Detection of antibiotic residues in groundwater with a validated multiresidue UHPLC-MS/MS quantification method

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Abstract

The occurrence of antibiotic residues in the environment has received considerable attention because of their potential to select for bacterial resistance. The overuse of antibiotics in human medicine and animal production results in antibiotic residues entering the aquatic environment, but concentrations are currently not well determined. This study investigates the occurrence of antibiotics in groundwater in areas strongly related to agriculture and the antibiotic treatment of animals. A multiresidue method was validated according to EU Regulation 2021/808, to allow (semi-)quantitative analysis of 78 antibiotics from 10 different classes: β-lactams, sulfonamides, tetracyclines, lincosamides, amphenicols, (fluoro)quinolones, macrolides, pleuromutilins, ansamycins and diaminopyrimidines using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). This method was used to test different storage conditions of these water samples during a stability study over a period of 2 weeks. Sulfonamides, lincosamides and pleuromutilins were the most stable. Degradation was most pronounced for β-lactam antibiotics, macrolides and ansamycins. To maintain stability, storage of samples at -18 °C is preferred. With the validated method, antibiotic residues were detected in groundwater, sampled from regions associated with intensive livestock farming in Flanders (Belgium). Out of 50 samples, 14% contained at least one residue. Concentrations were low, ranging from < LOD to 0.03 µg/L. Chloramphenicol, oxolinic acid, tetracycline and sulfonamides (sulfadiazine, sulfadoxine, sulfamethazine and sulfisoxazole) were detected. This study presents a new method for the quantification of antibiotic residues, which was applied to investigate the presence of antibiotic residues in groundwater in Flanders.

Keywords: Antibiotic residues, environment, agriculture, groundwater, UHPLC-MS/MS, validation, stability

1. Introduction

Over time, antibiotics have demonstrated their benefits in treating infectious diseases. Yet, the pressing issue of antimicrobial resistance (AMR) has been increasing globally, overshadowing the effectiveness of treatments and posing a severe threat to public health. Overuse, both in the human and veterinary sectors, increases the spread of antibiotic resistance and may result in untreatable infections. This contributes to the worrying prediction of antibiotic resistance as the global primary cause of death by 2050 (Jim O'Neill, 2016). Already in Europe, 541 000 deaths (10.9 % of global mortality) were associated with AMR in 2019 (European Collaborators Antimicrobial Resistance, 2022). In the context of "One Health", it is crucial to acknowledge the affiliation between both human and veterinary medicine and the spread of AMR within the environment. Antibiotic residues (ABRs) and antibiotic resistance genes disseminate into the environment, where allochthonous bacteria can mix with autochthonous bacterial communities, resulting in a possible horizontal gene transfer on plasmids or transposons (Robinson et al., 2016). Besides, when antibiotic-resistant bacteria from the environment are taken up by humans, antibiotic-resistance genes may be transferred to bacteria belonging to the human gut microbiota, including pathogens (Leonard et al., 2018). The contamination of antibiotic residues and antibiotic resistance in the aquatic environment may occur in a variety of ways, including the entry along wastewater treatment plants (WWTPs) (Blaak et al., 2015; Sabri et al., 2020), hospital effluents (Voigt et al., 2020), aquaculture facilities (Geetha Preena et al., 2019; Thiang et al., 2021) and pharmaceutical production sites (González-Plaza et al., 2019). It is also associated with antibiotic use in veterinary medicine amid the spread of manure on arable lands, especially in countries with high livestock concentrations, such as Belgium (3rd overall in Europe) (Eurostat, 2023). Manure is proven to be a source of antibiotic residues and resistance in the environment (Rasschaert et al., 2020; Van den Meersche et al., 2016), through which the antibiotic (resistance) load can end up in the soil (Huygens et al., 2022), taken up by plants (Tasho and Cho, 2016) or end up in the aquatic environment. Residues may reach surface water due to run-off and draining, whilst infiltration can lead to dissemination into groundwater (Gao

et al., 2020; Szekeres et al., 2018). In addition to ecological effects, human and animal exposure to antibiotics through drinking water and the consumption of irrigated crops, using groundwater reservoirs, remains of concern (Murphy et al., 2017). The release of residues in groundwater and the following spread can lead to a selection of antibiotic resistance genes, increasing the worldwide concern of environmental contamination (Gao et al., 2020). Extensive data, for both antibiotic residues and AMR in the aquatic environment, specifically in groundwater, remain relatively scarce in Europe and especially in Flanders, resulting in a need to investigate the topic.

