Wastewater-borne exposure of limnic fish to anticoagulant rodenticides

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A B S T R A C T
The recent emergence of second-generation anticoagulant rodenticides (AR) in the aquatic environment emphasizes the relevance and impact of aquatic exposure pathways during rodent control. Pest control in municipal sewer systems of urban and suburban areas is thought to be an important emission pathway for AR to reach wastewater and municipal wastewater treatment plants (WWTP), respectively. To circumstantiate that AR will enter streams via effluent discharges and bioaccumulate in aquatic organisms despite very low predicted environmental emissions, we conducted a retrospective biological monitoring of fish tissue samples from different WWTP fish monitoring ponds exclusively fed by municipal effluents in Bavaria, Germany. At the same time, information about rodent control in associated sewer systems was collected by telephone survey to assess relationships between sewer baiting and rodenticide residues in fish. In addition, mussel and fish tissue samples from several Bavarian surface waters with different effluent impact were analyzed to evaluate the prevalence of anticoagulants in indigenous aquatic organisms.

Hepatic AR residues were detected at 12 out of 25 WWTP sampling sites in the low µg/kg range, thereof six sites with one or more second-generation AR (i.e., brodifacoum, difenacoum, bromadiolone). 14 of 18 surveyed sites confirmed sewer baiting with AR and detected hepatic residues matched the reported active ingredients used for sewer baiting at six sites. Furthermore, second-generation AR were detected in more than 80% of fish liver samples from investigated Bavarian streams. Highest total hepatic AR concentrations in these fish were 9.1 and 8.5 µg/kg wet weight, respectively and were observed at two riverine sampling sites characterized by close proximity to upstream WWTP outfalls. No anticoagulant residues were found in fish liver samples from two lakes without known influences of effluent discharges.

The findings of our study clearly show incomplete removal of anticoagulants during conventional wastewater treatment and confirm exposure of aquatic organisms via municipal effluents. Based on the demonstrated temporal and spatial coherence between sewer baiting and hepatic AR residues in effluent-exposed fish, sewer baiting in combined sewer systems contributes to the release of active ingredients into the aquatic environment.

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1. Introduction
Anticoagulant rodenticides (AR) are used worldwide to control commensal rodents for hygienic and public health reasons (Buckle and Smith, 2015). Eight anticoagulants are currently approved in the European Union (EU) for biocidal use under the EU Biocidal Products Regulation No. 528/2012 (European Union, 2012), thereof three first-generation anticoagulants with maximum permissible concentrations of 0.079% (warfarin), 0.0375% (coumatetralyl), and 0.005% (chlorophacinone), and five second-generation AR with maximum permissible concentrations of 0.0075% (difenacoum), 0.005% (bromadiolone, brodifacoum, flucoumafen), and 0.0025% (difethialone) of active ingredient in bait formulations, respectively. In recent years, EU-wide application of second-generation AR has

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been increasingly restrained because of human and environmental risks and their classification as persistent, bioaccumulative, and toxic substances (Regnery et al., 2019; van den Brink et al., 2018). Until lately, however, risk mitigation measures during rodent control focused almost exclusively on the terrestrial environment (Berny et al., 2014), despite considerable acute toxicity of several AR to aquatic species. As summarized in Regnery et al. (2019), LC$_{50}$ values (i.e., lethal AR concentration for 50% of test subjects after 96 h of exposure) for rainbow trout (Oncorhynchus mykiss) are in the range of 40 μg/L (brodifacoum), 51 μg/L (difethialone), 65 μg/L (difenacon), 70 μg/L (floucomafen), and 2860 μg/L (bromadiolone). Furthermore, bromdifacoum, difethialone, and floucomafen exhibit very high bioaccumulation potential in fish (eCA, 2016a, c, d).

The recent emergence of AR residues in the aquatic environment, amongst others their widespread occurrence in liver tissue of freshwater fish (Kothoff et al., 2018), emphasizes the relevance and impact of aquatic exposure pathways that had previously been underestimated (Regnery et al., 2019). Several studies hypothesized that pest control in and around municipal sewer systems by local authorities and commissioned pest control professionals is one important emission source of AR in urban and suburban settings (Gómez-Canela et al., 2014a; Kothoff et al., 2018). In Germany, the annual domestic use of AR in sewer baiting scenarios was projected as approximately 600 metric tons of bait material and 50 kg of active ingredients, respectively according to survey results from 2008 (Krüger and Solas, 2010). These quantities appear minor compared to sales volumes of common human or veterinary pharmaceuticals that are frequently detected in effluent-impacted surface waters (Ashton et al., 2004; Thomas et al., 2007). Although AR bait formulations authorized for use in sewers mainly consist of wax or fat, active ingredients are not chemically bound to the bait material and can be released upon disintegration of baits, e.g., during prolonged exposure to moist or wet conditions. From the sewers, exposure of the aquatic environment most likely occurs via wastewater treatment plant (WWTP) effluents or stormwater overflow structures in combined sewer systems that discharge highly diluted but untreated sewage directly into receiving surface waters when precipitation causes a surcharge within the system. A Spanish study reported sporadic occurrence of AR in WWTP samples in the low ng/L and μg/kg range, respectively but failed in establishing meaningful input and elimination routes (Gómez-Canela et al. 2014a, 2014b; Gómez-Canela and Lacorte, 2016). Despite shortcomings of their analytical approach (Regnery et al., 2019), results pointed toward incomplete removal of AR during activated sludge treatment and potential discharges into receiving surface waters at trace level. In laboratory tests, all AR were shown to be hydrolytically stable in water under environmentally relevant conditions and were not readily biodegradable as summarized in Regnery et al. (2019). However, a strong tendency to adsorb to organic matter combined with low water solubility and a high degree of photo-instability suggest that second-generation AR are unlikely to remain in the aqueous phase during conventional wastewater treatment. Their residues are more likely to persist and accumulate in (organic-rich) sediments, activated sludge, suspended particulate matter, and biosolids.

