Heavy rainfall provokes anticoagulant rodenticides’ release from baited sewer systems and outdoor surfaces into receiving streams

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Abstract

Prevalent findings of anticoagulant rodenticide (AR) residues in liver tissue of freshwater fish recently emphasized the existence of aquatic exposure pathways. Thus, a comprehensive wastewater treatment plant and surface water monitoring campaign was conducted at two urban catchments in Germany in 2018 and 2019 to investigate potential emission sources of ARs into the aquatic environment. Over several months, the occurrence and fate of all eight ARs authorized in the European Union as well as two pharmaceutical anticoagulants was monitored in a variety of aqueous, solid, and biological environmental matrices during and after widespread sewer baiting with AR-containing bait. As a result, sewer baiting in combined sewer systems, besides outdoor rodent control at the surface, was identified as a substantial contributor of these biocidal active ingredients in the aquatic environment. In conjunction with heavy or prolonged precipitation during bait application in combined sewer systems, a direct link between sewer baiting and AR residues in wastewater treatment plant influent, effluent, and the liver of freshwater fish was established. Moreover, study results confirmed insufficient removal of anticoagulants during conventional wastewater treatment and thus indirect exposure of aquatic organisms in receiving streams via tertiary treated effluents and combined sewer overflows. Nevertheless, further research is required to determine the ecological implications and risks for aquatic organisms as well as fish-eating predators from chronic AR exposure at environmentally relevant concentrations.

Keywords: Biocides, combined sewer overflow, PBT, rat management, sewer baiting, wastewater treatment
1. Introduction

In recent years, European Union (EU)-wide application of anticoagulant rodenticides (ARs) to control commensal rodents for hygienic and public health reasons has been increasingly restrained because of human and environmental risks. Second-generation ARs are classified as (very) persistent, (very) bioaccumulative, and toxic (PBT or vPvB, respectively) substances (Regnery et al. 2019a, van den Brink et al. 2018). In order to minimize environmental exposure due to the toxicological relevance of anticoagulants at trace concentrations, national best practice guidelines and mandatory instructions for use of ARs were implemented in Germany from 2012 (Umweltbundesamt 2019). Nevertheless, prevalent findings of AR residues in liver tissue of freshwater fish (Kotthoff et al. 2019, Regnery et al. 2019b) from streams in Germany recently highlighted the emergence of aquatic exposure pathways. So far, worldwide monitoring of AR residues mainly focused on terrestrial and avian non-target species and their routes of exposure (Elmeros et al. 2018, Koivisto et al. 2018, Serieys et al. 2019, van den Brink et al. 2018). As discussed in detail in a review by Regnery et al. (2019a), AR residue screening in aquatic compartments is challenging, and accordingly little is known about direct and indirect exposure routes as well as anticoagulants’ distribution and fate in the aquatic environment.

Three first-generation (i.e., warfarin, coumatetralyl, and chlorophacinone) and five second-generation ARs (i.e., difenacoum, bromadiolone, brodifacoum, flocoumafen, and difethialone) are currently approved in the EU for biocidal use under the EU Biocidal Products Regulation (BPR) No. 528/2012 (European Union 2012) with maximum permissible concentrations in bait formulations in the range of 0.0025% (difethialone) and 0.079% (warfarin). While pest control professionals are presumed to be among the main users of biocidal ARs in Germany, agribusinesses, local authorities, and household consumers also represent important user groups (Regnery et al. 2019a). Considering the lack of detailed market data (Regnery et al.
2019a) as well as manifold applications of ARs in urban and suburban areas (Meyer and Kaukeinen 2015), evidence of specific emission sources and aquatic exposure pathways is not straightforward. Several potential rodenticide emission scenarios for different environmental compartments have been described by the European Chemicals Agency. For example deployment of baits in the immediate vicinity of watercourses represents a likely direct emission source of ARs 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, ECHA 2018b). Rodent control in and around municipal sewer systems by local authorities and commissioned pest control professionals is assumed to be another important emission source of ARs into the aquatic environment in urban and suburban settings (Gómez-Canela et al. 2014a, Kotthoff et al. 2019, Regnery et al. 2019b). In a recent survey in Germany (Regnery et al. 2020), the annual domestic use of ARs in municipal sewer baiting scenarios in 2017 was estimated at approximately 225 metric tons of bait material and thus 32 kg of active ingredients (thereof 21.4 kg of warfarin, 5.6 kg of difenacoum, 3.1 kg of brodifacoum, and 1.8 kg of bromadiolone). From the sewers, exposure of the aquatic environment presumably occurs directly via baited storm drains or combined sewer overflows (CSOs) that discharge highly diluted but untreated sewage directly into receiving surface waters when stormwater runoff causes an increase within the system, or indirectly via wastewater treatment plant (WWTP) effluents (ECHA 2018b). In their retrospective biological monitoring study, Regnery et al. (2019b) confirmed exposure of aquatic organisms via municipal effluents and thus incomplete removal of anticoagulants during conventional wastewater treatment. A study in Spain also reported incomplete removal of ARs during activated sludge treatment and their discharges into receiving streams at trace level (Gómez-Canela et al. 2014a, 2014b, Gómez-Canela and Lacorte 2016). Nevertheless, comprehensive monitoring data on the occurrence and fate of ARs in WWTPs and receiving surface waters during or shortly after widespread chemical rodent control in and around municipal sewer systems are not available. Such data
are required to establish robust relationships and causative associations as previously shown for the unintended poisoning of terrestrial non-target organisms (Geduhn et al. 2015).

To further investigate the above-mentioned potential emission pathways, a comprehensive WWTP and surface water monitoring campaign was conducted at two urban catchments in Germany in 2018 and 2019. At both monitoring sites, common sewer baiting schemes (ECHA 2018b, Regnery et al. 2020) such as bi-annual, annual, or biennial preventive rodent control measures using second-generation ARs had been used for years. However, the receiving streams Queich and Moselle differ in size and thus effluent load at studied WWTP outfalls. Over several months, the occurrence and fate of all eight authorized biocidal anticoagulants was monitored in a variety of aqueous, solid, and biological environmental matrices during and after widespread sewer baiting with AR-containing bait. This study contributes valuable information to future risk assessments of ARs and assists in developing more effective and practical risk mitigation measures to protect the aquatic environment.

