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Exposure assessment of anticoagulant rodenticides in the liver of red foxes (*Vulpes vulpes*) in Slovenia

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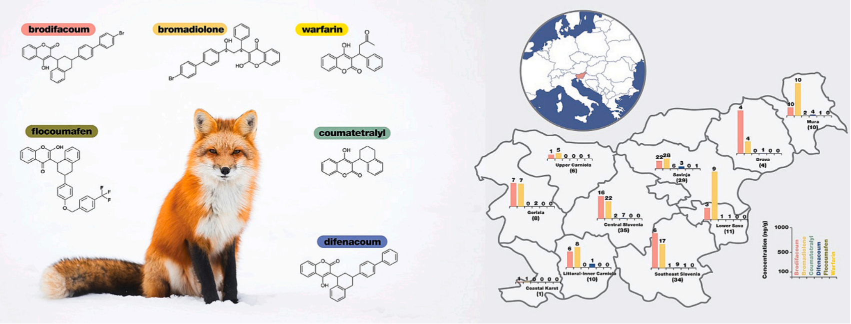
HIGHLIGHTS

- This is the first study of ARs in non-target animals in the Western Balkans.
- The results show a serious toxicological risk for red foxes in Slovenia.
- A potential environmental problem in this region is indicated.
- Much higher AR residue levels were found compared to those at other monitoring studies.

GRAPHICAL ABSTRACT



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ABSTRACT

The study deals with the environmental residues of anticoagulant rodenticides (ARs) in Slovenia to evaluate the toxicological risk of secondary poisoning of red foxes (*Vulpes vulpes*) as representatives of non-target wildlife, and in relation to the investigated use patterns of ARs and specific local parameters in Slovenia. From 2019 to 2022, 148 liver tissue samples of adult red foxes were collected from almost all state geographical regions. The samples were extracted with methanol/water (2:1, v/v), cleaned-up using a solid supported liquid-liquid extraction, and measured by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) with reporting limits of 0.5 to 5.0 ng/g. Residues of at least one rodenticide were detected in 77.7 % of the samples. The second generation ARs of bromadiolone, brodifacoum and difenacoum were the most frequently found, appearing in 75.0, 51.4, and 18.9 % of the samples, respectively. Concentrations of pooled ARs ranged from 1.5 to 2866.5 ng/g with mean and median values of 601.4 and 350.2 ng/g, respectively. We determined bromadiolone and brodifacoum at concentrations of ≥800 ng/g in 10.8 and 10.1 % of the samples, and 1.4 and 0.7 % of the samples contained residues >2000 ng/g, respectively. These concentrations are much higher than those found in comparable studies in Europe and elsewhere in the world. Residues of ARs were detected in all monitored statistical

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regions of Slovenia, with higher concentrations in the eastern parts of the country. First generation ARs were found in only 9.5 % of samples, and residues were below 10 ng/g with one exception (coumatetralyl with 55 ng/g). The results of the study indicate a serious toxicological risk for red foxes in Slovenia as part of the Western Balkans, and will contribute to the growing body of knowledge about the protection of European ecosystems, as wildlife is not limited by national borders.

1. Introduction

Anticoagulant rodenticides (ARs) are the principal pesticides used to control commensal rodents, being antagonists of the vitamin K metabolism. They thus impair and even prevent blood clotting and cause lethal bleeding in rodents. Inhibition of the hepatocyte predominant membrane protein vitamin K epoxide reductase (VKOR) by ARs prevents the reduction of vitamin K epoxide and vitamin K to their biologically active form vitamin K hydroquinone, which is required for carboxylation of the blood clotting factors II, VII, IX and X (Furie et al., 1999). Such a toxic effect is common to all vertebrates, so ARs can also be dangerous to non-target organisms such as predators and scavengers in the environment, especially birds, wildlife, and domestic mammals, which usually experience secondary poisoning when they eat weakened or dead rodents affected by ARs (Sage et al., 2008; Rattner et al., 2014). Even exposure to low doses of ARs results in subtle behavioural and pathological changes, and such animals can be weakened in the long term and have slower responses, and are thus more susceptible to accidents, predation, and infection (King, 1983; Fournier-Chambrillon et al., 2004; Brakes and Smith, 2005). Indeed, recent findings suggest possible correlations between infectious diseases and concentrations of ARs in affected non-target animals (Carrera et al., 2023).

In this context, the analysis of ARs in liver samples from foxes and other species addresses the problem of the bioaccumulation of anthropogenic substances in the animal food chain (Elliott et al., 2016; Geduhn et al., 2015, 2016; Walther et al., 2021; Badry et al., 2021). This is important, as 58 % of avian and mammalian predators worldwide have been shown to contain the residues of one or more ARs (López-Perea and Mateo, 2018; Shore et al., 2018). In particular, the second generation ARs (SGARs), so-called superwarfarins (Feinstein et al., 2016), are highly persistent, bioaccumulative and toxic, which indicates their increased environmental risk. In France, SGAR residues were found in 95 % of common buzzards (*Buteo buteo*) (Berny et al., 1997; Lambert et al., 2007) and 88 % of foxes (*Vulpes vulpes*) (Berny et al., 1997), in Denmark in 93 % of tawny owls (*Strix aluco*) (Christensen et al., 2012), and in Spain in 58 % of stone martens (*Martes foina*) (Sánchez-Barbudo et al., 2012). Some studies have already shown the effects of SGAR with regard to reducing the number of foxes in areas where ARs have been used to control rodent populations (Proulx and MacKenzie, 2012; Jacquot et al., 2013). Exposure of non-target organisms to ARs – mainly through secondary poisoning, which is particularly hazardous on a long-term basis (Berny, 2007) – and their biological concentration, is therefore a major problem. However, research on this and related issues is currently scarce or insufficient, the consequences of such exposure remain uncertain, and there is no general alternative approach for the sustainable management of rodents (Coeurdassier et al., 2014). A holistic risk assessment of the factors involved in secondary poisoning is therefore necessary, and should be based on biomonitoring of environmental contamination with ARs and assessment of their toxic effects on non-target species in the environment (van den Brink et al., 2018).

