L and on the growth of *Hyalella azteca* with a NOEC of 33 μg/L.

These studies demonstrate that the life history and reproduction of some organisms are indeed affected by fluoxetine. However, to understand the impacts of fluoxetine on entire aquatic ecosystems, a holistic insight is necessary that includes assessing the effects of fluoxetine on the structure of aquatic populations, communities and ecosystem functions. Artificial stream experiments have shown some effects of fluoxetine (0.02 and 20 μg/L) on algae and biofilms, resulting in reduced primary production and increased aquatic insect emergence after 2–3 weeks exposure (Richmond et al., 2016, 2019; Robson et al., 2020), highlighting the capacity of fluoxetine to disrupt ecosystem processes. However, knowledge on how fluoxetine affects other ecosystem processes and taxonomic groups is necessary to better understand the impact on the environment. Consequently, this calls for more research investigating the effects of fluoxetine on additional ecological endpoints (Richmond et al., 2019) and longer exposure durations.

Traditionally, monitoring studies investigating the effects of chemical stressors on ecological endpoints in aquatic ecosystems have relied on taxonomic inventories of various organism groups based on the morphological identification of species (Birk et al., 2012). However, morphological assessments have a limited capacity in assessing the effects of chemical stressors on certain species groups such as bacteria and planktonic organisms, is time consuming and prone to observer bias. In addition, although taxonomic inventories provide information on human disturbances on species diversity (Cao and Hawkins, 2019), they provide limited information on how ecosystem functioning is affected (Verdonschot and van der Lee, 2020) and on the potential stressor causing the observed effect (Lemm et al., 2019). To overcome these current limitations, many new tools have been developed in the last decade to improve water quality assessment by providing complementary information (Altenburger et al., 2019; Schuijt et al., 2021).

Over the past decades, environmental DNA (eDNA) has proven itself as a useful and time-efficient technique for detecting species and communities in a legion of different environments (Ruppert et al., 2019), despite its difficulty to translate sequence data into absolute abundances of individual taxa (Beng and Corlett, 2020). eDNA refers to any DNA (e.g. derived from cells, mucous, feces, etc.) left in an environment by the species living there, which can be subsequently extracted from an environmental sample, in many cases without having to isolate it from target organisms directly (Taberlet et al., 2012). Due to its sensitivity and time-effectiveness, eDNA techniques allow researchers to detect cryptic or rare species (Jerde et al., 2011), increase the taxonomic resolution but also easily increase sampling efforts in space and time (Ruppert et al., 2019; Beng and Corlett, 2020). Furthermore, eDNA metabarcoding enables the inclusion of entire organism groups, such as microorganisms, that are not easily included in morphological assessments.

However, not only structural attributes of aquatic ecosystems can be affected by chemical stressors, but also functional attributes respond to ecosystem disturbances. Therefore, it is important to include functional measures next to the structural inventories in the assessments. Particularly, organic matter breakdown has been suggested as a sensitive endpoint for assessing the effects of anthropogenic stress (Gessner and Chauvet, 2002; Young et al., 2008).

Next to adopting molecular tools in structural measures and including functional measures in the assessment, there is also a recognized need to improve the diagnostic value of water quality assessment approaches. Specifically, both *in vitro* as *in vivo* ecotoxicological testing methods have been incorporated to provide a bridge between chemical and ecological assessment (Lam, 2009; Wernersson et al., 2015; Altenburger et al., 2019; Brack et al., 2019). Ecotoxicological tests include biological components (e.g. cells or individuals) that are exposed to an environmental medium and subsequently evaluate the biological effects of chemical stressors (Schuijt et al., 2021). These tests include *in vitro* bioassays, measuring sub-organismal responses to chemicals in cell cultures or subcellular systems, and *in vivo* bioassays that measure whole-organismal responses of single species exposed in the laboratory or the field (*in situ*).

In this study, we assessed the impact of long-term fluoxetine exposure on aquatic communities in mesocosms by using traditional assessment methods in combination with non-traditional monitoring tools. Studying the effects of fluoxetine on aquatic communities by a combination of different methods may not only help to identify potential threats of this antidepressant to aquatic ecosystems but also improve our understanding of the diagnostic power of non-traditional monitoring tools including eDNA metabarcoding and *in vitro* and *in vivo* bioassays. To this purpose, we performed an outdoor freshwater mesocosm experiment where we applied fluoxetine for two months, followed by a two month recovery period. We assessed the effects of fluoxetine by morphological assessment of macroinvertebrates, eDNA-based assessments of microorganisms, phytoplankton, zooplankton and three macroinvertebrate phyla, functional assessment of organic matter decomposition and effect-based assessment by using *in vitro* and *in vivo* bioassays.

2. Material and methods

2.1. Experimental design

An outdoor experiment was conducted at the Sinderhoeve Experimental Station in Renkum (www.sinderhoeve.org), the Netherlands, from mid-June to mid-October 2019 (4 months). We used 18 outdoor mesocosms (diameter 1.8 m, total depth 0.8 m, water depth 0.6 m) that were lined with a watertight non-toxic layer of black polyethylene. The cosms contained a 10 cm layer of fine sandy clay sediment and ca. 1530 L of water originating from the experimental station's supply basin. Three months before the start of the experiment, each cosm received aliquots of macroinvertebrates, zooplankton, phytoplankton, macrophytes (*Elodea nuttallii* and *Myriophyllum spicatum*) and some additional sediment (for (resting) eggs, diaspores and microbial community) collected from uncontaminated freshwater basins at the Sinderhoeve. Over the next three months, the community had time to colonize and establish itself. Extra nutrients as KH2PO4 and NH4NO3 (90 μg N/L and 15 μg P/L) were added once a month to the cosms to stimulate phytoplankton growth, as nutrients tend to disappear quickly from the water column since they are being used by macrophytes or stored in the sediment. During the pre-treatment period all cosms were interconnected by tubes and water was circulated by a pump to achieve the development of a similar biocoenosis in the test systems. Once a week (on Fridays) water level was checked and adjusted if it deviated by more than 3 cm from the reference depth (indicated by a marker). When the water level was too low, groundwater was added.

The experimental design consisted of a solvent control (6 cosms) and four different fluoxetine exposure concentrations (0.2, 2, 20 and 200 μg/L) with three replicates each and all treatments were randomly assigned to the cosms. This concentration range included concentrations detected in aquatic environments and wastewater effluents (Mole and Brooks, 2019; Correia et al., 2022; Salahinejad et al., 2022; Wilkinson et al., 2022) and higher concentrations that have previously shown to have ecological effects (Richmond et al., 2016). Concentrations were maintained at the intended concentration for eight weeks by weekly application of the chemical after measuring the residual levels.

2.2. Fluoxetine application, sampling and analysis

Fluoxetine was applied every Tuesday for eight weeks in a row in the late afternoon between 16:00 and 17:00 from June 25 till August 13, 2019. The stock solutions were made using fluoxetine hydrochloride (Sigma-Aldrich, Product PHR1394, LOT#LRAA9180, concentration 99.95%), and were prepared with acetone to improve the solubility. Next, dosing solutions of about 2000 mL were prepared by diluting 10 mL of a stock solution in acetone with tap water. In this way, equal amounts of acetone were added to all experimental units (10 mL acetone in 1530 L systems). The solvent controls received about 2000 mL of tap water containing 10 mL of pure acetone. After manual shaking of the dosing solution, duplicate samples for analytical verification of the fluoxetine concentration were taken in 5 mL HDPE bottles, diluted into a 5-mL PP tube with ultrapure water and spiked with 100 μl internal standard solution of fluoxetine-d5 (Sigma Aldrich, article 613347, Lot#LS-68-131) in methanol (1 mg/mL). Each subsample was stored both prior and after analysis in a freezer at approximately −20 °C.

The dosing solution was poured evenly over the water surface and mixed through the water column by stirring with a steel rod. To analytically measure fluoxetine concentrations by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) (see SI 1), water samples were taken from all cosms 1 h after application and 24 h before the next application. Depth-integrated water samples were collected using a Perspex® sampling tube. Immediately after sampling, a sub-sample of 4.0 mL was transferred into a 5-mL PP tube and spiked with 100 μL internal standard fluoxetine-d5 with a concentration of 200 ng/mL in methanol. Both the sample and sub-sample were stored in the freezer at −20 °C until further analysis (see SI 1). Dosing concentrations of the next application were adjusted based on the concentration measured 24 h before, to achieve intended maximum concentrations. Additionally, water samples were collected one day (about 24 h) and 3 days (about 65 h) after every application moment from the systems of the 2 μg/L and 200 μg/L treatments, to assess the dissipation of the antidepressant in the mesocosm water.

To measure the fate of fluoxetine in the sediment, sediment samples were taken three times after the first application on days 29, 57 and 113 and before the first application (day −11) by using sediment containers (100 mL HDPE containers). The sediment containers were filled with 2 cm of sediment collected from a spare cosm which was not used during this study. During each sampling day, 3 sediment containers were retrieved from each cosm, the overlying water was gently removed and after that, the sediment was stored frozen (<-20 °C) until further analysis (see SI 1).

