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Screening for ecotoxicological effects of antiepileptic drugs in biologically treated waste water originating from an epilepsy ward by *Danio rerio* embryos

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Abstract

Background: Pharmaceuticals, like antiepileptic drugs, are found regularly in surface waters, and consequently, advanced waste water treatment technologies are discussed for substance elimination. Because antiepileptic drugs have shown to transform to more toxic substances, their behavior in these treatment processes and resulting effects on ecotoxicity should be investigated. To validate if waste water from an epilepsy ward of a neurological hospital is appropriate for these investigations, it was treated with a membrane bioreactor (MBR), analyzed for antiepileptic drugs and screened for ecotoxicological effects with *Danio rerio* embryos. Further, the behavior of antiepileptic drugs in MBR treatment was estimated.

Results: Treatment of raw hospital waste water by the pilot scale MBR was successful regarding the low dissolved organic carbon concentration in the effluent and allowed ecotoxicological testing with *D. rerio*. According to the estimated behavior, partial elimination of 10-hydroxy-10,11-dihydrocarbamazepine (10-OH carbamazepine) and rufinamide and some release of lamotrigine, oxcarbazepine and, possibly, primidone occurred. The other investigated substances did not considerably change concentrations due to treatment. The highest concentrated substances found were 10-OH carbamazepine, lamotrigine, and oxcarbazepine. The complex mixture of the treated waste water had no effect on *D. rerio* morphology and did not change its primary and secondary motor neurons (indicator for developmental neurotoxicity). Oxcarbazepine did not show morphological effects on *D. rerio* at 8.7 mg L⁻¹.

Conclusions: Biological treatment was not sufficient to significantly eliminate the load of antiepileptic drugs investigated. No effects on *D. rerio* embryos were observed. Biologically treated waste water, originating from an epilepsy ward, is appropriate for the investigation of the fate of antiepileptic drugs in advanced treatment processes.

Keywords: Oxcarbazepine; MBR; Membrane bioreactor; Hospital; Fish; Motor neurons; LC-TOF-MS

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Background

The first findings of pharmaceuticals, an important group of anthropogenic compounds, in the environment occurred in the 1970s (e.g., by Garrison et al. [1] and Hignite et al. [2]). Since then, pharmacologically active compounds were reported in numerous studies investigating rivers and lakes, as well as ground and drinking water.

Pharmaceuticals are excreted after administration in metabolized and unmetabolized forms, and subsequently reach waste water treatment plants (WWTPs) via sewer networks. Many pharmaceuticals are not sufficiently eliminated in WWTPs; thus, they reach receiving waters. Exemplary, concentrations of pharmaceuticals found in WWTP effluents and rivers are compiled in Table 1.

In order to assess the possible effects of substances on organisms, different ecotoxicological test systems are used; test systems for aquatic organisms include bacteria, algae, crustacean, and fish. Effect concentrations below 1,000 $\mu\text{g L}^{-1}$ for pharmaceuticals found in WWTP effluents and rivers (Table 1) are given in Table 2. Effect concentrations found in ecotoxicological tests are in the range of environmental concentrations for the substance groups such as lipid regulators (bezafibrate), antiphlogistics (diclofenac), betablockers (metoprolol), and antiepileptic drugs (carbamazepine).

Due to numerous data on pharmacologically active compounds in the environment and the rising concern about possible chronic effects on aquatic organisms, treatment processes for elimination of these compounds were investigated. To improve elimination rates in WWTPs, not only the influence of sludge age and dosing of activated charcoal but also advanced treatment processes like ozonation, chlorination, UV-radiation, and combinations such as ozone/UV and H_2O_2 /UV were investigated and discussed (e.g., [18-20]). Because oxidation processes can lead to the formation of toxic degradation products, possible effects of advanced treatment processes were investigated (e.g., by [21-23]).

The formation of toxic degradation products in advanced treatment processes was shown for the antiepileptic drugs carbamazepine and oxcarbazepine (e.g., by [24-27]). In [26], the authors found an elevation of acute toxicity on *Daphnia magna* due to UV treatment of oxcarbazepine, as they presumed, due to the formation of acridine. In [27], the authors found similar results for UV treatment of carbamazepine.

The prevalence of epilepsy is estimated at 0.91%, and antiepileptic drugs were used by 634,566 patients in Germany in 2009 according to [28]. Since most epilepsy patients are in ambulant treatment, antiepileptic drugs can be detected in most, if not all, WWTP effluents, though data do not exist for all antiepileptic drugs. Hence, chemical transformations of antiepileptic drugs, which will occur if advanced treatment processes are

Table 1 Maximum concentrations of pharmaceuticals in municipal WWTP effluents and rivers

Analyte	Concentration at WWTP effluents ($\mu\text{g L}^{-1}$)	Concentration in rivers and streams ($\mu\text{g L}^{-1}$)
Lipid regulators		
Bezafibrate	4.6 [3]	3.1 [3]
Gemfibrozil	1.5 [3]	0.51 [3]
Metabolites of lipid regulators		
Clofibrac acid	1.6 [3]	0.55 [3]
Fenofibrac acid	1.2 [3]	0.28 [3]
Antiphlogistics		
Diclofenac	2.1 [3]	1.2 [3]
	1.76 [4]	
Acetylsalicylic acid	12.1 [4]	
Ibuprofen	3.4 [3]	0.53 [3]
Acetaminophen	6.0 [3]	n.d. [3]
Mefenamic acid	1.36 [4]	
Paracetamol	24.5 [5]	1.5 [5]
Tramadol	97.6 [5]	5.97 [5]
Metabolites of antiphlogistics		
Salicylic acid	0.14 [3]	4.1 [3]
Gentisic acid	0.59 [3]	1.2 [3]
Betablockers		
Metoprolol	2.2 [3]	2.2 [3]
Bisoprolol	0.37 [3]	2.9 [3]
Stimulant		
Caffeine	3.18 [4]	
Antibiotics		
Lincomycin	45.7 [4]	
Sulfathiazole	2.77 [4]	
Trimethoprim	2.0 [4]	
Ciprofloxacin	2.05 [4]	
H_2 receptor antagonist		
Cimetidine	9.4 [5]	0.2 [5]
Antiepileptic drugs		
Gabapentin	42.6 [5]	1.9 [5]
Carbamazepine	6.3 [3]	1.1 [3]
	21.6 [4]	0.3 [5]
	4.6 [5]	

n.d., no data; WWTP, waste water treatment plant. Data were taken from the reviews and screenings [3,6-8]. Empty fields: concentrations are not given in [3,6-8].