A first part of this work is the development of a multiresidue method to analyze antibiotics from different classes in an aquatic matrix. For this purpose, liquid chromatography, coupled with mass spectrometry, is often used as a method to identify and quantify antibiotic residues in water samples (Borecka et al., 2013; Gbylik-Sikorska et al., 2015; Goessens et al., 2020; González-Gaya et al., 2018; Mirzaei et al., 2017; Zhou et al., 2012). Specifically for groundwater, Burke et al., (2016) explored the occurrence of 26 antibiotics in the groundwater of a drinking water catchment, whereas Balzer et al., (2016) and Kivits et al., (2018) studied veterinary antibiotics, respectively 22 and 23 analytes, in groundwater under locations with high livestock density (Balzer et al., 2016; Burke et al., 2016; Kivits et al., 2018). Still, there was a need for optimization of existing methods in terms of scope, sensitivity and efficiency. To cover a wider range of antibiotics, while simultaneously maintaining a high sensitivity, a new method was developed which is the most comprehensive UHPLC-MS/MS approach to our knowledge. This study presents an efficient multiresidue method for the quantification of 78 antibiotics (10 classes) in various aquatic samples and is validated according to the Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021). Special attention was placed in this research on the stability of antibiotic residues during transport after sampling and storage before analysis. So far, some work has been conducted on the behavior of a variety of antibiotics during storage (Gbylik-Sikorska et al., 2015; Llorca et al., 2014), but data on the degradation of residues in environmental matrices for a broad spectrum of antibiotics must be strengthened,

especially for short storage terms of life-like samples. There are several degradation mechanisms in the environment, abiotic (hydrolysis, photodegradation, oxidation and reduction) and biotic (biodegradation) (Polianciuc et al., 2020; Yang et al., 2021). In water, mainly hydrolysis will play a part in the rapid breakdown of some antibiotics, highly dependent on the chemical properties (Huang et al., 2011). The investigation of this degradation is essential to ensure proper storage and enable an accurate determination of antibiotic residues. Finally, this method is put to use for the analysis of groundwater samples. The objective is to assess on which scale antibiotic residues are present in groundwater in areas characterized by intensive livestock production. In The Netherlands, Kivits et al. (2018) detected antibiotic residues in 7 out of 10 sampled wells; next to sulfonamides, chloramphenicol and ciprofloxacin were detected in low concentrations (< 0.018 µg/L) (Kivits et al., 2018). In Germany, 3 different sulfonamides could be quantified (0.01-0.1 µg/L) in groundwater in rural areas. Seven out of 48 investigated locations were positive (Balzer et al., 2016). The occurrence of antibiotic residues in agricultural areas in Flanders will be related to the usage of antibiotics and the fate of antibiotics in the soil-water nexus.

2. Materials & methods

2.1. UHPLC-MS/MS method

2.1.1. Chemicals

Methanol (MeOH), acetonitrile (MeCN) and acetic acid are purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$) and ammonium acetate ($NH_4CH_3CO_2$) are obtained from Merck (Darmstadt, Germany). Utilized water in this research is high-performance liquid chromatography (HPLC) grade. The (internal) standards used are purchased from LGC standards (Molsheim, France), Merck, WITEGA laboratorien Berlin-Adlershof GmbH (Berlin, Germany), HPC standards (Borsdorf, Germany) and Toronto Research Chemicals (Toronto, Canada). Prior to each experiment working solutions were made at 1 µg/L, diluted in (50:25:25 v/v H_2O:MeCN:MeOH + 0,05% acetic acid). For validation and quantification purposes, a stream river in Melle, Belgium, was chosen as the blank matrix. Repeated analyses made sure no residues were present above the limits of detection. A sample was taken in Oostende, Belgium to test the suitability of the multiresidue method for the marine environment.

2.1.1. Study area and sample collection

2.1.2. Extraction and clean-up

A multiresidue method is developed and optimized in order to quantify 78 antibiotics from different classes **(Table 1)** in an efficient way. Almost all veterinary antibiotics, as mentioned in Regulation (EU) 37/2010 (European Commission, 2010), dealing with residues in foodstuffs of animal origin, are included. After thawing the water samples, to 100 mL water of each sample, 3 mL Na_2EDTA (0.1 M) is added before adjusting the pH to 3 with HCl (3.5 M). Internal standards (cefotaxime, ceftiofur-D3, clindamycin, metacycline, piperacillin, roxithromycin, sulfadimethoxine 13C6, threo-chloramphenicol-D5 and trimethoprim-D9) are added. OASIS HLB