2.3. Water quality parameters

Water quality parameters including dissolved oxygen concentration, water temperature, pH and electrical conductivity were measured by a multimeter (Hach HQ40d) in the morning (8 a.m.) every week before and during the application period (on days −8, 2, 6, 13, 20, 27, 34, 41, 48, 55) and once every two weeks during the recovery period (days 62, 76, 90, 104). Turbidity was measured in the water column, and water samples, to analyze nutrients, were collected once every two weeks during the application period (days −8, 6, 20, 34, 48) and once a month in the recovery period (days 76, 104). A segmented flow analyzer (Skalar 5100 Autoanalyzer, Breda, The Netherlands) was used to determine dissolved (samples filtered with 0.45 μm) and undissolved nutrients; nitrite and nitrate (NO2 + NO3), ammonia (NH3), phosphate (PO4) and total phosphorus (P) and total nitrogen (N). Turbidity in the water column was measured at three different spots in each cosm by an Algae Torch (ATo 04–050, bbe Moldaenke GmbH, Schwentinental, Germany).

2.4. Primary producers

Chlorophyll-a concentration in the water was determined by using the Algae Torch (3 different spots per cosm) and was used as a proxy for the suspended algae community. Macrophyte cover per cosm was classified from 0% (no plants) up to 100% (mesocosm sediment surface was totally covered by plants). Chlorophyll-a concentration and macrophyte cover were measured on sampling days −8, 2, 6, 20, 34, 48, 76, 104. Additionally, at the end of the experiment (at day 114) all macrophytes were harvested, washed to remove adhering particles and organisms, dried at 60 °C and the total dry weight was determined.

2.5. Macroinvertebrates morphological identification

In order to collect pelagic and benthic macroinvertebrates, two sampling methods were used; pebble baskets and sediment traps. Two pebble baskets (17 cm × 17 cm × 11 cm) positioned on concrete tiles on the sediment surface of each cosm were gently retrieved using a net (mesh size 0.3 mm) after a colonization period of 2 weeks. During the pre-treatment period, 12 sediment traps (diameter 26 cm, height 3 cm) filled with a sediment layer of 2 cm were positioned over the sediment surface of each cosm. The sediment originated from an experimental ditch previously not used for chemical pollution studies. On each sampling day, two sediment traps were gently collected from each cosm by using a net (mesh size 0.3 mm) and sieved using 0.5 and 1 mm sieves. The invertebrates sampled from each mesocosm with the two sampling methods were morphologically identified and counted alive. Afterward, all invertebrates from both sampling methods were returned to their original cosm. All invertebrate samples were taken at sampling days −7, 7, 21, 49, 77 and 105 relative to the first application of fluoxetine.

2.6. Decomposition

Decomposition rates were measured using litterbags with *Populus* leaves (2 g dry weight, dried at 60 °C). We deployed two types of litterbags in each cosm just above the sediment substrate; one litterbag with a fine mesh (width 500 μm) and one with a coarse mesh (width 5 mm). The first type excluded invertebrates and is used to assess decomposition "solely" due to the microbial community (bacteria and fungi), while the second type allowed macroinvertebrates to pass through and assessed the overall decomposition. Every 2 weeks, (days 13, 27, 41, 55, 69, 83, 97, 111) two litterbags were retrieved from each cosm and gently washed to remove adhering particles and organisms. The remaining organic leaf material was dried in pre-weighed aluminum foil at a temperature of 60 °C for 96 h and weighed to determine their dry weight and new litterbags were placed in the cosms.

2.7. Phytoplankton and invertebrate eDNA water sampling

Environmental DNA metabarcoding was used to obtain a higher taxonomic resolution for eukaryotic phytoplankton, in addition to chlorophyll-a measurements, zooplankton and some specific invertebrate taxa for which effects were observed based on the morphological identification. Environmental DNA sampling was performed in all cosms at two time points, sampling day 48 (end of the application period, i.e. after the last application) and day 112 (end of the experiment) to characterize the taxonomic composition for phytoplankton, zooplankton, Chironomidae, Mollusca and Odonata. However, we did not take samples at day 0 (before fluoxetine exposure), and consequently, we cannot make statements on the composition of the different communities at the start of the experiment.

Plastic sterile syringes were used to take five water samples of 50 mL right beneath the surface and five water samples of 50 mL close to the sediment (without disturbing the sediment) from each mesocosm. Subsequently, the ten 50 mL water samples belonging to a mesocosm, were filtered through the same 0.45 μm polyethersulphone (PES) filter membranes (47 mm diameter, Sartorius) placed in sterilized 47 mm filter holders. After filtration, membranes were stored in 700 μl CTAB at −20 °C until further analysis (see SI 2).

2.8. Bacterial DNA sampling

The bacterial community composition was monitored at four sampling days (−1, 27, 56, 111) on leaf material, as changes in the leaf-associated bacterial community might affect leaf litter decomposition and sediment samples since fluoxetine might bind to the sediment and potentially impact bacterial community composition. To monitor the bacterial community on leaf material, we used nylon litterbags containing *Populus* leaves consisting of a fine mesh (as described above). In each cosm, four nylon bags were deployed at 20 cm depth seven days before the first application. During the sampling days, the nylon bags were retrieved from the cosms, opened and leaves were carefully transferred into plastic bags and stored at < -20 °C until further processing. For the sediment sampling, we used 100 mL HDPE containers filled with 2 cm of sediment collected from the surplus cosm, not assigned to any treatment. In each cosm, 8 containers were deployed and 2 containers were retrieved during each sampling, the overlying water was gently removed and the sediment sample was stored frozen (<-20 °C) until further analysis (see SI 3).

2.9. Bioassays

A battery of *in vitro* CALUX assays (cytotox, anti-AR and anti-PR; Table S3) and whole organism bioassays (Daphniatox, Rotox and Algaltox) was applied to test for ecotoxicological effects in the cosms at the end of the application period (sampling day 48). From each cosm, we collected water samples in two dark HDPE bottles of 1 L (one bottle for the CALUX assays and one for the whole organism bioassays) and stored in the freezer at −20 °C until further analysis (see SI 4).

2.10. Statistical analyses

The dissipation rate coefficients (k) of fluoxetine were calculated using linear regression of the ln-transformed concentrations assuming first-order kinetics. Next, the half-lives (DT50) of fluoxetine were calculated for each treatment by dividing Ln(2) by k. We calculated time-weighted average fluoxetine concentrations using the equations described by Roessink et al. (2013).

Treatment-related responses for water quality parameters (oxygen, pH, EC, temperature, turbidity and nutrients), primary producers (macrophyte cover and chlorophyll-a concentration), decomposition rates and macroinvertebrate abundances were derived by calculating NOECs ($p \leq 0.05$) using the Williams test (ANOVA; Williams, 1972) by comparing the treatments to the control. We considered effects to be consistent when we found statistically significant deviations for at least two consecutive sampling days pointing in the same direction (to reduce type 1 error) and when abundance values were high enough (the numbers in the controls should be ≥ 3 individuals/sample for macroinvertebrates) on the sampling date of the calculated NOEC. In addition, the Williams test was used to compare responses of the *in vivo* bioassays in the treatments to the control treatment. Before statistical analysis, abundance data were ln-transformed ($\ln[2 \times +1]$), whereas eDNA data were arcsine transformed. The Williams tests were performed with the Community Analysis computer program, version 4.3 (Hommen et al., 1994).

Treatment-related changes in macroinvertebrate community composition (morphological identification and eDNA) and zooplankton community composition (eDNA) data were assessed using the multivariate Principal Response Curves (PRC) method (Van den Brink and Braak, 1999). The overall significance of the effect of the fluoxetine treatment on the variation in the community composition was tested by performing 499 Monte Carlo permutations. The corresponding taxa scores (b_k) allowed interpretations of the PRCs at the taxa level. Specifically, taxa with high b_k values are indicated to follow the PRC pattern, whereas taxa with a low negative score were inferred to follow the opposite pattern. Taxa with scores close to 0 were presumed to be unrelated to the PRC pattern. In addition to the PRC, Monte Carlo permutation tests under the Redundancy Analysis (RDA) option were performed to assess the significance of the effect of fluoxetine on the macroinvertebrate community for each sampling day and for each treatment separately. The multivariate analyses were performed using the CANOCO software, version 5 (ter Braak and Smilauer, 2012). All tests were performed based on a significance level (α) of 0.05.

In vitro bioassay responses were compared to effect-based trigger

values (EBTs) for interpreting the effects of fluoxetine. EBTs previously defined by Escher et al. (2018) (anti-AR), De Baat et al. (2020) (anti-PR) and van der Oost et al. (2017) (cytotox) were used. In addition, we calculated the potentially affected fraction (PAF) based on acute and chronic toxicity data using the PAF calculator developed by "Kennis Impuls Water Kwaliteit" (https://www.sleutelfactortoxiciteit.nl/verdieping/werken-met-het-chemiespoor/aan-de-slag-met-de-chemie-rekentool). The acute and chronic PAF values indicate which fraction of the species have an acute (assessed using EC50s) or chronic (NOECs) toxicity values lower than the exposure concentration (in our case nominal treatment levels for the acute PAF values and 7-day' time-weighted average concentrations for the chronic PAF values), which were calculated using the species sensitivity distribution concept (Posthuma et al., 2019).

3. Results

3.1. Fluoxetine fate

Measured fluoxetine concentrations 1 h after application where on average 105% of the intended concentration. For the 2 and 200 μg/L fluoxetine treatments, we calculated first-order half dissipation times (DT50) of 2.1 ± 0.57 and 3.9 ± 1.0 days and dissipation rate constants of 0.35 ± 0.11 and 0.19 ± 0.07 d-1 (mean ± SD), respectively. These values are based on the fluoxetine concentrations measured 1, 3 and 6 d after application 1 to 7 (Table S4). After the last application 8, we monitored the fluoxetine concentration for all treatments until it dropped below the detection limit (Fig. 1A) and calculated the half dissipation time and dissipation rate constant if possible (Table S4). The two highest test concentrations showed that the degradation of fluoxetine was fastest in the first week, and thereafter the degradation rate declined (Fig. 1A). For the lowest test concentration of 0.2 μg/L, we were not able to calculate the DT50 and rate constant since the concentration quickly dropped below the detection limit.

