**Impact of two pharmaceuticals on aquatic mesocosms evaluated with a combination of effect-based methods and structural-functional endpoints**

**Achtergronddocument beschikbare kennis bij de sleutelfactor Toxiciteit**

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**Dit document is vertrouwelijk, en mag daarom niet verder verspreid worden.**

Colofon

Deze notitie is geschreven in het kader van het project Toxiciteit van de Kennisimpuls Waterkwaliteit. In de Kennisimpuls werken Rijk, provincies, waterschappen, drinkwaterbedrijven en kennisinstituten aan meer inzicht in de kwaliteit van het grond- en oppervlaktewater en de factoren die deze kwaliteit beïnvloeden. Daarmee kunnen waterbeheerders en andere partijen de juiste maatregelen nemen om de waterkwaliteit te verbeteren en de biodiversiteit te vergroten.

In het programma brengen partijen bestaande en nieuwe kennis bijeen, en maken ze deze kennis (beter) toepasbaar voor de praktijk. Hiermee verstevigen ze de basis onder het waterkwaliteitsbeleid. Het programma is gestart in 2018 en duurt vier jaar. Het wordt gefinancierd door het ministerie van Infrastructuur en Waterstaat, STOWA, waterschappen, provincies en drinkwaterbedrijven.

Nederlandse samenvatting

Geneesmiddelen worden over heel de wereld gebruikt voor het behandelen van mensen en dieren, en eindigen daardoor regelmatig in het aquatische milieu. Eenmaal daar beland kunnen ze door hun sterke biologische activiteit zelfs bij lage concentraties voor schadelijke effecten zorgen bij niet-doelorganismen. Deze effecten zijn vaak niet-lethaal, maar beïnvloeden het gedrag, de reproductie en de groei van deze organismen. De exacte consequenties van deze effecten op ecosysteem niveau zijn echter nog niet voldoende onderzocht. Het doel van deze studie was daarom om de effecten van twee geneesmiddelen (de antidepressiva fluoxetine (FLU), en de antibiotica sulfamethoxazole(SMX)) op het aquatische ecosysteem te onderzoeken. Dit hebben we gedaan met behulp van 30 semi-gecontroleerde aquatische testsystemen. Zes van deze systemen diende als controle, 12 hebben we blootgesteld aan FLU (0.2, 2, 20, en 200 µg/L, in sets van drie replica’s per concentratie), en 12 hebben we blootgesteld aan SMX (0.15, 1.5, 15, en 150 µg/L, in sets van drie replica’s per concentratie). De blootstelling duurde 7 weken. De systemen zijn enerzijds geëvalueerd aan de hand van structureel-functionele eindpunten zoals de macro-invertebraten, zoöplankton en fytoplankton samenstelling, en anderzijds aan de hand van een diverse set bioassays. De gezamenlijke evaluatie van zowel structureel-functionele eindpunten en bioassays helpt om inzicht te krijgen in het vermogen van de reeks geteste bioassays om de bioactiviteit en effecten van de twee geneesmiddelen te detecteren.

Uit onze resultaten blijkt dat er voor beide stoffen een grote overlap zit tussen het algemene beeld die de structureel-functionele eindpunten geven en die de bioassay geven. Echter geven de structureel-functionele eindpunten een completer en gedetailleerder beeld geven van de aard en omvang van de veranderingen die optreden in een gemeenschap onder chemische blootstelling, dan de reeks geteste bioassays. Zo was er een afname van Zygoptera en *Radix* soorten in de FLU-behandelingen van respectievelijk 20 en 200 µg/L. Voor SMX was er een stijging van *Dugesia* sp. en *Chaoborus* sp. bij respectievelijk 15 en 1,5 µg/l. Effecten op de relevante bioassays werden echter pas gevonden bij 200 µg/l voor FLU en waren helemaal afwezig bij SMX. In het geval dat individuele soorten een response geven, zou nader onderzoek naar de ecologische rol, de wettelijke beschermingsstatus en het herstelvermogen van deze soorten richtinggevend moeten zijn voor een beslissing over de aanvaardbaarheid van het gevonden effect. Een dergelijk effect zou echter niet worden opgemerkt met alleen bioassay resultaten.