2. Experimental

2.1. Monitoring site A at River Queich

A schematic map providing an overview of all WWTP and surface water sampling locations at monitoring site A is shown in Figure 1. The studied WWTP at monitoring site A (hereafter referred to as WWTP A) serves the medium-sized town of Landau in der Pfalz, Rhineland-Palatinate, Germany and employs conventional treatment (i.e., mechanical, biological, chemical) with a treatment capacity of 90,000 person equivalent. WWTP A discharges into the small stream Queich after it flows through the urban center of Landau. The small stream’s mean discharge is in the range of 1.75 m³/s with an estimated 7% effluent contribution of WWTP A near its outfall under dry weather and average flow conditions.
Annual preventive rat control measures in the WWTP’s associated sewer system (i.e., mostly combined, with a total length of approximately 230 km and 4900 sewer manholes) were carried out during a three-week period in May 2018 through commissioned and trained pest control professionals. A total of approximately 2000 brodifacoum-containing bait blocks (i.e., 225 g each containing 0.005% of active ingredient) were deployed in the town’s combined sewer system. As a benchmark, approximately 2500 brodifacoum-containing bait blocks had been applied in 2017, whereas no widespread sewer baiting occurred in 2019. Baits were lowered into the manhole to (short above) the berm and were attached to the manhole’s gully trap or step irons by wire to prevent direct wastewater contact during normal sewer operation as well as dragging off by rats or being flushed away. According to provided information, remaining baits were generally not collected for disposal after the baiting campaign ended. In addition, a total of approximately 60 bromadiolone-containing baits (i.e., 200 g each containing 0.005% of active ingredient) were deployed above ground in tamper-resistant bait stations near watercourses throughout the urban center.

During the monitoring campaign a total of 10 samplings were carried out between March and July 2018. Activated sludge grab samples and 24-hour composite samples of raw wastewater and corresponding treated effluent as well as operational and water quality parameters were kindly provided by WWTP staff. Deposited solids from the bottom of two different CSO structures were also provided by WWTP staff. All of the riverine sampling sites were situated in a rural setting downstream of the town’s urban center and were chosen based on their ease of access and their position in relation to the outfall of WWTP A (Figure 1). Sediment grab samples of Queich were obtained from 0 – 5 cm depth near the bank using a small stainless-steel shovel whereas surface water grab samples were scooped midstream by lowering a bucket from adjacent pedestrian bridges. Surface water quality parameters such as temperature, dissolved oxygen concentration, pH, and electrical conductivity were measured
in situ with sensors using a Multi 3630 IDS handheld (WTW, Weilheim, Germany). Other water quality parameters such as dissolved organic carbon and nutrient levels as well as characteristics of solid samples were determined in laboratory according to Standard Methods (Wasserchemische Gesellschaft and Normenausschuss Wasserwesen im DIN 2020). Suspended particulate matter (SPM) in the stream was collected over defined time intervals (generally 14 days) using passive sedimentation boxes (Schulze et al. 2007). Upstream and downstream of the WWTP A outfall, two sedimentation boxes were suspended in the water from overhanging structures using steel cables (i.e., bankside, approximately 0.2 m above ground). Fish liver and filet samples (frozen at -20°C) from the investigated stream were kindly provided by the Upper Fisheries Authority, Structural and Approval Directorate South, Rhineland-Palatinate. A total of 15 individuals of brown trout (Salmo trutta fario), perch (Perca fluviatilis), roach (Rutilus rutilus), chub (Squalius cephalus), and common gudgeon (Gobio gobio) had been caught at the same time from the same river stretch downstream of the WWTP A outfall in July 2019, approximately one year after the initial WWTP and surface water monitoring campaign ended (Table S1).

2.2. Monitoring site B at River Moselle

The studied WWTP at monitoring site B (hereafter referred to as WWTP B) serves the city of Trier, Rhineland-Palatinate, Germany and employs conventional treatment (i.e., mechanical, biological, chemical) with a treatment capacity of 170,000 person equivalent. WWTP B discharges into Moselle, a tributary of River Rhine. The mean discharge of Moselle at gauge Trier is 277 m³/s. Under average flow conditions the overall effluent contribution of Moselle is less than 5% (Karakurt et al. 2019), with an estimated 0.1% effluent contribution of WWTP B near its outfall during dry weather conditions.
Until 2017, city-wide preventive rat control measures in the sewers had been carried out through commissioned and trained pest control professionals once a year during an eight-week period in spring or fall using second-generation AR-containing bait (e.g., approximately 3500 bait blocks with 0.005% brodifacoum in fall 2017). Since then, rat control in sewers had been switched to a more targeted approach based upon reported sightings of rats. A total of approximately 1055 bait blocks (i.e., 200 g each containing 0.0029% of brodifacoum) were successively deployed in different urban districts of the city’s combined sewer system (i.e., approximately 550 km total length with 15,000 sewer manholes) between January and May 2019 by public services through trained municipal staff. Similar to monitoring site A, bait blocks were attached to the manhole’s gully trap or step irons by wire. However, remaining baits were now removed from the manholes and collected for appropriate disposal at the end of baiting campaigns according to personal communication. Moreover, baits were not applied in stormwater channels.

A total of 7 samplings were carried out at the WWTP between April and June 2019. To study the fate of ARs during wastewater treatment, grab samples included activated sludge, biosolids, sludge liquor, prewashed mineral material from the grit chamber, and grit chamber solids washing water. Twenty-four-hour composite samples of raw wastewater and corresponding treated effluent as well as operational and water quality parameters were kindly provided by WWTP staff. Sediment grab samples from sand traps of two different stormwater retention basins discharging into small tributary creeks were also provided by public services. Due to expected substantial dilution of effluent discharges in the receiving stream, surface water grab samples upstream and downstream of WWTP B were collected less frequently than WWTP samples. As described earlier, surface water quality parameters and characteristics of solid samples were either measured in situ with sensors or determined in laboratory according to Standard Methods (Wasserchemische Gesellschaft and
Normenausschuss Wasserwesen im DIN 2020). Downstream of WWTP B, two sedimentation boxes were suspended in the river from overhanging structures or buoys near river kilometer (rkm) 167.0 and 186.1, respectively, using steel cables. SPM samples were retrieved after defined time intervals (i.e., 14 days) throughout the sampling campaign. In addition, monthly composites of SPM were obtained from five permanent water quality monitoring stations along Moselle upstream (Perl at rkm 241.9, Palzem at rkm 229.8, and Trier at rkm 196.0) and downstream (Fankel at rkm 59.4 and Koblenz at rkm 2.0) of WWTP B over the course of six months (January – July 2019) by the Federal Institute of Hydrology’s Radiology and Monitoring Department (refer to https://geoportal-wasser.rlp-umwelt.de for details). Aliquots of sediment grab samples that had been collected within a permanent state monitoring program at locations upstream (at rkm 196.0) and downstream (at rkm 184.0) of WWTP B during this period were also kindly provided. An overview of all WWTP and surface water sampling locations at monitoring site B is shown in Figure 2. Due to the lengthy spatial distance, monthly composite SPM sampling locations (with the exemption of Trier at rkm 196.0) are not illustrated in Figure 2. Fish liver (n = 35) and filet (n = 6) samples of chub (S. cephalus), perch (P. fluviatilis), European eel (Anguilla anguilla), European catfish (Silurus glanis), pike-perch (Sander lucioperca), and round goby (Neogobius melanostomus) were received between 2017 – 2020 from three different sites along a 6 km stretch of Moselle approximately 25 km downstream of WWTP B (Table S1). Fish had been caught for food consumption by local fishermen in compliance with German fishing regulation. Handling of fish samples followed a standardized protocol (Bayerisches Landesamt für Umwelt 2012). Tissue samples were individually wrapped in aluminum foil and immediately frozen at -20°C. Sampling, measuring, and dissection of quagga mussels (Dreissena bugensis) followed general procedures described in Schäfer et al. (2012). In general, ten individuals of mussels were pooled per sample to provide enough soft body sample material for subsequent analyses.
One additional sampling site for mussels at rkm 1.0 (i.e., near the confluence of Moselle and Rhine in the city of Koblenz; Table S1) is not depicted in Figure 2.