To date, detailed information about the fate of anticoagulants other than warfarin during conventional or advanced wastewater treatment is lacking. Moreover, multiple challenges of AR residue screening in aquatic environmental compartments were recently highlighted by Regnery et al. (2019). Notably, expected AR concentrations in WWTP effluent and receiving surface waters may be out of reach for current analytical methods and routine monitoring schemes according to worst-case predicted environmental concentrations (European Chemicals Agency, 2018; Regnery et al., 2019). Thus, we initiated a retrospective biological monitoring to assess whether trace levels of AR will occur in tertiary treated wastewater effluents and thereby cause exposure of aquatic organisms in receiving streams. We analyzed tissue samples of fish (Cyprinus carpio) from 25 different WWTP fish monitoring ponds in Bavaria, Germany that were provided by the Bavarian Environment Agency. These fish monitoring ponds are exclusively fed by tertiary treated municipal effluents and annually stocked with fish for six months to enable monitoring of trace level residual wastewater contaminants that might concentrate in aquatic organisms. Moreover, information about rodent control in associated sewer systems shortly before or during the respective bioaccumulation period in these fish monitoring ponds was collected by telephone survey of municipal pest control officials at selected sites in 2018 to assess potential relationships between sewer baiting and AR residues in fish. Names and exact geographic locations of WWTP sampling sites in this study are nondisclosed to preserve individual privacy of investigated WWTP and associated municipalities. To further evaluate the occurrence of anticoagulants in indigenous aquatic organisms as a function of wastewater effluent discharges, mussel and fish tissue samples from seven Bavarian streams with different degrees of municipal effluent contribution as well as two lakes without effluent discharges were also provided by the Bavarian Environment Agency and were screened for anticoagulant residues.

2. Materials and methods

Anticoagulants (i.e., eight rodenticides and two pharmaceuticals) in biological tissues were analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) in negative electrospray ionization (ESI) mode after ultra-sound assisted solvent extraction and dispersive solid phase extraction (dSPE) clean-up following a QuEChERS (quick, easy, cheap, effective, rugged, and safe) approach. Fish liver samples as well as several corresponding fillet samples were analyzed instead of whole-body samples because anticoagulants bind strongly to vitamin K epoxide reductase (i.e., liver is presumed to be the main organ of accumulation). Quantification of target analytes was achieved by means of individual deuterated internal standards.

2.1. Chemicals

Analytical grade standards of biocidal (i.e., warfarin, chlorophacinone, coumatetalyl, bromadiolone, difenacoum, brodifacoum, difethialone, and floucomafen) and pharmaceutical (i.e., phenprocoumon, acenocoumarol) anticoagulants were purchased from Sigma-Aldrich (Steinheim, Germany) and Tokyo Research Chemicals (TRC, North York, Ontario, Canada), respectively. Depending on availability, compound-specific deuterated analogs were used as internal standards for quantitative analysis, namely difenacon-d$_4$, brodifacoum-d$_4$, floucomafen-d$_4$, phenprocoumon-d$_5$ (all TRC), bromadiolone-d$_5$, warfarin-d$_5$, and chlorophacinone-d$_4$ (all C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada). Difethialone-d$_4$ was custom-synthesized (TLC, Aurora, Ontario, Canada), but delivery was delayed until completion of analyses. Stock solutions of individual compounds were prepared in methanol and aliquots were taken to compose respective mixtures of natives and isotopes at the 200 ng/mL level in methanol. Organic solvents and ultrapure water used for preparation of solutions, extraction, and chromatography were HPLC grade. Reagents utilized for sample preparation were analytical grade except magnesium sulfate and sodium chloride (reagent grade, Agilent Technologies, Waldbronn, Germany).
2.2. Sampling sites and handling of samples

All biological tissue samples analyzed in this study were kindly provided by the Bavarian Environment Agency. Sample material from the Bavarian Specimen Bank (frozen at −20 °C, homogenized, wrapped in aluminum foil and vacuum-sealed) was shipped overnight on dry-ice to the Federal Institute of Hydrology laboratory to ensure an uninterrupted cool chain. Parameters such as species, total length, total weight, organ weight, age, gender, Fulton’s condition factor (CF), hepatosomatic index (HSI), and gonadosomatic index were made available for each fish sample.