The calculated 7-day' time-weighted average fluoxetine concentrations were 0.1, 0.8, 7.6 and 116 μg/L, for the lowest to the highest treatment level. For the sake of clarity, results are referred to nominal values (0.2, 2, 20 and 200 μg/L). Over time, fluoxetine accumulated in sediment during the fluoxetine application period (Fig. 1B). Fluoxetine concentrations in the sediment only showed a slight decrease in the recovery period (Fig. 1B).

3.2. Water quality parameters and primary producers

Although there was some fluctuation in the daily average temperature, a gradual decrease from 22.5 to 11 °C was observed between June and October (Fig. 2). During the two month application period the temperature ranged from 22.5 to 18 °C whereas in the recovery period it varied between 21 and 11 °C. In the cosms treated with the two highest fluoxetine treatments (20 and 200 μg/L), significant decreases were observed for the oxygen concentration, pH level and chlorophyll-a concentration (Fig. 2, S2, Table S5). In addition, a significant decrease in turbidity was found for the 200 μg/L treatment whereas the EC level and ammonia concentration significantly increased (Fig. 2, S2, S3, Tables S5 and S6). Hence, parameters related to photosynthetic activity and primary production by the suspended algae community were significantly affected by the two highest fluoxetine treatments. Effects of fluoxetine on macrophyte coverage (Table S5) and biomass (Fig. S2, One-way ANOVA: $F(4,13) = 7.3$, $p = 0.59$) were not observed.

3.3. Macroinvertebrate responses (morphological identification)

The macroinvertebrate community in the mesocosms was dominated by Diptera, Amphipoda, Ephemeroptera and Isopoda at the start of the experiment, but this changed over time to Ephemeroptera, Diptera and Odonata (Fig. S4). Fluoxetine-treated cosms were generally associated with lower total abundances during the exposure period (Fig. S5), however, this was only significant on day 21 for the two highest concentrations (20 and 200 μg/L). Fluoxetine did not lead to a decrease in diversity (Fig. S5) but impacted the macroinvertebrate community structure. The Principal Response Curves (PRC) showed significant treatment-related effects of fluoxetine on the macroinvertebrate community (Monte Carlo p-values = 0.014; Fig. 3). Monte Carlo Permutation tests for each individual sampling date showed a significant difference from the control for the highest concentration at days 7, 21 and 105 (NOEC = 20 μg/L; Table S7). The species weights (b_k) of the PRC were highest for *Radix* sp. and Zygoptera, indicating that these taxa decreased most in abundance when fluoxetine concentrations increased. The lowest b_k values were found for *Asellus aquaticus* and Asellidae juveniles, indicating a treatment-related increase in abundance.

The Williams test revealed statistically significant treatment-related increases in abundance for two consecutive sampling days for *Asellus aquaticus* and Asellidae juveniles, whereas statistically significant decreases in abundance were observed for *Radix* sp., Zygoptera, and Sphaeriidae (Table S8). For *Radix* sp. a NOEC of 2 μg/L was found during

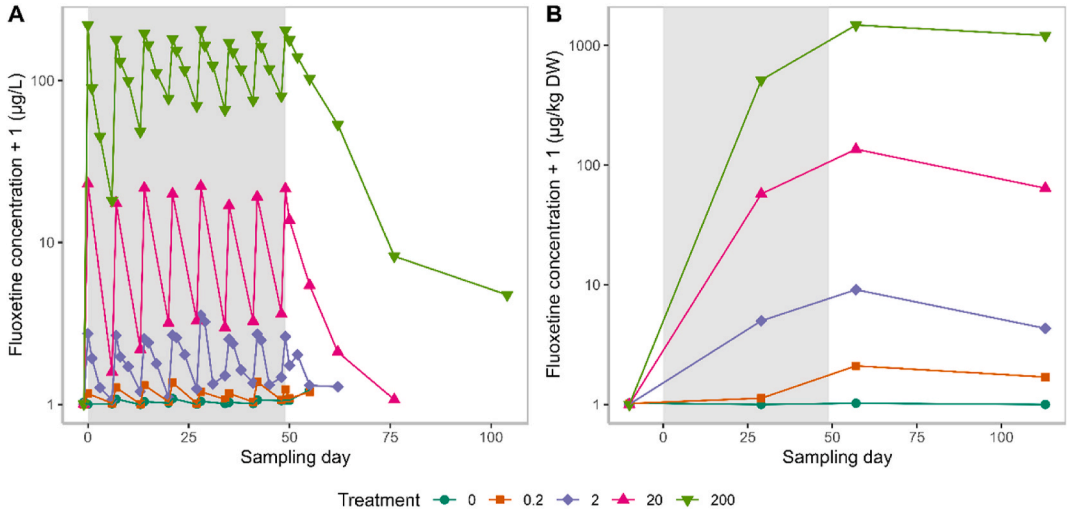

Fig. 1. Average fluoxetine concentrations measured in the water column (A) and the sediment (B) of the outdoor mesocosms over the experimental period. The grey-shaded areas in the graphs indicate the fluoxetine application period and the treatments are based on nominal fluoxetine concentrations (μg/L).

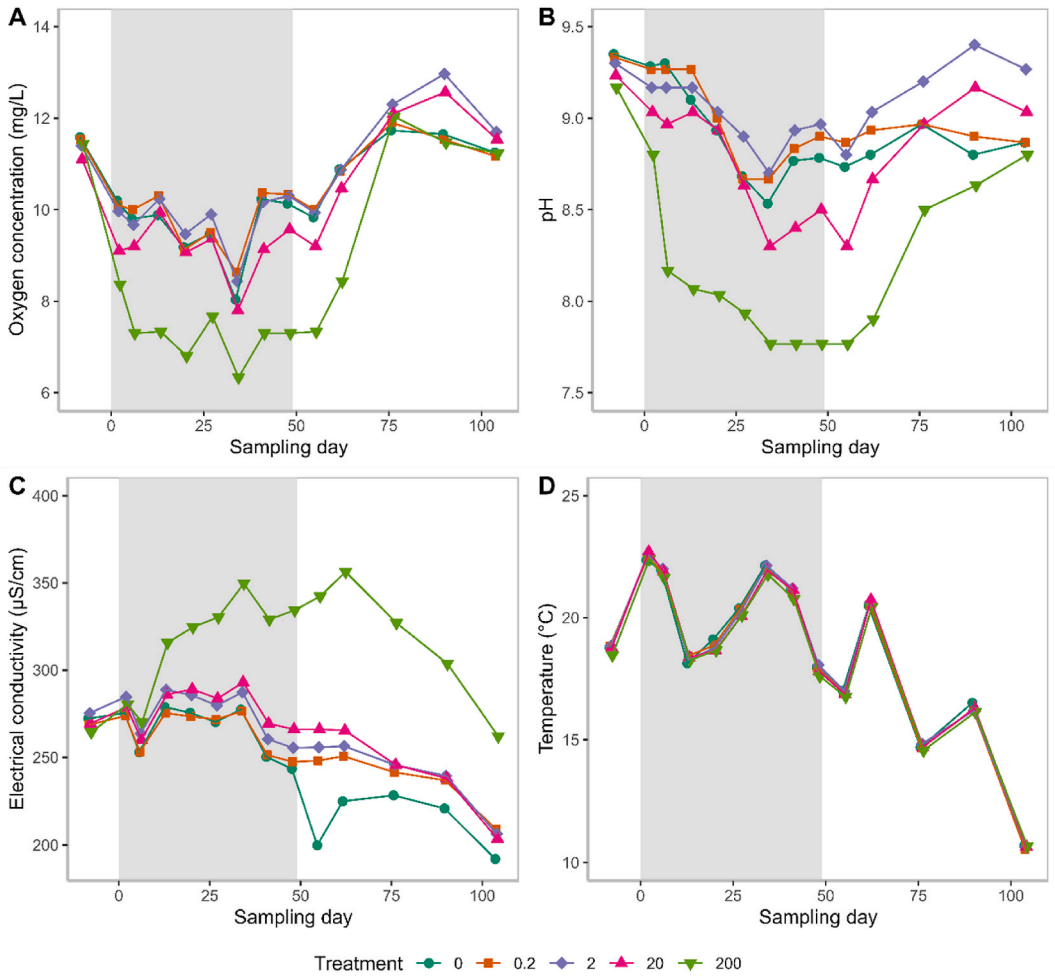

Fig. 2. Water quality parameter dynamics including average oxygen concentration (A), pH (B), electrical conductivity (C) and temperature (D) in the mesocosms for the different fluoxetine treatments. The grey-shaded areas in the graphs indicate the fluoxetine application period and the treatments are based on nominal fluoxetine concentrations (µg/L).