2.3. Analysis of anticoagulants in environmental matrices

Overall, all eight active ingredients used in biocidal ARs in Germany (i.e., warfarin, chlorophacinone, coumatetralyl, bromadiolone, difenacoum, brodifacoum, difethialone, and flocoumafen) as well as two pharmaceutical anticoagulants (i.e., phenprocoumon, acenocoumarol) were targeted in this monitoring study. Aqueous samples were extracted by solid phase extraction with hydrophilic-lipophilic balanced sorbent material using a modified method by Gómez-Canela et al. (2014b) as summarized in the Supplementary Material (SM). Ultra-sound assisted solvent extraction and dispersive solid phase extraction (dSPE) were used for the extraction and clean-up of WWTP solids (e.g., activated sludge, biosolids, and prewashed mineral material from grit chamber). Details can be found in the SM. Extraction of SPM and sediment samples followed the same procedure but omitted further clean-up by dSPE. Mean recoveries and standard deviations for each analyte are provided in Tables S2 – S3 for selected matrices. Ultra-sound assisted solvent extraction and dSPE cleanup procedures for all biological tissue samples (i.e., soft body mussel, fish liver, fish filet) are described in detail in Regnery et al. (2019b). Total lipid content in homogenized tissue samples was determined according to Smedes (1999). Additional information about all biological samples analyzed in this study is provided in Table S1 in the SM.

All sample extracts were analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) in negative electro-spray ionization mode using an Agilent 1260 Infinity LC (Waldbronn, Germany) coupled with a Sciex 4500 QTrap MS/MS system (Darmstadt, Germany). Instrument specifications and details of the analytical method are provided elsewhere (Regnery et al. 2019b). Individual deuterated internal standards were used for
quantification of target analytes, namely difenacoum-d4, brodifacoum-d4, flocoumafen-d4, phenprocoumon-d5 (all Toronto Research Chemicals, North York, Ontario, Canada), bromadiolone-d5, warfarin-d5, chlorophacinone-d4 (all C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada), and difethialone-d4 (TLC, Aurora, Ontario, Canada). Coumatetralyl and acenocoumarol were quantified based on warfarin-d5. 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. Samples with residual AR concentrations outside the calibration standard range (i.e., 0.01 – 5 ng/mL) were diluted accordingly and reanalyzed. Reported analyte concentrations in biological tissues are based on wet weight, whereas those in all other solids are based on dry weight. Method quantification limits (MQL) were in the low ng/L and ng/g range respectively for all analytes and are summarized in Table S4 in the SM. Values above the method detection limit (MDL) but below the respective MQL are denoted (i.e., parenthesized) when provided in Tables 1 and 2 and Table S1 in the SM.

2.4. Statistical analyses

Statistical analyses were performed using Origin 2017G, version b9.4.0.220 (OriginLab Corporation, Northampton, MA, USA). The significance of the differences of total hepatic AR concentrations in fish between groups was assessed through one-way ANOVA followed by Tukey-Kramer post-hoc test or through student’s t-test when two groups were compared. Statistical differences were considered significant when \( p < 0.05 \).

3. Results and discussion

3.1. Occurrence and fate of anticoagulants in WWTPs and receiving streams

Overall, a total of 242 environmental samples were screened for residues of eight ARs and two pharmaceuticals. Given the multitude of different sample matrices and analytes, Figures 3
and 4 illustrate the detection frequencies of all anticoagulant residues above their MQL in all samples collected at monitoring sites A and B over the course of this study. A summary of average operational and water quality parameters at the investigated WWTP A and WWTP B during respective monitoring campaigns is provided in Table S5. In addition, their daily raw wastewater inflow rates and total organic carbon loads, individual sampling dates, and daily total precipitation in the area recorded at the nearest weather station are depicted in Figures 5 and S1, respectively. Additional water quality parameters of Moselle and Queich as well as characteristics of select solid samples are summarized in Tables S6–S8 in the SM.

 Phenprocoumon was the only target substance that was frequently detected above its MQL in raw and treated wastewater at both WWTP (Table 1). Phenprocoumon is extensively metabolized in humans by hepatic microsomal enzymes (e.g., cytochrome P450 2C9) and is excreted almost entirely as a glucuronide conjugate, with less than 10% of the dose as parent compound (Kasprzyk-Hordern 2010). Higher concentrations of phenprocoumon in effluent compared to corresponding influent samples at WWTP A might be explained by cleavage of glucuronide conjugates during biological treatment. Similar findings were reported by Du et al. (2014) for warfarin. Activated sludge samples revealed no residues of phenprocoumon above MQL at WWTP A. At WWTP B, phenprocoumon was frequently detected at very low levels in samples of activated sludge (0.3 ± 0.1 ng/g dry weight) and biosolids (0.2 ± 0.1 ng/g dry weight). It was also present in sludge liquor (46.8 ± 21.8 ng/L) and grit chamber solids washing water (4.8 ± 1.7 ng/L) (Figure 4). Based on its physicochemical properties (i.e., water solubility of 12.9 mg/L at 20°C and n-octanol-water partition coefficient log $P_{OW}$ of 3.6 at neutral pH), phenprocoumon presumably ranks between first-generation and second-generation ARs. Their water solubility and estimated log $P_{OW}$ at neutral pH are in the range of 267 – 460 mg/L and 0.7 – 2.4 (first-generation) and 0.1 – 18.4 mg/L and 3.8 – 8.5 (second-generation), respectively (Regnery et al. 2019a). Although a slight reduction (i.e., 12 ± 8%) of
phenprocoumon concentrations in wastewater was observed after treatment at WWTP B (Table 1), results indicate poor biodegradability. This is in good agreement with predicted values (i.e., not readily biodegradable according to EPI Suite™ (US EPA 2012)) and previous observations (Regnery et al. 2019b, Wode et al. 2015). Most likely because of dilution effects, its concentrations in surface water samples from Queich and Moselle were five- and fortyfold lower, respectively compared to discharged effluent concentrations (Table 1). Despite short predicted photolytic half-lives, anticoagulants were shown to be hydrolytically stable in water under environmentally relevant conditions and are not expected to partition to the atmosphere (Regnery et al. 2019a).