Over het algemeen tonen de huidige resultaten van deze studie aan dat bioassays en structureel-functionele eindpunten resulteren in een vergelijkbare toxiciteitsbeoordeling, hoewel een meer uitgebreide kalibratie in sommige gevallen noodzakelijk blijft. Het zou mogelijk zijn om bioassay-batterijen te gebruiken in combinatie met landschapsscenario's of chemische monitoring om mogelijke ecotoxicologische risico's aan te geven. In een dergelijk schema kunnen landschapsscenario's of chemische monitoring helpen bij het bepalen van de reeks verwachte chemicaliën en de bijbehorende MOA's. Deze zouden kunnen helpen bij het construeren van een bioassay-batterij die gevoelig genoeg is om de volledige set van mogelijk aanwezige MOA's te dekken. Grootschalige relaties tussen bioassayresponsen en ecologische effecten zullen echter nodig blijven om deze bioassays te kalibreren naar ecotoxicologisch relevante eindpunten, en zullen daarom ontwikkeld moeten worden. Dit document zal worden bijgewerkt wanneer de ontbrekende informatie beschikbaar is, aangezien sommige metingen zijn vertraagd door de Covid19-pandemie.

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1. Introduction

The pharmaceuticals that help cure billions of people all around the world often end up in the aquatic environment where they may induce adverse effects on non-target organisms even at low concentrations (De Lange et al., 2006). Pharmaceuticals, such as antidepressants and antibiotics, have been detected in surface waters ranging from 0.001 to 10 µg/L in rivers and up to 100 µg/L in sewage water (Desbiolles et al., 2018). These environmental concentrations are often not sufficient enough to induce lethal effects on organisms. Nonetheless, they are known to alter ecological traits such as behaviour, activity levels, development and growth in non-target species (De Lange et al., 2006; Peeters et al., 2009; Fursdon et al., 2019).

In the past decades, the consumption of antidepressants has increased (Tisler et al., 2019). This group of pharmaceuticals pose a potential high risk to the aquatic ecosystem, both because of their high occurrence and environmental persistence. Fluoxetine (FLU) is one of the most commonly prescribed antidepressants (Ford et al., 2018), and falls within the class of Selective Serotonin Reuptake Inhibitors (SSRIs). SSRIs are known to inhibit the reuptake of serotonin from the synaptic cleft, and thereby increase the signals between neurones. Effects at the individual level have primarily been assessed on fish, and has revealed changes in behaviour and decreased reproduction and growth (Ford et al., 2018). However, community-level risks associated with FLU occurring in aquatic ecosystems are far from known (Gonzalez-Rey et al., 2006).

Another example of a commonly found pharmaceutical in surface waters is sulfamethoxazole (SMX). This antibiotic belongs to the sulphonamides, and is considered to be one of the most hazardous classes of pharmaceuticals for the environment (Desbiolles et al., 2018). In the Netherlands, SMX is detected in 30% of the ditches (Straub, 2016). The antibiotic is very mobile in the environment and could disturb the balance of ecosystems by eliminating bacteria (Rademaker and De Lange, 2009) and fungal communities (Bundschuh et al. 2009). However, also for this pharmaceutical, risks for aquatic communities and ecosystem functioning are still largely unknown.

The objective of this study is to investigate the effects of the chronic application of both pharmaceuticals on the aquatic ecosystem under semi controlled conditions by using mesocosms. We assessed the individual effects of FLU and SMX on key components of aquatic ecosystems including macroinvertebrate, zooplankton, phytoplankton and microbial communities and organic matter decomposition, a measure of ecosystem functioning. Additionally, we determined the ability of a suite of bioassays to detect bioactivity and effects of the specific compounds used in this study. For the Kennisimpuls project, the main focus lies on comparing bioassay results with results on structural-functional endpoints.

2. Material and Methods

## 2.1. Experimental design

The experiment was conducted at Sinderhoeve Experimental Station in Renkum (the Netherlands) from mid-June till mid-October 2019 (4 months). In total, we used 30 outdoor mesocosms whose dimensions were 1.8 x 0.8 m (diameter x depth), containing a total water volume of 1,500L and 10 cm of sediment. Three months before the application of FLU, each cosm received aliquots of macroinvertebrates, zooplankton, phytoplankton, macrophytes (Elodea and Myriophyllum) and some additional sediment (for (resting) eggs, diasphores and microbial community) collected from uncontaminated freshwater basins at Sinderhoeve. Over the next three months, the community had time to colonize and acclimatize.

Mid-June we started applying FLU (99.95%, from Sigma Aldrich) and SMX (100%, from Sigma Aldrich) with each 4 different exposure concentrations and a solvent control treatment (10 ml acetone) (Table 1). This concentration range included ecologically relevant chronic exposures (below the predicted no effect concentration (PNEC), provided in Table 1) and higher concentrations. Concentrations (3 replicates for each concentration of the pharmaceuticals) were maintained at intended concentration for 8 weeks by weekly application of the chemical on Tuesday after measuring the remaining levels on Monday. A solvent control treatment (10 ml acetone, 6 replicates) was maintained in the same manner, applying solvent concentrations at a weekly interval. Each treatment was randomly assigned to the cosms. FLU and SMX were applied every week for 2 months and thereafter we had a 2 month recovery period (Fig. 1).