The red fox (*Vulpes vulpes*) is the most widespread and adaptable wild carnivore in the world (Lindsø et al., 2022; Kobryn et al., 2023), and can be found all over Europe (Delcourt et al., 2022), including Slovenia (Rataj et al., 2010), having adapted its activity level and behaviour to many different habitats (Gil-Fernández et al., 2020). As a result of the successful control of rabies by oral vaccination, an increase in the population density of the red fox has been observed in most European countries (Deplazes et al., 2004; Holmala and Kauhala, 2006). Consequently, foxes have increasingly expanded their range to urban and suburban areas in recent decades (Deplazes et al., 2004). This rise in the red fox population can also be seen in Slovenia, where the hunting bag of red foxes killed increased from 7906 in 2005 to 15997 in 2021 (Slovenia-Forest-Service, 2022). The red fox is an opportunistic predator and scavenger that preys mainly on small mammals, lagomorphs, and numerous birds (Delcourt et al., 2022; Kämmerle and Storch, 2019), as well as carrion, plants, invertebrates, and reptiles (Soe et al., 2017). In agricultural areas of Europe rodents are the main diet of foxes (Soe et al., 2017), while in rural areas small mammals are the second most important component after anthropogenic food (Panek and Budny, 2017). Overall, microtus voles appear to be the main prey group of foxes in Europe (Goldyn et al., 2003; Dell'Arte et al., 2007; Pagh et al., 2015; Panek and Budny, 2017; Lanszki et al., 2023).

An examination of the relevant literature revealed that data on the monitoring of secondary exposure to ARs among wildlife have been obtained for in North America, Western Europe, Australia and New Zealand (López-Perea and Mateo, 2018), with a relative lack of such data in the rest of the world. Therefore, the aim of the present study was to open up this topic for Slovenia as a part of Europe and the Western Balkans region, using red foxes as a representative non-target species, and thus to make a contribution to European environmental protection as a whole.

1.1. *Authorisation and use of anticoagulant rodenticides in Slovenia*

ARs are subject to authorisation in the European Union (EU) under the Biocides Regulation (EU) No. 528/2012 (European Union, 2012). From a historical perspective, when it gained independence in 1991 Slovenia adopted the related law of the former Yugoslavia, the Act on Trade in Poisons, published in the Official Gazette of the Republic of Slovenia (RS), No. 16/1991. This was then later replaced by the Act on Biocidal Products, published in the Official Gazette of RS, No. 61/2006, 77/2011 and 25/2014, and today all ARs are authorised as biocides in Slovenia (Republic of Slovenia, 2014b, 2018). By January 9, 2023, a total of 5369 biocidal products (trade names) are authorised, of which 152 contain ARs (Table 1) (Republic of Slovenia, 2023a). The number of such products has increased by 11 % since 2021.

Regarding the consumption of ARs in Slovenia, the only relevant data on estimated professional use were obtained from the authorised performers of disinfection, disinsection and deratisation (DDD) in the country, to whom we sent the questionnaire on their use of ARs in the period 2016–2020, and the summarised results are presented in this paper. Of the total 44 providers of DDD activities authorised by the Ministry of Health of the Republic of Slovenia, 33 (75 %) responded, and of the total 46 concessionaires for the performance of DDD activities in

Table 1
Current number of authorised biocidal products (trade names) with ARs in Slovenia (Republic of Slovenia, 2023a).

Active substance	Number of products
Brodifacoum	51
Bromadiolone	64*
Difenacoum	28*
Difethialone	2
Flocoumafen	7
Coumatetralyl	1
*One product contains two substances	Sum 152*

the veterinary field authorised by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia, 44 (96 %) responded. Twenty-two respondents were authorised by both of these ministries. The data on estimated professional use of ARs (Fig. 1) indicate a general growth in the use of ARs, with a total of 1.677 kg used in 2020, and generally reflect the range of authorisations of these substances (Table 1). Bromadiolone, brodifacoum and difenacoum, ARs with the most registrations, were also the most widely used in Slovenia from the professional point of view, with the total use in 2020 of 1.054, 0.186 and 0.252 kg, respectively. As the share of the use of bromadiolone and difenacoum in the total use of ARs was relatively constant in the period 2016–2020, averaging 62 and 15 %, respectively, an increasing trend can be observed for the use of brodifacoum, from 90 g to 201 g in the period 2016–2019, which is an increase of 223 %.

2. Materials and methods

2.1. Sample collection

Liver tissue samples from 148 red foxes of different ages and sexes (male, n = 89; female, n = 59) were collected between 2019 and 2022. Fox carcasses were collected as part of the regular annual harvest. Volunteer gamekeepers and professional game wardens from 55 hunting districts and five special purpose state hunting grounds provided fox carcasses. Ten (out of 12) geographical regions of Slovenia were included in the survey, namely Mura (n = 10), Drava (n = 4), Savinja (n = 29), Lower Sava (n = 11), Southeast Slovenia (n = 34), Littoral Inner Carniola (n = 10), Central Slovenia (n = 35), Upper Carniola (n = 6), Gorizia (n = 8) and Coastal Karst (n = 1) (Republic of Slovenia, Statistical Office, 2023a; Perko and Ciglič, 2020). A total of 83 carcasses were collected from the Lower Sava, Southeast Slovenia, Littoral Inner Carniola, Central Slovenia, and Upper Carniola regions in the period from October 2019 to January 2020, and a total of 65 carcasses were collected from the Mura, Drava, Savinja, Gorizia, Coastal Karst, Central Slovenia, Upper Carniola and Littoral Inner Carniola regions in the period from September 2021 to February 2022. Both time sampling intervals can be treated as a single interval because there was no major variation in the natural environment of red foxes that could affect their contamination with ARs in the overall period. Carcasses were collected by the Institute of Pathology, Wild Animals, Fish and Bees (IPWFB) at the Veterinary Faculty of University of Ljubljana, Slovenia, or by the Veterinary Hygiene Service, which operates in affiliation with the Veterinary Faculty. The date, sex, age class, and location of each carcass were recorded in an Excel dataset (Table S1). The carcasses were necropsied and sampled immediately after their acceptance at the IPWFB. Carcasses with visible signs of decomposition (n = 6) or with severe injuries to internal organs from vehicle collisions (n = 7) or firearms (n = 7) were excluded from sampling due to damaged or missing organs. All samples were collected post-mortem, and thus approval from the national ethics committee/social welfare agency was not required. Liver samples were collected from each carcass and stored in a freezer at −80 °C for at least one month before processing to avoid contamination with viable *Echinococcus* eggs. After processing at the IPWFB, the samples were transported in dry ice to the Julius Kühn Institute in Berlin, where they were stored at −80 °C until chemical analysis.

The age of the animals was determined by counting the increment layers of the secondary dental cementum of a cut lower canine root and, in young animals, by the size of the pulp cavity according to the method of Roulichova and Andera (2007). The fact that the foxes were born in spring was also taken into account as an additional time factor. The age of the animals included in this study ranged from <1 year to >1 year, considering the springtime of birth from 0.4 to 5.7 years, with an average value of 1.6 year, while their body mass ranged from 3 to 9.5 kg, with average value of 6 kg. The distributions of the age and body mass of the foxes in the statistical regions are shown in Fig. S1.