While warfarin was frequently detected in wastewater and surface water samples at trace level, its concentrations rarely exceeded MQL at both monitoring sites (Figures 3 and 4 and Table 1). The maximum concentration measured in raw wastewater and sludge liquor at WWTP B was 1.5 ng/L. Acenocoumarol was not detected above MQL at all. As warfarin is the only anticoagulant that is concurrently authorized for biocidal and pharmaceutical use, its presence in wastewater and receiving surface waters can be linked to rodent control measures (Regnery et al. 2019b, 2020) as well as consumption of blood-thinning medication by resident population (Ajo et al. 2018, Regnery et al. 2019a, Santos et al. 2013). Like phenprocoumon, warfarin is extensively metabolized in humans and only about 2% of the typical daily prescription dose is excreted as unchanged active ingredient (Crouse et al. 2012, Park 1988). According to IQVIA MIDAS, the annual domestic pharmaceutical use of these blood-thinning agents accounted for 801 kg of phenprocoumon and 26 kg of warfarin in Germany in 2018 (IQVIA 2019). While acenocoumarol is commonly used in neighboring European countries (e.g., The Netherlands, Italy, Switzerland), no product with the active ingredient acenocoumarol has been marketed in Germany since 1993 (DIMDI 2020).
The active ingredients brodifacoum and bromadiolone, which had been applied for rodent control by the municipality, were sporadically detected in samples from various WWTP and environmental compartments at monitoring site A, but concentrations rarely exceeded MQL (Figure 3). After several rain events caused elevated water levels due to runoff (Figure S2), bromadiolone was detected above MQL in a surface water grab sample (4.3 ng/L) from Queich during sampling S8. In addition, it was detected in one SPM sample at sampling S9 just under the MQL of 1.0 ng/g dry weight. At WWTP A, brodifacoum was detected in one 24-hour composite sample of raw wastewater characterized by high proportions of stormwater runoff during sampling S5 (2.3 ng/L) as well as three 24-hour composite samples of treated effluent during samplings S5, S6, and S9 (max. 2.7 ng/L, Figure 5). Brodifacoum was also detected in sandy material obtained from the bottom of a CSO structure (0.5 ng/g dry weight) as well as two grab samples of raw wastewater (max. 2.5 ng/L) that had been directly collected from the main sewer of a baited district at sampling S9. Despite such few quantifiable detections of both ARs in aqueous and solid samples, brodifacoum and bromadiolone were prevalent in liver tissue samples of fish caught in the small stream Queich in 2019 (Figure 3). Residues of difethialone and coumatetralyl were also detected in these fish liver samples, whereas difenacoum, flocoumafen, and chlorophacinone were not detected above MQL in any of the samples at monitoring site A (Figure 3). In contrast, corresponding filet samples contained no anticoagulant residues (Table S1).

At WWTP B, solely chlorophacinone occurred in one biosolids sample (3.2 ng/g dry weight). With the exemption of warfarin, none of the other biocidal anticoagulants were detected above MQL in any of the samples collected at WWTP B. However, brodifacoum (0.5 ng/g dry weight) was detected in sandy material that had been removed from the sand trap of a baited district’s stormwater retention basin during maintenance operations. This stormwater retention structure generally discharges into one of Moselle’s small tributary creeks. Although
none of the analyzed surface water, SPM, sediment, and mussel samples from Moselle revealed quantifiable AR concentrations, residues of second-generation ARs were frequently observed in the liver of indigenous fish, first and foremost brodifacoum and difenacoum. Analyzed corresponding filet samples revealed no residues (Figure 4).

3.2. Evidence of anticoagulant rodenticide emission sources and pathways

Brodifacoum-containing bait blocks were exclusively used during sewer baiting at both communities in the respective year of monitoring (as well as the previous baiting campaign). Applying the calculations of the recently revised emission scenario document for biocides used as rodenticides for combined sewer systems (ECHA 2018b), the worst-case predicted direct and indirect emissions of active ingredient to WWTPs during sewer baiting (i.e., in the time period between initial bait placement and first inspection) amount to approximately 0.1 kg brodifacoum/day at WWTP A and less than 0.03 kg brodifacoum/day at WWTP B (based on provided information such as bait amount and product specifics, refer to sections 2.1 and 2.2). Hence, worst-case predicted brodifacoum concentrations in raw wastewater influent were expected to be in the range of 5.2 ng/L at WWTP A and 1.1 ng/L at WWTP B according to mean daily discharge rates (Table S5). Notably, worst-case predicted brodifacoum concentrations remained below its MQL in wastewater at WWTP B (Table S4).

Few detections of brodifacoum in the 2 ng/L concentration range in raw wastewater and treated effluent at WWTP A confirmed that sewer baiting can lead to indirect (e.g., via rat carcasses, urine and feces) and direct (e.g., via scouring and spillage) release of active ingredients into wastewater. A previous study provided crucial evidence that anticoagulants are not completely removed during conventional wastewater treatment and will enter the aquatic environment by way of effluent discharges (Regnery et al. 2019b). Nonetheless, information about the fate of ARs during conventional or advanced wastewater treatment is
scarce. Baits deployed in the combined sewer system connected to WWTP A faced an increased risk of scouring (e.g., when precipitation causes a sudden increase within the combined sewer system) as remaining baits were usually not removed from the sewers at the end of annual preventive baiting campaigns according to provided information. 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. As depicted in Figure 5 for the monitoring period in 2018, stormwater runoff repeatedly caused a surcharge in the combined sewer system and increased discharge rates at WWTP A (e.g., during sampling S5 when brodifacoum was detected in the influent). Thus, a substantial number of deployed baits were repeatedly immersed in wastewater in the combined sewer due to elevated wastewater levels or backwater. Diffuse release of active ingredients into stormwater runoff from rodent control measures at the surface by residents or pest control professionals (e.g., around buildings) were likely additional emission sources throughout the urban area (Spahr et al. 2020).

Notably, a short-duration extreme precipitation event occurred at monitoring site A on June 11, 2018 (i.e., between samplings S8 and S9) and caused severe flooding of the downtown area due to backwater in the combined sewer system and stormwater channels (Figure 5). Capacities were greatly exceeded at WWTP A and several intermittent CSO retention structures, which resulted in confirmed CSO discharges into Queich. Moreover, WWTP staff reported a large number of dead rats and mounting wires from disintegrated baits that were retained at the screen during this event. It was assumed that the majority of baits in the sewer was affected by scouring (‘worst-case-scenario’) at this event and thus emitted brodifacoum. Obviously, the flash flood also immersed the tamper-resistant bait stations used for surface rat control with bromadiolone that had been deployed near watercourses throughout the urban center. In good agreement, brodifacoum (0.5 ng/g dry weight) as well as traces of
bromadiolone (i.e., just under the MQL) were detected in deposited solids from the bottom of two different CSO structures after this precipitation extreme.