Bavarian state regulation requires the operation of ponds for an active fish monitoring (herein after referred to as bioaccumulation ponds) by municipal WWTP with equal to or more than 100,000 person equivalents. The majority of these WWTP facilities employ conventional treatment (i.e., mechanical, biological, chemical). The surface area size of bioaccumulation ponds is mostly in the range of 20–130 m² with an average depth of 1 m and a hydraulic retention time of more than 3 d (Bayerisches Landesamt für Umwelt, 2012a). They are exclusively fed by municipal effluents and annually stocked with 10 carp (C. carpio) (i.e., individuals of the same age and bloodline from the fish rearing ponds at the Bavarian Environment Agency) for a six months exposure period to enable active monitoring of potential adverse effects and bioaccumulation of residual contaminants (Bayerisches Landesamt für Umwelt, 2012b). All of the stocked carp are self-feeding and not allowed to be fed throughout the bioaccumulation period to prevent contamination and bias.

Of the active monitoring in 2015 (bioaccumulation period April through October), 31 liver and 12 corresponding filet samples of individual carp were received from 25 different WWTP bioaccumulation ponds (herein referred to as WWTP A – WWTP Y) for analysis in 2017 and 2018. Tissue samples of three individuals from the same bioaccumulation pond were analyzed as replicates at three sites (WWTP A, WWTP B, and WWTP C). At site WWTP C, one liver sample of the 2014 bioaccumulation period was also investigated. Moreover, one wastewater unexposed carp liver sample was obtained from the Bavarian Environment Agency's fish rearing ponds as a reference. Pooled zebra mussel samples (Dreissena polymorpha, n = 2) and individual fish liver (n = 14) and filet (n = 3) samples of chub (Squalius cephalus), perch (Perca fluviatilis), and pike (Esox lucius) had been collected from seven Bavarian streams (i.e., Amper, Danube, Iller, Isar, Lech, Main, Vils) and two lakes (i.e., Starnberger See, Weitnsee) in 2013–2016 as part of the EU Water Framework Directive 2000/60/EC monitoring program. Detailed information about all samples is provided in the supplementary material (SM, Tables S1 and S2). Though not necessarily in close proximity to WWTP outfalls, the riverine sampling sites were situated upstream and downstream of several WWTP that were part of the active biological WWTP monitoring. A general map highlighting all surface water sampling sites can be found in the SM (Fig. S1).

2.3. Determination of total lipids in biological tissue samples

Total lipid content in homogenized tissue samples was determined according to Smedes (1999). A detailed description is provided in the SM. Percent lipid for each sample was determined by dividing the lipid weight for each sample by the initial wet weight of each individual sample.

2.4. Sample extraction and clean-up

The chosen QuECHERS approach followed general procedures described by Vudathala et al. (2010) and Morrison et al. (2016). Approximately 1–2 g wet weight of homogenized fish liver or filet sample was suspended in 3.2 mL acetonitrile and 0.8 mL acetone acidified with 0.1% formic acid (v/v) in a 50 mL polypropylene centrifuge tube using a vortex shaker (MS2 Minishaker, IKA). For extraction of pooled soft body mussel samples, approximately 0.3 g of freeze-dried material was used. Internal standard mix (25 μL of 200 ng/mL each in methanol) as well as 0.2 g magnesium sulfate and 0.2 g sodium chloride salts (Agilent Technologies) were added to the sample tube. The tube was capped tightly and immediately vortexed for 60 s. Following 30 min in an ultra-sonication bath at 20 °C, 4 mL of fresh acidified acetone was added to the sample and the extraction step was repeated. Subsequently, sample tubes were stored in a freezer at −20 °C overnight to enhance protein precipitation. Afterwards, samples were centrifuged for 5 min at 2000 rcf (relative centrifugal force) and the crude extract was transferred to a 15 mL polypropylene centrifuge tube for further clean-up via dSPE. dSPE facilitated removal of co-extracted compounds (e.g., phospholipids) and helped reduce matrix interferences during LC-MS/MS analysis. The amount of applied dSPE bulk sorbents varied depending on tissue type. dSPE of liver extracts was carried out using 0.3 g magnesium sulfate together with 0.1 g each of primary- secondary amine bonded silica, end-capped C18 material, florisor (60–100 mesh), and basic alumina. Clean-up of mussel extracts required less sorbents (i.e., half the amount used for liver tissue). Prior use, florisor and basic alumina had been activated in an oven at 350 °C for 12 h followed by the addition of 2% (v/v) ultrapure water. The dSPE tubes were tightly capped and immediately vortexed for 60 s. After 5 min rest, samples were centrifuged. The organic phase was retrieved, evaporated to dryness under nitrogen, and resolved in 500 μL methanol. A 200 μL subsample thereof was diluted with ultrapure water at a ratio of 1:1 (v/v) and transferred to a Thomson Single Step filter vial (PTFE membrane, 0.45 μm pore size) for LC-MS/MS analysis.