2.2. Chemical residue analysis

The sample preparation and the measurement of the rodenticide concentrations were carried out according to the method described in Geduhn et al. (2014). Approximately one gram wet mass of liver was weighed and fortified with a surrogate (Surr) mixture, consisting of 100 ng each of acenocoumarol and diphacinone-d4. ARs were extracted twice with an Ultra-Turrax T25 (IKA, Staufen, Germany), at first in 20 ml methanol for 2 min, and secondly for 1 min after 10 ml water were added to the methanol extract. The homogenate in methanol/water (2:1, v/v) was then centrifuged for 5 min at room temperature and 4500 ×g (Megafuge 16, Thermo Fisher Scientific, Waltham, Massachusetts, USA). An aliquot of 15 ml of the supernatant was mixed with 5 ml of a sodium chloride solution (20 %, w/v), and the mixture was transferred to a diatomaceous earth Chem Elut solid-support liquid extraction cartridge, unbuffered, 20 ml (Agilent Technologies, Santa Clara, CA, USA). After waiting for at least 15 min, the ARs were eluted with 100 ml of dichloromethane. An aliquot of 10 ml of the eluate was concentrated to

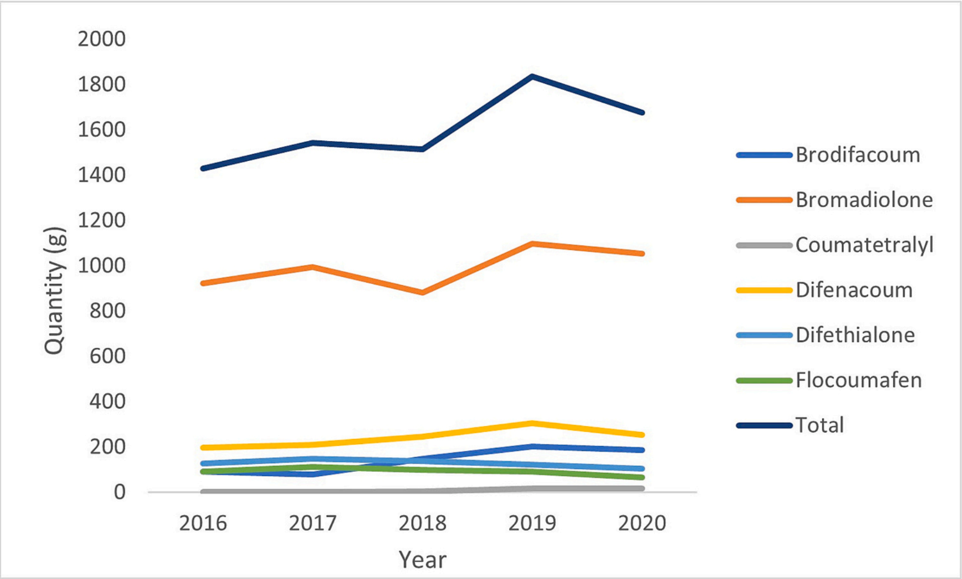

Fig. 1. Estimated professional use of ARs in Slovenia in the period 2016 to 2020; the data were obtained through a survey of the authorised performers of DDD.

dryness by vacuum evaporation at 37 °C and redissolved with 1 ml methanol/water (1:1, v/v) containing the internal standards (IS) chlorophacinone-d4 and warfarin-d5 at a concentration of 5 ng/ml each. The vortexed solution was filtered through a PTFE syringe filter (Ø 13 mm, 0.2 μm) into an autosampler vial and stored at −20 °C until measurement.

Measurements of ARs were performed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS), using the 1290 Infinity II liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to the QTRAP 6500+ high performance triple quadrupole/linear ion trap mass selective detector (SCIEX, Framingham, MA, USA). Five μl of the sample extract were injected on a Zorbax Eclipse C18 (1.8 μm, 50 mm, 2.1 mm i.d.) (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed at 40 °C, using mobile phases A and B, consisting of water with the addition of 1 mmol NH_4F and methanol/acetonitrile (65/35, v/v), respectively. The mobile phase was pumped at a flow rate of 0.5 ml/min. The proportion of the organic phase increased linearly from 2 to 98 % (v/v) at time 0–3 min, held at 98 % (v/v) at time 3–5 min, reverted to 2 % (v/v) at time 5–5.1 min, and held at 2 % (v/v) at time 5.1–6 min. The measurement of ARs was performed in electrospray ionisation negative mode with a source temperature of 550 °C and an ion spray potential of −4500 V. Identification and quantification were carried out with two characteristic precursor (Q1) – product ion (Q3) – transitions of each AR (with the exception of chlorophacinone) by Multiple Reaction Monitoring (MRM), as presented in Table S2. Furthermore, enhanced product ion spectra (EPI) were recorded in the ion trap mode of the mass spectrometer with dynamic filling time to confirm the AR findings (Table S3). The threshold value for the acceptance of an AR was an agreement of >80 % between the enhanced product spectra of the sample and the corresponding standards.

The concentrations of ARs were calculated according to peak areas using Analyst 1.7.1. software (SCIEX, Framingham, MA, USA) and were neither surrogate nor recovery corrected. The calibration curves were linear with $r^2 > 0.99$ over the whole concentration range of matrix-matched standards (0.01–20.0 ng/ml). All samples were measured twice. The reporting limits (RLs) refer to the lowest calibration level with a signal to noise ratio > 6:1 and relative standard deviation <20 % in the sequence, and are presented in the Supplementary Data, together with the recovery levels ± relative standard deviations (RSDs) (Tables S4, S5). No interferences (<RL) were observed in the blank quality control samples, which were run in the same batch as the fortified quality control samples. All open land fox liver samples were fortified with the surrogate mixture for ongoing validation of the analytical performance. Overall recovery ± RSD values for 148 samples were 104.9 ± 17.8 % and 113.6 ± 16.2 % for acenocoumarol and diphacinone-d4, respectively.

2.3. Data preparation and methods for statistical analysis

We observed the number and concentration of each AR and the sum values (separately for each generation and for both generations combined) using descriptive statistics (with frequencies, percentages, and standard measures such as median and mean, and also standard deviation to measure variability between individual samples). These descriptive statistics were additionally produced separately for sex, statistical region, cohesion region (eastern and western Slovenia), and groups of statistical regions due to the degree of urbanisation (according to the EUROSTAT typology there are no urban regions in Slovenia, and all regions are classified as intermediate or predominantly rural) (EUROSTAT, 2023). For the visual representation of a small number of samples, we used a stripchart (one-dimensional scatterplot) as an alternative to a boxplot.