Due to extensive impervious surfaces, densely populated urban and suburban areas are prone to flash floods during short-duration (i.e., hourly) precipitation extremes. A recent study estimated the extent and frequency of environmental impacts due to such heavy rainfall events in Germany between 2005 and 2017. CSOs and thus discharge of highly diluted but untreated sewage directly into receiving surface waters occurred in 65% of the investigated heavy rainfall events (Kind et al. 2019). A substantial increase in short-duration precipitation extremes as a consequence of a changing climate has been predicted (Lenderink and van Meijgaard 2008). 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. According to representative survey results, about 30% of German municipalities that applied rat control in their sewer systems in 2017 also applied AR-containing baits in stormwater channels (Regnery et al. 2020).

As implied by low predicted worst-case concentrations (ECHA 2018b) at WWTP B, ARs were not detected above MQL in WWTP sample matrices throughout the treatment train during normal operation of the associated combined sewer system. Nevertheless, brodifacoum was detected in deposits from the sand trap of one of the city’s stormwater retention basins. Emissions likely resulted from diffuse release of active ingredients into stormwater runoff during outdoor rodent control as AR-containing bait was generally not applied in or near the city’s stormwater infrastructure according to personal communication. Though no samples of deposits from CSO retention structures became available for analysis at monitoring site B during the sampling period, CSO discharges at WWTP B after heavy or prolonged rainfall most likely contributed to emissions of ARs into Moselle. Similar to what had been observed
at monitoring site A in 2018, heavy rainfall at site B (i.e., 37 mm rainfall in one hour) caused flash flooding in several baited city districts shortly after the end of the monitoring campaign (Figure S1). As illustrated in Figure 2, several smaller conventional municipal WWTP (mostly cluster WWTP) discharge into Moselle downstream of WWTP B. The neighboring association of municipalities located North-East of Trier (approximately 28,200 inhabitants total) is connected to the next smaller WWTP along this stretch of Moselle. When surveyed via telephone, their municipal pest control official confirmed the annual use of approximately 300 bait blocks (i.e., 200 g each containing 0.005% of difenacoum) for sewer baiting among all 19 municipalities in 2019. After reported rat sightings above ground by residents, trained municipal workers applied baits in the combined sewer system of the respective municipality. According to this communication, difenacoum-containing baits had been used for sewer baiting since 2013. Hence, municipal sewer baiting activities can explain the observed hepatic brodifacoum and difenacoum residues in fish from monitoring site B (Table 2). In good agreement, mainly baits containing brodifacoum, difenacoum, or bromadiolone were used for sewer baiting in Germany in 2017 (Regnery et al. 2020), whereas difethialone and coumatetralyl are preferably used in agriculture (Koivisto et al. 2018, Regnery et al. 2019b).

Previous studies suggested that unlike high-volume pharmaceutical anticoagulants, biocidal anticoagulants are difficult to capture in routine surface water or WWTP monitoring schemes due to the transient character of AR input rates as well as elevated detection limits compared to predicted environmental concentrations for these compartments. In Germany, difenacoum, brodifacoum, and bromadiolone were estimated to exhibit the highest market shares of AR active ingredients based on registered commercial biocidal products. Still, actual quantities of these active ingredients applied as rodenticides appear minor compared to other high-volume chemicals (Regnery et al. 2019a). Results summarized in Figures 2 and 3 on the one hand corroborate the assumption that actual AR concentrations in WWTP influent, effluent, and
receiving surface waters are generally either too low or/and sporadic to be routinely monitored using current analytical methods, even with extensive sample enrichment and cleanup. On the other hand, they demonstrate that analyses of biological tissue samples provide crucial information regarding the burden of the aquatic environment with these PBT/vPvB substances as fish reflect an average exposure to AR emissions over time.

### 3.3. Hepatic residues of anticoagulant rodenticides in fish

Recent research demonstrated that second-generation ARs bioaccumulate in fish liver under environmentally realistic conditions and exposure scenarios (Kotthoff et al. 2019, Regnery et al. 2019b). Anticoagulants’ high protein binding capacity and the persistence of specifically second-generation ARs in liver tissues of terrestrial wildlife is well documented (Horak et al. 2018). So although it is generally difficult to link hepatic AR residues in wild fish to distinct exposure events, there is an undeniable relationship between flushed away brodifacoum-containing baits during sewer baiting in 2018 at site A and elevated brodifacoum residues (5.2 – 29.9 ng/g wet weight) in the liver of all analyzed fish from the small stream Queich in 2019 (Table 2). As mentioned earlier, the effluent contribution of WWTP A under dry weather condition is approximately 7%. Moreover, frequent detections of hepatic bromadiolone residues (max. 1.0 ng/g wet weight) confirmed the assumption that outdoor surface baiting in the vicinity of watercourses also represents a prominent emission source of ARs into the aquatic environment. All fish from Queich were captured at the same time and location approximately 2 km downstream of WWTP A’s discharge point one year after the flash flood incident (Figure 1). Distinct hepatic phenprocoumon residues (0.03 – 0.22 ng/g wet weight) due to the high-volume use and release of phenprocoumon indicated their frequent exposure to high WWTP effluent contributions in the small stream. Yet, the median concentration of phenprocoumon in these samples is < 1% that of the concentration of brodifacoum (Table 2). Unlike second-generation ARs, previous findings and an estimated bioconcentration factor
(BCF) of 122.3 L/kg in fish (BCFWIN v2.17, US Environmental Protection Agency) already pointed towards marginal bioaccumulation potential of phenprocoumon in native aquatic organisms (Regnery et al. 2019b). In contrast, the estimated BCF for brodifacoum in fish is 35,648 L/kg (Regnery et al. 2019a). In order to provide catches for recreational fishing, the Queich is stocked with hatchery-reared brown trout (i.e., individuals between 30 – 35 cm total length and 0.3 – 0.5 kg total weight) each year in late February/early March by the local fishing association. The analyzed brown trout individuals with total hepatic anticoagulant residues of 19.0 and 32.6 ng/g wet weight had likely been released in spring 2018 based on their total length of 37 and 40 cm, respectively (Table S1). Notably, no anticoagulant residues were detected in liver samples of unexposed hatchery-reared fish analyzed as reference material (Regnery et al. 2019b).