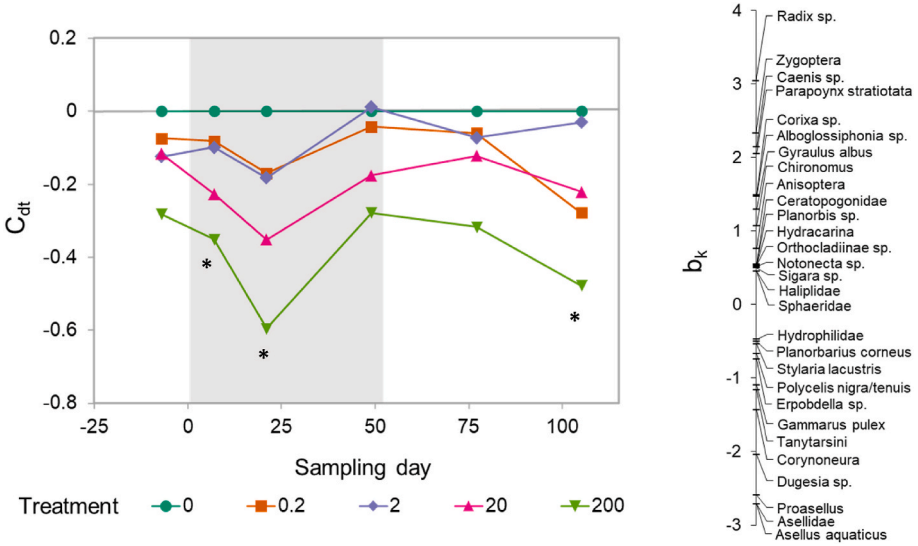

Fig. 3. Principal Response Curve (PRC) showing the effect of fluoxetine on the macroinvertebrate community as characterized by morphological identification. Of all variance, 37% could be attributed to the sampling date; this is displayed on the horizontal axis. 16% of all variance could be attributed to treatment. Of this variance, 21% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of a given taxon with the Principal Response Curves. Taxa with a species weight between 0.45 and −0.45 are not shown. The Monte Carlo permutation test indicated that a significant part of the variance explained by treatment is displayed in the diagram ($p = 0.03$). * indicates a significant difference ($p \leq 0.05$) from control treatment. The grey-shaded area in the graph indicates the fluoxetine application period and the treatments are based on nominal fluoxetine concentrations (µg/L).

sampling day 7, however differences in *Radix* sp. abundances were already found during the pre-exposure sampling (day −7; Fig. S6A). Zygoptera showed a treatment-related decrease in abundance at sampling days 21 and 49 (NOEC = 2 μg/L; Fig. S6B). For Sphaeriidae decreases in abundances were observed at the end of the experiment (sampling day 77 and 105, NOEC = 20 μg/L; Fig. S6C). In contrast, a treatment-related increase in abundance was found for *Asellus aquaticus* starting from day 21 until the end of the experiment (NOEC = 20 μg/L; Fig. S6D).

3.4. Invertebrate and phytoplankton responses (eDNA metabarcoding)

We assessed the effect of fluoxetine on the macroinvertebrate, zooplankton and phytoplankton community composition by eDNA metabarcoding at two sampling days during the experiment: sampling day 49, which is at the end of the two months fluoxetine exposure period, and at sampling day 114, which is at the end of the experiment (after 4 months). The PRCs indicate that fluoxetine treatment might explain a significant part of the variation in zooplankton community composition (p = 0.038; Fig. 4B), whereas for phytoplankton community the fluoxetine treatment did not explain a statistically significant part of the variation observed (p = 0.1; Fig. 4A). According to the species weights (b_k), the phytoplankton class Cryptophyceae and the zooplankton family Cyclopidae showed the largest response to the fluoxetine treatment (Fig. 4). The Monte Carlo permutation tests for individual sampling days indicated significant effects on the phytoplankton community for the highest fluoxetine concentration during both sampling days, and for the zooplankton community a significant effect of the highest fluoxetine concentration at sampling day 49 (Table S9). The results of the PRC analyses did not show a significant effect of fluoxetine on the Mollusca (p = 0.086; Fig. S7) and Odonata (p = 0.324; Fig. S8) communities, whereas this was significant for the

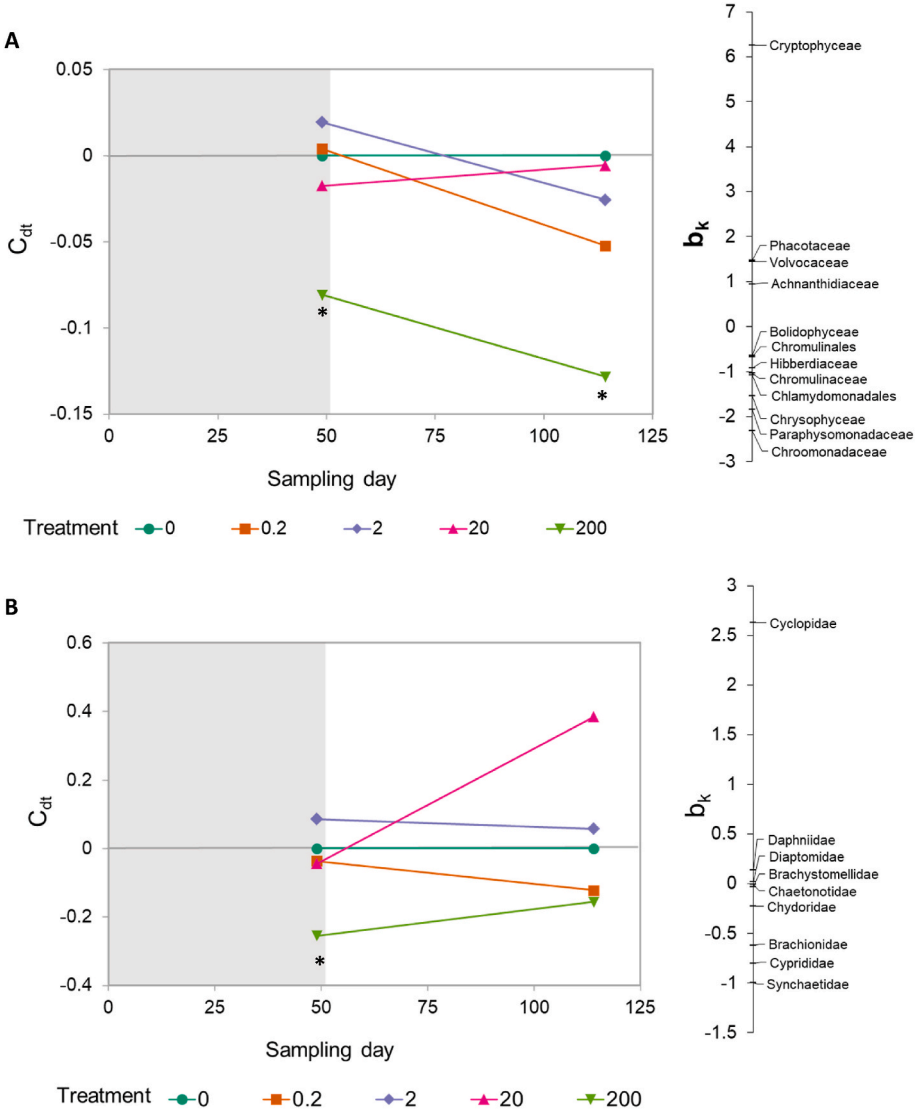

Fig. 4. Principal response curves (PRCs) showing the effect of fluoxetine on the phytoplankton (A) and zooplankton (B) community as characterized by eDNA metabarcoding. Of all variance, 17% for the phytoplankton data and 25% for the zooplankton data could be attributed to the sampling date; this is displayed on the horizontal axis. 27% of the phytoplankton and 30 of the zooplankton community variation could be attributed to treatment. Of this variance, 50 % for the phytoplankton and 54% for the zooplankton community treatment-related variation is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves. Phytoplankton taxa with a species weight between 0.6 and −0.6 are not shown. The Monte Carlo permutation test indicated that for the zooplankton community a significant part of the variance explained by treatment is displayed in the diagram (p = 0.04), whereas this is not significant (p = 0.1) for the phytoplankton community. * indicates a significant difference ($p \leq 0.05$) from control treatment. The grey-shaded areas in the graphs indicate the fluoxetine application period and the treatments are based on nominal fluoxetine concentrations (μg/L).

Chironomidae ($p = 0.03$) community with a treatment-related increase of *Tanytarsus* sp. (Fig. S9).

3.5. Bacterial responses

In total 1965 different amplicon sequencing variants (ASVs) were detected in the sediment samples with Cyanobiaceae and Bacillaceae being the predominant families. In the leaf samples, 2215 ASVs were detected in total and the most predominant family was the Comamonadaceae.

The PRC analysis indicated significant effects of fluoxetine on the bacterial community at the family level (Monte Carlo p-values = 0.006; Fig. 5). Highest species weights with the PRC (b_k) were found for Cyanobiaceae and Leptolyngbyaceae, indicating that these cyanobacteria families responded negatively to fluoxetine. The cyanobacteria families Nodosilineaceae, Microcystaceae, and some Cyanobacteria taxa that could not be identified at the family level, also had relatively high b_k values, indicating that relative abundance decreased, particularly in the highest fluoxetine treatment (Fig. S10). In turn, the unclassified Oxyphotobacteria belonging to the cyanobacteria received the highest negative species weight (Fig. 5) and increased in relative abundances in the highest fluoxetine concentration (Fig. S10). Monte Carlo Permutation tests for each individual sampling date showed a significant difference from the control for the highest concentration at day 56 and 112 (Table S10), whereas for day 28 this was not significant ($p = 0.09$).

The result of the PRC analysis did not show a significant effect of fluoxetine on the bacterial families in the leaf samples (Monte Carlo p-values = 0.5; Fig. S11). However, Monte Carlo Permutation tests showed significant differences for the 0.2 and 200 μg/L treatments on the last sampling day (Table S10).

