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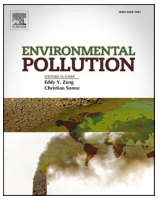

Assessing ecological responses to exposure to the antibiotic sulfamethoxazole in freshwater mesocosms☆

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ABSTRACT

Antibiotics are a contaminant class of worldwide concern as they are frequently detected in aquatic ecosystems. To better understand the impacts of antibiotics on aquatic ecosystems, we conducted an outdoor mesocosm experiment in which aquatic communities were exposed to different concentrations of the antibiotic sulfamethoxazole (0, 0.15, 1.5, 15 and 150 μg/L). These concentrations include mean (0.15 μg/L) and maximum detected concentrations (15 and 150 μg/L) in aquatic ecosystems worldwide. Sulfamethoxazole was applied once a week for eight consecutive weeks to 1530 L outdoor mesocosms in the Netherlands, followed by an eight-week recovery period. We evaluated phytoplankton-, bacterial- and invertebrate responses during and after sulfamethoxazole exposure and assessed impacts on organic matter decomposition. Contrary to our expectations, consistent treatment-related effects on algal and bacterial communities could not be demonstrated. In addition, sulfamethoxazole did not significantly affect zooplankton and macroinvertebrate communities. However, some effects on specific taxa were observed, with an increase in Mesostoma flatworm abundance (NOEC of <0.15 μg/L). In addition, eDNA analyses indicated negative impacts on the insects Odonata at a sulfamethoxazole concentration of 15 μg/L. Overall, environmentally relevant sulfamethoxazole concentration did not result in direct or indirect impairment of entire aquatic communities and ecological processes in our mesocosms. However, several specific macroinvertebrate taxa demonstrated significant (in)direct effects from sulfamethoxazole. Comparison of the results with the literature showed inconsistent results between studies using comparable, environmentally relevant, concentrations. Therefore, our study highlights the importance of testing the ecological impacts of pharmaceuticals (such as sulfamethoxazole) across multiple trophic levels spanning multiple aquatic communities, to fully understand its potential ecological threats.

1. Introduction

Antibiotics are used therapeutically in human and veterinary medicine for treating infections with pathogens and/or prophylactically to increase yields in aquaculture and livestock farming (Kümmerer, 2009). In turn, antibiotics are dispersed into the aquatic ecosystem largely via wastewater effluents, aquaculture residues or runoff from veterinary use (Kovalakova et al., 2020), and have been detected in surface waters worldwide (Wilkinson et al., 2022; Danner et al., 2019; Duan et al., 2022). Once present in the aquatic environment, non-target organisms are inevitably exposed to these antibiotics. These non-target organisms, including microorganisms and invertebrates, are part of communities and they are involved in several ecological processes, such as nutrient cycling, primary production or degradation of organic material. Hence,

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by disturbing non-target organisms these ecological processes might be subsequently disturbed as well.

Non-target organisms that are likely to be most affected by antibiotics are prokaryotes, as antibiotics are developed to inhibit bacterial growth (Brandt et al., 2015). Indeed, some prokaryotes, including cyanobacteria and ammonium oxidizing bacteria, were found to have some of the highest sensitivity towards antibiotics (Le Page et al., 2017; Välitalo et al., 2017; Grenni et al., 2018). Additionally, antibiotics might also have indirect effects on higher trophic levels (Friman et al., 2015; Wan et al., 2020). For example, Hahn and Schulz (2007) demonstrated in a food-selection experiment that the amphipod *Gammarus pulex* preferred leaves that were not exposed to antibiotics.

To date, our knowledge of the direct and indirect effects of antibiotics and their consequences for aquatic communities and ecological processes is still limited (Rico et al., 2014; Danner et al., 2019). A suitable method for investigating these impacts is by using model ecosystems (e.g. mesocosms), as they allow the study of direct and indirect effects of antibiotic exposure on communities, as well as their recovery. Mesocosms are intended to simulate a simplified form of a natural environment by including multiple aquatic communities, food webs and interspecies interactions (Clements and Newman, 2003). Hence, mesocosms provide a higher level of ecological complexity than laboratory tests, while at the same time, they allow control over relevant variables (e.g. antibiotic concentrations) as well as statistical evaluation through replication (Culp et al., 2000).

Among the detected antibiotics in surface waters, sulfamethoxazole is one of the most prominent, with a detection frequency of about 40% in water samples (Wilkinson et al., 2022). Sulfamethoxazole is a broad-spectrum sulfonamide antibiotic that inhibits the growth of bacteria and other microorganisms that depend on folate synthesis used for the synthesis of pyrimidine and purine nucleotides (Mc Dermott et al., 2003; Straub, 2016). Members of aquatic bacterial communities seem to be the most sensitive organisms to sulfamethoxazole in freshwater ecosystems, as effects on bacterial communities in biofilms have been observed at concentrations as low as 0.5 μg/L after 28 d exposure (Kergoat et al., 2021) and bacterial biomass accrual in leaves was found to decrease after exposure to 5 μg/L sulfamethoxazole for 16 d (Paumelle et al., 2021). While single species tests for some zooplankton species exist, including *Ceriodaphnia dubia*, *Daphnia magna* and *Brachionus calyciflorus* (with EC50s of 210, 123,000, 9630 μg/L; Ferrari et al. (2004); Flaherty and Dodson (2005); Isidori et al. (2005); Park and Choi (2008)), effects of sulfamethoxazole on aquatic invertebrate communities and impacts on the interactions between communities have not been investigated yet.

Therefore, in this study, we investigated ecological responses to sulfamethoxazole exposure by assessing changes in invertebrate population abundances, microbial (algal and bacterial) and invertebrate community composition, macrophyte abundance, abiotic water conditions and decomposition rates. We conducted a mesocosm experiment using sulfamethoxazole concentrations of 0, 0.15, 1.5, 15 and 150 μg/L. These concentrations include mean and maximum detected concentrations worldwide. In a previous study, mean measured concentrations of about 0.1 μg/L have been detected in aquatic ecosystems worldwide (Wilkinson et al., 2022). However, also higher concentrations have been reported in surface water with levels up to 12 μg/L in Europe (Ginebreda et al., 2010) and 140 μg/L in Africa (Kairigo et al., 2020). As prokaryotes are expected to be the most sensitive organisms to antibiotics (Le Page et al., 2017; Välitalo et al., 2017), we expected that we would observe changes in bacterial community composition with subsequent indirect influences on decomposition rates. Sulfamethoxazole has dissociation constants of pKa1 = 1.6 and pKa2 = 5.7 (Boreen et al., 2004), meaning that at a pH of 5.7 half of the molecules will be neutral and half anion. Higher pH values of the medium enhances the ionization of the sulfamethoxazole and lowers its toxicity. As the pH in our control mesocosms ranges between 8.5 and 9.5 we expect to assess the effects of sulfamethoxazole in its anion form. Although this is the least toxic form, this has field relevance as only pH values of <6 will have a substantial (>50% increase) influence on the toxicity of sulfamethoxazole (Sun et al., 2020). In addition, we provided a complete overview of the effects of sulfamethoxazole on aquatic ecosystems as described in the literature and discuss whether the currently used concentration-response relationships are suitable for describing the effects of sulfamethoxazole.