***Table 1****. Nominal concentrations of fluoxetine and sulfamethoxazole used in this experiment, as well as their chronic predicted no effect concentration (PNEC) according to the NORMAD database1. In brackets are the number of cosms assigned to the different treatments. Both chemicals shared the same solvent control cosms.*

|  |  |
| --- | --- |
| **Fluoxetine,**  **PNECchronic= 0.1 µg/L** | **Sulfamethoxazole,**  **PNECchronic= 0.6 µg/L** |
| Solvent control (6) | |
| 0.2 µg/L (3) | 0.15 µg/L (3) |
| 2 µg/L (3) | 1.5 µg/L (3) |
| 20 µg/L (3) | 15 µg/L (3) |
| 200 µg/L (3) | 150 µg/L (3) |

1Chronic PNEC values were extracted from the Norman Ecotoxicology Database ([www.norman-network.com](http://www.norman-network.com/)) on the 6th of January 2021.

Water samples of all cosms were taken directly after application (t=0) and 24 h (t=6) before the next application. Dosing was adjusted based on the concentration measured 24h before next application, to achieve intended concentrations. Additionally, we collected water samples one day and 3 days after every application moment from the FLU treatments 2 μg/l and 200 μg/l and SMX treatments 1.5 μg/l and 150 μg/l in order to establish the dissipation curve.



***Figure 1****. Schematic overview of the experimental design of the present study including the exposure regimes and sampling weeks (for macroinvertebrates). In week 7 we collected water samples for the bioassay battery.*

## 2.2. Structural and functional endpoints

The structural endpoints we assessed included physico-chemical parameters, primary producers, macroinvertebrate community, zooplankton community, and microbial community, with as functional endpoint decomposition rates. Effects of FLU on physico-chemical parameters were assessed by following patterns of dissolved oxygen, pH, electrical conductivity and temperature over time. With regard to primary producers, we measured the amount of chlorophyll-a in the water and estimated macrophytes cover per cosm by classifying it from 0% (no plants) until 100% (cosm surface totally covered by plants). Zooplankton and macroinvertebrate communities were assessed by evaluating species composition over time. Organic decomposition rates were studied by the use of litterbags.

## 2.3. Bioassay battery

After two months of exposure (week 7), we collected depth-integrated grab samples from several locations in each mesocosm by using a Perspex® tube in clean 1L HDPE bottles and stored these in a freezer at -20°C until processing. This water was extracted and used for the bioassay battery (except the Daphnia in situ). For the extraction method, see Appendix A.

A battery of 10 bioassays was applied in this study (Table 2). The whole organism bioassays with crustacea *Daphnia magna*, rotifer *Brancionus calyciflorus*, algae *Raphidocelis subcapitata* and bacteria *Vibrio fischeri* were performed by Wageningen Marine Research (Den Helder, The Netherlands), the *in vitro* CALUX assays by BioDetection Systems (Amsterdam, The Netherlands) and MEA will be performed at the laboratory of IRAS (Utrecht, The Netherlands).

***Table 2.*** *Bioassay battery applied to assess the toxicity of the mesocosm water. Effect-based trigger (EBT) values were defined by Escher et al., 2018 (anti-AR), De Baat et al., 2020 (Anti-PR) and Van der Oost et al., 2017 (Bacterial growth inhibition, Sulfonamides).*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Bioassay** | **Endpoint** | **Reference compound** | **EBT (unit)** | **Unit** |
| In vivo | Daphnia in situ | Immobilization and mortality | NA | 20 | % |
|  | Daphniatox | Immobilization | NA | 20 | % |
|  | Rototox | Mortality | NA | 20 | % |
|  | Algatox | Algal growth inhibition | NA | 20 | % |
|  | Microtox | Luminescence inhibition | NA | 20 | % |
| In vitro | Cytotox | Cytotoxicity | TBT |  | µg TEQ/L |
|  | Anti-AR | Antiandrogenic activity | flutamide | 14.4 | µg FEQ/L |
|  | Anti-PR | Antiprogestagenic activity | Ru486 (Mifepriston) | 13 | ng REQ/L |
|  | SULFlux | Bacterial growth inhibition  (Sulfonamides) | sulfamethoxazole | 100 | ng SEQ/L |
|  | MEA | Neurotoxic activity | \* | \* | \* |

\* data not yet available

## 2.4. Statistical analyses

The macroinvertebrate data set was analysed using the principal response curve (PRC) method in Canoco version 5.0 (Van den Brink & Ter Braak, 1999; Ter Braak & Šmilauer, 2018). For this, abundance data were Ln(Ax +1) transformed prior to analysis, where ‘x’ is the abundance value and A equals 2. To check for pharmaceutically induced macroinvertebrate community level effects, Monte Carlo permutation tests (p ≤ 0.05) were used to test each treatment against the control for each sampling week.