Elevated hepatic residues of brodifacoum (max. 19.8 ng/g wet weight) and difenacoum (max. 16.5 ng/g wet weight) were also detected in fish from the considerably larger stream Moselle that had been caught between 2017 and 2019 about 25 – 30 km downstream of WWTP B (Table 2), presumably as a consequence of sewer baiting as mentioned above. Available experimental (1100 L/kg) and estimated (451, 9010, and 35,645 L/kg) BCF of difenacoum in fish are similar to those of brodifacoum (Regnery et al. 2019a). Interestingly, no AR residues were detected above MDL in pooled mussel samples from five different Moselle sampling sites. Two sites thereof corresponded with sampling locations of fish with hepatic AR residues. This is in good agreement with findings from Main River (Regnery et al. 2019b) and suggests that AR bioaccumulation processes fundamentally differ among these aquatic organisms and are not just driven by lipophilicity (Table S1).

Unfortunately, substantial data gaps exist regarding the understanding of AR uptake routes (e.g., aqueous uptake of water-borne chemicals, dietary uptake by ingestion of contaminated
food, prey, or particles) in freshwater environments. Overall, hepatic AR residues were found in fish species from all sections of the investigated surface water bodies: sub-surface (e.g., chub), mid-water (e.g., perch, roach, brown trout), and benthic (e.g., gudgeon, round goby). It has been assumed that the abundance of ARs in liver of fish species is likely correlated with their feeding habits (Regnery et al. 2019a). At the small stream Queich, highest brodifacoum concentrations of 25.1 and 29.9 ng/g wet weight liver were observed in common gudgeons (Table S1), which inhabit freshwater habitats with sandy or gravelly bottoms and predominantly feed on benthic invertebrates (e.g., worms, aquatic insects and larvae, small mollusks). Gammarids exposed to wastewater effluents (Miller et al. 2019, Munz et al. 2018) or terrestrial invertebrates feeding on AR-containing bait may function as vector in the environment (Masuda et al. 2014, Pitt et al. 2015). Based on their physicochemical properties (e.g., not readily biodegradable, low water solubility, high lipophilicity, and photolytic instability) at ambient environmental conditions, second-generation ARs might occur particle-bound in wastewater effluents rather than freely dissolved. The potential formation of non-extractable residues of second-generation ARs in organic-rich matrices such as activated sludge under environmentally realistic exposure is thus another aspect that should be considered. Irreversible binding of phenolic compounds to humic substances and activated carbon is well documented (Burgos et al. 1996). Interactions of natural organic matter and AR can involve numerous mechanisms depending on functional groups (e.g., phenolic hydroxyl group) and environmental conditions (e.g., pH, ionic strength). In addition to hydrophobic interactions, complexation may result from ion exchange reactions, ion bridging, and hydrogen bonds (Andre et al. 2005, Delle Site 2001). Covalent binding reactions such as biologically-mediated or mineral surface-catalyzed oxidative coupling may account for irreversible binding of ARs to natural organic matter after adequate exposure (Burgos et al. 1996); bindings that might be reversed through metabolic processes in fish after ingestion (Tao et al. 2011). Two primary classes of enzymes involved in these biological coupling
reactions, peroxidase and phenol oxidase, for example belong to the microbial community functional structure of activated sludge in municipal WWTPs (Wang et al. 2014). Bioavailability of non-extractable pesticide residues and their potential transfer along the food chain was already suggested for soil-dwelling organisms such as earthworms (Barois et al. 1993, Gevao et al. 2001). If this assumption extrapolates to fish, benthivorous fish potentially play a key role in the transfer of second-generation ARs through the trophic levels of the aquatic food web.

Highest total AR concentrations measured in omnivorous feeders such as roach and chub from Queich (11.3 and 10.2 ng/g wet weight, respectively) and Moselle (9.2 ng/g wet weight) were similar to those observed in the liver of chub individuals from other German streams with comparable effluent contributions (Regnery et al. 2019b). Fish with a predominantly piscivorous diet (i.e., pike-perch and perch with a total length exceeding 15 cm) experienced on average significantly higher total hepatic AR concentrations (20.4 ± 7.8 ng/ng wet weight, n = 13) compared to omnivorous feeders (5.8 ± 4.0 ng/g wet weight, n = 13) in this study, t(18) = 6.04, p < 0.001. A one-way analysis of variance showed that total hepatic AR concentrations differed significantly among different fish species caught at the same monitoring site (Figure 6), F(4, 29) = 30.46, p < 0.001. In Moselle, adult perch primarily prey on round goby, an overabundant invasive species utilizing benthic habitats (Borcherding and Gertzen 2016). Post-hoc comparisons using the Tukey-Kramer test indicated significant differences between both fish species (p < 0.001) as shown in Figure 6 and illustrate the biomagnification potential of very persistent second-generation ARs such as brodifacoum in the aquatic food chain when released into the aquatic environment.

3.4. Potential risks for fish and fish-eating predators
During environmental risk assessment of ARs under the BPR, no unacceptable risks had been identified for the aquatic compartment despite considerable acute toxicity of several ARs to aquatic species. The predicted environmental concentration (PEC) of the active substances in surface water calculated for the sewer baiting scenario (ECHA 2018b) was below the corresponding predicted no-effect concentration (PNEC), above which adverse effects to aquatic organisms were to be expected (Umweltbundesamt 2019). Experimental monitoring data for the aquatic environment and WWTP effluent (this study and reviewed in Regnery et al. (2019a)) corroborate these minor surface water PEC. Concomitantly, biota monitoring demonstrates the widespread emergence of ARs in liver tissue of freshwater fish.

AR’s toxic mode of action in warm-blooded vertebrates (mammals, birds) is caused by the inhibition of vitamin K epoxide reductase, which results in the disruption of the carboxylation of clotting factors and, subsequently, the clotting cascade (Rattner et al. 2014). In terrestrial non-target species, hepatic AR residue levels of > 100 to 200 ng/g wet weight were associated with lethality (Fourel et al. 2017). In comparison, highest total hepatic AR levels measured in fish were 35 ng/g wet weight (Figure 6 and Table S1). While species-specific differences between fish and mammals regarding their sensitivity towards AR likely exist (Riegerix et al. 2020), sub-lethal effects may impair the fitness of individuals (i.e., anticoagulants could act as stressors). To date, direct links to physiological effects at environmentally relevant concentrations other than those directly caused by impaired blood coagulation are unknown (Rattner et al. 2014, 2020) especially with regard to chronic exposure with multiple active ingredients. According to Rattner et al. (2014), adverse effects associated with AR exposure of non-target wildlife are manifold, including factors such as impaired body condition and reproduction, increased susceptibility to disease, reduced resilience to extreme weather conditions, sensitivity to other contaminants, and disturbance of population dynamics.
Anticoagulants entering the aquatic environment and accumulating in indigenous freshwater fish are likely to be transferred in the food chain, potentially affecting the health of fish-eating predators (e.g., protected species such as European otter and common kingfisher). While the European otter diet is dominated by fish (85% of consumed biomass), the common kingfisher is an almost exclusive fish-eater. Similar to other fish-eating birds swallowing their prey as a whole, the upper size limit of consumed fish is clearly dependent on its body shape. Total hepatic AR residues detected in fish from Moselle and Queich illustrate not only a substantial AR burden in large predatory fish but also small-growing fish species with a shorter life span (i.e., less than 5 years) such as round goby and gudgeon (Figure 6 and Table S1). The gudgeon for instance is a common prey of many fish-eating predators like European otter or common kingfisher (Čech and Čech 2015, Lyach and Čech 2017). A case study from a lowland trout stream in the Czech Republic revealed that otters mostly preyed upon small-growing fish species. The authors reported that gudgeon was the most important otter prey and represented 38% of consumed biomass (Lyach and Čech 2017). So far, several monitoring studies reported residues of ARs in the livers of avian and mammalian predators with a fish-eating diet, e.g., in European otter (Serieys et al. 2019), American mink (Ruiz-Suarez et al. 2016), white-tailed sea eagle, and osprey (Hughes et al. 2013).