2. Material and methods

2.1. Experimental design

Experiments were carried out in 18 outdoor mesocosms (diameter 1.8 m, total depth 0.8 m, water depth 0.6 m, water volume ca. 1530 L and 10 cm layer of fine sandy clay sediment) at the Sinderhoeve Experimental Station in Renkum (the round polymer type show at www.sinderhoeve.org), the Netherlands, from mid-June till mid-October 2019 (4 months). Mesocosms were filled with water originating from the experimental station's supply basin and received aliquots of phytoplankton, zooplankton, macroinvertebrates, macrophytes (*Elodea nuttallii* and *Myriophyllum spicatum*), and some additional uncontaminated sediment (for microbial community, (resting) eggs and diaspores) three months before the first application. In this pre-treatment period, we also added some extra nutrients as KH_2PO_4 and NH_4NO_3 (90 μg N/L and 15 μg P/L) to the cosms monthly and interconnected all cosms by using tubes and a pump to circulate the water to achieve the development of a similar biocoenosis. Next to that, 12 invertebrate sediment trays (diam. 26 cm, height 3 cm) filled with a sediment layer of 2 cm, originating from an uncontaminated experimental ditch, were positioned on the sediment surface of each cosm. In addition, each cosm received 18 sediment containers (100 mL HDPE) filled with 2 cm of sediment collected from a spare cosm not used in the experiment, for the sampling of sulfamethoxazole fate and bacterial community in the sediment. Two weeks before the start of the experiment, each cosm received two pebble baskets, positioned on concrete tiles on the sediment surface, for invertebrate sampling. Seven days before the first application, four litterbags with a fine mesh (width 500 μm) containing *Populus* leaves (5 g dry weight, dried at 60 °C) were deployed at 20 cm depth, to monitor changes in bacterial community structure present in leaf material. Additionally, to study the effects of sulfamethoxazole on decomposition rates, a litterbag with a fine mesh (width 500 μm) and with a coarse mesh (width 5 mm; allowing macroinvertebrate to pass through) filled with *Populus* leaves (2 g dry weight, dried at 60 °C) were deployed in each cosm at the start of the experiment.

We randomly divided the 18 cosms into five experimental treatments: control (6 cosms), and four different sulfamethoxazole exposure concentrations (0.15, 1.5, 15 and 150 μg/L) with three replicates each. Concentrations were maintained at the intended concentration for eight weeks by weekly application of sulfamethoxazole after measuring the remaining levels and correcting the water level of the cosms by adding groundwater. The latter, since this was free of planktonic organisms and therefore did not interfere with the measurements of plankton endpoints.

2.2. Sulfamethoxazole application, sampling and analysis

Sulfamethoxazole was applied to the mesocosms weekly (around 4 p.m.) at a nominal concentration of 0.15, 1.5, 15 or 150 μg/L for a total period of eight weeks. Sulfamethoxazole was applied in 12 cosms (3 replicates per concentration), while the remaining six cosms were used as control. Sulfamethoxazole stock solutions were made with sulfamethoxazole (Sigma-Aldrich, Product PS7507, LOT#BCBP8794V, concentration 100%) and acetone. Next, dosing solutions were prepared by diluting 10 mL of the stock solution in 2000 ml of tap water. The control cosms received 2000 mL tap water containing 10 mL pure acetone, to ensure that the same amount of acetone was added to all cosms (10 mL acetone in 1570 L systems). After manual shaking, the dosing solution

was poured evenly over the water surface and mixed by stirring with a steel rod in all cosms.

To determine the concentration of sulfamethoxazole in the dosing solution, duplicate samples in 5 mL in HDPE bottles were taken, subsequently diluted into 5-mL PP tubes with ultrapure water and spiked with 100 μl internal standard sulfamethoxazole-(phenyl-$^{13}C_6$) (99.8%; Sigma Aldrich, article 32,514, Lot#BCBW7218) in acetone (concentration of 200 ng/ml). To determine the sulfamethoxazole concentration in the cosms, depth-integrated water samples were collected using a Perspex® sampling tube. Water samples of all mesocosms were taken at t = −1 day, t = 0 (1 h after application) and t = 6 (about 24 h before the next application). In addition, at t = 1 (about 24 h after application) and t = 3 (about 65 h after application), only water samples were taken from the cosms receiving the 1.5 μg/L and 150 μg/L treatment. Immediately after all sampling times, a sub-sample of 4.0 mL was transferred into a 5-mL PP tube, spiked with 100 μL internal standard sulfamethoxazole-(phenyl-$^{13}C_6$) and stored in the freezer (about −20 °C) until further analysis. Before the next application, the concentrations of the dosing solutions were adjusted based on the concentration measured at t = 6 in the water samples, to achieve intended concentrations of 0.15, 1.5, 15 and 150 μg/L.

The concentration of sulfamethoxazole in the sediment was determined using sediment containers (100 mL HDPE jars filled with 2 cm of sediment) which had been added to each cosm before the first application. During each sampling event, three sediment containers were retrieved from each cosm. Two of the three containers were pooled and mixed and subsequently duplicate samples of about 5 g of wet sediment were used for extraction. For the sulfamethoxazole sediment extraction and water and sediment sample analysis is referred to appendix A.

2.3. Ecological responses

We measured the abiotic ecosystem properties including dissolved oxygen concentration, water temperature, pH and electrical conductivity by a HACH multimeter (HQ40d) in the morning (8 a.m.) on sampling days −8, 2, 6, 13, 20, 27, 34, 41, 48, 55, 62, 76, 90 and 104 relative to the first application of the antibiotic. We collected filtered (pore size: 0.45 μm) and unfiltered water samples for analysis of nitrite and nitrate ($NO_2^- + NO_3^{2-}$), ammonia (NH_3), phosphate (PO_4^{3-}) and total phosphorus (P) and total nitrogen (N) by a segmented flow analyzer (Skalar 5100 Autoanalyser, Breda, The Netherlands) on sampling days −8, 6, 20, 34, 48, 76 and 104.

Chlorophyll-a concentration in the water and turbidity was determined by using an ALGAETORCH (3 different locations per cosm). Macrophyte cover was classified from 0% (no plants) up to 100% (mesocosm sediment surface was totally covered by plants) for each cosm. Chlorophyll-a concentration, turbidity and macrophyte cover were determined on sampling days −8, 2, 6, 20, 34, 48, 76 and 104. In addition, we harvested all macrophytes at the end of the experiment (day 114), and measured the total dry weight after washing off adhering particles and organisms and drying the plants in the oven at 60 °C for 96 h.

Macroinvertebrates were collected using two pebble baskets (17 cm × 17 cm × 11 cm) and two sediment trays (diam. 26 cm, height 3 cm) from each cosm at sampling days −7, 21, 49, 77 and 105 relative to the first application of sulfamethoxazole. Pebble baskets were gently retrieved using a net (mesh size 0.3 mm) after a colonization period of 2 weeks, and sediment trays were gently collected with the same net and subsequently sieved using 0.5 and 1 mm sieves. The invertebrates of the pebble baskets and sediment trays were identified and counted and afterward returned alive to their original cosm.

Decomposition rates were measured by using litterbags with fine (width 500 μm) and coarse (width 5 mm) mesh sizes. Every 2 weeks (days 13, 27, 41, 55, 69, 83, 97, 111), we retrieved the bags, gently washed them to remove adhering particles and organisms and placed new litterbags in the cosms. To determine the dry weight, the organic leaf material in the retrieved litter bags was dried in pre-weighed aluminum foil at a temperature of 60 °C for 96 h.

Environmental DNA samples were collected for more detailed information on the taxonomic composition of phytoplankton, zooplankton and Odonata, next to chlorophyll-a measurements and macroinvertebrate sampling described earlier. We selected these groups as we expected the effects of sulfamethoxazole on phytoplankton community composition based on the available literature (e.g. Kergoat et al. (2021)), with possible indirect effects on the zooplankton community. Odonata were selected as they showed the largest response to the sulfamethoxazole treatment as assessed using morphological identification (see results). Furthermore, for this we collected water samples of 50 ml at ten different positions in the cosm, 5 samples close to the surface and 5 samples close to the bottom) adding up to a total volume of 500 ml per mesocosm on sampling day 48 (at the end of the application period) and day 112 (at the end of the experiment). The water samples were filtered by 0.45 μm polyethersulphone (PES) filter membranes (47 mm diameter, Sartorius) placed in sterilized 47 mm filter holders, and membranes were stored in 700 μl CTAB at −20 °C until further analysis (see Appendix B). Unfortunately, a study limitation was that we did not take samples at day 0, and therefore the composition of the different communities at the start of the experiment is not known.