The Williams test (p <0.05) was performed on macroinvertebrate data to determine No-Observed-Effect Concentrations (NOECs) on macroinvertebrate abundances (Rico, 2015; Sumon et al., 2018). For this, the Community Analysis computer program version 4.3.05 was used (Hommen et al., 1994).

The pysico-chemical data were additionally assessed with linear mixed models (LMMs) using SPSS Advanced Statistics version 25 (IBM Corporation 1989, 2013). The abiotic values where first checked for normal distribution and the homogeneity of variance. For oxygen, pH and temperature the repeated covariance type Compound Symmetry was used, because their structure had a constant variance and constant covariance. Electrical conductivity was analysed with the heterogeneous covariance type Compound Symmetry, because the covariance structure was heterogenous and had a constant correlation between elements. The microcosm were treated as a random effect and therefore nested. When the p-values of the LLM were significant, the post-hoc Sidak test was used. The Sidak post-hoc test adjusts the significance level and provides tighter bounds than the Bonferroni test (IBM Corporation 1989, 2013).

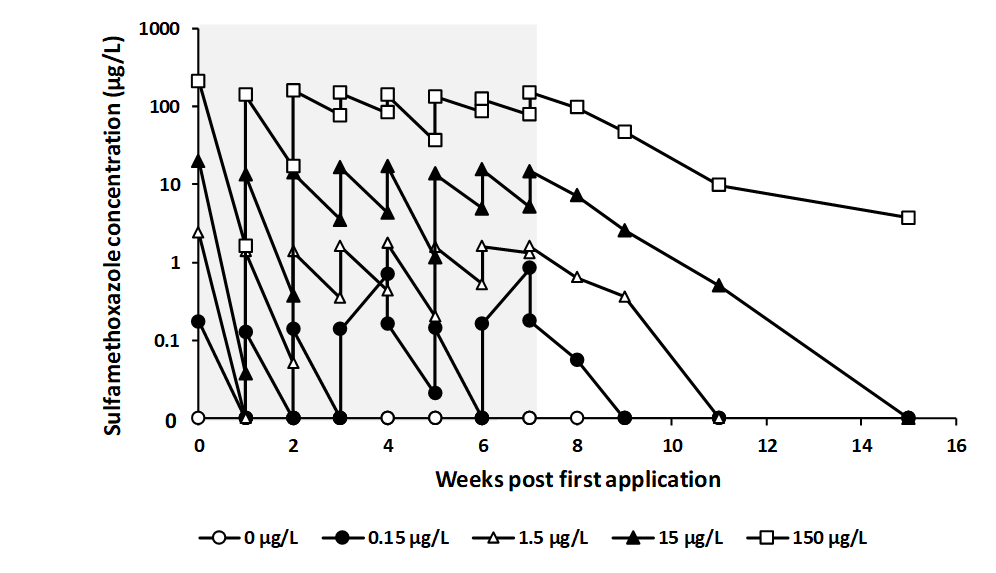
3. Preliminary results

## 3.1. Chemical dynamics

Preliminary results demonstrate that FLU and SMX dissipated rapidly from the water phase (Figure 2). The dissipation of FLU from water phase has been reported to be largely dominated by sorption to sediments (Kwon et al., 2006) and photodegradation for SMX (Isidori et al., 2005). However, with our weekly application scheme we managed to maintain intended time weighted average concentrations (Figure 2). At the end of the experiment (day 105) only in the highest treatment FLU and SMX concentrations where still above the detection limit (average of 3.7 μg/L for both).

**A**

**B**



***Figure 2.*** *Dynamics of fluoxetine (A) and sulfamethoxazole (B) concentrations in mesocosm water. The application period (week 0 to 7) is shown in grey and the recovery period (week 8 to 15) in white.*

## *3.2. Structural and functional responses*

We found a treatment-related decrease in dissolved oxygen (DO) concentrations and pH in the highest FLU treatment. This decrease could be observed during the entire application period and also the first two weeks of the recovery period (from week 0 till week 9). During the same period, we found elevated levels of electrical conductivity (EC) in the highest FLU treatment. Table 3 shows the average values of the physico-chemical parameters of sampling week 7.