3.6. Decomposition

A higher leaf mass loss, compared to the control, was observed for the highest fluoxetine concentration for two consecutive sampling days at the end of the application period for the litterbags with a coarse mesh size (NOEC = 20 μg/L; Table S5, Fig. S12). For the fine-mesh-sized litterbags, we did not observe a consistent change in leaf mass for at least two consecutive sampling days (Table S5).

3.7. Bioassays responses and PAF values

Responses to fluoxetine were found for both *in vivo* and *in vitro* bioassays (Fig. 6). Cytotoxicity and anti-AR responses were found to be above the effect-based trigger values (EBT) for one cosm in the highest treatment. As the water samples used for the *in vivo* bioassays were extracted and thus concentrated, the exposure concentrations in these bioassays were higher than the concentrations in the cosms. Measured fluoxetine concentrations in the medium of *in vivo* bioassays corresponded to 0.7 ± 0.2 (mean ± SD), 2.6 ± 0.6, 96 ± 40 and 3493 ± 162 μg/L for the 0.2, 2, 20 and 200 μg/L treatment, respectively. Significant differences compared to the control were found for the highest concentration in the Daphniatox, Rotox and Microtox bioassays (NOEC = 20 μg/L treatment, actual exposure concentration of 96 μg/L), whereas a 100% growth inhibition of *Raphidocelis subcapitata* was found for the 20 and 200 μg/L treatments (actual concentrations of 96 and 3493 μg/L, respectively) in the Algatox assay (NOEC = 2 μg/L treatment with an actual exposure concentration of 2.6 μg/L; Fig. 6).

The potentially affected fraction based on acute toxicity data of aquatic species was higher than 5% at a fluoxetine concentration of 200 μg/L, whereas the PAF based on chronic toxicity data was higher than 5% for the 20 μg/L fluoxetine nominal treatment level, corresponding to a 7-day' time-weighted average concentration of 7.6 μg/L and higher (Table S11).

4. Discussion

Worldwide, freshwater ecosystems receive inputs of pharmaceuticals, including antidepressants, and these compounds have potential (sub-lethal) ecological consequences (Wilkinson et al., 2022). In this study, we explored the effects of long-term exposure to different concentrations of the antidepressant fluoxetine (0.2, 2, 20 and 200 μg/L) on freshwater communities by using both traditional and non-traditional endpoints. In this case long-term refers to the time-window of the applications as the exposure in the field may be much longer, but also varying with time. Moreover, we obtained a pulsed exposure by weekly additions of fluoxetine, while in the field exposure is more constant but may also vary, depending on parameters like emissions and rainfall. We found effects of fluoxetine on bacteria, algae, zooplankton, macroinvertebrates and decomposition rates, mainly for the highest treatment

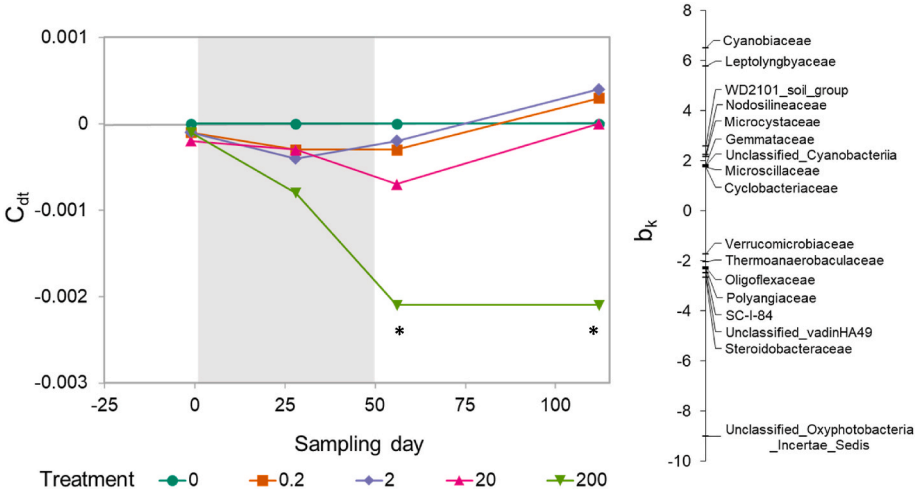

Fig. 5. Principal response curves (PRCs) showing the effect of fluoxetine on the bacterial community in sediment samples. Of all variance, 36% could be attributed to the sampling date; this is displayed on the horizontal axis. 20% of the variation could be attributed to treatment. Of this variance, 37% of the treatment-related variation is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves. Bacterial families with a species weight between 1.7 and −1.7 are not shown. The Monte Carlo permutation test indicated that for the bacterial community a significant part of the variance explained by treatment is displayed in the diagram ($p = 0.006$). * indicates a significant difference ($p \leq 0.05$) from control treatment. The grey-shaded area in the graph indicates the fluoxetine application period and the treatments are based on nominal fluoxetine concentrations (μg/L).

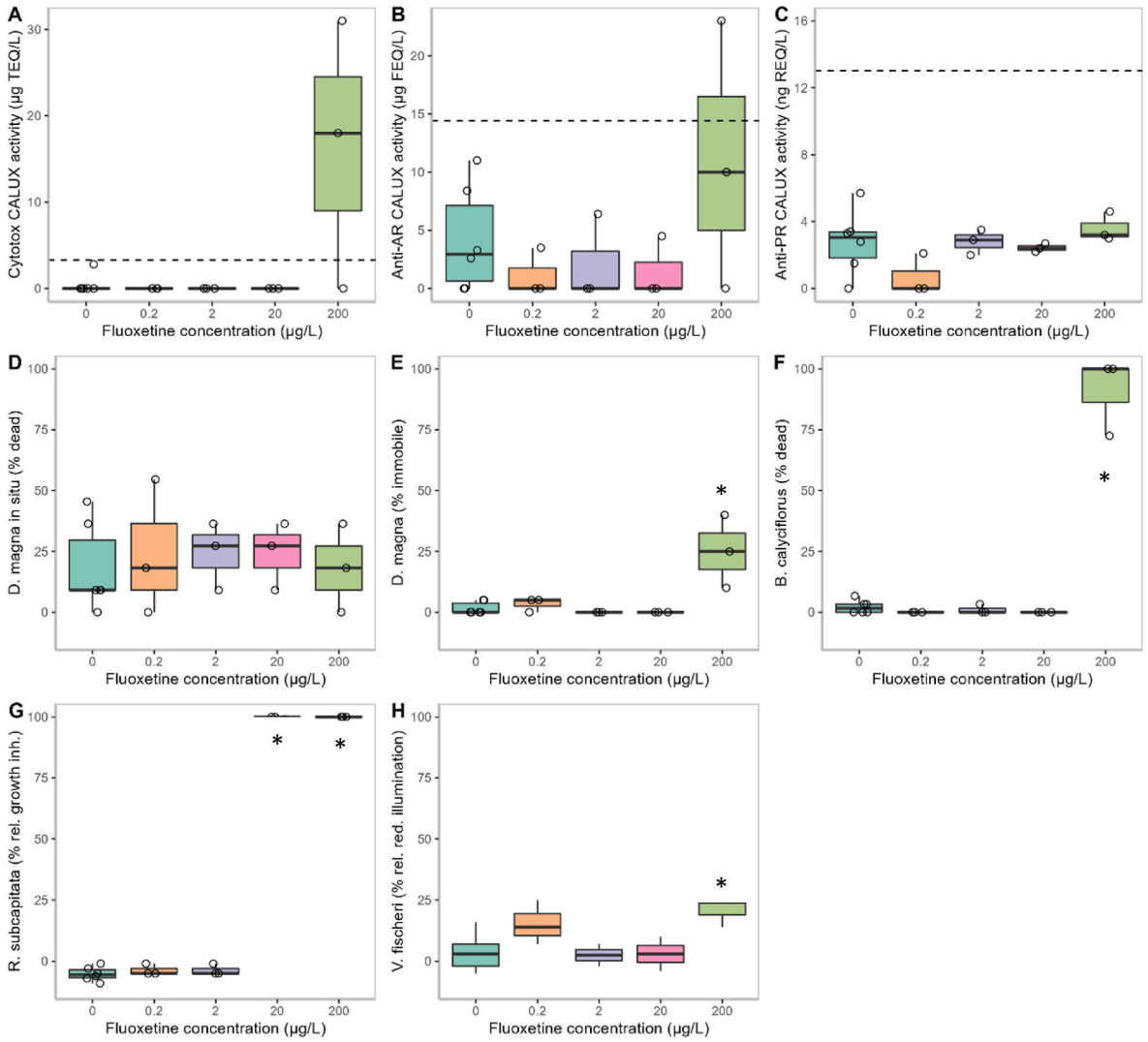

Fig. 6. Mesocosm water extracts tested on a battery of *in vitro* and *in vivo* bioassays including cytotox CALUX (A), anti-AR CALUX (B), anti-PR CALUX (C), Daphnia in situ test (D), Daphniatox (E), Rotox (F), Algaltox (G) and Microtox (H). The dashed lines indicate the effect-based trigger values of the *in vitro* bioassays. Open circles are individual data points. The treatments are based on nominal fluoxetine concentrations (µg/L). *P < 0.05.

concentration. Furthermore, using non-traditional assessment tools (including functional measures, eDNA and bioassays) in combination with traditional morphological identification of invertebrate taxa, provided complementary information and enabled a more comprehensive assessment of the effects on aquatic biota and ecosystems.