Aliquots of pooled homogenized residue-free fish livers (n = 6), filets (n = 3), and freeze-dried mussel soft bodies (n = 4) were fortified with target substances at low concentration (i.e., 1–2 g of wet fish tissue or 0.3 g of freeze-dried mussel tissue spiked with 25 μL of 200 ng/mL standard mix) and were analyzed to validate the optimized extraction and clean-up procedure. Mean recoveries and standard deviations for each analyte are provided in Table S3, SM. Besides residue-free reference tissues (procedural blanks), each batch of samples included a low-level fortified matrix control that was processed in the same way as samples. To prevent cross-contamination of samples, all glass ware was rinsed with acetone prior cleaning in the dishwasher and heated at 350 °C for several hours afterwards. Polypropylene centrifuge tubes were only used once and were discarded after extraction and clean-up.

2.5. Analysis of anticoagulants by LC-MS/MS

LC-MS/MS analysis was performed on an Agilent 1260 Infinity LC system equipped with a high-precision liquid autosampler and temperature-controlled column compartment (40 °C) coupled with a Sciex 4500 QTrap MS/MS system. The sample injection volume was 10 μL. Prior injection, a 20 s needle wash with isopropanol was performed at the flush port to minimize sample carry-over. A binary gradient at a flow rate of 0.6 mL/min was used to separate compounds on a Phenomenex 50 × 2 mm Luna PFP column with 3 μm particle size and upstream steel guard cartridge. Chromatographic separation of individual AR stereoisomers was not intended. Eluents consisted of (A) 4 mL ammonium acetate solution in water and (B) methanol with the following gradient: 20% B held for 0.5 min, stepped to 90% B at 3.5 min, then held at 90% B for 0.5 min before returning to 20% B at 4.5 min. A 2.5 min equilibration step at 20% B resulted in a total run time of 7 min. Two mass
transitions (i.e., quantifier and qualifier) were monitored for each analyte in ESI negative mode using scheduled multiple reaction monitoring. The monitoring window for each transition was 60 s with a target scan time of 1 s. Monitored mass transitions and compound specific tuning parameters are summarized in Table S4, SM.

Qualitative and quantitative analyses were performed in Analyst (version 1.6.3) and MultiQuant (version 3.0). An internal standard calibration was used for quantification. Eight calibration standards over the concentration range of 0.01–5 ng/mL were analyzed within each LC-MS/MS sequence run. Analytes without isotope-labeled analogs were quantified based on bromadiolone-d5 (difet- thialone) and warfarin-d5 (coumatetralyl, acenocoumarol). Analyte peaks with a signal-to-noise ratio of less than 10 or 3 of the mass transitions used for quantification and confirmation, respectively or shifted retention time compared to their respective isotope-labeled analogs were discarded from further data evaluation. All reported analyte concentrations in biological tissues are based on wet weight and account for analyte loss and ion suppression during sample extraction, clean-up, and LC-MS/MS analysis. Accuracy and precision of the method was checked within each measurement series by repeated injections of reference samples (i.e., procedural blanks, low-level fortified matrix controls). Method detection limits for all analytes in tissue materials ranged between 0.01 μg/kg and 0.3 μg/kg wet weight.

2.6. Sewer baiting survey at selected sites

Operators and administrators in charge of municipal pest control at 11 WWTP sampling sites with evidence of AR residues were surveyed in May 2018 to retrospectively obtain information about rodent control in associated sewer systems shortly before or during the 2015 bioaccumulation period of carp in respective bioaccumulation ponds. Furthermore, 7 WWTP samplings sites without evidence of AR residues in fish were contacted. Surveyed information covered relevant WWTP operational parameters, sewer system specifics, applied pest control schemes within municipal purview (i.e., mechanical or chemical, frequency, duration, types of active ingredients, bait amount and placement), as well as other known (or assumed) sources of AR in the catchment area. The narrow selection of surveyed sites as well as variable quality of mined data allowed for qualitative but not quantitative statistical analysis of survey results.