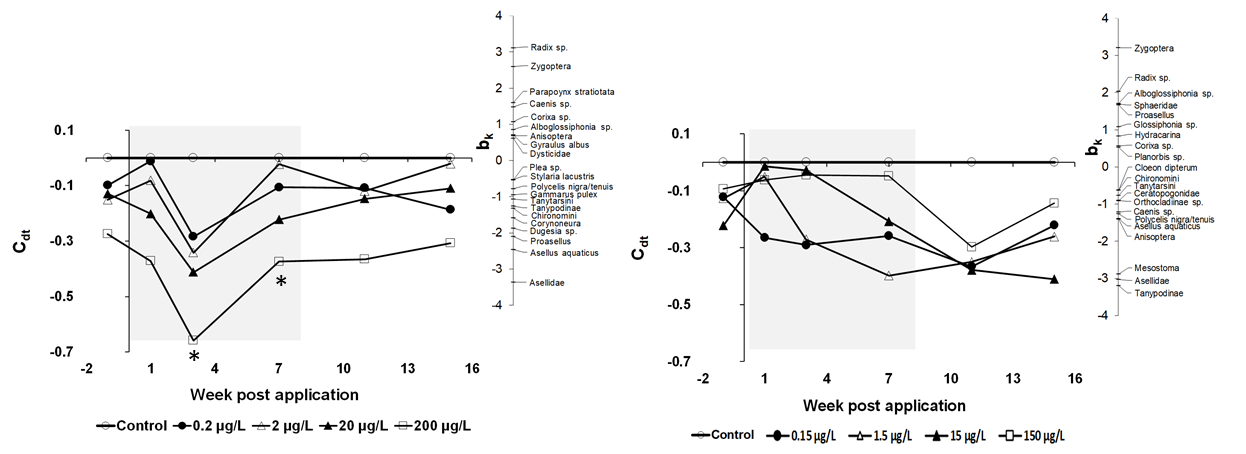
***Table 3.*** *Mean values for the functional and structural endpoints measured in* ***sampling week 7*** *of the mesocosm experiment. Macroinvertebrate Cdt values give the regression coefficients of the first Principle Component of the macroinvertebrate treatment effects deviating from the control. In red are the results that significantly differ compared to control treatment. Temperature is not shown.*

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Chemical** | **Conc**  **(µg/L)** | **EC**  **(µS/cm)** | **pH** | **DO**  **(mg/l)** | **Turbidity**  **(FTU)** | **Micro-**  **organ.** | **Algae**  **(µg chl-a/L)** | **Macrophytes**  **(% cover)** | **Zoo-**  **plankton** | **Macroinvert**  **(Cdt)** |
| **Control** | **0** | 244 | 8.8 | 10.1 | 3.9 | \* | 3.6 | 14 | \* | 0 |
| **FLU** | **0.2** | 248 | 8.9 | 10.3 | 4.2 | \* | 3.7 | 22 | \* | -0.11 |
| **FLU** | **2** | 255 | 9.0 | 10.3 | 3.3 | \* | 3.3 | 17 | \* | -0.02 |
| **FLU** | **20** | 266 | 8.5 | 9.6 | 3.4 | \* | 2.8 | 25 | \* | -0.22 |
| **FLU** | **200** | 334 | 7.8 | 7.3 | 3.7 | \* | 1.6 | 20 | \* | -0.37 |
| **SMX** | **0.15** | 263 | 9.0 | 10.3 | 3.3 | \* | 4.0 | 20 | \* | -0.26 |
| **SMX** | **1.5** | 233 | 9.5 | 11.2 | 3.2 | \* | 4.7 | 13 | \* | -0.40 |
| **SMX** | **15** | 230 | 9.5 | 11.3 | 3.0 | \* | 2.6 | 25 | \* | -0.21 |
| **SMX** | **150** | 258 | 8.9 | 10.5 | 3.0 | \* | 3.6 | 3 | \* | -0.05 |

\* data not yet available

The decrease in oxygen concentration and pH suggests a reduction in phytoplankton. Indeed, we found a decrease in chlorophyll-a concentration (Table 3). A reduction in chlorophyll concentrations was also observed in the SMX 15 ug/L treatment, although this effect was not significant. However, in the highest SMX treatments, there was a significant decrease in macrophyte cover (Table 3).

A total of 75 different macroinvertebrate taxa were identified in the microcosms during the whole experimental period. We analysed the macroinvertebrate dataset using principal response curves (PRC) (Van den Brink & Ter Braak, 1999; Ter Braak & Šmilauer, 2018). The PRC for SMX did not indicate any treatment related effects (Monte Carlo p-value > 0.05; Figure 4b), whilst the PRC for FLU did indicate treatment related effects (Monte Carlo p-value = 0.014; Figure 4a) on the macroinvertebrate community structure for the highest treatment level (Table 3). For FLU, Zygoptera and *Radix* sp. had the highest bk values, indicating a prominent decrease in abundance in the higher treatments due to FLU. Indeed, univariate analysis showed that Zygoptera (NOEC = 2 μg/L) and *Radix* sp. (NOEC = 20 μg/L) were the most affected macroinvertebrate taxa by FLU (see Table B1 in the Appendix). For SMX, *Dugesia* sp. (NOEC = 1.5 μg/L) and *Chaoborus* sp. (NOEC = 0.15 μg/L) showed an increase in abundance (Table B2).