First, we will discuss in section 4.1 the effects found in this study and compare them to the effects of fluoxetine on aquatic ecosystems as described in the literature. For this, we gathered data of published studies that measured endpoints on the whole-organismal and higher levels of biological organizations which are compiled, together with data from this paper, in Table S12 and visualized in Fig. 7. In section 4.2 we discuss the different effect assessment tools used. We end the discussion with a complete overview of the effects of fluoxetine on aquatic ecosystems (including fish and endpoints not yet discussed in section 4.1), discuss whether the currently used concentration-response relationships are suitable for describing the effects of fluoxetine and the environmental relevance of the reported impacts of fluoxetine.

4.1. *Effects of fluoxetine on structural and functional ecosystem attributes*

Fluoxetine is an ionizable compound and shows a predominance of the species with positive charge at pH values below its pKa of 10 and will reach a maximum positive overall charge at pH values 2 units below its pKa (Silva et al., 2019). The ionized species is presumably more easily dissolved in water than a neutral species. Thus, the octanol/water distribution coefficient decreases with the reduction in pH, because of the decrease in hydrophobic nonionized species. Therefore, at higher pH, the BCF of fluoxetine will increase and, herewith, its toxicity (Nakamura et al., 2008). As many studies indicate that ionizable substances are more toxic and bioaccumulative in their neutral state than in their charged ones (e.g. Rendal et al., 2011), we expected that fluoxetine toxicity was the highest at the highest pH levels observed in our study and lower in, for instance the highest treatment level which showed a considerably lower pH in the treatment period (Fig. 2A).

This lower pH in the highest treatment is probably the result of an impact of fluoxetine on parameters related to photosynthetic activity and primary production by the suspended algae community (including oxygen, pH and chlorophyll-a concentrations; Fig. 2; Table S5). This result was expected, as fluoxetine has been shown to reduce the growth of green algae with an EC50 (120 h) of 24 µg/L (Brooks et al., 2003) and the majority of studies (but not all) found that fluoxetine exposure decreased algal populations and primary production (Fig. 7C; Table S12). More specifically, Johnson et al. (2007) reported a decrease in DO concentrations, pH levels and phytoplankton abundance in

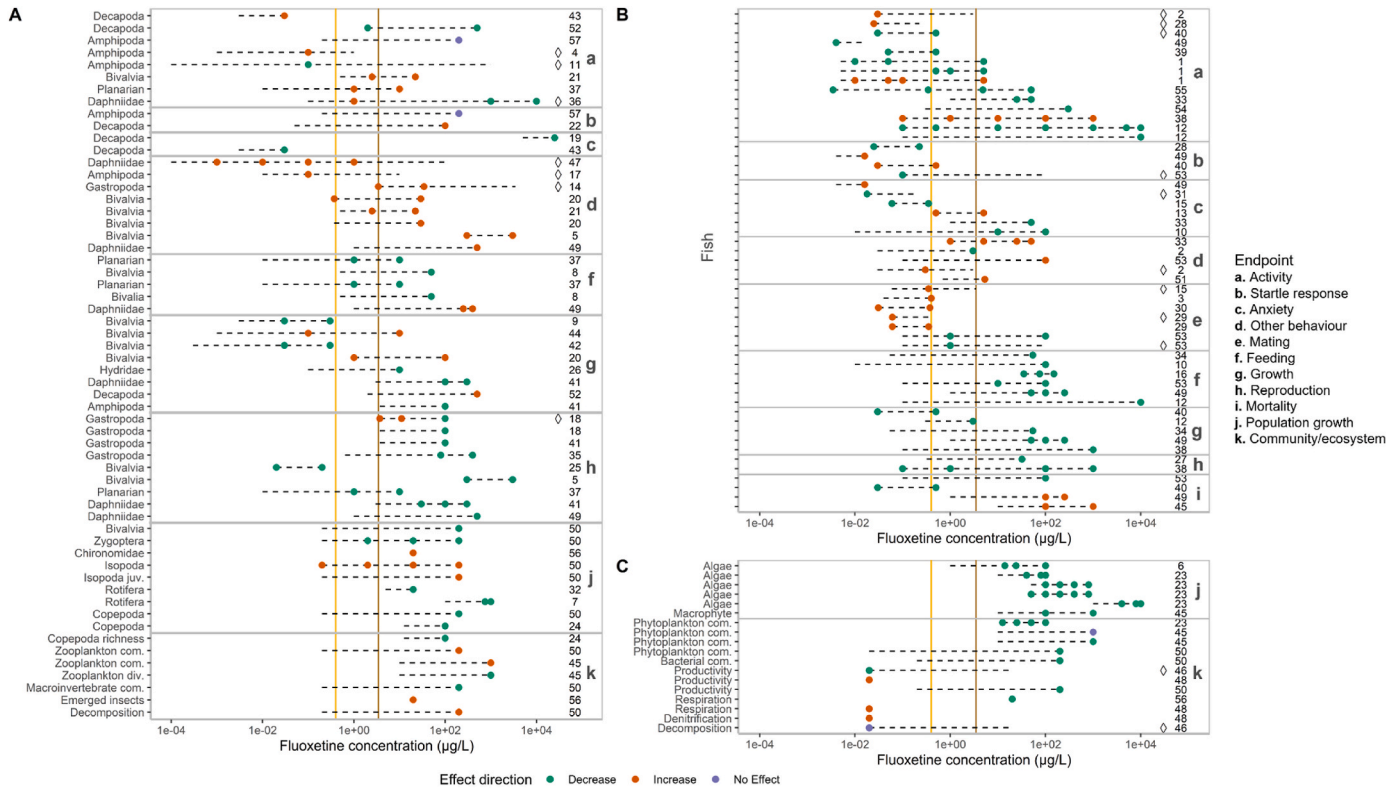

Fig. 7. Effects of different fluoxetine concentrations on aquatic invertebrates (A), fish (B) and primary producers and microorganisms (C) from the literature and this paper. The dots indicate the effects of fluoxetine for the corresponding taxa and endpoint. Green dots indicate a decrease of the measured endpoint, orange dots an increase and purple dots no effect (but only for results from this paper (50) and Schuijt et al., 2023 (57)). The dashed lines show the concentration range that has been tested. The endpoints are categorized into 11 groups (a–k). References to the different studies are indicated by different numbers and details can be found in Table S12. The vertical lines show the maximum fluoxetine concentration measured in surface water (yellow) and wastewater effluent (brown). ◊ indicate a nonmonotonic concentration-response relationship. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

aquatic microcosms exposed to an SSRI mixture, including fluoxetine (12.5–100 µg/L, 35 d). Next to that, Richmond et al. (2016) reported a reduction in the rate of primary production of rock biofilms in artificial streams after 2 weeks of fluoxetine exposure (20 µg/L). In a follow-up study, Richmond et al. (2019) found effects on biofilm chlorophyll-a in a lower fluoxetine treatment (0.02 µg/L) after two weeks but not in the 20 µg/L treatment and the effects disappeared after 3 weeks. However, different from the previous study, they started with bare rocks instead of pre-colonized algae-covered rocks and therefore fluoxetine might have caused delays in algal colonization rather than growth inhibition. Furthermore, two previous studies examined the impacts of fluoxetine exposure, in a mixture with other pharmaceuticals, on phytoplankton community structure (Richards et al., 2004; Johnson et al., 2007). Both studies found a decrease in phytoplankton diversity and community abundances, with a LOEC of 100 µg/L and 11 µg/L after 7 d in Richards et al. (2004) and Johnson et al. (2007), with *Cryptomonas ovata* (Class: Cryptophyceae) showing the largest negative response to the treatments (Richards et al., 2004; Johnson et al., 2007). This is in agreement with our study where we found for eukaryotic algae the class Cryptophyceae to be most affected, as we found a lower proportion of reads in the eDNA assessment.

In addition, the bacterial community analysis showed impacts of fluoxetine on cyanobacteria in sediment samples. Whereas the cyanobacteria families Cyanobiaceae and Leptolyngbyaceae decreased in relative abundances in the highest treatment, unclassified taxa within Oxyphotobacteria increased (Fig. 5). To our knowledge, this is the first study showing effects of fluoxetine on the bacterial community (Fig. 7C). However, our results are in agreement with a study that used Sertraline hydrochloride, also an SSRI antidepressant, where they found that the antidepressant disturbed cyanobacteria species by inhibiting the growth of certain taxa but stimulating the growth of other taxa (Yang et al., 2019).