In addition, we performed several statistical tests for comparisons. In the statistical analysis, residue concentrations were considered in two ways: as continuous values (measured in ng/g) or as categories, where we used a categorisation following Geduhn et al. (2015) (i.e. five categories, I: not detected (n. d.), II: n. d. < c < 200 ng/g, III: 200 ng/g ≤ c < 800 ng/g, IV: 800 ng/g ≤ c < 2000 ng/g, V: c ≥ 2000 ng/g). Possible relationships between AR residues and external variables for statistical regions and municipalities as local administrative units were observed, such as population density, number of farms per square kilometre of municipality area, and the number of livestock units (LU) per hectare of utilised agricultural area (UAA), and the data used refer to the year 2020 (Republic of Slovenia, Statistical Office, 2023b, 2023c). First, due to the limited sample size, we examined pairwise statistical relationships between concentrations of ARs and these potentially related variables. The Shapiro-Wilk normality test was used to test assumptions about the normal distribution (Shapiro and Wilk, 1965). When assumptions for parametric tests were not met, we used nonparametric tests (Hollander and Wolfe, 2015). Correlations were tested using Spearman and Kendall rank correlation coefficients. The Wilcoxon or Mann-Whitney rank sum test was used to test differences between two groups, and the Kruskal-Wallis rank sum test was used for comparisons between more than two groups. The chi-square test for independence was used to test whether two categorical variables were related, and the Fisher's exact test was used when the frequencies were very small and the chi-square approximation might not have been correct. To gain a more comprehensive insight into the relationships between all these variables, generalised linear models (GLM) were used to determine the presence or absence of ARs (logistic regression with GLM, family = binomial, link function = logit) and to better reveal their interrelated influence on concentration (GLM for logarithmic values of concentrations, family = Gaussian). In the generalised models, the age calculated on the basis of the expected time of birth in spring was used.

The statistical analysis was performed in R version 4.2.3 (R Core Team, 2022).

3. Results

3.1. Animals

Sampling of foxes did not show any visible macroscopic lesions in body cavities or organs that would indicate possible poisoning by rodenticides. Nevertheless, it has to be taken into account that the foxes were usually shot with lead cartridges or died due to injuries to internal organs caused by traffic accidents, which could mask any haemorrhages resulting from poisoning.

3.2. Descriptive statistics

Residues of at least one AR were detected in 115 of the 148 analysed fox livers (77.7 %). Of these, 33 samples (22.3 %) contained one active AR substance, 53 samples (35.8 %) contained two substances, 25 samples (16.9 %) contained three substances, three samples (2.0 %) contained four substances, and one sample (0.7 %) contained five substances. SGARs occurred significantly more often than FGARs and were found in 77.7 % of the samples, where bromadiolone, brodifacoum and difenacoum were determined in 75.0, 51.4, and 18.9 % of the samples, respectively. Concentrations of pooled ARs ranged from 1.5 to 2867 ng/g with a mean and median values of 601.4 and 350.2 ng/g, respectively (Table 2). Bromadiolone and brodifacoum were the most common AR substances and also had the highest residue levels. Bromadiolone was determined at concentrations of ≥200 ng/g in 56 of 148 fox livers (37.8 %), at concentrations of ≥800 ng/g in 16 samples (10.8 %), while two samples (1.4 %) contained bromadiolone residues of >2000 ng/g (2639 and 2049 ng/g) (Table S1, Fig. 2, Table 2). Brodifacoum was determined at concentrations of ≥200 ng/g in 39 of 148 fox livers (26.4 %), at concentrations of ≥800 ng/g in 15 samples (10.1 %), while one sample (0.7 %) contained >2000 ng/g (2642 ng/g) of its residues (Table S1, Fig. 2, Table 2). Concentrations of difenacoum and flocoumafen of <200 ng/g were found in 28 samples for both substances, and two samples contained residues with maximum levels of 110.2 and 2.5 ng/g, respectively (Table S1, Fig. 2, Table 2). FGARs coumatetralyl and warfarin were found in only 9.5 % of the samples (Table 2) and at concentrations below 10 ng/g, with one exception of coumatetralyl at a concentration of 55 ng/g (Table S1, Table 2).

The distributions of bromadiolone and brodifacoum showed no significant differences across sex and body mass.

3.3. Regional differences

Residues of ARs were detected in all monitored statistical regions of Slovenia with a prevalence of 56 to 100 % (Fig. S2), with higher concentrations in the eastern parts of the country (Fig. 3). The number of samples with residues of ARs is very different between statistical regions, and the pooled concentrations of ARs between them are statistically significantly different (Kruskal-Wallis rank sum test without Coastal-Karst region with only one sample, $p = 0.035$).

Statistical regions of Slovenia can be grouped into two bigger cohesion regions: Eastern and Western Slovenia (Republic of Slovenia, 2023b). The difference in the concentration of pooled ARs ($n = 115$) and separately for brodifacoum ($n = 76$) and bromadiolone ($n = 111$) between the total eastern and western cohesion regions was not statistically significant (Table S6), although, as with the statistical regions, statistically significant differences in age and body mass were found between the cohesion regions. The concentrations of the AR residues vary considerably between the samples, especially in the eastern part of Slovenia. The mean and median values of the bromadiolone concentration are higher in the eastern cohesion area, while both values for brodifacoum are higher in the western part, although slightly less than half the number of the samples with brodifacoum were found in the western cohesion area than in the eastern one (nWest = 25, nEast = 51). On the other hand, all three samples in which the highest detected concentration of a single AR was >2000 ng/g (one with brodifacoum and two with bromadiolone) were found in the eastern part.

Similar comparisons were made for the two groups of statistical regions according to the international typology of the degree of urbanisation: Intermediate (which includes the statistical regions from the western cohesion region, except for Gorizia) and Predominantly Rural (all other statistical regions) (EUROSTAT, 2023). None of the differences in residue concentrations of pooled ARs, bromadiolone, and brodifacoum between these two groups were statistically significant.

Since the official statistical offices provide a lot of additional data that could be related to the use of ARs, we have additionally considered possible relationships between residue concentration of ARs and some other external variables related to the local administrative units (LAU), i.e., municipalities, such as population density, number of farms per square kilometre of municipality area, and the number of LU per hectare of UAA (Republic of Slovenia, Statistical Office, 2023b, 2023c). To examine the distribution of sample concentrations in relation to these variables, we divided the values of these variables into four groups using quartiles calculated from all municipalities. A significant relationship was found only between brodifacoum and the groups based on the number of farms per area (nQ1 = 23, meanQ1 = 370.4 ng/g, medianQ1 = 170.8 ng/g, nQ2 = 17, meanQ2 = 458.8 ng/g, medianQ2 = 309.4 ng/g, nQ3 = 11, meanQ3 = 193.4 ng/g, medianQ3 = 218.2 ng/g, nQ4 = 25, meanQ4 = 269.8 ng/g, medianQ4 = 96.8 ng/g, Kruskal-Wallis rank sum test: $p = 0.082$; Fisher exact test for count data with simulated p-value: $p = 0.04$). The differences between these four groups in median values and in distributions are presented in Fig. 4.