**A**

**B**

***Figure 4.*** *Effects of fluoxetine (A) and sulfamethoxazole (B) on the macroinvertebrate community (PRC). For fluoxetine, 29% of all variance could be attributed to sampling date (displayed on the horizontal axis) and 18% could be attributed to treatment. Of all variance of the macroinvertebrate community for the sulfamethoxazole treatments, 28% could be attributed to sampling date (displayed on the horizontal axis) and 18% could be attributed to treatment (of this variance is 18% displayed on the vertical axis). The PRC diagram shows on its horizontal axis time and on its vertical axis the regression coefficient (cdt) of the first Principle Component of the treatment effects deviating from the control. The bk parameter, located at the right side of the diagram, indicates the weight species. \* indicate significantly different from control (p<0.005).*

## *3.3. Bioassay battery responses*

Out of the 10, 9 bioassays have been successfully performed up till now, only the MEA assays still needs to be performed. Responses of the bioassays for the different treatments applied in this study are given in Table 4.

For the *in vivo* bioassays, effects were observed for FLU but not for the SMX treatments (Table 4). Algae growth inhibition was 100% for the two highest treatments compared to the controls, meaning that algae (*Raphidocelis subcapitata*) showed to be the most sensitive organisms to FLU in the bioassay battery. In the highest FLU treatment, on average 90% mortality was observed for the rotifera (*Brachionus calyciflorus*) and 25% of crustacea *Daphnia magna* where immobile (Table 4).

Considering the *in vitro* bioassays, cytotoxicity was only observed in the cosm samples that received the highest dose of FLU. Anti-AR CALUX activity was observed in all treatment groups, including control. However, on average, the highest anti-AR CALUX activity was observed in the FLU 200 ug/L mesocosms, although this activity did not exceed the trigger value in table 2. In the FLU treated mesocosms a dose-dependent increase in anti-AR activity was found whereas a slight dose-dependent decrease in anti-PR activity was observed for SMX. As expected for the SULFlux assay, only the samples which contained SMX showed activity (Table 4). Average values of the activity measured by the SULFlux assay exceeded the trigger value of 100 ng SEQ/L in all SMX treatments.

***Table 4.*** *Bioassay analysis results (shown as average) for extracted mesocosm samples collected in week 7 for each treatment. Depicted in white are the* in vivo *bioassays, and in grey the* in vitro *bioassays. In red are the bioassay responses exceeding the effect-based trigger value (EBT).*

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ***In vivo*** | | | | ***In vitro*** | | | | | |
| **Treatment** | **Conc**  **(µg/L)** | **Daphniatox**  **% effect** | **Rototox**  **% effect** | **Microtox**  **% effect** | **Algatox**  **% effect** | | **Cytotox**  **(µg TEQ/L)** | **Anti-AR**  **(µg FEQ/L)** | **Anti-PR**  **(ng REQ/L)** | **SULFlux**  **(ng SEQ/L)** | **MEA**  **assay** |
| **Control** | **0** | 1.7 | 2.2 | -0.8 | -5.2 | | <LOD | 4.9 | 2.9 | <LOD | \* |
| **FLU** | **0.2** | 3.3 | 0.0 | 15.3 | -3.7 | | <LOD | 2.1 | 1.2 | <LOD | \* |
| **FLU** | **2** | 0.0 | 1.1 | -4.7 | -3.7 | | <LOD | 3.6 | 2.8 | <LOD | \* |
| **FLU** | **20** | 0.0 | 0.0 | 3.0 | 99.7 | | <LOD | 2.4 | 2.4 | <LOD | \* |
| **FLU** | **200** | 25.0 | 90.8 | 23.7 | 100.0 | | 9.3 | 11.8 | 3.6 | <LOD | \* |
| **SMX** | **0.15** | 3.3 | 0.0 | 2.7 | -1.7 | | <LOD | 7.6 | 1.8 | 139 | \* |
| **SMX** | **1.5** | 0.0 | 2.2 | -13.0 | -1.7 | | <LOD | 4.4 | 2.2 | 264 | \* |
| **SMX** | **15** | 0.0 | 0.0 | 8.0 | -4.3 | | <LOD | 2.9 | <LOD | 1906 | \* |
| **SMX** | **150** | 3.3 | 0.0 | 5.3 | -3.7 | | <LOD | 3.5 | <LOD | 29084 | \* |

\* data not yet available

4. Discussion and conclusions

The main objective of this study under the Kennisimpuls project lies on the bioassay results. Therefore, the discussion and conclusion section is focused on comparing the bioassay results with results on structural-functional endpoints.