The highest concentration of fluoxetine did significantly alter zooplankton and macroinvertebrate community composition (Fig. 3; 4B). A treatment-related decrease in abundance was found for damselfly larvae (Zygoptera) during the exposure period and for the bivalve family Sphaeriidae at the end of the experiment. Whereas fluoxetine concentrations in the water were only still measurable in the highest treatment at the end of the experiment, concentrations in the sediment remained high in all treatments (Fig. 1). Sphaeriidae are benthic organisms, living on the sediment and siphoning detritus from the sediment surface. Therefore, Sphaeriidae could still be exposed by food to high fluoxetine concentrations at the end of the experiment, which might be a possible explanation for the decline in abundance at the last two sampling days. Actually, few data exist regarding the presence of fluoxetine or other antidepressants in sediment (Schultz et al., 2010). Concentrations of about 20 µg/kg were found in an effluent-impacted stream (Schultz et al., 2010) which are close to values found in the 20 µg/L treatment in this study. However, concentrations detected in the water of the effluent impacted stream were much lower (0.001 µg/L), showing the high adsorption capacity of fluoxetine to organic material (Kwon and Armbrust, 2006) and accumulation in the sediment. Accumulation of fluoxetine has also been found in tissue of effluent exposed caged bivalves (79.1 µg/kg wet weight after 14 d, Bringolf et al. (2010)). These concentrations in bivalve tissue were substantially higher than those measured in different fish species (range of 0.1–1.6 ng/g wet weight) in effluent impacted waters (Salahinejad et al., 2022), hence indicating a higher risk for bivalves. In addition, known effects of fluoxetine on

bivalves include stimulation of mantle display behavior (96h-LOEC = 300 μg/L (Bringolf et al., 2010), 67d-LOEC = 2.5 μg/L (Hazelton et al., 2014), and 28d-LOEC = 29.3 μg/L (Hazelton et al., 2013)), disruption of reproduction (96h-LOEC = 300 μg/L (Bringolf et al., 2010) and 6d-LOEC = 0.02 μg/L (Lazzara et al., 2012)), alteration of enzyme activities involved in xenobiotic metabolism with negative impacts on the overall health (96h-LOEC = 0.03 μg/L; Cortez et al. (2019)), increased activity (67d-LOEC = 2.5 μg/L; Hazelton et al. (2014)), reduction in shell growth and gonadosomatic index (90d-LOEC = 0.03 μg/L; Peters and Granek (2016)) and increased metamorphosis (24h-LOEC = 1 μg/L (Hazelton et al., 2013)) (see also Table S12 and Fig. 7A). However, the translation of these effects to ecological outcomes, such as population growth, is not well understood. Hence, this study provides some evidence that long-term exposure to fluoxetine may impact bivalves at the population level.

Next to the effects on bivalves, a treatment-related decrease in the abundance of damselfly larvae (Zygoptera) was found (Table S8). Since we did not find a direct effect of fluoxetine (concentrations ranging from 0.02 to 200 μg/L after 9 weeks exposure) on mortality of damselfly larvae in a laboratory experiment (unpublished data), this result might be caused by indirect effects. For example, fluoxetine might have caused a more rapid emergence resulting in a lower number of individuals, as Richmond et al. (2016) found a (nonsignificant) increase in aquatic insect emergence in streams exposed to fluoxetine (20 μg/L) after 14d (Table S12).

Whereas, in general, we observed a treatment-related increase in the relative abundance of zooplankton taxa for the highest fluoxetine concentration, a decrease was found for Cyclopidae (Fig. 4B). A microcosm study by Laird et al. (2007) also found that fluoxetine, in a mixture with other SSRIs, significantly reduced the abundance and species richness of copepod populations at 100 μg/L after 35 d exposure (Fig. S7A, study 24). Another microcosm study, by Richards et al. (2004), used a mixture of pharmaceuticals including fluoxetine (10–1000 μg/L, 35d), and observed a zooplankton community abundance increase as well, similar to this study (Fig. S7A, study 45). However, they found a reduction of Cladocera and rotifers, which we did not find (Fig. S7A).

Concerning functional ecosystem attributes, we observed an increase in leaf mass loss in the presence of invertebrates for the 200 μg/L fluoxetine treatment (Table S5). One possible explanation for this response could be an increase in foraging rates of decomposers in the presence of fluoxetine, which could have led to greater rates of leaf litter decomposition. This may be supported by the significant increase in Asellidae juveniles and *Asellus aquaticus* individuals (Table S8). *Asellus aquaticus* is a common detritivore in temperate lentic freshwaters (Zimmer and Bartholmé, 2003) and feeds on litter by scraping the leaf surface (Graça et al., 1993). The decrease of damselfly larvae (Zygoptera), preying on benthic macroinvertebrates including *A. aquaticus* (Lagesson et al., 2016), might have caused the increase in Asellidae juveniles and *A. aquaticus* individuals, leading in turn to an increase in leaf mass loss.

In contrast, there was no difference in leaf mass loss when invertebrates were excluded. The observed no effects of fluoxetine on microbial breakdown rates are consistent with the study by De Castro-Català et al. (2017) who did not detect effects of fluoxetine (0.1 μg/L) in either leaf litter decomposition or fungal colonization. In contrast, Richmond et al. (2019) found an increase in microbial decomposition after fluoxetine exposure (0.02 μg/L and 20 μg/L) (Fig. 7C, study 46). Actually, effects of pharmaceuticals on decomposition rates are not well studied yet, and the effects on leaf-associated microbial communities are poorly understood (Rossi et al., 2019). Among the limited research on this topic, no effects of pharmaceutical compounds on microbial decomposition, as assessed in watersheds (mostly antihypertensives, anxiolytics and analgesics such as irbesartan, oxazepam and tramadol were detected (Rossi et al., 2019)), in the laboratory (anti-inflammatories, beta-blockers and antibiotics (Hughes et al., 2016)) and microcosms (clotrimazole and terbinafine (Pimentão et al., 2020)), were reported. Furthermore, we did not find major changes in the structure of leaf-associated bacterial communities after fluoxetine exposure, except for the final sampling day (Table S10). The higher richness (as shown by the number of AVSs) of the leaf-associated bacterial community compared to the sediment community might make them more resilient to fluoxetine exposure (Girvan et al., 2005).

4.2. Traditional and non-traditional assessment tools

We aimed to investigate the applicability and capacity of relatively new and non-traditional water quality assessment tools including eDNA metabarcoding and bioassays (*in vitro* and *in vivo*) for assessing the impacts of a chemical stressor on aquatic ecosystems. For phytoplankton, eDNA provided additional insights next to chlorophyll-a and abiotic measurements. We found that all measured parameters related to the suspended algae community were significantly affected by the highest fluoxetine treatment (Table S5; Fig. 4A). eDNA assessment added extra information to this by showing a change in eukaryotic algae community composition in the highest fluoxetine treatment, with algae belonging to the class Cryptophyceae being the most negatively affected taxa to fluoxetine. Furthermore, assessment of the bacterial community showed that fluoxetine disturbed the balance of cyanobacteria taxa by stimulating the growth of specific cyanobacteria and inhibiting others (Fig. 5).

For macroinvertebrates, not all eDNA results are in line with observed patterns found by the morphological assessment. Whereas morphological identification of sampled macroinvertebrates indicated a decrease in Sphaeriidae for the highest fluoxetine treatment at the end of the experiment (Table S8), we did not observe any compositional changes for Mollusca by the eDNA assessment (Fig. S7). A possible explanation for this discrepancy might be that the primers used are not optimal for the detection of Mollusca. Indeed, out of the total 36 samples, in 10 samples only 1 mollusk taxa could be detected (*Lymnaea* sp.), whereas in 11 samples no mollusks were detected at all. Consistently, a previous study by Beentjes et al. (2019) also found the lowest overlap for Mollusca when comparing morphological and eDNA-based assessments indicating that the poor performance of the mollusk detection might be attributed to the primers. For Odonata and Chironomidae, however, eDNA assessment complemented the morphological assessment of macroinvertebrates by increasing taxonomic resolution (Fig. S8; S9). For Chironomidae, the PRC analysis based on morphological identification indicated an increase in abundance of Tanytarsini in response to fluoxetine exposure, with eDNA assessment showing that the species *Tanytarsus* sp. (of the tribe Tanytarsini) showed the largest relative increase related to the fluoxetine treatments. Furthermore, morphological assessment of alive organisms only allowed the identification of Odonata to the suborder level, showing a treatment-related effect of fluoxetine for Zygoptera. The eDNA analyses indicated that *Enallagma* sp. of the suborder Zygoptera might be the most negatively affected by fluoxetine as it could not be detected in the highest treatment on day 49. These examples show that eDNA can be a useful tool when used in combination with morphological identification. However, eDNA and morphological identification should not be considered alternative methods for assessing and monitoring biodiversity, since they give different information (Beng and Corlett, 2020).

While *in vivo* bioassays are used routinely in water quality assessment, *in vitro* bioassays are increasingly applied, since they overcome the limitations of measuring only a limited number of target compounds (De Baat et al., 2020; Schuijt et al., 2021). The aim of using bioassays is to identify potential risks associated with unknown compounds and mixtures present in the water of which the ecological effects are unknown. In this study, responses were observed in both *in vitro* and *in vivo* bioassays (Fig. 6). Effect-based trigger value exceedance for the cytotoxicity was observed at 200 μg/L for two cosms and in one cosm for the anti-AR CALUX bioassay. This shows that those bioassays are able to identify ecotoxicological risks in some but not all cosms that received the highest fluoxetine concentration. It should be noted, of course, that

bioassays are not performed on the original water sample, but extracts of them, leading to much higher exposure concentrations in the test media. On the other hand, the test duration of the bioassays (minutes to a couple of days), is much shorter than the duration of our study, hampering a direct comparison of the observed effects in the bioassays and the mesocosms. The *in vivo* bioassays showed a similar pattern when compared to the structural endpoints. In the Daphniatox and Rotox bioassays, effects on immobilization and mortality, respectively, were found for the highest concentration, which matches with the results of the invertebrate community composition analysis and the zooplankton eDNA metabarcoding results. However, for some individual taxa, effects were already observed at lower concentrations (e.g. NOEC <0.2 μg/L for *Asellus aquaticus* and a NOEC = 0.2 μg/L for Zygoptera) by morphological identification. Thus, effects on biota were found at lower fluoxetine concentrations by structural assessment compared to the bioassays, indicating that the bioassay endpoints used in this study are less sensitive compared to the structural measures.