solid-phase extraction (SPE) cartridges (6 cc barrel size, 500 mg sorbent weight, 60 µm particle size and 80 Å pore size) are used for the clean-up of the samples and were previously conditioned with 5 mL MeOH and 5 mL H_2O. After passing 100 mL of the sample through the column, a washing step with 5 mL H_2O and subsequent drying of the column is needed. The retained antibiotics are eluted with 5 mL MeOH and the obtained extract is evaporated under N_2 at 40°C until full dryness. The residues are then resolved in 1 mL of the reconstitution liquid (50:25:25 v/v H2O:MeCN:MeOH + 0,05% acetic acid) and filtered through a 0.22 µm PVDF filter (Merck) into a vial (Chrom4, Suhl Germany).

2.1.3. UHPLC-MS/MS

Separation is performed in an ACQUITY UPLC H-class (Waters) over a reversed-phase ACQUITY UHPLC BEH C18-column (2.1 x 150 mm; 1.7 µm, 100 Å) (Waters, Milford USA). Elution follows a gradient with solvent A (H_2O + 0.05% acetic acid) and solvent B (MeCN:MeOH 50:50 v/v + 0.05% acetic acid) at a rate of 0.4 mL/min and for 23 min at 45 °C. For the first 2.45 min, no solvent B is used, followed by a linear increase of solvent B to 95 % from min 2.45-14.45. This is held for 4.5 min and re-equilibration of the gradient at 0% of solvent B is maintained from 18.95-23 min. This UPLC H-class system is coupled to a Xevo TQ-XS spectrometer (Waters), equipped with a tandem quadrupole, monitoring at least 2 transitions for the antibiotics included in this method and only one product-ion for internal standards. Ions are generated in positive mode (ESI +) or negative mode (ESI -) with optimized cone voltage and collision energy **(Table 1)**. As a lot of antibiotics are analyzed in one method, a prior screening is needed, meaning that the transition of the precursor ion to only one fragmentation ion is followed. To prevent peak distortion, 1 µL (ESI +) or 10 µL (ESI -) of the extracted sample is injected. In case a signal is obtained, the sample is reinjected and a minimum of 2 fragmentation ions are followed for confirmation. Only if this requirement is fulfilled, the analyte can be identified. For the following validation, only one fragmentation ion with the highest signal over noise (S/N) was selected. Generated data is processed with MassLynx software version 4.2 (Waters).

Table 1: Optimum mass spectrometric parameters of antibiotic residues in UHPLC-MS/MS method: ionization mode, precursor and fragmentation ion (m/z), voltage (V), and collision energy (eV) and retention time (t_R) (min). Every class of antibiotics is accompanied by internal standards (IS). Antibiotics belonging to group B are indicated in italics to make a distinction from group A. Substances belonging to group A are quantifiable between 0.01 µg/L and 20 µg/L, group B on the other hand follows a calibration curve between 0.1 µg/L and 40 µg/L.

Component	Ionization mode	Precursor ion [m/z]	Fragmentation ion [m/z]	Cone voltage [V]	Collision energy [eV]	**t_R (min)**
β-lactam (penicillins)						
amoxicillin	ESI+	366.01	113.94	12	28	4.51
			133.96		26	
			207.93		12	
ampicillin	ESI+	349.95	106.02	18	22	6.59
			160.05		12	
			191.99		15	
benzylpenicillin (pen G)	ESI+	335.07	114.02	10	25	10.21
			160.00		15	
			176.00		17	
cloxacillin	ESI+	436.02	160.00	10	15	11.59
			177.98		17	
			288.00		13	
dicloxacillin	ESI+	470.10	160.01	15	10	12.25
			310.91		15	
nafcillin	ESI+	414.97	114.98	16	68	12.04
			170.95		42	
			198.97		12	
oxacillin	ESI+	402.01	143.98	10	38	11.14
			159.97		10	
			242.93		12	
phenoxymethylpenicillin (pen V)	ESI+	350.97	160.02	25	10	10.86
			192.09		8	
piperacillin (IS)	ESI+	540	181.94	20	26	10.07
β-lactam (cephalosporines)						
cefacetril	ESI+	361.98	177.90	20	12	6.7
			257.95		12	
cefadroxil	ESI+	364.05	113.91	14	20	5.28
			207.87		10	
cefalexin	ESI+	347.93	157.97	12	8	6.61
			173.96		18	
			190.99		8	
cefalonium	ESI+	459.04	151.90	15	20	6.9
			157.95		18	
			336.99		10	
cefapirin	ESI+	424.05	151.97	20	22	6.29
			292.03		14	
			319.90		12	
cefazolin	ESI+	455.04	155.93	15	16	7.66
			294.91		16	
			322.93		12	