The bacterial community composition was monitored on leaf material and sediment at four sampling days (−1, 27, 56, 111). We were interested in potential changes due to sulfamethoxazole in bacteria colonizing leaf material as this might be linked to changes in decomposition rates. In addition, we investigated bacteria in the sediment as they play important roles in the natural biogeochemical processes, and therefore changes in bacterial community structure induced by sulfamethoxazole could have important influences on ecological processes. During the sampling days, four litterbags with a fine mesh (width 500 μm) containing *Populus* leaves (5 g dry weight, dried at 60 °C) were retrieved from the cosms, and leaves were carefully transferred into plastic bags and stored at −20 °C until further processing. For the sediment sampling, 2 sediment containers were retrieved, the overlying water was gently removed and containers were stored frozen (<-20 °C) until further analysis (see Appendix C).

2.4. Statistical analyses

We calculated the dissipation rate coefficients (k) of sulfamethoxazole in the water by means of linear regression of the ln-transformed concentrations, assuming first-order kinetics. In addition, sulfamethoxazole half-lives were calculated by dividing Ln(2) by k, and time-weighted average sulfamethoxazole concentrations were calculated by using the equations described in Roessink et al. (2013).

No observed effect concentrations (NOECs) were calculated by using Dunnett's test, which does not assume a monotonic increasing effect with increasing concentration, for all abiotic ecosystem properties, chlorophyll-a concentration, macrophyte cover and biomass, decomposition rates, macroinvertebrate taxa and diversity indices by comparing the treatments to the control. To reduce type 1 error, effects were considered to be consistent in case the following two prerequisites were met: (i) abundance values of controls of the calculated NOEC sampling day were >3 individuals/sample for macroinvertebrate taxa and (ii) statistically significant deviations point in the same direction for at least two consecutive sampling days. Before the analysis, macroinvertebrate abundance data were ln-transformed (ln[2 × + 1]), while eDNA data were arcsine transformed. Dunnett's tests were performed with the Community Analysis computer program version 4.3.05 (Hommen et al., 1994).

The macroinvertebrate (morphological identification data and eDNA data), zooplankton (eDNA data) and microbial datasets were analyzed by the multivariate Principle Reponses Curves (PRC) method (Van den Brink and ter Braak, 1999) using the CANOCO software, version 5 (ter Braak and Š; milauer, 2012). By performing 499 Monte Carlo

permutations, we tested the overall significance of the effect of the sulfamethoxazole treatment on the community composition. The corresponding b_K scores were used to interpret whether taxa followed the PRC pattern (those taxa had high, positive b_K values), or the opposite pattern (taxa with a low, negative score). Next, we ran Monte Carlo permutation tests under the Redundancy Analysis (RDA) option to test the significance of the separate sulfamethoxazole treatments on the community composition for each sampling day. For all statistical analyses, a significance level (α) of 0.05 was used.

3. Results and discussion

3.1. Sulfamethoxazole fate

Measured sulfamethoxazole concentrations were on average 104% of the intended concentration (range: 71–166%) 1 h after application. Only in cosms receiving the highest treatment, sulfamethoxazole could still be detected at the end of the experiment (Fig. 1A). Time (7-days) weighted average sulfamethoxazole concentrations were 0.1, 0.8, 7.2 and 91 μg/L, for the lowest to the highest treatment level. In the control mesocosms all concentrations were below the level of quantification (0.045 μg/L; Appendix A). For the sake of clarity, we will refer in the following to nominal values (0.15, 1.5, 15 and 150 μg/L).

The average half-life was 3.3 and 5.5 d for the 1.5 and 150 μg/L treatment, respectively, and the individual half-lives after each application are shown in Table A.3. The DT50 increased towards the end of the application period, probably due to a decrease in temperature (Fig. A1). The observed half-life of sulfamethoxazole was lower compared to other cosm studies, as an average half-life of 19 d was found in outdoor cosms (Lam et al., 2004) and a half-life of 18 d in indoor streams (Liu et al., 2019). The half-life time reported in an eel pond water-sediment system (4.9 d under light conditions) was consistent with our findings (Lai and Hou, 2008). Additionally, Lai and Hou (2008) observed that half-lives of sulfamethoxazole were lower under light conditions compared to the dark, when sediment was added to the system and in the presence of microbes (nonsterile). Hence, in surface waters, photolysis and especially biodegradation are expected to be the dominant pathways for degradation (Radke et al., 2009; Straub, 2016).

Accumulation of sulfamethoxazole in the sediment was below the limit of quantification (<100 ng/kg dry weight) for the 0.15 and 1.5 μg/L treatment. The highest sulfamethoxazole concentration in the sediment was found for the highest treatment on sampling day 57 with an average concentration of 6.7 μg/kg (Fig. 1B). When compared to the multiple applications of 150 μg/L of water, the accumulation to the sediment is low. Based on the log Koc value (2.41; Duan et al. (2022)) and the log Kow (0.89; Duan et al. (2022)), sulfamethoxazole is indeed expected to have high mobility and a low binding capacity to sediment. The sulfamethoxazole accumulation to the sediment in our cosms is also lower when compared to a study by Pesce et al. (2021) where they contaminated the water with 5 μg/L sulfamethoxazole and measured maximum sulfamethoxazole concentrations in the sediment of 2.9 μg/kg.

3.2. Abiotic water condition, algal and bacterial responses

We found a significant decrease in turbidity after sulfamethoxazole exposure for all treatments (Fig. 2A, Table A.4). This decrease was found during the application period (NOEC of 0 μg/L) and turbidity was significantly lower until two weeks after the last application for the three highest concentrations (1.5, 15 and 150 μg/L). An increase in pH was observed for the 0.15 and 15 μg/L treatment during the recovery period (Fig. 2D). Oxygen concentration, electrical conductivity, nutrient concentrations and chlorophyll-a were not significantly affected by sulfamethoxazole for two or more consecutive sampling days (Fig. 2, A.1, A.2, Table A.4, A.5).

The PRC analysis did not show a significant overall treatment-related effect on the eukaryotic phytoplankton community (Monte Carlo test p-value = 0.54; Fig. 3). When performing a Monte Carlo Permutation test for the individual sampling days, a significant effect was found for the 15 μg/L compared to the control treatment at the final sampling day 114 (Table A.6). This effect was mainly caused by a decrease in the algae class Cryptophyceae and an increase in Chrysophyceae (Fig. 3 and A3). Also, we did not find overall effects of sulfamethoxazole on the bacterial community composition as indicated by the PRC analysis (Fig. 4, A4). Again, when performing Monte Carlo Permutation tests for the individual sampling days we did find a significant effect for the final