For FLU, the structural-functional endpoints provide similar results as the *in vivo* bioassays. For instance, we find a reduction in algae abundance at the two highest concentrations, both in the chlorophyll measurements (Table 3) and in the Algatox bioassay (Table 4). Additionally, we find a change in the macroinvertebrate community at the highest concentration, both in the macroinvertebrate structure (Table 3) and in the invertebrate bioassays (Daphniatox and Rototox, Table 4). Considering the *in vitro* bioassays, the response of the cytotox bioassay corresponds with Daphniatox, Rototox, and Microtox bioassays. The results of the microorganisms and zooplankton measurements, along with the MEA bioassay, can provide confirmation that both structural-functional endpoints and effect-based methods are able to assess community-level effects of FLU.

For SMX, it is difficult to compare structural-functional and bioassay results, primarily due to the absence of the results of the microbial community analysis. Since SMX is an antibiotics, microorganisms are expected to be the most sensitive taxonomic group. Indeed, few structural-functional effects have been observed in other taxonomic groups, with only a reduction in macrophyte coverage at the highest concentration. This response could not be observed in the bioassay results, probably due to the absence of a macrophyte bioassay. The Algatox bioassay was phylogenetically and functionally the closest bioassay out of all the bioassays tested, but also gave no response to SMX. The SULFlux bioassay gave a response that exceeded the EBT threshold at all concentrations, clearly increasing with increased SMX concentration (Table 4). This bioassay is therefore a good exposure bioassay. Once the results of the microbial community are known, the EBT threshold of the SULFlux bioassay can be calibrated on the most sensitive structural-functional endpoints, making it more predictive of community level effects.

Evidently, structural-functional endpoints provide a more complete and detailed view of the nature and extent of the changes that occur in a community under chemical exposure than a range of bioassays does. For instance, there was a decline in Zygoptera and *Radix* species in the FLU treatments of respectively 20 and 200 µg/L (Table B1). For SMX, there was an increase in *Dugesia* sp. and *Chaoborus* sp. at respectively 15 and 1.5 µg/L (Table B2). So for both chemicals, the effects found for individual species was at lower concentrations than the effects found for the whole community (Table 3), or for the invertebrates tested in the bioassay battery (Table 4). In such a case, further research into the ecological role, legal protection status and the ability to recover of these species should guide a decision on the acceptability of the effect found. However, such an effect would not be picked up with bioassay results only.

Overall, the contemporary results of this study show that bioassays and structural-functional endpoints result in a similar toxicity assessment, although a more extensive calibration remains necessary in some cases. Potentially it would be possible to use bioassay batteries in combination with either landscape scenarios or chemical monitoring to indicate potential ecotoxicological risks. In such a scheme, either landscape scenarios or chemical monitoring can help determine the set of expected chemicals, and their associated MOAs. These could assist in constructing a bioassay battery that is sensitive enough to cover the entire set of potentially present MOAs. However, large scale assessment of relationships between bioassay responses and ecological effects will remain necessary to calibrate these bioassays to ecotoxicologically relevant endpoints. This document will be updated when the missing information is available as some measurements were delayed due to the Covid19 pandemic.

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Appendix A

Oasis HLB SPE cartridges have been used to extract fluoxetine, sulfamethoxazole and possible transformation products from the water samples for bioanalysis. The extraction protocol is based on Houtman et al. (2018). First, the water samples have been filtered with 0.7 μm pore size glass fibre filters (47 mm diameter, Whatman, United Kingdom) and the SPE cartridges (500 mg, 6 cm3, 30 μm) have been conditioned with 4 mL methanol/ethyl acetate (50:50 v/v) and equilibrated with 5 mL MilliQ. For each cartridge, 1 L of sample has been extracted by passing the sample under vacuum. After drying, the compounds have been eluted from the cartridges in three steps with 3 mL methanol/ethyl acetate (50:50 v/v). The combined extracts have subsequently been evaporated at 35 °C with N2 until dryness and resolved in 100 μL DMSO for the bioassay analysis. Extracts have been stored in the freezer at -20°C until use.