3. Results and discussion

3.1. Residues of anticoagulants in fish from wastewater treatment plant bioaccumulation ponds

Although effluent-dominated systems such as bioaccumulation ponds represent worst-case exposure scenarios for fish, they provide valuable insight regarding the bioaccumulation potential of effluent-sourced contaminants in indigenous aquatic organisms. At 12 out of 25 studied sampling sites, AR residues were detected in the livers of individual carp in the low μg/kg range after being exposed to municipal effluents for approximately six months. No distinct correlation between AR concentration in fish and WWTP treatment capacity (i.e., population equivalents) was observed (Fig. 1). A total of six sites revealed hepatic residues of one or more second-generation AR. Due to the high frequency of non-detect data (i.e., less-than values) throughout the samples, analyte detection frequencies, median, 95th percentile, and maximum concentrations are listed in Table 1. Average biometric parameters of the analyzed two-year old carp (14 male, 8 female, 10 undetermined) were 35.7 ± 3.7 cm total length, 769 ± 258 g whole-body weight, and 6.2 ± 3.3% lipid content in liver tissue (Table S2, SM).

Interestingly, the first-generation AR coumatetralyl was detected most frequently in carp from bioaccumulation ponds (Table 1). It was followed by the second-generation AR bromadiolone, brodifacoum, and difenacoum, all of which had higher detection limits than coumatetralyl (Table 1). Notably, multiple individuals from the same sampling site had matching distributions and comparable concentrations as shown for treatment facilities WWTP A, B, and C (Tables 2 and S2, SM), pointing towards identical exposure of fish individuals via non-dietary and/or dietary routes. In contrast, difethialone was solely observed in one liver sample at WWTP B, which was more likely due to individual dietary uptake rather than non-dietary routes. Few studies hypothesized that terrestrial invertebrates feeding on AR containing bait may function as vector in the environment (Masuda et al., 2014; Pitt et al., 2015). No residues of floccouafen and chlorophacinone were detected in any of the analyzed samples from the bioaccumulation ponds (Tables 1 and 2) and no anticoagulant residues were detected in carp liver of a wastewater unexposed sibling that had been analyzed as a reference (Table S2, SM). With the exemption of coumatetralyl, none of the analyzed corresponding filet samples (on average 0.9 ± 0.5% lipid content) contained anticoagulant residues (Table 2). At WWTP C traces of coumatetralyl were observed in filet samples of all three individuals. The coumatetralyl residues in filet tissue were an indication for ongoing exposure at the time of sampling as active ingredient not yet bound to protein (e.g., in the liver or blood plasma) is expected to quickly depurate in fish based on laboratory bioconcentration studies (eCA, 2016b). In good agreement, corresponding liver samples revealed substantial coumatetralyl residues (Table 2). WWTP C also exhibited highest hepatic concentration of total AR in a single organism (4.6 μg/kg, Fig. 1). In comparison, lower hepatic concentrations of total AR (1.1 μg/kg) and fewer active ingredients were detected during the 2014 bioaccumulation period at site WWTP C. While residues of difenacoum (1.0 μg/kg), bromadiolone (0.1 μg/kg) as well as phenprocoumon (0.36 μg/kg) were in the range of concentrations observed in liver samples of the 2015 bioaccumulation period (Table 2), no other AR residues were present. This implies that even at the same site wastewater-borne rodenticide emissions will vary over consecutive years due to variable usage patterns and multiple emission sources. Differences in the diversity of AR residues over time was recently reported by
Hepatic warfarin residues were only observed at trace level in less than 10% of samples with a maximum of 0.05 μg/kg (Table 1). The more pronounced hepatic phenprocoumon residues in effluent-exposed fish can be explained by the higher frequency and amplitude of contaminant loading considering that both substances are extensively metabolized in the human body. Only about 2% of the typical daily warfarin prescription dose is excreted as unchanged active ingredient (Crouse et al., 2012; Park, 1988). Phenprocoumon is excreted almost entirely as a glucuronide conjugate, with less than 10% of the dose as unchanged drug (Kasprzyk-Hordern, 2010). The presence of warfarin in wastewater has mainly been linked to the consumption of blood-thinning medication by resident population as warfarin is the only biocidal anticoagulant that is concurrently authorized for pharmaceutical use (Ajo et al., 2018; Regnery et al., 2019; Santos et al., 2013). To date, the 4-hydroxycoumarin derivatives phenprocoumon and acenocoumarol are primarily used across continental Europe instead of warfarin to prevent and treat thromboembolic diseases besides direct coagulation factor inhibitor drugs (Fan et al., 2018; Lin et al., 2013). Medical consumption of phenprocoumon exceeds that of warfarin by approximately factor 40 according to German prescription statistics (Regnery et al., 2019). Congruently, hepatic residues of phenprocoumon were detected in 76.9% of carp from bioaccumulation ponds with a maximum level of 1.8 μg/kg whereas hepatic warfarin residues were only observed at trace level in less than 10% of samples with a maximum of 0.05 μg/kg (Table 1). The more pronounced hepatic phenprocoumon residues in effluent-exposed fish can be explained by the higher frequency and amplitude of contaminant loading considering that both substances are extensively metabolized in the human body. Only about 2% of the typical daily warfarin prescription dose is excreted as unchanged active ingredient (Crouse et al., 2012; Park, 1988). Phenprocoumon is excreted almost entirely as a glucuronide conjugate, with less than 10% of the dose as unchanged drug (Kasprzyk-Hordern, 2010).