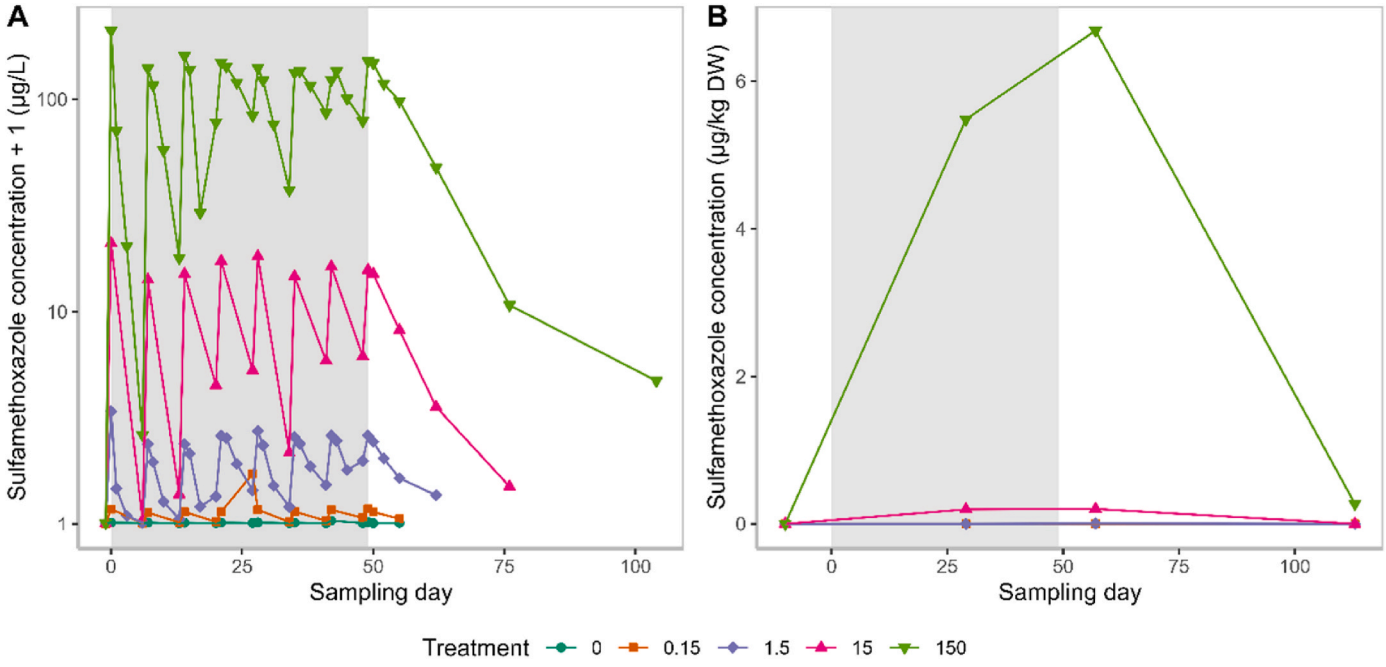

Fig. 1. Average sulfamethoxazole concentration measured in water (A) and in sediment (B) of the cosms over the course of the experiment. The grey-shaded areas in the graphs indicate the sulfamethoxazole application period and the treatments are based on the nominal sulfamethoxazole concentrations (μg/L).

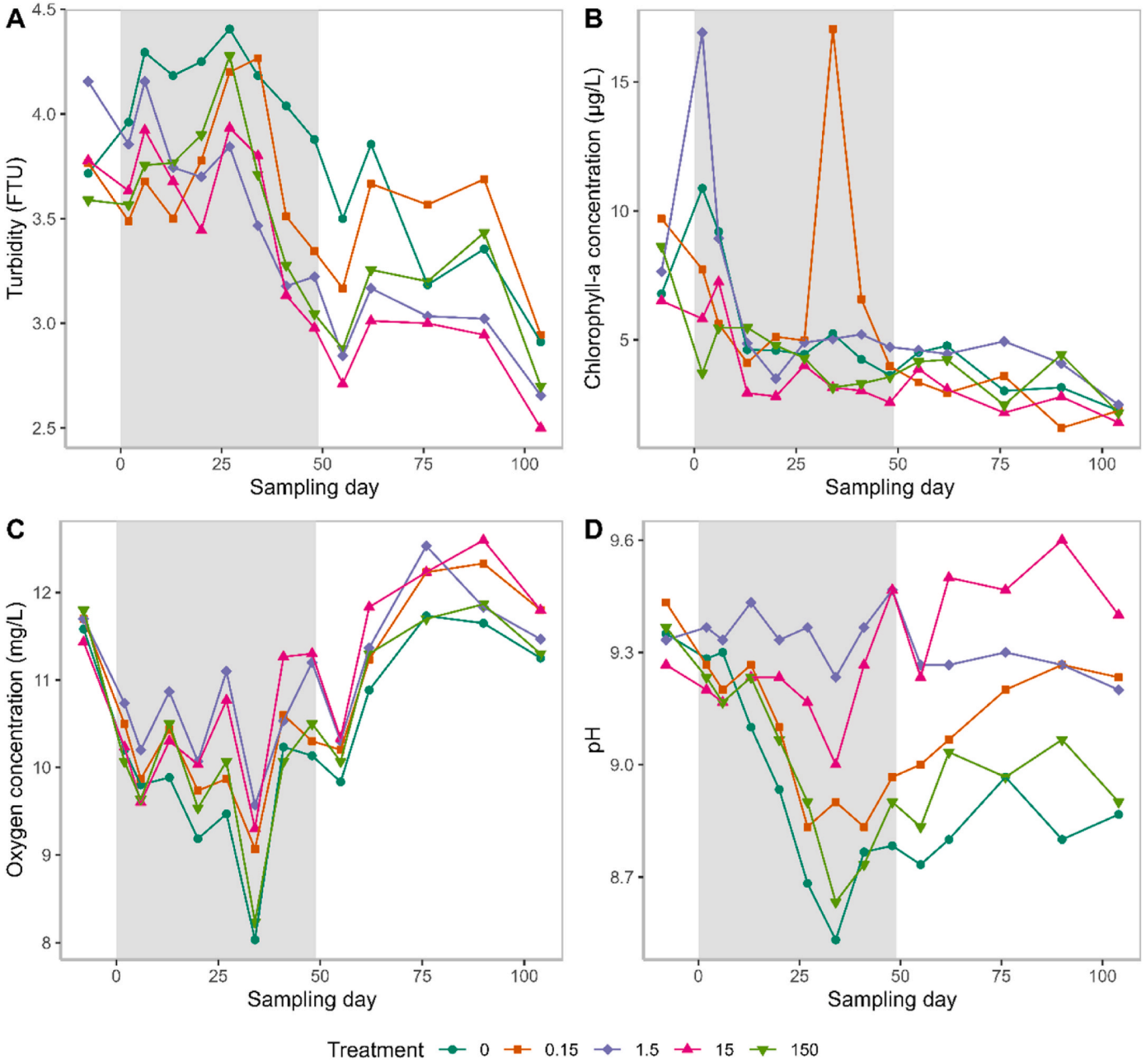

Fig. 2. Average (±SE) turbidity (A), chlorophyll-a concentration (B), oxygen concentration (C), and pH (D) measured in the outdoor mesocosm over the experimental period. The grey-shaded areas in the graphs indicate the sulfamethoxazole application period and the treatments are based on the nominal sulfamethoxazole concentrations (µg/L).

sampling day 114, this time for the bacterial community in the leaf samples of the highest treatment (150 µg/L; Table A.7), but no substantial changes in relative bacterial abundance were observed (Fig. A5D). It should be noted that eDNA metabarcoding yields semi-quantitative data, while quantitative data are needed to analyze effects of stressors on biological communities (Van den Brink and Ter Braak, 1998).

The observed lack of major shifts in bacterial community composition in response to sulfamethoxazole exposure was unexpected as antibiotics are designed to impact bacteria (Le Page et al., 2017). Also, previous experiments investigating bacterial communities reported impacts of sulfamethoxazole (Johansson et al., 2014; Patrolecco et al., 2018; Grenni et al., 2019; Kergoat et al., 2021). Specifically, impairment of biofilm bacterial functions was observed when exposed to sulfamethoxazole concentrations of 0.5 and 5 µg/L, though these functions recovered within four weeks (Kergoat et al., 2021). In the same study, the authors also found bacterial community structure to be affected by sulfamethoxazole, suggesting that sulfamethoxazole exposure acted as a selection pressure by selecting the most tolerant species being able to maintain bacterial functions (Kergoat et al., 2021). Another study found structural changes in river bacterial communities in response to sulfamethoxazole exposure (Patrolecco et al., 2018; Grenni et al., 2019), but in this case, a higher concentration was used (500 µg/L for 28 d) compared to our study. Finally, Johansson et al. (2014) found that sulfamethoxazole affected the marine periphytic bacterial carbon source metabolization (NOEC of 4 d is 38 µg/L), but this was not caused by major shifts in bacterial biodiversity and/or function.