To gain a more comprehensive insight into the relationships between the presence of ARs and external variables, we applied generalised linear models (GLM) for the possible influence of many different combinations of already mentioned potential explanatory variables on the concentration of pooled ARs, as well as separately for the concentration of bromadiolone and brodifacoum as two of the most frequently detected ARs in our data. In these models, the logarithm of the concentration was used as the response variable. We examined the potential influence of the external variables on the concentration for all samples, and also only for those samples with the detected concentration. In the first approach, we replaced the original n.d. values with each AR having half of its RL value. In the second, the model was examined only with regard to the samples with the detected concentration. Observing pooled ARs, the only statistically significant external variable in both approaches was the number of farms per square kilometre of municipality area.

The application of logistic regression to determine the presence or absence of each AR shows the statistically significant relationships between the presence of bromadiolone and the number of farms per area (positive relationship, $p < 0.01$). Further statistical analyses (using GLM for the logarithm of the bromadiolone concentration with the Gaussian family) on all samples showed a certain influence of the number of farms on the bromadiolone concentration. Running the same model for 111 samples with a detected concentration above the RL beside the influence of the number of farms also showed an additional positive influence of body mass on bromadiolone concentration ($p = 0.032$), and surprisingly also a negative influence of the indicator LU ($p = 0.022$). Results are discussed in the next section. Although both variables related to local administrative units are positively correlated in our data, the variance inflation factor (VIF) still remained acceptable (below 1.5), and therefore we leave both in the model.

For the presence of brodifacoum, statistically positive relations were observed with age of foxes and number of farms per area and a negative relation was found with body mass. The separate observation of the influences on the brodifacoum concentration (the second most detected AR, $n = 76$) by applying the GLM on all samples revealed the influences of age (positive) and body mass (negative), but the usage of this method only on samples with a concentration above its RL did not show a significant influence of any of the external variables.

4. Discussion

This work presents the first evidence of AR exposure in wildlife in Slovenia. The widespread use of ARs in Slovenia and consequent distribution in the environment has led to widely perceived contamination and potential secondary poisonings of red foxes as representatives of non-target wild animals that interact closely with other animals and human life.

Table 2
Descriptive statistics of AR distribution in red fox liver samples in Slovenia (2019–2022).

Sample size = 148	n	n out of 148 (%)	Min (ng/g)	Max (ng/g)	Mean (ng/g)	Median (ng/g)	Sample st. dev. (ng/g)
Brodifacoum	76	51.4	8.17	2642.28	331.44	210.55	428.18
Bromadiolone	111	75.0	1.59	2638.79	390.73	206.48	500.01
Coumatetralyl	12	8.1	0.50	55.34	6.52	1.16	15.54
Difenacoum	28	18.9	1.46	110.19	18.15	10.35	24.92
Flocoumafen	2	1.4	0.65	2.46	1.56	1.56	1.28
Warfarin	2	1.4	1.44	5.49	3.47	3.47	2.86
Sum (all 6 ARs)	115	77.7	1.46	2866.47	601.37	350.17	661.82
Sum (FGARs = 2 ARs)	14	9.5	0.50	55.34	6.08	1.24	14.36
Sum (SGARs = 4 ARs)	115	77.7	1.46	2866.47	600.63	350.17	661.65

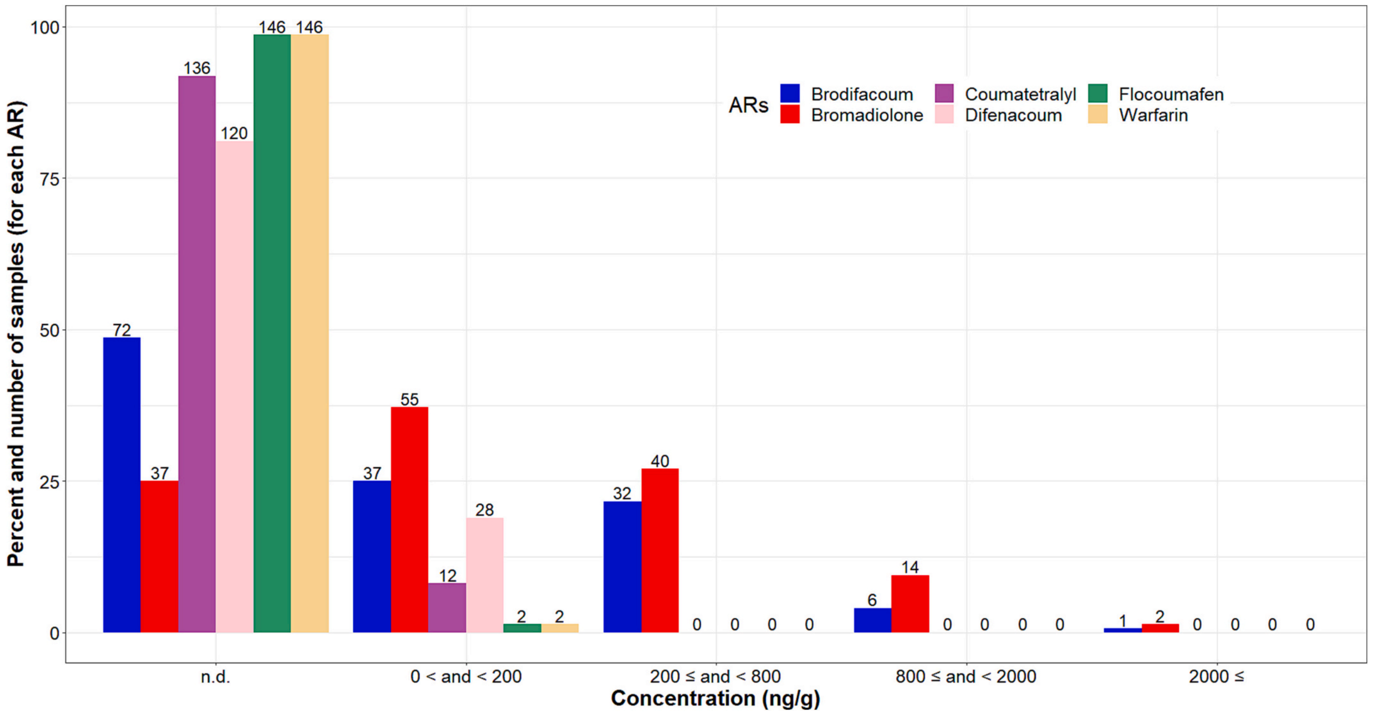

Fig. 2. Residues of ARs in 148 red fox liver samples in the period 2019 to 2022 in Slovenia with the percentage and number of samples per concentration category; n.d. – not detected.