used, will probably influence the toxicity of waste water. To assess this possible influence, an investigation and treatment of waste water loaded with antiepileptic drugs and corresponding metabolites should be done. The

Table 2 Toxicological data for substances of Table 1 with effect concentrations below 1,000 µg L⁻¹

Test	Species	Endpoint (time)	Endpoint	Effect concentration (µg L ⁻¹)
Bezafibrate				
Mussels	<i>D. polymorpha</i>	LOEC (7 days)	piGST transcript expression	0.236 [9]
Diclofenac				
Fish (adult)	<i>O. mykiss</i>	LOEC (28 days)	Cytopathology of the liver, kidney, and gills	1 [10]
Metoprolol				
Fish (adult)	<i>O. mykiss</i>	LOEC (28 days)	Cytopathology of the liver	1 [11]
Carbamazepine				
Rotokit (rotifers)	<i>B. calyciflorus</i>	LOEC (48 h)	Reproduction	754 [12]
Phytoplankton	<i>S. obliquus</i>	EC ₅₀ (30 days)	Chlorophyll a synthesis	800 [13]
Crustaceans				
	<i>G. pulex</i>	LOEC (1.5 h)	Behavior	0.01 (ns) [14]
	<i>C. dubia</i>	LOEC (7 days)	Reproduction	100 [12]
Mussels	<i>M. galloprovincialis</i>	LOEC (7 days)	Membrane stability	0.1 [15]
Fish (adult)				
	<i>D. rerio</i>	LOEC (6 weeks)	Egg production, oocytes, and kidney	0.5 [16]
	<i>D. rerio</i>	LOEC (3, 7, and 15 days)	DNA integrity	0.31 [17]
	<i>C. carpio</i>	LOEC (28 days)	Cytopathology of the kidney	1 [11]

LOEC lowest observed effect concentration, ns not significant.

influence of other pharmaceuticals on this waste water and its dilution should be as low as possible.

For an effective ozonation, a low concentration of dissolved organic carbon (DOC) is important [29], and this is most likely true for other oxidative treatment processes. Therefore, the waste water loaded with antiepileptic drugs should be treated biologically prior to advanced treatment steps. A membrane bioreactor (MBR) is appropriate to eliminate DOC and additionally, due to filtration, emits only a small amount of suspended particles (which interfere with irradiation and oxidative processes).

In the study presented here, a hospital ward, specialized on epilepsy treatment, was chosen as source for waste water highly loaded with antiepileptic drugs. The waste water was treated biologically, analyzed for antiepileptic drugs, and tested for ecotoxicological effects. A standard test system for the assessment of ecotoxicity of treated waste water is the zebrafish (*Danio rerio*) embryo test [30,31]; additional endpoints are given in [32] and other publications. The central nervous system (CNS) of zebrafish has similarities with the human CNS, and some mammalian neurotransmitter systems are present [33]; the site of action of antiepileptic drugs is the human CNS.

Although the concentrations of antiepileptic drugs to be expected in the hospital waste water would not reach the effect concentrations for antiepileptic drugs on *D. rerio* (Table 3), possible effects of this complex mixture should be investigated. On the one hand, ecotoxicological data exist only for carbamazepine and phenytoin; on the other hand, according to [34], substances with

similar targets can add their effects to each other and cause effects below the single substance effect concentrations. Due to the expected low dilution, possible additive effects, the incomplete data in the existing literature, and the similarity of CNS, toxic effects on *D. rerio* embryos seemed possible and worth investigating.

Because antiepileptic drugs take effect on the neuronal system and show some neurotoxicological effects (e.g., [37]), the biologically treated waste water was also tested with a newly introduced test for developmental neurotoxicity with *D. rerio* according to [38], where the motor neurons are stained.

To validate, if the chosen waste water is appropriate for further investigation of advanced treatment processes, the following steps were performed:

- Waste water loaded with antiepileptic drugs was treated with a MBR.
- Concentrations of antiepileptic drugs were determined in order to assess potential variations due to MBR treatment.
- Ecotoxicological tests with *D. rerio* were applied to MBR-treated waste water.
- In order to assess the developmental neurotoxicological effects in *D. rerio*, motor neurons were stained.

To our knowledge, this is the first work regarding biologically treated waste water of a ward in a neurological hospital specialized on epilepsy treatment which is screened for antiepileptic drugs and tested with *D. rerio* embryos.

Table 3 Toxicological data for antiepileptic drugs, investigated in this study, regarding *D. rerio* embryo test

Test	Species	Endpoint (time)	Endpoint	Effect concentration ($\mu\text{g L}^{-1}$)
Carbamazepine				
Fish (early life stage)	<i>D. rerio</i>	LOEC (10 days)	Embryos and larvae mortality	50,000 [12]
		EC ₅₀ (72 h)	Growth retardation	86,500 [35]
		EC ₂₀ (72 h)	Tail malformation	17,500 [36]
		EC ₅₀ (72 h)	Tail malformation	52,500 [36]
Phenytoin				
Fish (early life stage)	<i>D. rerio</i>	EC ₂₀ (72 h)	Tail malformation	10,000 [36]
		EC ₅₀ (72 h)	Tail malformation	97,400 [36]

Results and discussion

Characterization of the raw waste water

The results for the chemical oxygen demand (COD), given in Additional file 1: Table a1, show a high heterogeneity of the sampled raw waste water. This may be explained by the frequency of toilet flushes and occurrence of feces.

Biological treatment

Since the purpose of the biological treatment is the reduction of readily biodegradable substances and DOC concentration, no process monitoring was performed. The weighted mean of the COD was 222 mg L⁻¹. The mean dried matter content of 2.1 g L⁻¹, the reactor volume of 107 L, and a load of 315 L raw waste water in 10 days resulted in a biochemical oxygen demand (BOD₅) load of 16 mg BOD₅ (g TS)⁻¹ day⁻¹ (assuming a COD/BOD₅ ratio of 2:1). The membrane flux was approximately 21 L (h bar m²)⁻¹. The detention period of theoretically 80 h was much higher than the typical 8 h of a municipal waste water treatment plant.