Most parameters related to photosynthetic activity and primary production by the suspended algae community (chlorophyll-a, oxygen concentration and conductivity) were not affected by sulfamethoxazole

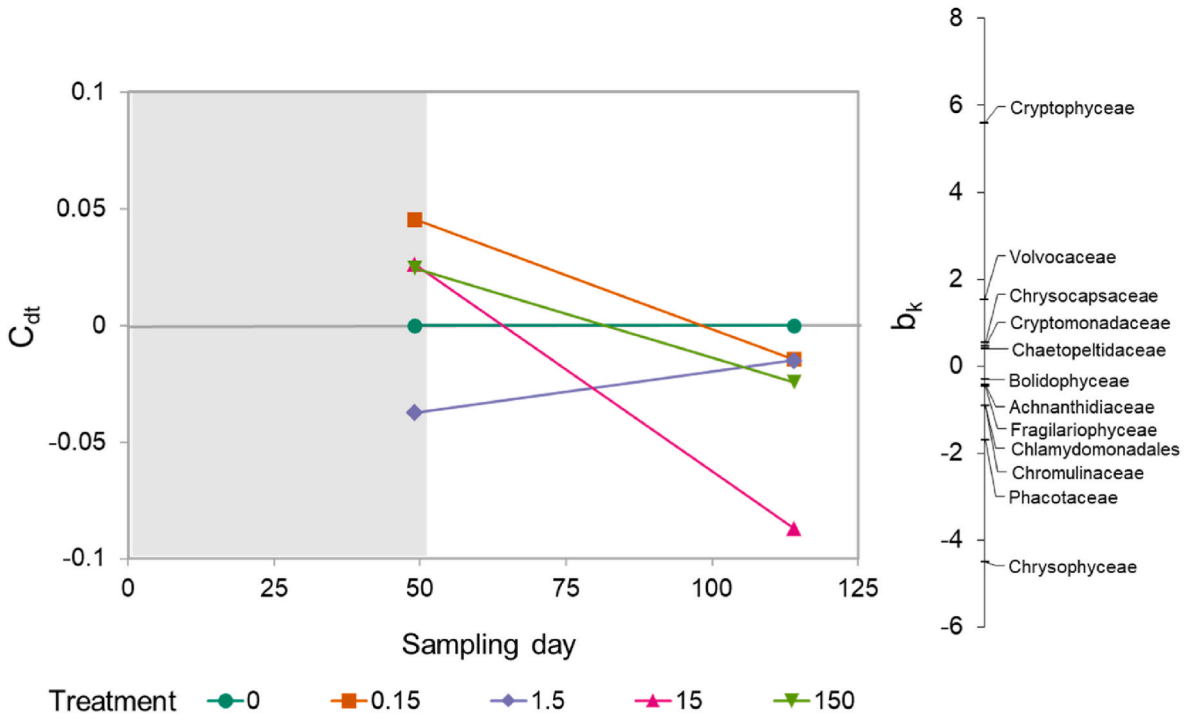

Fig. 3. Principal Response Curve showing the effect of sulfamethoxazole treatments (in μg/L) on the phytoplankton community structure (eDNA metabarcoding). Of all variances, 20% could be attributed to the sampling date; this is displayed on the horizontal axis. 19% of all variances could be attributed to treatment. Of this variance, 49% 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 0.3 and −0.3 are not shown. The Monte Carlo permutation test indicated that the displayed part of the variance explained by treatment in the diagram is not significant ($p = 0.42$). The grey-shaded area in the graph indicates the sulfamethoxazole application period.

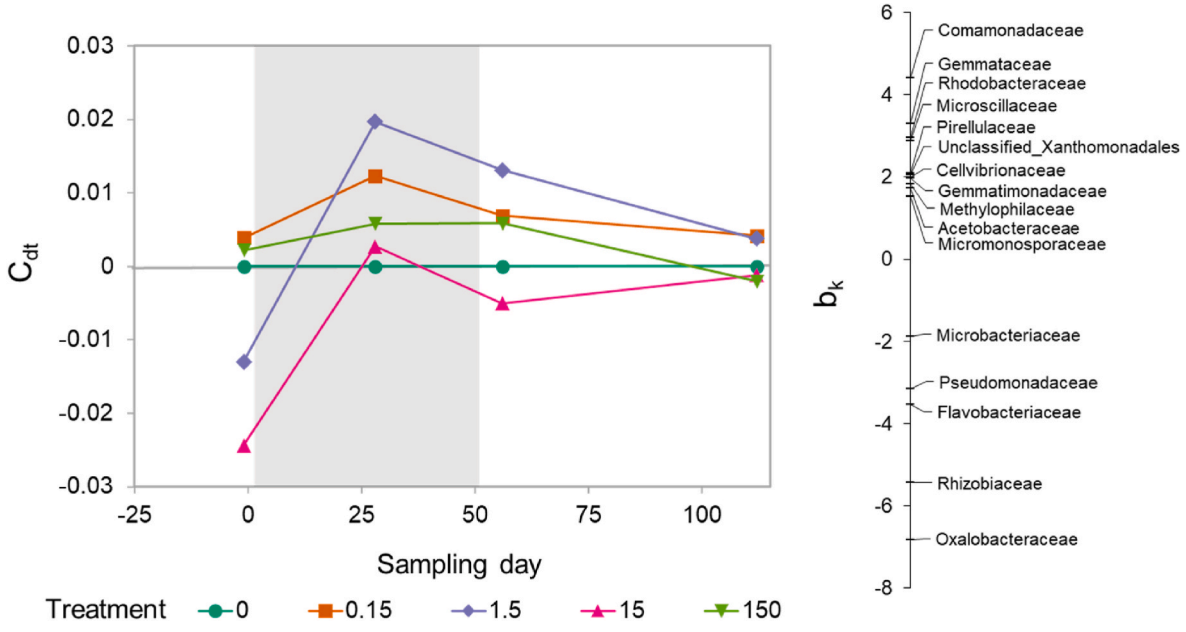

Fig. 4. Principal response curves (PRCs) showing the effect of sulfamethoxazole treatments (in μg/L) on the bacterial community structure in leaf samples. Of all variances, 54% could be attributed to the sampling date; this is displayed on the horizontal axis. 12% of the variation could be attributed to treatment and 28% of this variance 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. Bacterial families with a species weight between 1.5 and −1.5 are not shown. The Monte Carlo permutation test indicated that the displayed part of the variance explained by treatment in the diagram is not significant ($p = 0.21$). The grey-shaded area in the graph indicates the sulfamethoxazole application period.