Historically, a common way to express ecotoxicological risks is the potentially affected fraction (PAF) (Posthuma et al., 2001), which is used to calculate the HC_5 (i.e., hazardous concentration for 5% of the species) that is often used in environmental risk assessment (Maltby et al., 2005). Following this, fluoxetine concentrations lower than 2 μg/L (based on PAF based on chronic toxicity endpoints) are not expected to adversely impact aquatic communities in natural ecosystems. This is partly in accordance with our results found, as most effects were observed for the higher concentrations. However, for certain macroinvertebrate taxa, including Zygoptera and *Asellus aquaticus*, negative and positive effects at 2 μg/L or lower concentrations were observed, respectively. Next to that, it is important to take into consideration that this study was based on a single compound whereas in the environment aquatic communities are exposed to a mixture of stressors that might cause interaction effects.

4.3. Effects of fluoxetine found in literature at the whole-organismal level

As described in section 4.1, fluoxetine can promote changes in ecosystem structure and function, including changes in productivity and phytoplankton community composition, decomposition and invertebrate population dynamics (Fig. 7). However, most of the studies found and included in Table S12 focus on effects at the at the whole-organismal level.

We found several studies investigating behavioral changes in response to fluoxetine exposure in invertebrates and fish. Overall, the finding of these studies show shifts in invertebrates and fish behavior after fluoxetine exposure (Fig. 7). However, the direction of behavioral changes are contradictory for some endpoints. For example, amphipod swimming activity was found to increase (Bossus et al., 2014), decrease (De Lange et al., 2006), or remained unchanged (Schuijt et al., 2023) when exposed to fluoxetine. Interestingly, the majority of studies investigating the effects of fluoxetine on anxiety behaviors reported a decrease in anxiety-related behaviors such as increased exploratory or phototactic behavior (Fig. 7). Behavioral changes might be a result of fluoxetine affecting neurotransmitters levels in the brain. At least for fish, evidence exists that fluoxetine can cause altered neurotransmitter levels in the brain, which consequently result in altered behavioral responses (Correia et al., 2022).

Changes in behavior can influence feeding, growth, reproduction and survival with profound impacts on individual fitness (Ford et al., 2021). These whole-organismal responses can, in turn, have consequences for population dynamics, species interactions, and ecosystem function (Saaristo et al., 2018) by altering, for example, demographic parameters. Thus, behavioral changes of species might have cascading impacts from the whole-organismal level to higher levels of biological organization. However, whether and how changes in behavior, caused by fluoxetine exposure, translate into ecological effects remains poorly studied. Therefore, when assessing the possible impacts of fluoxetine or other chemical stressors on behavior, ideally, a holistic approach should be followed by integrating complementary laboratory, semi-field, and field experiments (Bertram et al., 2022). Whereas behavioral studies in the laboratory are crucial for deriving a mechanistic understanding, allowing explicit control of the variables and providing valuable insights in a standardized setting, semi-field- and field-based ecotoxicology is crucial for assessing the impacts of chemicals in dynamic, complex and large-scale natural systems (Ford et al., 2021; Bertram et al., 2022).

Furthermore, other observed effects of fluoxetine on the whole-organismal level in the literature include reductions in fish growth and reproduction (Fig. 7B). A possible explanation for the growth impairment might be the anorexigenic and inhibitory effects of fluoxetine on feeding (Mennigen et al., 2011; McDonald, 2017) and thus a consequence of changes in behavior. Indeed, a reduction in fish feeding rate has been observed in the included studies (Fig. 7B). For invertebrates, both a decrease as well as an increase in individual growth have been observed, though most included studies reported a decrease in feeding and reproduction (Fig. 7A).

4.4. Concentration-response relationships for fluoxetine

Nonmonotonic concentration-response relationships have been reported by some previous studies with antidepressants (De Lange et al., 2006; Painter et al., 2009; Guler and Ford, 2010; Barry, 2013; Bossus et al., 2014; Fong and Ford, 2014; Ford and Fong, 2016; Martin et al., 2017, 2019; Saaristo et al., 2017; Bertram et al., 2018; Aulsebrook et al., 2022), and also with other pharmaceuticals (e.g. Calabrese and Baldwin (2001); Vandenberg et al. (2012); Fong and Ford (2014); Wilkinson et al. (2016)). A definition of a nonmonotonic concentration-response relationship is that the slope of the curve changes in the sign (positive or negative) over the range of concentrations tested (Kohn and Melnick, 2002; Lagarde et al., 2015). In other words, it means that effects are observed at low concentrations, but not at higher ones (Vandenberg et al., 2012).

The majority of studies included in Fig. 7 found monotonic concentration-response relationships for fluoxetine (this type of response was found in the current study as well), but also some studies reported nonmonotonic responses when exposed to fluoxetine. Specifically, seven out of a total of 57 responses for invertebrates were nonmonotonic, eight out of 53 responses for fish, and two out of 18 responses were nonmonotonic at the ecosystem-level. All, except for one, observed nonmonotonic responses showed effects only at low concentrations, whereas at high concentrations tested endpoints (such as behavior, growth and mortality) did not differ from control. Or, as in Weinberger II and Klaper (2014), specific behavioral changes occurred at different concentrations (tested concentrations: 0.1, 1, 10 and 100 μg/L), meaning that different responses were triggered at lower fluoxetine concentrations than at higher ones. Gust et al. (2009) showed evidence of hormesis effects of fluoxetine, with an increase in reproduction at 11.1 μg/L and a decrease at 100 μg/L (tested concentrations of 3.7, 11.1 and 100 μg/L). A potential mechanism for the observed nonmonotonic responses could be the desensitization of receptors at low fluoxetine concentrations and/or the induction of negative feedback, as has been shown for natural hormones and endocrine-disrupting chemicals (Vandenberg et al., 2012).

4.5. Environmental relevance of impacts of fluoxetine found in studies and this paper

A wide array of research shows that fluoxetine concentrations typically range from <0.001 to 0.35 μg/L in surface waters in Europe and North America whereas in wastewater effluent fluoxetine concentrations can be as high as 3.5 μg/L (Salahinejad et al., 2022). When comparing the highest fluoxetine concentrations in surface waters and waters near wastewater treatment plants, it becomes evident from the available data that exposure to environmentally realistic concentrations

can have consequences at the whole-organismal-, population and ecosystem-level (Fig. 7).

At environmentally relevant concentrations, impacts on invertebrates mainly include changes in Amphipoda and Decapoda behavior and Bivalvia growth and reproduction (Fig. 7B). However, most effects of fluoxetine on invertebrates were found at higher concentrations than typically occur in aquatic environments. For fish, effects on behavior can be found at low concentrations whereas most impacts on feeding, growth and mortality have been reported at higher concentrations than found in the surface water (Fig. 7B). Actually, 26 out of the 42 included behavioral endpoints that show changes in response to environmentally relevant fluoxetine concentrations, suggesting that fluoxetine exposure in aquatic ecosystems can have consequences to fish behavior. At the ecosystem-level, some effects on productivity, respiration and decomposition have been found at these low concentrations (Fig. 7C), though this was not found in our study.

So what can be concluded regarding the expected impacts of fluoxetine on aquatic ecosystems? It is evident that at environmentally relevant concentrations impacts of fluoxetine on aquatic organisms can be found, especially for fish. Those impacts include effects on behavior, which in turn might have direct or indirect ecological consequences (Brodin et al., 2014). This might not be surprising as fish serotonin transporters (the target for fluoxetine) have a high affinity to SSRIs, including fluoxetine (McDonald, 2017). However, variability and discrepancies can be found concerning the direction of change of the measured endpoint, variability in the sensitivity between different studies, as well as in the type of concentration-response relationships that are observed. Part of the discrepancies might be caused by differences in experimental conditions and methodology between studies and are context specific. Next to that, the majority of studies focus on assessing structural endpoints and impacts on functional endpoints are scarce. Also, how sublethal effects might propagate to population- and eventually ecosystem-level effects (Schuijt et al., 2021) and affect multiple generations are still open questions.

5. Conclusions

We found effects of the antidepressant fluoxetine on bacterial, algal, zooplankton and macroinvertebrate communities, but mainly for the highest concentration (200 μg/L). However, on the population level, impacts of fluoxetine at lower concentrations (2 and even 0.2 μg/L) were observed for certain macroinvertebrate taxa. These findings indicate and are also supported by previous evidence, that the impacts of fluoxetine on certain aquatic species can be observed at environmentally realistic concentrations. The combination of traditional morphological identification and non-traditional assessment tools (e.g. eDNA and functional measures) provided complementary information by, for example, providing abundance data of taxa and increasing taxonomic resolution with occurrence data. However, since the different methods give different information, they should not be considered as alternative methods for assessing the effects on aquatic communities. While a remaining key question to be answered concerns the influence of mixtures of multiple pharmaceuticals on aquatic ecosystems, this study provides a valuable step in understanding the effects of the antidepressant fluoxetine at the population-, community- and ecosystem-level.

CRediT authorship contribution statement

Lara M. Schuijt: Conceptualization, Investigation, Project administration, Writing - original draft, Formal analysis. **Jasper van Smeden:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Chantal K.E. van Drimmelen:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Laura L. Buijse:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Dailing Wu:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Marie-Claire Boerwinkel:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Dick J.M. Belgers:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Arrienne M. Matser:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Ivo Roessink:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Ineke Heikamp-de Jong:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Kevin K. Beentjes:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Krijn B. Trimbos:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Hauke Smidt:** Conceptualization, Writing - review & editing, Methodology, Investigation. **Paul J. Van den Brink:** Conceptualization, Supervision, Investigation, Writing - review & editing, Formal analysis, Project administration.

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

We made al raw data available onfigshare (https://doi.org/10.6084/m9.figshare.24451714).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140706.

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