Characterization of the biologically treated waste water

The total amount of biologically treated waste water was approximately 320 L. The modeled fractions originating from raw waste water of the hospital was 59%. The biologically treated waste water, stored at 4°C, showed a concentration of dissolved organic carbon of 5 to 6 mg L⁻¹ (quadruple determination), a pH of 7.3, and an electrical conductivity of 0.71 ms cm⁻¹ (single determinations). The results of the multi-elemental analysis (single determination), given in Additional file 1: Table a2, show a low Cu load, which is important for ecotoxicological test systems.

Estimation of the behavior of antiepileptic drugs during MBR treatment

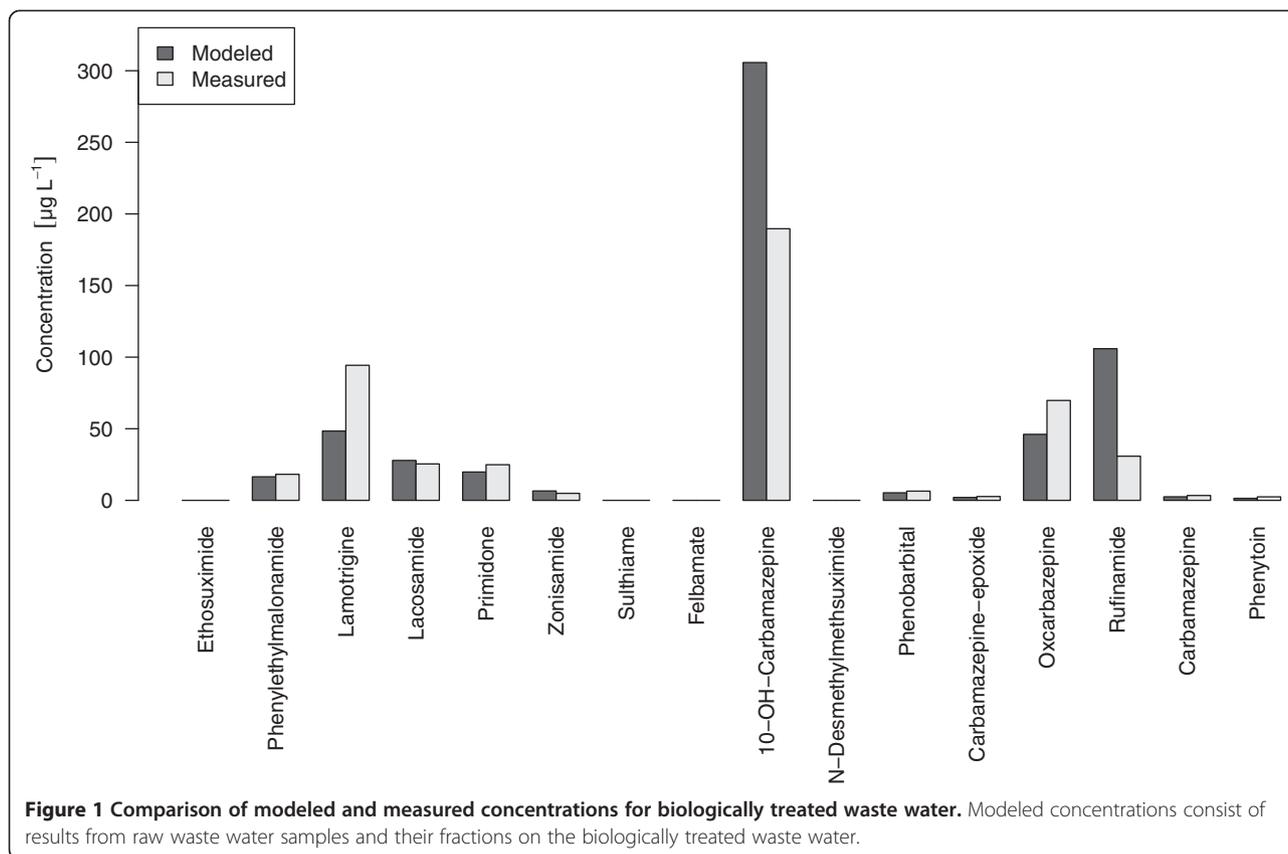
Based on the measured concentrations of antiepileptic drugs in the raw waste water samples and their modeled fractions of the biologically treated waste water, the concentrations to be expected in the mixed sample after MBR passage without transformation were calculated.

These modeled concentrations were compared to the concentrations measured in the biologically treated waste water in Figure 1. The underlying results of raw waste water samples, given in Additional file 1: Figure a1, show high variations of antiepileptic drug concentrations over time.

For interpretation of Figure 1, it has to be taken into account that the measured concentrations are based on single determinations. Additionally, raw waste water contains much higher concentrations of organic compounds, which could overload the solid phase extraction (SPE) cartridges and cause matrix effects in the mass spectrometer. The internal standard 10,11-dihydrocarbamazepine was used to account for cartridge capacity. Because the aim of the analysis was to perform an estimation on the behavior and the expected analyte concentrations were high, the use of surrogate standards for elimination of possible matrix effects was omitted.

According to the estimation, partial elimination of 10-hydroxy-10,11-dihydrocarbamazepine (10-OH carbamazepine) and rufinamide and some release of lamotrigine, oxcarbazepine and, possibly, primidone occurred. The other investigated substances did not distinctly change concentrations due to this biological treatment in the MBR.

The elimination of 10-OH carbamazepine is contrary to the findings in [39], wherein the authors found a release of 10-OH carbamazepine in a WWTP. For lamotrigine, no data about its behavior in WWTP were found in the literature, but the authors in [40] found a large portion of lamotrigine in WWTP effluent in the 2-*N*-glucuronidated form (see Table 4). Deglucuronidation, which occurs in WWTPs (e.g., [41]), presumably led to the release of lamotrigine. The release of oxcarbazepine is also contrary to the literature; in [39], the authors found an elimination rate of 25% to 73% in WWTPs. In [42], the authors found no elimination of primidone and a release of phenylethylmalonamide and phenobarbital in a WWTP, which is in general accordance with the presented data. For rufinamide, lamotrigine, lacosamide, and zonisamide, no data were found in the literature.