Data summarized in Table 1, Fig. 1, and Table S5 corroborate the assumption that AR input rates are of transient character and will fluctuate depending on site-specific factors such as usage patterns and runoff regimes in urban and suburban catchments, hydro-meteorological conditions, and WWTP operational parameters and performance. All of the investigated municipal WWTP in this study applied tertiary treatment, i.e., each treatment train consisted of mechanical treatment followed by biological treatment stages.
and chemical dosing for enhanced nutrient removal. Treatment capacities varied between 100,000 and 2,000,000 population equivalents (Bayerisches Landesamt für Umwelt, 2012b) with average daily dry weather effluent discharges in the range of 5000 to 570,240 m³/d (Table S5, SM). Yet, discharges could quadruplicate during wet weather at facilities with mainly combined sewer systems connected (majority of investigated sites). None of the treatment facilities with confirmed second-generation AR residues in fish (WWTP A – F, Table S5, SM) applied further advanced treatment. Downstream advanced treatment stages that are focused on elimination of refractory wastewater-borne trace organic chemicals, such as ozone, advanced oxidation processes, membrane filtration, or activated carbon filtration, are still not common at full-scale facilities in Germany (Schaar and Kreuzinger, 2017). Nevertheless, several neighboring WWTP sites discharging into the same stream (i.e., along the upper and middle stretch of Isar River) operate downstream biological active sand filters (Table S5, SM). Although some of these facilities also run ultra-violet (UV) disinfection units during bathing season to meet microbial bathing water quality requirements, effluents feeding the bioaccumulation ponds had been diverted prior to disinfection according to personal communication with WWTP operators. While no second-generation AR residues were detected in fish samples from sampling sites with further advanced treatment (e.g., WWTP N, O, R, and S), low levels of hepatic phenprocoumon residues were frequently observed, indicating incomplete retention of this hydroxycoumarin derivative in biological active sand filters. Given their aforementioned physicochemical properties (e.g., not readily biodegradable, low water solubility, high lipophilicity, and photo-lytic instability) at ambient environmental conditions, second-generation AR might occur particle-bound in wastewater effluents rather than freely dissolved, although a previous study implied enhanced solubility or co-solubility of second-generation AR in organic-rich water (Pitt et al., 2015). Depending on the treatment train of investigated facilities, remaining suspended particle loads in the discharged effluents were generally in the range of 2–10 mg/L under dry weather conditions (Table S5, SM).

A previous investigation observed no relationship between fish length, total weight, and WWTP treatment capacity, whereas factors such as pond size and type were identified as major driver for fish condition in the bioaccumulation ponds (Bayerisches Landesamt für Umwelt, 2012b). Yet, site-specific distributions of AR were further evaluated based on available physiological fish health parameters (e.g., length, weight, lipid content, organ weight) and are shown as a function of Fulton’s CF and HSI in Fig. 2. Overall, occurrence and distribution of AR residues expressed no distinct relationship with gender, lipid content, and physiological parameters of fish health such as CF or HSI. Fulton’s CF for carp should generally be higher than 1 to indicate adequate nutritional state. Values for CF and HSI were on average 1.6 ± 0.2 and 2.8 ± 0.8, respectively (Table S2, SM). Teubner et al. (2015) concluded earlier that Fulton’s CF and HSI might be no meaningful stress indicators. Nevertheless, adverse effects of chronic AR exposure in fish from these bioaccumulation ponds could have been masked by other stressors or influential factors that we were not able to account for in retrospect at the investigated 25 WWTP.

In summary, our results provide crucial evidence that anticoagulants are not completely removed during conventional biological wastewater treatment and thus confirm one important exposure pathway for indigenous aquatic organisms: anticoagulants will enter the aquatic environment by way of effluent discharges. Furthermore, these findings also show that second-generation AR can bioaccumulate in fish liver under environmentally realistic conditions and exposure scenarios.

3.2. Potential sources of anticoagulant rodenticides in wastewater

Considering anticoagulants’ high protein binding capacity and the persistence of specifically second-generation AR in liver tissues of terrestrial wildlife (Horak et al. 2018), it is difficult to link hepatic AR residues in fish to distinct exposure events. Besides the aforementioned release of pharmaceutical anticoagulants (e.g., phenprocoumon, warfarin) due to medical consumption, pest control in
and around municipal sewer systems in urban and suburban settings is assumed to be the major emission source of AR into wastewater. Notably, 78% of operators and administrators at the 18 surveyed sampling sites confirmed sewer baiting with AR, whereas 17% negated pest control enforcement in their sewers in 2015 (Table S5, SM). One municipality used a mechanical rat trap system. Based on available information, mainly bait blocks were used when baiting with AR. In general baits were attached to the manhole’s gully trap or step iron by wire to prevent dragging off or flushing away. At most surveyed sites, however, deployed baits remained in the sewers after baiting campaigns ended and were not removed for disposal. Individual annual quantities deployed in the sewers ranged between 500 and 2500 baits (mostly 200 g bait blocks) among the 11 municipalities that provided quantitative information (Table S5, SM). This corresponded to a total of approximately 3000 kg of bait material per 2,500,000 person equivalents. At all surveyed sites, products containing difenacoum, warfarin, bromadiolone, or brodifacoum were exclusively used (Table S5, SM). Oftentimes multiple products with different active ingredients were applied over the course of one control measure. Based on provided information by municipal pest control officials, detected AR residues in fish liver matched the reported active ingredients used for sewer baiting at six sampling sites (Fig. 3), namely at WWTP A, B, D, E, I, and K (Table S5, SM). Interestingly, traces of warfarin residues in carp from WWTP D, I, and K concurred with confirmed deployment of warfarin baits during sewer baiting in the associated sewer systems.