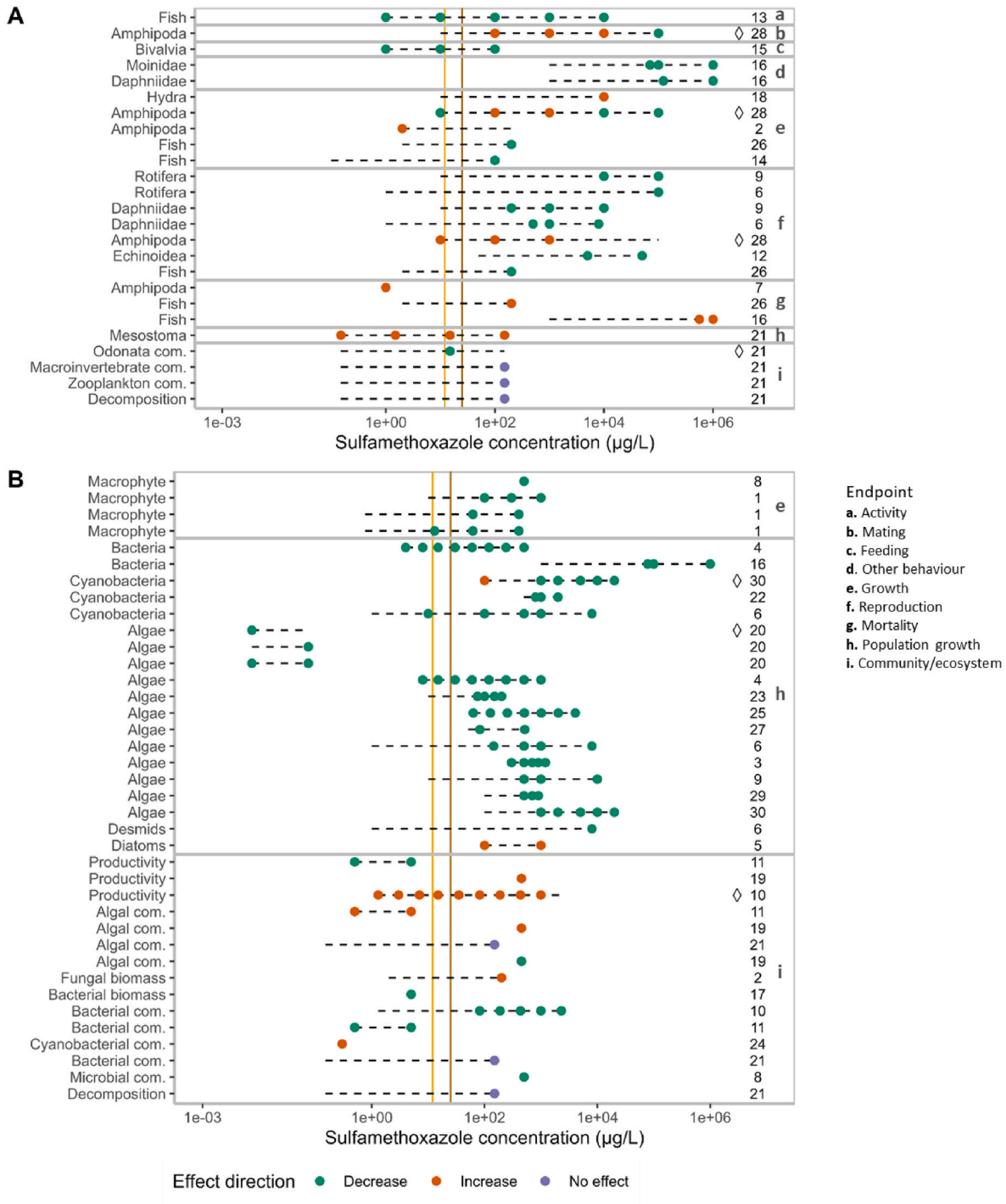

Fig. 5. Effects of different sulfamethoxazole concentrations on aquatic invertebrates and fish (A) and primary producers and microorganisms (B) from the literature and this paper. The dots indicate the effects of sulfamethoxazole for the corresponding taxa and endpoint. Green dots indicate a decrease of the measured endpoint, orange dots an increase and purple dots no effect (but only for data in this paper). The dashed lines show the concentration range that has been tested. The endpoints are categorized into 9 groups (a–i). References to the different studies are indicated by different numbers and details can be found in Table S7. The vertical lines show the maximum sulfamethoxazole concentration measured in surface water (yellow) and wastewater effluent (brown). ◊ indicate a nonmonotonic concentration-response relationship, and reference 21 are findings from the current experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Table A4). We also did not find clear effects of sulfamethoxazole on the phytoplankton community or cyanobacteria (Figs. 3 and 4, A.4). These results were contrary to what we expected as previous studies performing single species tests reported concentration-dependent algal growth inhibition for similar sulfamethoxazole concentrations as used in this study. Specifically, a LOEC for growth inhibition was observed for the green algae *Desmodesmus subspicatus* at 7.8 µg/L after 72 h (De Vasconcelos et al., 2017), for *Scenedesmus obliquus* at 63 µg/L after 24 h (Xu et al., 2022) and 75 µg/L after 11 d (Xiong et al., 2019), for *Raphidocelis subcapitata* at 82.4 µg/L (Yang et al., 2008) and for marine microalgae even at concentrations of 0.0075 and 0.75 µg/L after 3 weeks (Teixeira and Granek, 2017). Furthermore, a NOEC for growth

inhibition of 5.9 μg/L after 96 h was found for the cyanobacterium *Synechococcus leopolensis* (Ferrari et al., 2004).

Opposed to the mentioned single species tests, previous microcosm experiments found stimulatory effects rather than inhibiting effects of sulfamethoxazole on algae populations (Johansson et al., 2014; Kergoat et al., 2021; Duarte et al., 2023). Kergoat et al. (2021) found an increase in cyanobacteria, diatoms and green algae densities in biofilms exposed to 0.5 and 5 μg/L sulfamethoxazole for 14 d, and suggested that this stimulatory effect might be a consequence of a temporarily negatively impacted heterotrophic compartment. Also, Johansson et al. (2014) observed a general increase in periphytic algal biomass, which was found already at the lowest tested concentration of 1.3 μg/L. With increasing concentrations, the stimulation then decreased and returned to the control level at 760 μg/L (Johansson et al., 2014). Whereas Duarte et al. (2023) did not find effects on total phytoplankton community abundance, richness and diversity after 15 d exposure to 100, 500 and 1000 μg/L sulfamethoxazole, they found an increase in abundance for the algal group desmids. Interestingly, in our experiment, we also did find some indications for stimulatory effects of sulfamethoxazole on primary productivity, indicated by an increase in pH after exposure to 15 μg/L sulfamethoxazole during the recovery period (Fig. 2D). As we did not find impacts on chlorophyll-a concentrations in the water, a possible explanation for this enhanced primary productivity could be an increase in algae in periphytic biofilms, as has been observed by Johansson et al. (2014) and Kergoat et al. (2021). Periphyton biofilms grow on submerged surfaces that are abundant in mesocosms (i.e. walls) and therefore for future studies, it would be advisable to also include endpoints for periphyton, which are lacking in this study.

A possible explanation for the lack of effects found for bacteria and suspended algae in this study, in contrast to other studies, might be related to the high pH levels (above 8.5) measured in the water of our mesocosms. Sulfamethoxazole belongs to the sulfonamides, which are amphoteric but similar to weak acids (Rendal et al., 2011). Although we did not find studies about the effect of pH on the toxicity of sulfonamides on algae, a higher sensitivity to sulfamethoxazole at a lower compared to a higher pH was reported for *Daphnia magna* (Kim et al., 2010; Anskjær et al., 2013) and the bacterium *Pantoea agglomerans* (Rendal et al., 2011). In addition, Zarfl et al. (2008) showed, by using a mechanistic model for the transport of chemicals into cells, that no accumulation of any sulfonamide in bacterial cells at an extracellular pH of 8.5 occurred and accumulation increased with decreasing pH. Another cosm experiment with the antibiotic enrofloxacin, which is a weak acid, also unexpectedly did not find effects on the phytoplankton and periphyton community, and the authors hypothesized that this might indeed be caused by the high pH (8–10.7) measured in their outdoor microcosms (Rico et al., 2014).

Interestingly, we did find a treatment-related decrease in turbidity (Fig. 2, Table A4). Turbidity is primarily influenced by suspended particles, including microorganisms (e.g. bacteria, archaea, algae, viruses, fungi, protozoa), fragments of organisms and inorganic particles (Walch et al., 2022). As we did not find effects on suspended algae, a possible explanation for this decrease in turbidity might be a decline in other suspended microorganisms (such as bacteria, archaea, fungi or protozoa) in response to sulfamethoxazole. Even though we did not find changes in the bacterial community in the sediment, it is possible that bacterial biomass and abundance decreased in the water column, as sulfamethoxazole has a low affinity to the sediment and remains in the water (Fig. 1). Since we did not measure bacterial abundance in the water, we recommend to include this endpoint in future cosm experiments with antibiotics, as it might help to unravel the impacts on the bacterial community (Eckert et al., 2019). Furthermore, the antibiotic sulfamethoxazole is also used against protozoa and some fungal infections (Straub, 2016), next to Gram-positive and Gram-negative aerobic bacteria, thus, a decrease in fungi and/or protozoa might additionally be a possible explanation for the observed decreased turbidity found in this study, and assessing their biomass would also be advisable for future studies.

3.3. Macrophyte responses

We found no significant effects of sulfamethoxazole treatments on macrophyte coverage throughout the experiment or on the macrophyte biomass measured at the end of the experiment (Table A.4, Fig. A.6). Only two previous studies have been performed with sulfamethoxazole and macrophytes (Brain et al., 2004; Grenni et al., 2019). Both studies found reduced growth of the floating macrophyte *Lemna* sp. with a LOEC of 100 μg/L after 7 d (*Lemna gibba*; Brain et al. (2004)) and 500 μg/L after 28 d exposure (*Lemna minor*; Grenni et al. (2019)). In our experiment, we did not include floating macrophytes and did not observe effects on the submerged macrophytes *Elodea* sp. and *Myriophyllum* sp. However, for the 1.5 and 150 μg/L treatment a lower, but not significantly different, biomass was found (Fig. A6). This could indicate some inhibitory impacts of sulfamethoxazole, but as we measured macrophyte biomass only at the end of the experiment, after a two months recovery period, it is possible that we missed those effects as macrophyte biomass might have recovered by then.