The missing elimination of oxcarbazepine possibly resulted from missing sunlight under pilot scale conditions, since UV leads to degradation of oxcarbazepine according to [26]. Additionally to missing sunlight, the low BOD₅ load may have resulted in lower co-metabolization rates (e.g., [47]). However, results of oxcarbazepine showed a high variation (see Figure 2) and should be considered carefully.

The results suggest that biological treatment with the membrane bioreactor was not sufficient to considerably eliminate the antiepileptic drugs investigated, which is (fate of oxcarbazepine excepted) in good agreement with the data found in the literature.

In prospective biological treatments of loaded raw waste water, the dilution, caused by starting conditions of the MBR and sludge exchange, should be minimized by the use of a smaller reactor volume, bigger membrane area, and higher BOD₅ load. Sludge exchange should be avoided, if possible.

Antiepileptic drugs in the biologically treated waste water

Due to single determinations, the results of the previous analysis of biologically treated waste water are of limited significance. Therefore, the analysis of the stored (4°C),

biologically treated waste water was repeated with four different SPE procedures.

The calibration points using hydrophilic-lipophilic balance (HLB) and polar enhanced polymer (PEP) SPE cartridges were generally in good accordance so that they were used together in the calibration curve. The only exception was ethosuximide, whose mean recovery rate in SPE extraction was 38% with a high variation. Nevertheless, both SPE calibration points were used, to include the recovery rate, and the directly injected standards were discarded from the calibration curve of ethosuximide.

The results of the analysis are shown in Figure 2. The highest concentrated substances were 10-OH carbamazepine (which is a metabolite of carbamazepine and oxcarbazepine), lamotrigine, and oxcarbazepine.

Contrary to the previous analysis, now ethosuximide could be detected in the biologically treated waste water. The reason lies presumably in the low recovery rate, which may have led to a cartridge breakthrough due to the higher preconcentration factor in the previous analysis. Apart from that, the results for the biologically treated waste water obtained under the use of the two different SPE cartridges are in good agreement with the previous results (oxcarbazepine excepted).

Table 4 Maximum concentrations of antiepileptic drugs used in this study in municipal WWTP effluents and rivers

Analyte	Concentration at WWTP effluents ($\mu\text{g L}^{-1}$)	Concentration in rivers and streams ($\mu\text{g L}^{-1}$)
Carbamazepine	6.3 (DE) [3]	1.1 (DE) [3]
	21.6 (KR) [4]	0.3 (UK) [5]
	4.6 (UK) [5]	
Ethosuximide	No data	No data
Phenylethylmalonamide	0.37 (DE) [43]	0.11 (DE) [43]
Lamotrigine	0.488 (USA) [40]	0.108 (USA) [40]
	1.2 (USA) [6]	
Lamotrigine 2- <i>N</i> -glucuronide	0.209 (USA) [40]	0.195 (USA) [40]
Lacosamide	No data	No data
Primidone	0.71 (DE) [43]	0.18 (DE) [43]
Zonisamide	No data	No data
Sulthiame	No data	No data
Felbamate	No data	No data
	10-OH-Carbamazepine	No data
10-OH-Carbamazepine	1.17 (FR) [39]	
	1.9 (USA) [6]	
<i>N</i> -Desmethylmethsuximide	No data	No data
Oxcarbazepine	0.129 (FR) [39]	No data
	0.48 (USA) [6]	
Rufinamide	No data	No data
Phenytoin	0.25 (USA) [44]	0.004 (USA) [45]
Metabolites of antiepileptic drugs		
Phenobarbital	0.21 (DE) [43]	0.05 (DE) [43]
Carbamazepine epoxide	0.029 (FR) [39]	0.077 (NO) [46]

Exemplary data, not exhaustive. FR France, DE Germany, KR Republic of Korea, NO Norway, USA United States of America.

The total load of the analyzed pharmaceutically active substances was approximately $400 \mu\text{g L}^{-1}$, which is more than 15-fold higher than to be expected at WWTP effluents in Europe or the USA according to concentrations given in Table 3 (the mean concentration of $1 \mu\text{g L}^{-1}$ was assumed for substances without existing data). Considering the modeled portion of 59% hospital raw waste water in the biologically treated waste water, this rough estimation would result in a 25-fold higher load.

This confirms the low dilution due to missing rain water and due to the homogeneity of the group of patients. However, the contribution of the ward to the mass flow of the corresponding waste water treatment plant will be low due to the widespread use of antiepileptic drugs.

Due to the low concentration of dissolved organic carbon and the relatively high load of antiepileptic drugs, this biologically treated waste water, originating from an epilepsy ward, is appropriate for the investigation of the

behavior of antiepileptic drugs in advanced waste water treatment processes.

Embryo tests on biologically treated waste water

No morphological differences between the embryos exposed to biologically treated waste water and the controls in tests with durations of 48, 72, and 96 h could be detected. The overall mortality in valid tests was less than 7%. The results of heartbeat frequency after 96 h are shown in Figure 3.

Oxcarbazepine solution was used for comparison with a highly concentrated antiepileptic drug. The tested oxcarbazepine solution was saturated at 26°C ; a solubility test showed a concentration of $8.7 \pm 0.3 \text{ mg L}^{-1}$. Toxicity tests of 10-OH carbamazepine and lamotrigine, which were also present at high concentrations in the biologically treated waste water, should be performed with *D. rerio*. There were no significant differences regarding the primary and secondary motor neurons between embryos treated with biologically treated waste water and the control.

Weigt et al. [36] have shown that *D. rerio* embryos are able to metabolize (and therefore activate) proteratogens and conclude from their results that *D. rerio* embryos show phase I enzymatic activity at very early developmental stages. Therefore, a metabolization of test compounds during the test can be assumed. It should be tested if there is a significant change of antiepileptic drug concentrations due to metabolization.