Despite the demonstrated temporal and spatial coherence, evidence of specific emission sources and pathways remains challenging considering their wide application range. While existing data suggest that pest control professionals are among the main users of biocidal AR in Germany, agribusinesses, local authorities, and household consumers represent other important user groups (Regnery et al., 2019). For instance, the second-generation AR detected in fish liver at WWTP F did not match the second-generation AR supposedly used for sewer baiting by its largest connected municipality (Fig. 3 and Table S5, SM). It became apparent that WWTP F received wastewater from 11 additional communities, which were not part of the survey. Thus, the proportional dry weather discharge of the surveyed municipality was only in the range of approximately 30%. Moreover, at least four surveyed sites with confirmed second-generation AR sewer baiting in 2015 revealed no corresponding residues in fish. At two sites thereof (i.e., WWTP G and J), municipal pest control officials reported that untouched bait blocks had been removed from the sewers for appropriate disposal at the end of their baiting campaigns. In both samples solely traces of hepatic coumatetralyl residues were found. In Germany, none of the registered products containing coumatetralyl are permitted for use in sewer baiting scenarios. However, the use of first-generation AR such as coumatetralyl is less restricted. Information provided by German stakeholder groups and a recent study by Koivisto et al. (2018) suggest that coumatetralyl is more frequently used by agribusinesses and private consumers rather than pest control professionals or municipalities.

It can be concluded that sewer baiting contributes to the release of active ingredients into wastewater. Baits deployed in combined sewer systems and stormwater channels face a substantial risk of prolonged exposure to moist or wet conditions and thus scouring when precipitation causes a sudden surcharge within the system due to frequently occurring extreme weather events such as torrential downpours in urban and suburban areas. This is even more critical for the application of AR containing baits in stormwater channels that are not connected to retention basins or WWTP but discharge directly into natural water bodies. Nonetheless, the risk of active ingredient release during chemical pest control measures in sewer systems can be minimized if contact of bait material with water and wastewater is strictly excluded (e.g., by use of devices that keep the bait dry, deployment of baits exclusively in manholes free from backing-up/runoff pouring in, collection and appropriate disposal of remaining bait at the end of baiting campaigns).

Besides sewer baiting, additional emission sources of AR into sewer systems and WWTP are surmised and require further investigation. Potential other emission scenarios include baits or poisoned carcasses being flushed into the sewers during outdoor surface baiting (e.g., near storage facilities for goods or food production, public green space, private or communal garden plots), incorrect disposal of baits, landfill leachate, recirculate from sludge dewatering processes, or washing of disposed organic material containing active ingredients prior incineration. Deployment of baits in the immediate vicinity of watercourses represents another
likely emission source of AR into the aquatic environment, e.g., due to wash off from bank slopes, aboveground bait stations, or rodent burrows, respectively as well as contaminated run-off (European Chemicals Agency, 2018). Although mandatory instructions for use and risk mitigation measures were stipulated at EU-level and best practice guidelines were established during national product authorizations to minimize the risks of environmental exposure (Umweltbundesamt, 2019), the extent of compliance with these provisions and guidelines is largely unknown. Previous studies assumed that the typical use of AR commonly violates respective use and disposal instructions (Koivisto et al., 2016). It should be noted that anticoagulants are currently not approved as active ingredients in plant protection products in Germany. Operators at WWTP C confirmed that no sewer baiting with AR had been conducted in their associated combined sewer system over the past 10 years (Table S5, SM). Yet, WWTP C was the site with the highest number and total concentration of AR residues (i.e., brodifacoum, bromadiolone, difenacoum, and coumatetralyl) among the 2015 carp liver samples. Furthermore, bromadiolone and difenacoum were also detected in carp liver from the same site in 2014 as discussed earlier.