3.4. Invertebrate responses

The most abundant macroinvertebrate order was Diptera during the first two months of the experiment (Fig. A.7 A-D), while this changed to Ephemeroptera in the last two months (Fig. A7 E-F). Throughout the experimental period, the total abundance increased while the alpha diversity (both richness and Shannon index) decreased (Fig. A.8). We did not find the effects of sulfamethoxazole on macroinvertebrate total abundance and diversity (Table 1). We also did not find a significant effect on the composition of the macroinvertebrate community (Fig. A.9 and Table A.8) or the zooplankton community (Fig A.10; Table A.6) using the PRC method. According to the species weight (b_K) of the macroinvertebrate community, Zygoptera showed the largest response to the sulfamethoxazole treatment (Fig. A.9). For a higher taxonomic resolution, we additionally assessed the Odonata community by using eDNA data. The PRC indicated a significant treatment-related effect for the Odonata community (Fig. A.11), and Monte Carlo Permutation tests for each sampling date and concentration showed a significant difference between the control and the 15 μg/L treatment at days 49 and 114 (Table A6). Based on the species weight (b_K), *Enallagma* sp. of the suborder Zygoptera showed the most negative responses of the Odonata community to the sulfamethoxazole exposure. Furthermore, the univariate analysis indicated a significant increase in Mesostoma flatworms in the 1.5 and 15 μg/L sulfamethoxazole treatments (Table 1). We also found significant effects of sulfamethoxazole on some other macroinvertebrate taxa, however, these effects were observed for isolated sampling days and/or the abundance of these taxa was very low (Table 1).

The majority of the previously performed invertebrate single-species test reported effects at higher sulfamethoxazole concentrations than those tested in this experiment. For Daphniidae effects of sulfamethoxazole were found at concentrations ranging from 210 μg/L for *Ceriodaphnia dubia* up to 123,000 μg/L for *D. magna* (Ferrari et al., 2004; Flaherty and Dodson, 2005; Isidori et al., 2005; Park and Choi, 2008), while the rotifer *B. calyciflorus* seems even less sensitive with an EC50 for reproduction of 9630 μg/L (Isidori et al., 2005) and higher (>25,000 μg/L; Ferrari et al. (2004)). In addition, a LOEC of 10,000 μg/L was found for Hydra morphology after 96 h exposure (Quinn et al., 2008) and a decrease in reproduction for the sea urchin *Arbacia lixula* was found at 5000 μg/L after 4 h exposure to sulfamethoxazole (Lazzara et al., 2022).

However, also a few cases reported impacts on invertebrates at lower sulfamethoxazole concentrations. Liu et al. (2022) found suppression in the feeding rate of the bivalve *Corbicula fluminea* with a LOEC of 1 μg/L after 7 and 28 d exposure to sulfamethoxazole. Garcia-Galan et al.

Table 1
Calculated NOECs by Dunnet's test for the macroinvertebrate taxa over the experimental period. NOECs are in μg/L, arrows indicate a treatment-related increase (↑) or decrease (↓) and >150 indicates a NOEC higher than 150 μg/L.

		Sampling day					
Class/Order/Taxa		−7	7	21	49	77	105
Crustacea							
	Asellidae juveniles	>150	>150	>150	>150	0 ↑*	>150
	Asellus aquaticus	>150	>150	>150	>150	>150	>150
	Proasellus sp.	>150	>150	>150*	>150	>150	>150
	Gammarus pulex	>150	>150	>150	>150	>150	>150
Insecta							
	Anisoptera	0 ↓	>150*	>150	>150	>150	>150
	Caenis sp.	>150*	>150	>150	1.5 ↓	>150	>150
	Ceratopogonidae	>150*	>150*	>150*	>150*	>150*	>150*
	Chaoborus sp.	>150	0.15 ↑	>150	>150	>150	>150
	Chironomini	>150	>150	>150	>150	>150	>150
	Chironomus	>150	>150	>150	>150*	–	–
	Cloeon dipterum	>150	>150	>150	>150	>150	>150
	Corixa sp.	>150*	>150*	>150	>150*	>150*	>150*
	Dytiscidae	>150	>150	>150	>150*	>150*	>150*
	Gerris sp.	>150*	>150*	>150*	>150*	>150*	–
	Haliplidae	>150*	>150*	>150*	>150*	>150*	>150*
	Hydrophilidae	>150*	>150*	>150*	>150*	>150*	>150*
	Notonecta sp.	>150*	>150*	>150*	>150*	>150*	0 ↓*
	Orthocladiinae	>150*	>150*	>150*	>150*	>150*	>150*
	Parapoynx stratiotata	–	>150*	>150	>150*	>150*	>150*
	Plea sp.	>150*	>150	>150	>150	>150	>150
	Sigara sp.	>150*	–	>150*	>150*	>150*	>150*
	Tanypodinae	>150	>150	>150	>150	>150	>150
	Tanytarsini	>150	>150	>150	>150	>150	>150*
	Zygoptera	>150*	>150	>150	0 ↓	>150	>150
Platyhelminthes/Annelida							
	Alboglossiphonia sp.	>150*	>150*	>150*	>150*	>150*	>150
	Dugesia sp.	>150*	>150*	1.5 ↑*	>150*	>150*	>150*
	Erpobdella sp.	>150*	>150*	>150	>150	>150	>150*
	Glossiphonia sp.	>150*	>150*	>150*	>150*	>150*	>150*
	Mesostoma	>150	>150	>150	**0 ↑**	**0.15 ↑**	>150
	Polycelis nigra/tenuis	>150*	>150*	>150*	>150*	>150*	>150*
Mollusca							
	Gyraulus albus	>150	>150*	>150*	>150*	>150*	>150*
	Gyraulus crista	>150*	>150*	>150*	>150*	>150*	–
	Lymnaea sp.	>150	>150	>150	>150	>150	>150
	Planorbarius corneus	0.15 ↑*	>150*	>150*	>150*	>150*	>150*
	Planorbis sp.	>150	>150*	>150	>150*	>150*	0 ↓*
	Radix sp.	>150	0 ↓	>150	>150	>150*	>150*
	Sphaeridae	>150	>150	>150	>150	>150	>150
Other							
	Hydracarina	>150*	>150*	>150*	>150*	>150*	>150
Total abundance		>150	>150	>150	>150	>150	>150
Richness		>150	>150	>150	>150	>150	>150
Shannon index		>150	>150	>150	>150	>150	>150

-: taxon absent on that sampling day; *: mean abundance lower than 3 for that taxon at the indicated sampling day; Note: calculated NOECs on days −7 refer to deviations before the sulfamethoxazole application, and should not be interpreted as treatment-related effects.

(2017) found increased mortality of the amphipod *Gammarus fossarum* after 14 d exposure to 1 μg/L. However, this was not observed by Bundschuh et al. (2017) as they found an increase in growth after 24 d exposure to 2 μg/L sulfamethoxazole (in a mixture with other antibiotics). An increase in growth at low sulfamethoxazole concentration was also observed for the amphipod *Hyalella azteca* (at 0.1 and 1 μg/L after 35 d) but growth rates decreased at higher concentrations (10 and 100 μg/L) (Yu et al., 2019). In addition, an increase in reproduction for *H. azteca* was found at 0.01, 0.1, and 1 μg/L, while at higher concentrations reproduction did not differ from control. Contrary to this, in the current study we did not find effects of sulfamethoxazole on bivalve and amphipod populations but observed some effects on the Mesostoma flatworm population and Odonata community. All in all, the effects of sulfamethoxazole on invertebrates seem context-dependent, and different experimental conditions (such as differences in pH) might explain the differences between studies.