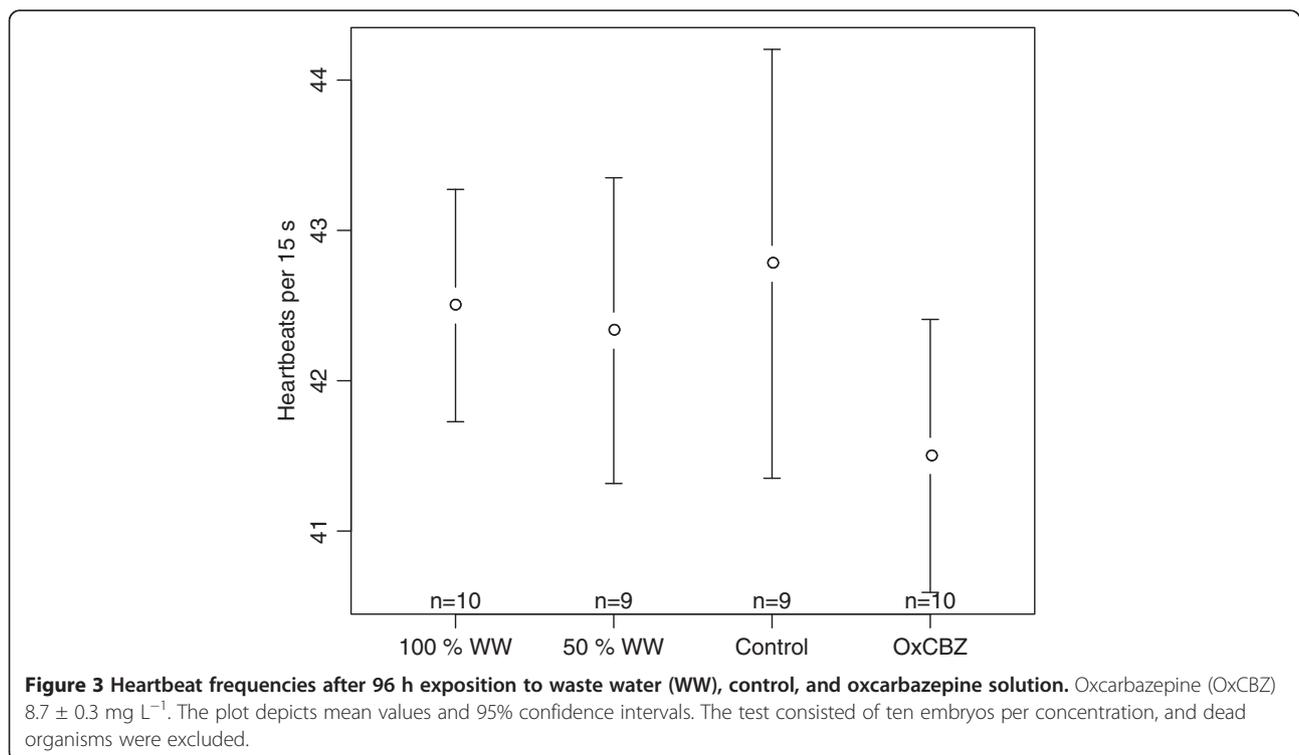
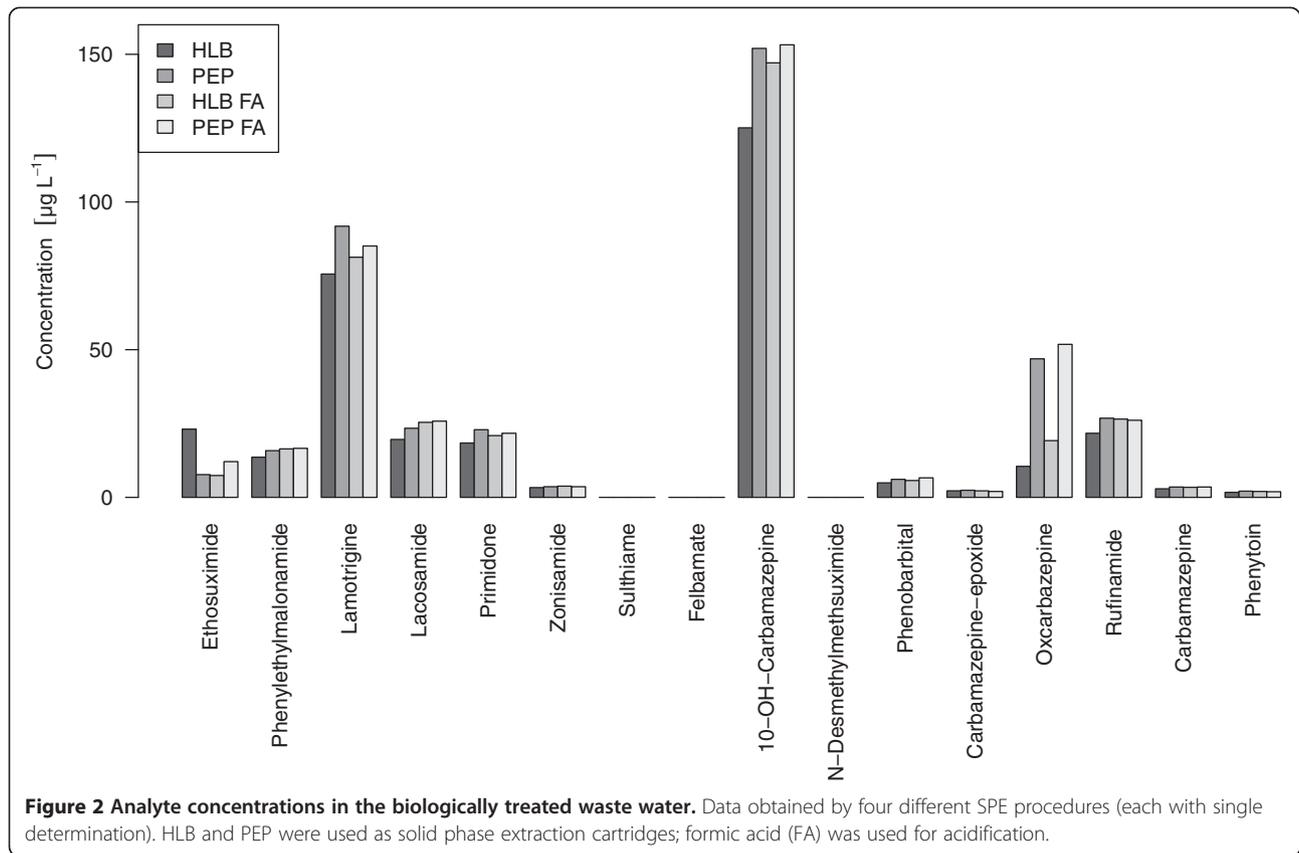
Until now, only a small amount of ecotoxicological data exist for antiepileptic drugs in the literature (exception: carbamazepine). Therefore, additional ecotoxicological tests should be conducted on this biologically treated waste water. A test system probably sensitive enough to show effects (derived from its sensitivity to carbamazepine) is *Mytilus galloprovincialis* [15], although this is a salt water organism.