### 3.3. Occurrence and fate of anticoagulants in fish and mussels from lakes and receiving streams

Prolonged input rates of anticoagulants from effluent loadings, even at trace level, can increase the effective exposure duration of organisms residing in receiving aquatic systems if input rates exceed environmental dissipation rates. In most upper river basins in Germany, wastewater effluent contributions during average flow conditions vary between 0 and 5% according to a recent study by Karakurt et al. (2019). Contributions of more than 5–10% and more than 10–20%, respectively are prevalent in river basins up- and downstream of urban centers as well as river stretches generally characterized by low-flow conditions (e.g., Main River). During low-flow conditions, however, effluent contributions of more than 10–20% are common for a large number of river basins nationwide, whereas several water-sheds exhibit wastewater effluent contributions of more than 20–30% (Karakurt et al., 2019).

As expected based on available information about their environmental fate and minor medical consumption in Germany, warfarin and acenocoumarol were not detected in any of the biological tissue samples from wild freshwater fish (Table 1). While phenprocoumon traces were detected in 83.3% of fish samples from receiving streams, its median concentration in liver tissue was only 0.04 μg/kg with a maximum of 0.2 μg/kg (Table 1), corroborating marginal bioaccumulation potential in indigenous aquatic organisms. In contrast, a 40-fold higher median concentration of brodifacoum was observed in these liver tissue samples. Overall, residues of second-generation AR were detected in more than 80% of fish liver samples (mainly chub, 4–11 years) from investigated Bavarian streams with different degrees of municipal effluent contributions (Fig. 4). Residues were detected in individuals from Amper (n = 1, approx. 0–5% wastewater effluent contribution during average flow conditions at this sampling site), Iller (n = 1, approx. 0–5%), Isar (n = 2, approx. 5–10% at both sites), Lech (n = 1, approx. 0–5%), and Main (n = 5, approx. 9–11% throughout sites), whereas no residues were observed in two individuals from sampling sites at Danube (approx. 5–10%) and Vils (approx. 0–5%), respectively. As summarized in Table 1, brodifacoum (66.7%) was most frequently detected followed by bromadiolone (41.7%), difenacoum (25%), flouxoum (25%), and difethialone (25%). The high detection frequency of hepatic brodifacoum residues in our study concurs with findings by Kotthoff et al. (2018) in 8–12 year old limnic bream. Likewise, no anticoagulant residues were found in liver samples of pike from two lakes (i.e., Starnberger See and

![Fig. 4.Measured concentration of anticoagulant rodenticides in 14 fish liver samples (i.e., chub, pike, perch) from 9 different surface waters (i.e., two lakes and 7 streams) in Bavaria, Germany. Analyte concentrations are reported in μg/kg relating to wet weight.](image-url)
Weißensee) without known influences of effluent discharges (Fig. 4).

The highest total AR concentrations were in the range of 9.1 and 8.5 µg/kg and were observed in an 8-year-old chub from the lower stretch of Isar near its confluence with Danube and an 11-year-old from Main near Rothwind (Fig. 4 and Table S1). Both sampling sites are characterized by close proximity to upstream WWTP outfalls according to their technical data sheets (accessed on 01/18/19 at https://www.gkd.bayern.de/de/fluessse/biologie/). In a 9-year-old individual from a second sampling site at the middle stretch of Isar near Moosburg (Table S1 and Fig. S1, SM), solely traces of hepatic individual from a second sampling site at the middle stretch of Isar near Moosburg (Table S1 and Fig. S1, SM), solely traces of hepatic residues of at least one second-generation AR. Their total AR concentrations ranged between 1.3 and 8.5 µg/kg (Fig. 4). It was estimated that portions of Main receive effluent contributions of more than 30–50% under low water conditions (Karakurt et al., 2019). Analyzed corresponding file samples of three individuals from Main River revealed no residues (Table S1, SM). Interestingly, no AR residues were detected above their respective method detection limits in pooled mussel samples from two Main sampling sites, thereof one site with confirmed hepatic AR residues in fish (Fig. S1 and Table S1, SM). As reported in previous studies, bioaccumulation processes can widely differ among aquatic species due to complex interactions between various routes of uptake, excretion, passive release, and metabolization (Streit, 1998). Furthermore, substantial data gaps exist regarding the understanding of exposure pathways and potential adverse effects of chronic exposure with multiple active ingredients (Rattner et al., 2014), making it nearly impossible at the moment to estimate the consequences of chronic AR exposure to freshwater fish. Nonetheless, very persistent second-generation AR such as brodifacoum will likely accumulate in the aquatic food chain when released into the aquatic environment and put predators at risk (Ruiz-Suarez et al., 2016; Seriesy et al., 2019).

4. Conclusions

Our results clearly indicate incomplete removal of AR during conventional wastewater treatment and confirm indirect exposure of aquatic organisms via WWTP effluents. Our findings also confirm high hepatic bioaccumulation potential and persistence of second-generation AR in indigenous limnic fish. Based on the demonstrated temporal and spatial coherence between sewer baiting and the aquatic environment and put predators at risk (Ruiz-Suarez et al., 2016; Seriesy et al., 2019).

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.

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Appendix A. Supplementary data

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References