3.5. Decomposition

Sulfamethoxazole did not affect macroinvertebrate litter decomposition (Fig. A.12, Table A4). This is in line with the study of Bundschuh et al. (2017), where no effects were found on leaf consumption by *G. fossarum* after exposure to 2 and 200 μg/L antibiotic mixture, including sulfamethoxazole. However, when *G. fossarum* was given the choice between leaves conditioned in a control medium or antibiotic mixture (200 μg/L), leaf consumption was higher for antibiotic-conditioned leaves (Bundschuh et al., 2009). Also, we did not find effects on microbial decomposition for all tested sulfamethoxazole treatments (Fig. A12 Table A4). This is in agreement with the study of Paumelle et al. (2021) where they did not find effects on the leaf-litter decomposition processes after exposure to sulfamethoxazole (5 μg/L) for 16 d.

3.6. Review on the impacts of the antibiotic sulfamethoxazole on aquatic ecosystems

In this section, we provide an overview of the impacts of sulfamethoxazole on aquatic organisms and ecosystems reported in the available literature in combination with the data of the current mesocosm experiment (Table A.9 and Fig. 5). This to evaluate the agreement or disagreement in effect thresholds between different experiments. This information is vital for the ecological risk assessment of sulfamethoxazole for freshwater ecosystems.

The included studies in Fig. 5 observed effects of sulfamethoxazole at the whole-organismal level on fish and invertebrate behavior (including activity, mating and feeding behavior), growth and reproduction, with the majority of effects being observed at sulfamethoxazole concentrations of 100 μg/L or higher (Fig. 5A). More specifically, for fish a decrease in swimming activity, feeding, and eventually survival was observed at very high concentrations (Table A9, Fig. 5). Regarding invertebrates, zooplankton taxa seem less affected by sulfamethoxazole compared to Bivalvia and Amphipoda. A decrease in the feeding of the bivalve *Corbicula fluminea* was observed after sulfamethoxazole exposure, and the amphipods *Hyalella azteca* and *Gammarus fossarum* showed a decrease in growth rate (Table A9). The abovementioned studies are all single-species experiments, which are useful for giving toxicity information, however, they only provide limited information about the expected impacts of sulfamethoxazole in complex natural systems. However, besides the current experiment, we did not find other studies at the population and community-level, investigating the effects of sulfamethoxazole on invertebrates, or fish.

For algae, bacteria and other microorganisms, the effects of sulfamethoxazole have been assessed on population growth, as well as on community structure and functioning. Whereas most single species tests showed a decrease in the population growth of green algae and cyanobacteria, two studies reported the opposite and showed an increase in cyanobacteria population growth at low concentrations (Fig. 5B). Cosm studies reported effects of sulfamethoxazole on microbial community structure, diversity and productivity, but these effects of sulfamethoxazole were not clearly found in the current experiment. That we did not find clear effects that might be caused by differences in experimental conditions and design as explained earlier. Whereas studies reported a decrease in bacterial diversity, algal diversity and bacterial community abundances after sulfamethoxazole exposure, fungal and algal community abundances, as well as productivity, seemed to increase (Fig. 5B). It would be particularly interesting to investigate whether the stimulatory effects found for (attached) algae are direct effects or indirect effects caused by changes in bacterial species composition and/or abundances.

Of the included studies in Fig. 5, also a few nonmonotonic concentration-response relationships have been observed after exposure to sulfamethoxazole (seven out of 63; Fig. 5). Specifically, an inverse U-shaped concentration-response curve for growth of the amphipod *Hyalella azteca* was observed by Yu et al. (2019). They found a decrease in growth at low (10 μg/L) and high (10,000 & 100,000 μg/L) sulfamethoxazole concentrations and an increase at intermediate (100 and 1000 μg/L) concentrations. Next to that, Johansson et al. (2014) found the maximum stimulatory effect on the productivity of the biofilm community to be at the lowest tested sulfamethoxazole concentration (1.3 μg/L), whereas this effect decreased with increasing concentration and disappeared at the highest concentration (2300 μg/L). In addition, Zhou et al. (2021) found low-dose stimulation (100 μg/L) and high-dose inhibition (100-20,000 μg/L) on population growth of the cyanobacterium *Microcystis aeruginosa*. These low-dose stimulatory effects could disturb ecosystems by enhancing the risks of algal blooms. Lastly, in the current study we observed an increase in Mesostoma flatworm abundances at low concentrations (0.15 and 1.5 μg/L) and changes in Odonata community composition at an intermediate concentration (15 μg/L), whereas no effects were observed for the highest concentration (150 μg/L). Overall, the observed nonmonotonic responses report a stimulating effect at low and/or intermediate concentrations, whereas high concentrations cause inhibitory or no effects. However, mechanisms behind these stimulatory effects of sulfamethoxazole are currently still unknown.

Of the studies that included environmentally relevant concentrations of sulfamethoxazole (see introduction), about half of the measured endpoints (21 out of 45) showed effects at these concentrations, whereas for the other half effects at higher concentrations were observed (Fig. 5, Table A9). At environmentally relevant concentrations, algae and bacteria seem to be the most susceptible group of species based on the available literature (Table A9), although we did not find major effects on suspended primary producers in the current experiment. In addition, also effects on invertebrate feeding, growth and reproduction have been found at environmentally relevant concentrations, as well as on fish behavior (Fig. 5). At the community and ecosystem-level, both increase and decrease in productivity have been reported as well as changes in bacterial and algal community composition at an environmentally relevant concentration (Fig. 5).

All in all, while some impacts of sulfamethoxazole on aquatic ecosystems were identified based on the available literature (mostly for algae and bacteria; Fig. 5), we also revealed inconsistent results between studies using comparable concentrations. Whereas single species tests observed inhibitory effects of sulfamethoxazole on cyanobacteria population growth (e.g. Ferrari et al. (2004); De Vasconcelos et al. (2017)), cosm experiments recorded stimulatory effects (e.g. Johansson et al. (2014); Kergoat et al. (2021); Duarte et al. (2023), current study). This could indicate that the impacts of sulfamethoxazole are context dependent. For example, it has been shown that sulfamethoxazole can be degraded by ultraviolet light (Fu et al., 2017; Oliveira et al., 2019) and that pH can influence the sensitivity toward this antibiotic (Kim et al., 2010; Rendal et al., 2011; Anskjær et al., 2013). Additionally, the majority of studies assessed the impacts of sulfamethoxazole on single species, or a specific ecosystem component (e.g. biofilm and/or microbial community). To our knowledge, besides this experiment, studies covering the impacts of sulfamethoxazole on both microbial and invertebrate communities are still lacking.

4. Conclusions

We expected that the antibiotic sulfamethoxazole would impact algal and bacterial freshwater communities and subsequent ecosystem responses, but observed only minor structural changes after exposure to different concentrations of sulfamethoxazole (0.15, 1.5, 15 and 150 μg/L). However, our study only addressed changes in bacterial community composition in sediment and leaves and did not focus on bacterial abundances and/or biomass in the water column. In addition, we focused on a single antibiotic, whereas in surface waters a whole universe of pharmaceutical compounds can be detected with complex interactive effects (Sumpter, 2009; Arnold et al., 2013; Mezzelani et al., 2023). With the vast numbers of pharmaceuticals found in aquatic ecosystems and the large number of possible pharmaceutical combinations, more research is required on the interactive effects of these combinations on the environment. Hence, the next step would be to extend the experiments to examine the effects of multiple antibiotics or pharmaceuticals on ecological endpoints to fully understand the potential ecological threats that the combination of antibiotics and other stressors poses.

CRediT authorship contribution statement

Lara M. Schuijt: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, Visualization. **Chantal K.E. van Drimmelen:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Laura L. Buijse:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Jasper van Smeden:**

Conceptualization, Investigation, Methodology, Writing – review & editing. **Dailing Wu:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Marie-Claire Boerwinkel:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Dick J.M. Belgers:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Arrienne M. Matser:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Ivo Roessink:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Kevin K. Beentjes:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Krijn B. Trimbos:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Hauke Smidt:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **Paul J. Van den Brink:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Supporting information

Supporting information to this article can be found online in the accompanying word file.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.123199.

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