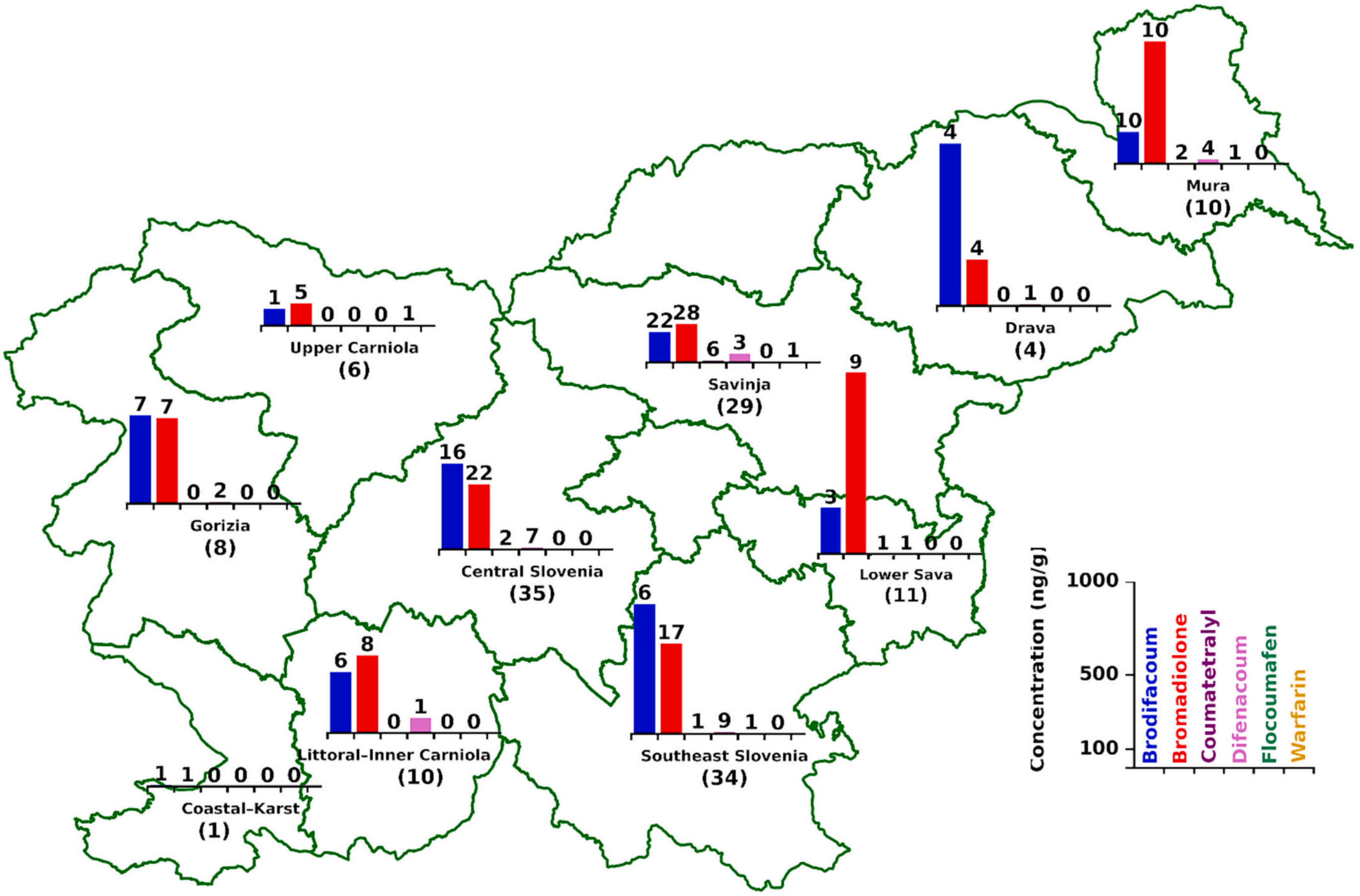

Fig. 3. Geographical distribution of positive samples of red fox liver for the presence of ARs in the period 2019 to 2022 in Slovenia. The height of each bar represents the mean concentration calculated from the corresponding number of positive samples indicated above the bars. In parentheses below the graphs is the number of samples taken in each statistical region.