For the investigation of advanced waste water treatment processes, the micro-algae *Nitzschia sigma* should additionally be tested because of its sensitivity to acridine (EC_{50} growth inhibition $80 \mu\text{g L}^{-1}$ [48]).

Conclusion

Biological treatment of the raw hospital waste water by a pilot scale membrane bioreactor was successful regarding the low dissolved organic carbon concentration and low oxygen consumption in the treated water. This allowed ecotoxicological testing of this highly contaminated water with embryos of *D. rerio*.

Biological treatment caused some elimination of 10-OH carbamazepine and rufinamide but was not sufficient for significant elimination of the load of antiepileptic drugs investigated, which is in general accordance with the



literature. The highest concentrated substances found were 10-OH carbamazepine, lamotrigine, and oxcarbazepine.

The complex substance mixture of the treated waste water from a ward located in a neurological hospital, which is specialized on epilepsy treatment, had neither toxic effects on *D. rerio* morphology nor on the development of the primary and secondary motor neurons (indicator for developmental neurotoxicity). An oxcarbazepine solution of $8.7 \pm 0.3 \text{ mg L}^{-1}$ had no morphological effects on *D. rerio* embryos. Biologically treated waste water originating from an epilepsy ward is appropriate for investigation of the fate of antiepileptic drugs in advanced treatment processes.

Methods

Waste water sampling

Sampling took place on Thursday, 28 June 2012, a hot dry summer day, from 12:05 p.m. until 16:30 p.m. at the waste water collector from an epilepsy ward and an epilepsy ambulance of an anonymous neurological hospital in Germany. The water was pumped out of the sewer with two 10-mm polytetrafluoroethylene (PTFE) tubes aligned parallel to the flow direction. A small barrier, located approximately 5 cm behind the openings of the tubes, was used to slightly raise the water level. With this strategy the more or less constantly flowing dilution water (e.g., from basins) was mainly discarded, and only the water of the toilet flushes was collected.

Because the concentrations of antiepileptic drugs to be expected depend on the patients present during sampling time, a generally high variability will occur. As a result of regular plugging of the tubes due to toilet paper and feces, continuous control of the pump was necessary. The aim of this sampling was to get waste water

highly loaded with antiepileptic drugs and not the description of representative mass flows of antiepileptic drugs from this hospital ward. Therefore, the sampling was done in 1 day. A total volume of ca. 320-L raw waste water was collected in new, RO water cleaned low-density polyethylene (LD-PE) canisters ($9 \times 25 \text{ L}$, $3 \times 30 \text{ L}$, filled above the nominal mark) and stored until treatment at 4°C .

Waste water treatment

The raw waste water was treated in a MBR with a ceramic membrane (200-nm pore size, Type G 200, kindly provided by ITN Nanovation AG, Saarbrücken, Germany). The flow chart of the MBR is shown in Figure 4. All materials with direct water contact after membrane passage were of PTFE, stainless steel, high-density polyethylene (HD-PE), or (in case of the membrane housing) polyether-sulfone (PES) and polyurethane (PU). The treatment cycle was as follows: After starting the re-filling process of the MBR with raw waste water, the aeration stopped for 30 min to reach anoxic conditions. Then, 2:30-min filtration with 0:10-min pause continued cyclic under constant aeration until the filtrate box was filled (5 L, duration ca. 3 h), which triggered the re-filling of the MBR. Re-filling was performed with a peristaltic pump from the clear supernatant of the raw waste water; the particle loaded bottom was discarded. The MBR had a volume of 107 L and was constantly stirred. It was initially filled with activated sludge from the last aeration tank before the clarifier of the waste water treatment plant in Giessen, Germany. Prior to use, it was washed twice with tap water via sedimentation to reduce the impact of the municipal waste water. The treatment was conducted in three separate batches (2.5, 4, and 3 days,

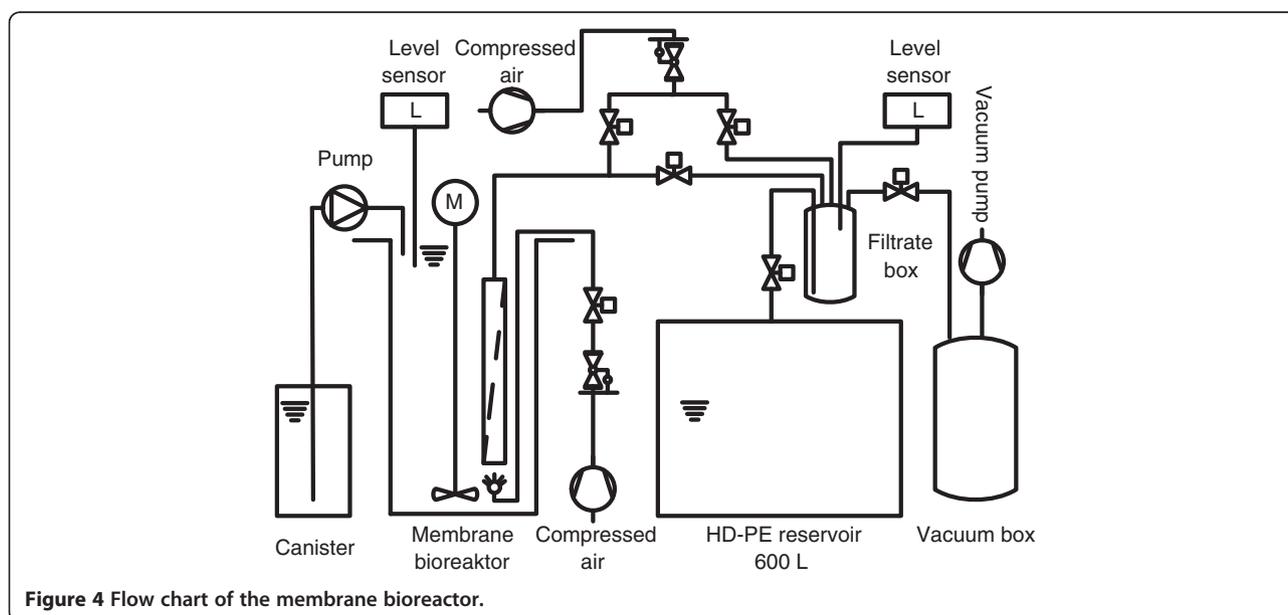


Figure 4 Flow chart of the membrane bioreactor.

respectively), for which fresh sludge was used; the decanted water of the prior batch was reused to reduce dilution of the waste water. The biologically treated waste water was collected in a 600-L HD-PE reservoir, which was aerated during treatment, and then stored at 4°C until further use. The term *biologically treated waste water* used in this publication refers to this mixed sample.