The high bioaccumulation potential of second-generation ARs via the aquatic food web therefore may pose an increased threat to (higher) aquatic organisms and fish-eating predators, which is also reflected in the environment risk assessment of secondary poisoning via the aquatic food chain. The corresponding PEC/PNEC ratios for rodenticide product formulations containing 0.0029% brodifacoum were 532.5 and 968, respectively, indicating high unacceptable risks of secondary poisoning for fish-eating mammals and birds (ECHA 2018a). However, these risk calculations are mostly based upon generic equations and default assumptions as experimental data for the determination of BCF and biomagnification factors
as well as chronic oral toxicity tests covering most sensitive aquatic focal predators for the
derivation of PNEC_{oral,predator} are lacking. Dedicated research to identify focal species of the
aquatic food chain is required to address these uncertainties and to evaluate the extent to
which apex predators are affected by ARs. Moreover, ecotoxicological consequences of
chronic rodenticide exposure to indigenous freshwater fish at concentrations relevant for
surface water bodies have not been identified yet.

3.5. Risk mitigation measures for the aquatic environment

Although mandatory instructions for use and risk mitigation measures for ARs were stipulated
at EU-level under the BPR and best practice guidelines were stipulated during national
biocidal product authorizations to minimize the risks of environmental exposure
(Umweltbundesamt 2019), the extent of compliance with these provisions in Germany is
largely unknown. Furthermore, weaknesses exist due to missing national legal provisions on
the sale of biocides (Regnery et al. 2019a). Several studies noticed that the typical use of ARs
commonly violates respective use and disposal instructions (Koivisto et al. 2016, Regnery et
al. 2019b, 2020). While almost 80% of 322 municipalities participating in a nationwide
survey applied chemical rat control in their sewer systems in 2017 in Germany, only 31% of
municipalities thereof conducted a monitoring prior application of rodenticides to confirm the
presence of rats in their sewer systems. Even less (i.e., about 26%) collected remaining baits
for appropriate disposal and efficiency control at the end of sewer baiting campaigns
(Regnery et al. 2020). Considering that bait intake during preventive rodent control measures
in sewers might not exceed 2% of baited manholes (Gras et al. 2012), it becomes apparent
that large amounts of bait face an unknown fate in the sewers. Yet, the implementation of
legally binding instructions for use and risk mitigation measures for ARs starting from 2012
already led to a substantial decrease in bait amounts used for sewer baiting over the last
decade in Germany (Regnery et al. 2020). However, temporal trend analysis (1992 – 2015) of
residual brodifacoum concentrations in fish liver samples from two German streams (Saar and Elbe) revealed no decreasing trend until 2015 (Kotthoff et al. 2019). Concordantly, regulatory restrictions and stewardship programs failed to prove an impact on AR residue abundances in terrestrial non-target predators in urbanized areas in Denmark (Elmeros et al. 2018) and the United Kingdom of Great Britain (CRRU UK 2020) until now. Recently, regulation (EU) 2016/1179 resulted in the market launch of second-generation ARs with reduced concentrations of active ingredient (i.e., below 0.003%) to circumvent limitations of use in the biocidal sector due to the classification of products as toxic for reproduction (Regnery et al. 2019a). The application of commercial products with lower doses that are still efficient in controlling rodents, e.g. as shown for brodifacoum (Frankova et al. 2019), may assist in reducing the ecological impacts and environmental residues of ARs.

The risk of active ingredient release during chemical rodent 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). Commercial paraffin-type rodent bait blocks are expected to have a long shelf life and thus efficacy (toxicity) of the active ingredient, even after extended placement in sewers (Papini et al. 2019). However, they disintegrate over time under common sewer conditions such as permanently high humidity or frequent contact with wastewater, resulting in unintended emissions to the WWTPs and receiving surface waters (ECHA 2018b). Hence, immediate collection and appropriate disposal of remaining bait at the end of baiting campaigns is crucial to prevent unintended direct emissions of the active ingredient into wastewater. As discussed earlier, unforeseen short-duration precipitation extremes will likely occur more often in the future (Lenderink and van Meijgaard 2008). Though mandated by the instructions for use, reactive approaches such as timely removal of all baits deployed by wire in combined sewers during widespread baiting
campaigns to prevent scouring are not feasible under such circumstances. In order to confine emissions solely to release from carcasses, urine, and feces of poisoned rats, proactive measures are advised to avoid any bait contact with water (ECHA 2018b). Such measures may involve the use of waterproof bait protection stations designated for the safe application of ARs in sewer systems, or advanced trap systems, which can be operated low maintenance for several months without using toxins. Traps and bait stations for controlling rats in sewers are often equipped with sensors and allow transmission of monitoring data to remote computers or smartphones, making manual inspections of baiting points unnecessary. This is a clear advantage as it substantially reduces time, labor, and human exposure, and provides a near real-time documentation of rat activity. Nevertheless, there is an urgent need for integrated, rat ecology based approaches (Byers et al. 2019, Traweger et al. 2006) alongside those technical innovations to achieve a major shift in rodent control practice and minimize the use of ARs in urban rat management.