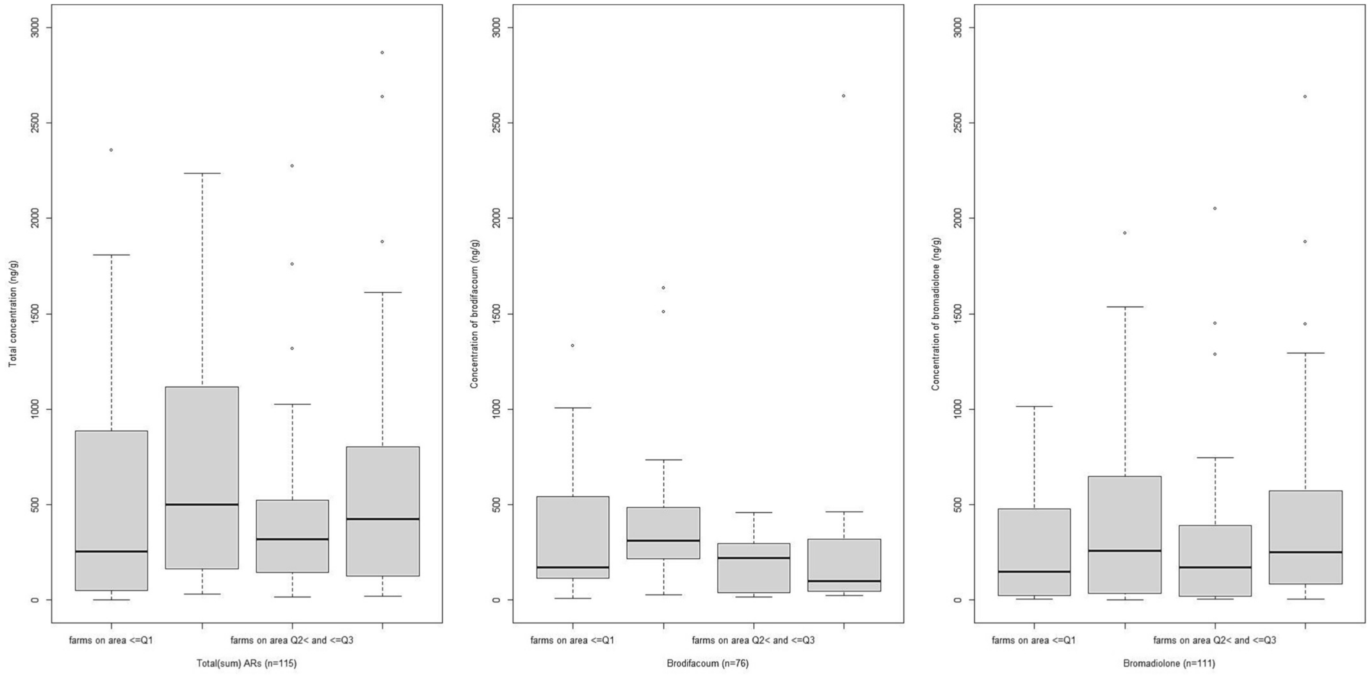

Fig. 4. Distribution of concentrations of total residues (sum) of ARs (n = 115) and separately for brodifacoum (n = 76) and bromadiolone (n = 111) in the liver of foxes in Slovenia in four groups of municipalities based on the number of farms per area, divided on the basis of quartiles calculated from all Slovenian municipalities.

4.1. *Anticoagulant rodenticide exposure in foxes and availability*

In most countries information about the sale of ARs is not available and kept confidential (Jacob and Buckle, 2018; Koivisto et al., 2018), and this is also the case for Slovenia. For this reason, the only way to at least roughly assess the use of these substances in Slovenia was to send a questionnaire to professional providers of DDD, most of whom responded. The summarised data for the entire Slovenian territory showed that in the period 2016 to 2020 (Fig. 1), the most frequently used substances were listed in the order bromadiolone, difenacoum, and brodifacoum, but in the fox liver we found a different order – bromadiolone, brodifacoum, and difenacoum.

A possible reason for the difference between the use of brodifacoum and difenacoum and the prevalence of their residues in liver was that we could not include private/retail use in the surveying for confidentiality reasons. We have received information that the use of brodifacoum in Slovenia is highly desirable due to its high efficacy and attractiveness to rodents, and because it is commercially available in the form of soft bait, which is also intended for general – i.e. non-professional – use. The development of more effective SGARs, such as brodifacoum, flocoumafen, and difethialone, offers some alternatives to bromadiolone and difenacoum, for which resistance has been found in rodents in many countries due to mutations in vitamin K epoxide reductase complex subunit 1 (VKORC1) (Buckle et al., 2012; Blažić et al., 2018), while their increased toxicity increases the risk to non-target species (Rodenticide Resistance Action Group (RRAG), 2018).

As our investigation with red foxes revealed, the confirmed large-scale contamination with ARs having a 78 % pooled exposure prevalence (Table 2) is not a local problem, but a nationwide and regional one in the Western Balkans, which coincides with the results of investigations already carried out in Western Europe (Koivisto et al., 2018; López-Perea et al., 2019; Seljetun et al., 2020; Lestrade et al., 2021) and other parts of the world (Elliott et al., 2022a; Serieys et al., 2019; Hong et al., 2019). Based on the authorisations (Table 1) and interviews with professional users (Fig. 1), the prediction that SGARs were more present in red foxes than FGARs was confirmed, which is consistent with the results of Geduhn et al. (2015). Our study found that bromadiolone was the most prevalent AR, at 75.0 %, which was also the case with a fox study in Northern Ireland that found a prevalence of 74 % (Tosh et al., 2011), and similar results were obtained in France (Lestrade et al., 2021) and Finland (Koivisto et al., 2018). This is in contrast to Norway (Seljetun et al., 2020) and Germany (Geduhn et al., 2015), where brodifacoum was the most common AR, with a 80 and 45.6 % prevalence, respectively. However, the latest trend of the increased use of brodifacoum in Slovenia (Fig. 1) suggests that there will be an increased occurrence of its residues in non-target wildlife and the environment in the future, which is of particular concern because this compound is much more toxic to living organisms, and particularly to birds (Elliott et al., 2022b).

Due to environmental risk, the European Chemicals Agency (ECHA) decides every five years whether to allow the further use of ARs. This requires joint research efforts among the EU Member States, which then have a direct impact on the ECHA's decisions. Knowledge about the consequences of ARs for ecosystems is limited. However, since wildlife animal species do not respect national borders, it is therefore in the common interest to generate basic knowledge for the protection of European ecosystems. This can only be done using monitoring data that reflect the diversity of European environmental conditions.

4.2. *Regional anticoagulant rodenticide distribution*

Due to the assumption that the frequency of occurrence of rodents and thus the use of ARs is higher in more populated areas and those where agricultural activity is more represented (Geduhn et al., 2015), and that the greater representation of livestock farming in the municipality may represent the need for greater use of ARs (Jacob and Buckle, 2018), we used some additional indicators at the municipality level in the analysis. "The population density of the municipality" reflects the character of the municipality, which can be more or less densely populated. Compared to many other parts of Europe, Slovenia does not have any very densely populated areas (Perpar et al., 2014). We nevertheless considered this external variable as a potential explanatory one due to the fact that more densely populated areas represent better conditions for rodents to live in than less populated ones, with more available food and buildings, facilities and so on where they can stay. Its logarithmic value was used in the models because of its rather large variability, but due to its correlation with the number of farms the density information was excluded in our final examined GLM. The intensity of agriculture was assessed on the basis of “the number of agricultural holdings in the municipality”, which, for the sake of comparability between municipalities was converted to the area (km^2) of the municipality's surface. In the GLM, a positive correlation was found between the presence of bromadiolone and the presence of brodifacoum and the number of farms per area. A larger number of farms means more opportunities for rodents. With a larger number of farms, these are usually smaller and their livestock farming is often based on old methods of animal husbandry (external manure storage), feed in outbuildings and barns is also accessible to rodents, there are more hiding places in barns and other farm buildings and storage areas, several manure pits, silos, etc. When additionally considering the possible influence on the concentration in combination with other external variables, the number of farms only showed an influence on bromadiolone.