Water analysis

The COD of filtrated (0.45- μ m polyethylene terephthalate (PET)) raw waste water samples, which were stored frozen before analysis, was quantified with cuvette tests (Hach-Lange, Düsseldorf, Germany). The DOC of the biologically treated waste water, which was stored at 4°C until analysis, was tested with cuvette tests (Hach-Lange). The cations of the biologically treated waste water were analyzed with an ICP-OES (720-ES, Varian, Darmstadt, Germany).

Estimation of antiepileptic drug degradation due to biological treatment

To allow the assessment of a possible biological degradation of antiepileptic drugs during MBR treatment, raw waste water and biologically treated waste water were analyzed for these substances. For preconcentration and cleanup, Oasis HLB (60 mg, 3 mL) SPE cartridges conditioned with 5 mL methanol and equilibrated with 5 mL Milli-Q (Merck Millipore, Darmstadt, Germany) water were used. The samples of raw waste water (stored at -32°C) were thawed; the treated waste water (stored at 4°C) was brought to room temperature. Twenty-five milliliters of decanted, unfiltered samples were spiked with 50- μ L solution of 10,11-dihydrocarbamazepine (12.5 mg L⁻¹) for internal standardization and forced, via peristaltic pump, through the cartridges with 1 ml min⁻¹. For identification and calibration of the analytes, ClinCal[®] serum calibrators 15013 and 15213 (Recipe, Germany) were diluted, mixed together, spiked with internal standard, and prepared as waste water samples to get a 2-point calibration with preconcentration factors of 0.6 and 0.3 for 15013 and 0.4 and 0.2 for 15213.

After extraction, the cartridges were dried for 30 min under vacuum and then eluted with 5 mL acetonitrile. The extract was evaporated to dryness under a gentle stream of nitrogen at room temperature. The analytes were then resolved in 200 μ L acetonitrile. After thorough rinsing of all surfaces, 300 μ L Milli-Q water was added; the sample was mixed and then filtrated with 0.45- μ m PTFE syringe filters. Calibration points were prepared twice and samples once.

For each raw waste water sample, the fraction on the biologically treated waste water was modeled, based on the canister volume, time of introduction in the MBR,

the sludge exchanges (after 2.5 and further 4 days, in which 47 L of water and sludge were exchanged due to treatment break), and removal from the system due to filtration. The fraction of each raw waste water sample on the biologically treated waste water was calculated with the sum of filtrated volumes. A water loss (without analyte loss) of 20 L due to evaporation was assumed. These modeled fractions were used to calculate the concentrations of antiepileptic drugs to be expected in the mixed sample after MBR passage considering the abovementioned factors without any transformation process.

Antiepileptic drugs in biologically treated waste water

To confirm the concentrations of antiepileptic drugs in the biologically treated waste water, an additional screening with two different SPE cartridges and a lower preconcentration factor was applied. Additionally to the uncontrolled conditions, the same conditions as in high-performance liquid chromatography (HPLC) gradient (0.1% formic acid) were used, resulting in a pH below 2.8.

For preconcentration and cleanup, Oasis HLB (60 mg, 3 mL) and Thermo HyperSep Retain PEP (60 mg, 3 mL) SPE cartridges, each conditioned with 5 mL methanol and equilibrated with 5 mL Milli-Q water, were used. For sample extraction, 10 mL of treated waste water were mixed with 15 mL Milli-Q water and forced via peristaltic pump through the cartridges with 1 ml min⁻¹. Drying, elution, evaporation, resolving, and filtration were performed as described above.

For identification and calibration of the analytes, Clintest[®] standards 15011, 14011, and 14111 (Recipe, Germany) were used. While 15011 was diluted and then preconcentrated like waste water samples (preconcentration factor 1, 1 \times HLB, 1 \times PEP), 14011 and 14111 were measured without further preparation.

Mass spectrometric analysis of antiepileptic drugs

Antiepileptic drugs and pharmacologically active metabolites were measured with liquid chromatography/time-of-flight mass spectrometry (LC-TOF-MS) consisting of a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Idstein, Germany) equipped with a Dionex Polar Advantage II column (2.1 \times 150 mm, 3 μ m). The detection was performed with a Bruker micrOTOF-QII (Bremen, Germany) in positive electrospray ionization (ESI) mode with a mass range of 50 to 500 m/z ; for mass calibration, Li formate was used.

The mobile phase consisted of 0.1% formic acid (FA) in Milli-Q water (A) and 90% acetonitrile with 10% of 0.1% FA in Milli-Q water (B); the gradient of A was 0 min 91%, 5 min 83%, 15 min 75%, 25 min 55%, 30 min 55%, 35 min 20%, 45 min 20%, 47 min 91%, and 60 min 91%. Identification of the analytes was performed with

the standards according to the mass signatures from literature given in Table 5 and (in case of equal masses) the elution order. To eliminate noise and artifacts as good as possible, the exact masses given in Table 5 were determined with the standards, and the mass window for quantification was set to $\pm 0.01 \text{ m z}^{-1}$.