4. Conclusions

This comprehensive monitoring study identified sewer baiting in combined sewer systems as substantial contributor of ARs in the aquatic environment. In conjunction with heavy or prolonged precipitation during bait application in combined sewer systems, a direct link between sewer baiting and AR residues in WWTP influent, effluent, and the liver of freshwater fish was demonstrated. Moreover, results confirmed insufficient removal of anticoagulants such as phenprocoumon, warfarin, and brodifacoum during conventional wastewater treatment and thus indirect exposure of aquatic organisms in receiving streams via WWTP effluents and CSOs. Outdoor surface baiting in the vicinity of watercourses and untreated storm drain in the presence of ARs also contributed to AR emissions in streams.
The high detection frequency of second-generation ARs in fish liver samples of up to 100% contrasted the low detection frequency of ARs above MQL in environmental samples from WWTP effluent, surface water, sediment, or SPM and clearly illustrates that environmental exposure involving PBT and vPvB substances such as second-generation ARs may be independent from the time and place of application of biocidal products. However, for substances where possible adverse effects are not correlated to immediate exposure, the PEC/PNEC approach as an indicator for environmental risks within the regulatory assessment of biocides reveals shortcomings. Thus, hazard assessments of PBT/vPvB substances are an appropriate and necessary instrument of the foresightful European chemical regulation. Although several criteria for exclusion are fulfilled to bar ARs from approval as biocidal active substances under the BPR, the European Commission decided that their non-authorization would have disproportionate adverse health impacts for human society in comparison to the predicted environmental risks arising from their use. Further research should investigate the potential risks and hazards of ARs in the aquatic environment in order to pave the way for scientific-based, targeted, and effective regulatory decisions. Until then, the ecological implications for aquatic organisms as well as fish-eating predators remain largely unknown.

Acknowledgement

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https://doi.org/10.1016/j.watres.2014.11.034
**Figure 1.** Generalized map of monitoring site A illustrating all sampling locations (orange arrows). Grey shapes indicate urban and suburban settlements. Abbreviations: CSO = combined sewer overflow; PE = person equivalent; SPM = suspended particulate matter; WWTP = wastewater treatment plant. Refer to Table S1 for further information about biological samples.

**Figure 2.** Generalized map of monitoring site B showing sampling locations (orange arrows) in the vicinity of the studied wastewater treatment plant (WWTP B). Grey shapes indicate urban and suburban settlements. Abbreviations: PE = person equivalent; rkm = river kilometer; SPM = suspended particulate matter. Refer to Table S1 for further information about biological samples.

**Figure 3.** Detection frequency of anticoagulant residues above their respective method quantification limits (MQL) in samples (total n = 90) from different WWTP and aquatic compartments collected at monitoring site A.

**Figure 4.** Detection frequency of anticoagulant residues above their respective method quantification limits (MQL) in samples (total n = 152) from different WWTP and aquatic compartments collected at monitoring site B.

**Figure 5.** Mean daily discharge (blue squares) and total organic carbon (TOC) load (black spheres) at WWTP A as well as individual sampling dates (S1 – S10). The green-shaded area illustrates the duration of the sewer baiting campaign. Daily total precipitation (grey bars) recorded at a nearby weather station was obtained from Agrar-Meteorologie Rhineland-Palatinate.

**Figure 6.** Box plots of total hepatic anticoagulant residue concentrations in individual fish (blue diamonds) at monitoring site B grouped by fish species and predominant diet (refer to Table S1 for details). The letters indicate statistical differences (Tukey-Kramer post-hoc test, $p < 0.05$) between the mean concentrations (grey squares) of perch (24.2 ± 7.3 ng/g), pike-perch (14.2 ± 4.9 ng/g), European eel (3.9 ± 2.6 ng/g), round goby (4.7 ± 3.1 ng/g), and chub (3.6 ± 3.5 ng/g).
Table 1. Mean concentrations and standard deviations of warfarin and phenprocoumon in corresponding 24-hour composite samples of raw and treated wastewater as well as surface water grab samples of receiving streams at monitoring sites A and B. ‘<’ indicates values below the respective method detection limits, values denoted in parenthesis are below method quantification limits. ND = not detected.

<table>
<thead>
<tr>
<th>Monitoring site</th>
<th>n</th>
<th>Warfarin (ng/L)</th>
<th>Phenprocoumon (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(ng/L)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WWTP influent</td>
<td>10</td>
<td>(0.5 ± 0.2)</td>
<td>20.0 ± 10.4</td>
</tr>
<tr>
<td>WWTP effluent</td>
<td>10</td>
<td>&lt;0.3</td>
<td>28.0 ± 15.9</td>
</tr>
<tr>
<td>Queich prior WWTP outfall</td>
<td>10</td>
<td>(0.1 ± 0.1)</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>Queich after WWTP outfall</td>
<td>10</td>
<td>(0.1 ± 0.1)</td>
<td>5.8 ± 3.2</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WWTP influent</td>
<td>7</td>
<td>(0.8 ± 0.4)</td>
<td>24.2 ± 11.4</td>
</tr>
<tr>
<td>WWTP effluent</td>
<td>7</td>
<td>&lt;0.3</td>
<td>21.1 ± 8.8</td>
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<tr>
<td>Moselle prior WWTP outfall</td>
<td>4</td>
<td>ND</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Moselle after WWTP outfall</td>
<td>4</td>
<td>(0.1 ± 0.0)</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>
Table 2. Analyte detection frequencies, median, 95th percentile, and maximum concentrations of anticoagulants in fish liver samples from two different streams. Analyte concentrations are reported in ng/g relating to wet weight. ‘<’ indicates values below the respective method detection limits, values denoted in parenthesis are below method quantification limits. ND = not detected.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Liver tissues from Queich, n = 11</th>
<th>Liver tissues from Moselle, n = 35a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency ( % )</td>
<td>Median ( ng/g )</td>
</tr>
<tr>
<td><strong>Rodenticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brodifacoum</td>
<td>100</td>
<td>12.2</td>
</tr>
<tr>
<td>Bromadiolone</td>
<td>90.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Difenacoum</td>
<td>36.4</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Flocoumafen</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Difethialone</td>
<td>45.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Chlorophacinone</td>
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<td>ND</td>
</tr>
<tr>
<td>Coumatrelatyl</td>
<td>81.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Warfarin</td>
<td>36.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Σ Rodenticides b</strong></td>
<td>100</td>
<td>12.7</td>
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<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>100</td>
<td>0.10</td>
</tr>
<tr>
<td>Acenocoumarol</td>
<td>45.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

a At least one of eight ARs detected. ARs were summed for each specimen, with median presenting the rank order 6th value for Queich River and the rank order 18th value for Moselle River.
b Reduced number of samples (n = 30) analyzed for pharmaceutical anticoagulants.
CRediT author statement

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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.
Graphical abstract

**Highlights**

- Anticoagulant rodenticides (AR) are emitted to the aquatic environment
- Chemical rodent control in combined sewer systems contributes to AR emissions
- AR are not sufficiently removed during conventional wastewater treatment
- Untreated storm drain in the presence of AR also contributes to emissions
- Second-generation AR accumulate in the liver of wild freshwater fish
Figure 4
Figure 5
Figure 6

Total hepatic AR conc. in ng/g wet weight

- Piscivore
  - Perch (8)
  - Pike-perch (3)
  - Europ. eel (11)
- Inverti-piscivore
  - Round goby (4)
- Omnivore
  - Chub (8)

Figure 6