Since rodents often appear on farms (in areas with manure, stables, feed storage, silos, etc.), we additionally used the indicator “number of LU” and converted it to the surface of the UAA in the municipality. We also found that the number of LU has a negative effect on the AR concentration in the liver of foxes. If there are fewer farms in an area, it means that they can be larger and usually raise more livestock, as they are more professional farms. This is more common in flat areas, as intensive livestock farming is also associated with the production of fodder in the fields (maize and other fodder crops). Larger farms today are generally modern and have large, well-maintained barns. They no longer have external manure storage, as the manure pits are located under the animals' stalls, so that rodents have no access. These farms are also better equipped in other respects; they have closed storage facilities for grain and heavy fodder (upright closed silos) so that rodents have almost no access. Slurry shovels and other modern equipment in the barn can also be a nuisance for rodents. Smaller, less arranged farms are a better habitat for them. In contrast to most European countries, Slovenia has a special farm structure. The average size of farms in 2020 was 7.0 ha of UAA, and on average 9.1 LU were kept on the farms. 39.9 % of all farms in 2020 had up to 3 ha of UAA, 54.5 % had 3 to 20 ha of UAA and only 5.6 % of farms were larger than 20 ha of UAA (Agricultural Institute of Slovenia, 2023).

Since the share of agriculture in the structure of economic activities is greater in the eastern cohesion region (Perpar et al., 2013), the probability of greater use of ARs may be greater in this part of Slovenia. Here, in the sowing structure of the fields, there is a greater representation of cereals and other crops, which could also mean better conditions for the development of rodents and, consequently, the need to use ARs. The eastern part of Slovenia, which is more rural, was statistically more exposed to ARs than the western part. Many more samples with at least one AR residue were found in the eastern part than in the western part (nEast = 79, nWest = 36), although the mean values of pooled ARs do not differ so much (for example, meanEast = 606.3, meanWest = 590.6). It should be mentioned that the differences between the eastern and western parts in the mean concentrations for bromadiolone and also for brodifacoum are not entirely negligible, but there is a very large variability between the samples inside each group. Comparing the two groups based on the urbanisation of the region, the median value of the brodifacoum concentration is higher in the so-called intermediate group than in the rural group, while the median value of the bromadiolone concentration is higher in the group with predominantly rural regions. It should be noted, however, that urban foxes were not included in our study because urban areas in Slovenia are non-hunting areas according to the Game and Hunting Act (Republic of Slovenia, 2014a). Nevertheless, 13 samples were found in the so-called metropolitan municipalities (Ljubljana, Kranj, Novo mesto, Velenje, Murska Sobota) and, for example, their mean concentration of bromadiolone was 1.8 times as high as the mean value for the remaining 98 foxes (meanM = 643.3 ng/g, meanNoM = 357.2 ng/g). Larger median values in metropolitan municipalities were found for total AR concentration, for brodifacoum and also for bromadiolone.

4.3. Toxicological and risk aspects

Interpretation of AR residues and their toxicity is challenging because of the many variables involved that have an impact on toxicity thresholds (Rattner and Harvey, 2021). The toxicity of ARs to non-targeted animals is reflected on the cellular level and in multiple organ systems, in whole animal and population responses (Rattner et al., 2014). The possible correlations between AR contamination, weakening of the organism and disease propensity upon AR exposure are the subject of ongoing investigations. A very recent study from Spain, limited to foxes with infectious pathologies and carried out in only one geographical region, found that 100 % of the red foxes examined tested positive for both bromadiolone and brodifacoum, and also for difenacoum (Carrera et al., 2023).

The results of our study confirm that the red fox is an appropriate sentinel predatory and scavenging species to evaluate the environmental risk of ARs due to its specialised consumption of rodents as target species. Samples were taken directly from the liver because ARs bioaccumulate in this organ (Fournier-Chambrillon et al., 2004), mostly as parent compounds, and because their retention time in the liver is very long – for example the half-life of brodifacoum in the liver of mammals is in the range of 282 to 350 days (ECHA, 2016).

Toxicological data for red foxes were examined for bromadiolone only, and are based on the results of a clinical study by Berny et al. (1997) in which 200 and 800 ng/g of residues in the liver were considered as the biological and toxicological thresholds, respectively. In our study these two thresholds were exceeded in 38 and 11 % of liver samples, respectively. A hepatic concentration of 2000 ng/g was evaluated as a poisoning threshold by experimental foxes included in Sage et al. (2008), in which some of the animals needed the antidote of vitamin K to survive the AR poisoning. In our study this level was exceeded in two animals out of 148, having bromadiolone concentrations at a maximum of 2639 ng/g, far above the highest levels reported in other monitoring studies with red foxes (Geduhn et al., 2015; Tosh et al., 2011; Seljetun et al., 2020). Moreover, the mean and median bromadiolone concentrations were 391 and 206 ng/g, respectively, far above the levels reported in other studies with red foxes and caracals (Geduhn et al., 2015; Tosh et al., 2011; Serieys et al., 2019; Seljetun et al., 2020), and both above the supposed biological threshold. No toxicological data for brodifacoum were evaluated for foxes, but this substance has been found to be more toxic to mammals than bromadiolone (Erickson and Urban, 2004). We thus conclude that the harmful effect limits for brodifacoum are lower than those given above for bromadiolone (Berny et al., 1997; Sage et al., 2008). In addition, our study again showed that the highest brodifacoum concentration of 2642 ng/g, and the mean and median values of 331 and 210 ng/g, respectively, were all higher than those found in comparable monitoring studies (Geduhn et al., 2015; Tosh et al., 2011; Serieys et al., 2019; Seljetun et al., 2020). Regarding the total (pooled) concentrations of ARs found in red foxes, one to five substances were detected in the samples tested, with 36 % of the samples tested being contaminated with two substances. This is of particular concern because of the potential synergistic/additive effects of different AR residues, which have not yet been investigated (Geduhn et al., 2015).

4.4. Conclusions

The results of our monitoring study demonstrate a serious toxicological risk to and endangerment of red foxes in Slovenia due to their high exposure to ARs. This conclusion is based on the AR residue concentrations found in their livers, allied with the long-lasting effects of these residues, and the known biological, toxicological and poisoning potential of ARs. In fact for all the investigated criteria, such as prevalence, maximum, mean and median levels of ARs, we found significantly higher values than comparable animal monitoring studies in Europe and elsewhere in the world, in which foxes were the most AR-exposed species. In order to mitigate the consequences of using ARs, it is also necessary to have an overview of the amount of ARs sold/used, as called for by earlier researchers (Koivisto et al., 2018). To get a more comprehensive insight into the environmental risks to non-target wildlife in Slovenia, it would be desirable to further assess data on the contamination with ARs of other sentinel animal species, e.g. wild raptors, in the future. Moreover, in the Slovenian context, and based on this initial study, questions are additionally raised about environmental threats in the Western Balkans region that future research could address.

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CRediT authorship contribution statement

Vesna Cerkvenik-Flajs: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Detlef Schenke:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Diana Žele-Vengušt:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Simona Korenjak-Černe:** Conceptualization, Data curation, Methodology, Software, Writing – original draft, Writing – review & editing, Funding acquisition. **Anton Perpar:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Gorazd Vengušt:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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