Solubility of oxcarbazepine

One of the toxicological tests was performed with a saturated oxcarbazepine solution. To reconstruct this concentration, a solubility test was conducted. An excess amount of oxcarbazepine (>98%, Sigma-Aldrich, St. Louis, MO, USA) was mixed in moderately hard synthetic freshwater [57] (target concentration 10 mg L^{-1}) in a light-protected flask. After agitation for 3 days at room temperature, the solution was tempered 3 days at $26^\circ\text{C} \pm 1^\circ\text{C}$ in a water bath without agitation. The solution was filtrated with tempered syringe filters ($0.45\text{-}\mu\text{m}$ PET) in tempered vials four times, and each was diluted 1:1 with methanol immediately to prevent precipitation. A 3-point calibration (1, 5, and 10 mg L^{-1}) was used. Concentrations of oxcarbazepine were measured with an Agilent 1200 HPLC (Waldbronn, Germany); detection was performed with a diode array detector (G1315B, Agilent) at 254.8 nm. Column and gradient were the same as in LC-TOF-MS analysis.

Table 5 Mass windows used for the measured analytes and concentrations found in the literature

Analyte	Retention time (min)	Used mass window ($\pm 0.01 \text{ m z}^{-1}$)	Mass given in the literature
Ethosuximide	6.6	142.085	142.0 [49]
Phenylethylmalonamide	6.7	207.112	207.0 [42]
Lamotrigine	9.1	256.013	256.1 [49]
Lacosamide	9.9	251.137	251.2 [50]
Primidone	10.1	219.111	219.1 [51]
Zonisamide	11.2	213.031	213.1 [49]
Sulthiame	11.5	291.044	291.0 [52]
Felbamate	12.9	178.085	178.2 [49,53]
10-OH-Carbamazepine	16.3	255.111	255 [54]
N-Desmethylnmethsuximide	17.2	190.086	190.1 [49]
Phenobarbital	17.8	233.090	231.2 [49], neg
Carbamazepine epoxide	19.2	253.095	253.2 [49]
Oxcarbazepine	21.4	253.095	253.1 [55]
Rufinamide	13.2	239.072	239.1 [56]
Carbamazepine	25.0	237.101	237.1 [49]
10,11-Dihydrocarbamazepine	25.1	239.116	
Phenytoin	27.2	253.095	253.2 [49]

neg negative polarity was used.

Toxicological tests

Fish maintenance

Approximately one hundred *D. rerio* were maintained in a 120-L tank at 26°C with 10:14-h light/dark cycle and fed two times daily with Tetramin® (Tetra, Melle, Germany) and once per workday with artemia. For egg production, a spawning dish, covered with a stainless steel mesh and artificial plants, was introduced in the tank before lights out and removed 30 to 45 min after lights on at the day of the test. The eggs were washed with control and then randomly put in a beaker filled with 20-mL test solution at the latest 2 h after lights on; from there, they were sorted to the test vessels (4-mL glass vessels with 3-mL test solution).

Tests with *D. rerio*

After controlling the vitality and morphology of the embryos, the vessels were put into the incubator at 26°C with 12:12-h light/dark cycle for the duration of the test. The control was prepared as moderately hard synthetic freshwater according to [57]. The test solutions were brought to test temperature and vented for a minimum of 15 min prior to exposition of the embryos. According to [31], the pH of the sample and control was not adjusted, given that it was within the limits of 6.5 to 8.5. For counting of the heartbeat, one vessel was removed from the incubator, put without delay on the inverse microscope (Olympus IM, Hamburg, Germany) for counting and, after examination of the morphology, put back into the incubator, so that the temperature of the vessel while counting was the same for each embryo. All examinations were done blinded.

Lethal endpoints, sublethal endpoints, and endpoints of teratogenicity in the fish embryo test (FET) were selected for 48 and 96 h according to [32] with emphasis on the heartbeat frequency. Tests were treated as valid when the mortality of the control was 10% or smaller.

Valid tests were conducted at 48 h (three samples + control with 20 embryos; two samples + control with 15 embryos^a), 72 h (three samples + control with 15 embryos^a), and 96 h (two samples + control with 10 embryos). No repetitions were done.

For assessment of developmental neurotoxicity, also the primary and secondary motor neurons of 48-h-old embryos were stained and rated according to the method previously published by Muth-Köhne et al. [38] which was adapted as follows: The fixed embryos were permeabilized and directly blocked in blocking solution (phosphate-buffered saline (PBS)/4% (v/v) Triton X-100/10% (v/v) normal goat serum) for 30 min at room temperature and constant agitation and then incubated with the primary antibody (5 $\mu\text{g/mL}$ in blocking solution) overnight at 4°C . The used chemicals and tools

were identical to the published method. The examination of the stained motor neurons and the rating of defects were done blinded. The test was conducted at 48 h (two samples + control with 15 embryos of which 13 to 14 were used).

Endnote

^aEmbryos were controlled for vitality and then fixed; morphology was assessed on fixed embryos.

Additional file

Additional file 1: Chemical oxygen demand, multi-elemental analytics, and antiepileptic drug concentrations. Table af1. Chemical oxygen demand (COD) of raw waste water samples. **Table af2.** Results of multi-elemental analytics for biologically treated waste water with ICP-OES (mg L⁻¹). **Figure af1.** Antiepileptic drug concentrations for raw waste water samples.

Abbreviations

BOD₅: Biochemical oxygen demand in 5 days; CNS: Central nervous system; COD: Chemical oxygen demand; DOC: Dissolved organic carbon; EC₂₀: Effect concentration at which 20% of the organisms show an effect; EC₅₀: Effect concentration at which 50% of the organisms show an effect; FA: Formic acid; HD-PE: High-density polyethylene; LC: Liquid chromatography; LD-PE: Low-density polyethylene; LOEC: Lowest observed effect concentration; MBR: Membrane bioreactor; OxCBZ: Oxcarbazepine; PBS: Phosphate-buffered saline; PES: Polyethersulfone; PET: Polyethylene terephthalate; PTFE: Polytetrafluoroethylene; PU: Polyurethane; SPE: Solid phase extraction; TOF-MS: Time-of-flight mass spectrometer; 10-OH carbamazepine: 10-hydroxy-10,11-dihydrocarbamazepine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CH and R-AD planned the study. CH constructed the MBR, conducted the sampling and treatment of the waste water, performed the LC-TOF-MS analytics, conducted the embryo tests, and interpreted the results. EM-K conducted the staining of the motor neurons and interpreted and rated the defects. CH took part in the staining of the motor neurons. CH drafted the manuscript. CH and R-AD reworked the manuscript critically. All authors read and approved the final manuscript.

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