Appendix B

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Table B1*** *The No Observed Effect Concentrations (NOECs) calculated for the different macroinvertebrate taxa measured on each sampling week for fluoxetine µg/L for the water sampling method (Williams test; p<0.05).* | | | | | | |
| **Macroinvertebrates** | **Sampling weeks** | | | | | |
|  | **-1** | **1** | **3** | **7** | **11** | **15** |
| *Asellidae* | > | 20↑ | 0↑ | 20↑ | 20↑ | 0↑ |
| *Gammarus pulex* | > | > | > | > | > | > |
| *Caenis sp.* | > | > | > | 20↓ | > | > |
| *Cloeon dipterum* | > | 20↓ | > | 20↑ | > | > |
| *Erpobdella sp.* | 20↑ | > | > | > | > | > |
| *Leaches other* | > | > | > | > | > | 20↓ |
| *Worms other* | > | 20↑ | 20↑ | - | - | - |
| *Hydracarina* | > | > | > | > | > | > |
| *Zygoptera* | > | > | 2↓ | 2↓ | > | > |
| *Anisoptera* | 20↓ | > | > | > | > | > |
| *Flatworms other* | - | - | - | > | - | - |
| *Dugesia sp.* | > | > | 2↑ | > | > | > |
| *Mesostoma* | > | > | > |  | > | > |
| *Polycelis nigra/tenuis* | > | > | > | > | > | > |
| *Tanypodinae* | > | > | > | > | 20↑ | > |
| *Tanytarsini* | > | > | > | > | > | > |
| *Chaoborus sp.* | > | > | > | > | > | > |
| *Chironomidae* | > | > | > | 20↑ | 20↑ | > |
| *Flies and mosquitoes other* | > | > | 0.2↑ | > | > | > |
| *Caddisfly sp.* | 20↑ | - | - | - | > | 0↓ |
| *Plea sp.* | > | > | > | 20↑ | > | > |
| *Heteroptera other* | > | > | > | > | > | > |
| *Dysticidae* | > | > | > | > | - | > |
| *Beetles other* | > | > | > | > | 20↑ | > |
| *Lymnaea sp.* | > | > | > | > | > | > |
| *Radix sp.* | > | 20↓ | 20↓ | > | > | > |
| *Snails other* | > | > | > | > | > | > |
| *Sphaeridae* | > | > | > | > | 20↓ | > |
| *Parapoynx stratiotata* | - | - | 20↓ | > | > | > |
| *Sialis lutaria* | - | - | - | - | - | - |
| *Triturus vulgaris* | > | > | 20↑ | > | > | - |
| *> = no signiﬁcant effect (NOEC > 200 ug/L); signiﬁcant decrease (-) compared to control.* | | | | | | |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Table B2*** *The No Observed Effect Concentrations (NOECs) calculated for the different macroinvertebrate taxa measured on each sampling week for sulfamethoxazole µg/L for the water sampling method (Williams test; p<0.05).* | | | | | | |
| **Macroinvertebrates** | **Sampling weeks** | | | | | |
|  | **-1** | **1** | **3** | **7** | **11** | **15** |
| *Asellidae* | > | > | > | > | > | 1.5↑ |
| *Gammarus pulex* | > | > | > | > | > | > |
| *Caenis sp.* | > | > | > | > | > | 0.15↑ |
| *Cloeon dipterum* | > | > | > | > | > | > |
| *Erpobdella sp.* | > | > | 15↓ | > | > | > |
| *Leaches other* | > | > | > | > | > | > |
| *Worms other* | > | 15↑ | - | - | > | > |
| *Hydracarina* | > | > | > | > | > | > |
| *Zygoptera* | > | > | > | > | > | > |
| *Anisoptera* | > | > | > | > | > | > |
| *Flatworms other* | > | - | - | - | - | > |
| *Dugesia sp.* | > | > | 1.5↑ | 15↑ | > | 15↑ |
| *Mesostoma* | > | 15↑ | > | > | 0↑ | > |
| *Polycelis nigra/tenuis* | > | > | > | > | > | 15↑ |
| *Tanypodinae* | > | > | > | > | 0.15↑ | > |
| *Tanytarsini* | > | > | > | > | > | 15↑ |
| *Chaoborus sp.* | > | 0.15↑ | 15↑ | > | > | > |
| *Chironomidae* | > | > | > | > | > | > |
| *Flies and mosquitoes other* | > | > | > | > | > | > |
| *Caddisfly sp.* | > | - | - | > | > | > |
| *Plea sp.* | > | > | > | > | > | > |
| *Heteroptera other* | > | > | > | > | > | > |
| *Dysticidae* | > | > | > | > | - | > |
| *Beetles other* | > | > | - | > | 15↑ | > |
| *Lymnaea sp.* | > | > | 0.15↑ | > | > | > |
| *Radix sp.* | > | > | > | > | > | > |
| *Snails other* | > | > | > | > | > | 15↓ |
| *Sphaeridae* | 15↓ | > | > | > | > | > |
| *Parapoynx stratiotata* | - | 15↑ | 1.5↓ | > | > | > |
| *Sialis lutaria* | - | - | - | - | - | - |
| *Triturus vulgaris* | > | > | > | > | > | - |
| *> = no signiﬁcant effect (NOEC > 200 ug/L); signiﬁcant decrease (-) compared to control.* | | | | | | |