cefoperazone	ESI+	668.4	165.00	40	25	8.43
			177.90		30	
			525.96		16	
cefquinome	ESI+	529.16	134.06	22	17	6.92
			166.91		26	
			395.92		12	
cefuroxime	ESI+	447.00	342.00	20	15	6.91
			386.00		12	
ceftiofur	ESI+	523.97	94.89	26	38	9.52
			125.05		58	
			240.90		16	
cefradin	ESI+	350	108.05	12	17	7.81
			158.02		10	
			176.11		15	
desacetylcefapirin	ESI+	381.89	111.86	24	44	9.51
			124.07		44	
			151.90		26	
cefotaxime (IS)	ESI+	455.9	124.99	20	50	7.52
ceftiofur-D3 (IS)	ESI+	527.06	244.09	27	16	9.51
sulfonamides						
dapsone	ESI+	248.9	92.03	22	22	7.5
			107.96		20	
			155.95		14	
sulfacetamide	ESI+	215.14	92.14	25	17	5.11
			108.14		15	
			156.11		10	
sulfabenzamide	ESI+	277.15	92.06	30	20	8.66
			108.13		20	
			156.03		10	
sulfachloropyridazine	ESI+	284.85	92.03	22	30	7.87
			108.03		26	
			155.95		14	
sulfaclozine	ESI+	284.92	92.03	24	32	8.93
			107.96		22	
sulfadiazine	ESI+	250.89	92.03	22	26	6.05
			107.96		22	
			155.63		14	
sulfadimethoxine	ESI+	310.91	92.03	30	30	9.12
			107.96		28	
			155.94		20	
sulfadoxine	ESI+	310.91	92.03	26	28	8.23
			108.03		26	
			155.94		18	
sulfaguanidine	ESI+	215.06	108.05	15	25	5.12
			156.01		15	
sulfamerazine	ESI+	264.91	92.04	24	24	6.85
			107.97		26	
			155.95		16	

sulfameter	ESI+	280.91	92.00	35	27	7.88
			107.91		23	
			126.01		23	
sulfamethazine	ESI+	278.92	92.03	26	30	7.46
			124.02		24	
			185.98		16	
sulfamethizole	ESI+	270.78	91.90	20	25	7.35
			107.90		20	
			155.90		15	
sulfamethoxazole	ESI+	253.89	92.03	22	28	8.07
			107.96		24	
			155.95		16	
sulfamethoxypyridazine	ESI+	280.97	92.04	26	28	7.28
			107.97		26	
			155.95		16	
sulfamonomethoxine	ESI+	281.09	108.00	22	24	7.88
			126.00		20	
			156.00		18	
sulfaphenazole	ESI+	315.22	108.08	45	22	9.03
			156.04		17	
			222.03		15	
sulfapyridine	ESI+	249.9	92.04	24	30	6.66
			107.97		22	
			155.96		16	
sulfaquinoxaline	ESI+	300.91	92.03	24	32	9.28
			108.03		26	
			155.94		16	
sulfathiazole	ESI+	255.85	92.03	20	24	6.44
			107.98		25	
			155.94		14	
sulfisomidine	ESI+	279.72	92.06	20	17	6.14
			108.20		17	
			156.03		15	
sulfisoxazole	ESI+	267.78	91.97	22	25	8.35
			112.97		15	
			155.97		12	
sulfadimethoxine-13C6 (IS)	ESI+	316.96	98.08	30	32	9.12
tetracyclines						
chlortetracycline	ESI+	479.02	98.01	24	39	8.56
			153.98		26	
			443.93		20	
doxycycline	ESI+	445.17	154.00	27	30	8.87
			320.98		30	
			428.97		20	
oxytetracycline	ESI+	460.99	200.98	20	42	7.37
			282.95		38	
			425.97		18	
tetracycline	ESI+	444.99	98.01	22	38	7.48

			153.98		26	
			409.98		18	
methacycline (IS)	ESI+	443.1	381.09	18	24	8.64
macrolides						
clarithromycin	ESI+	748.4	158.06	30	28	10.58
			558.18		20	
			590.19		20	
erythromycin	ESI+	734.43	82.99	22	52	9.76
			158.00		32	
			576.29		20	
josamycin	ESI+	828.33	108.88	30	40	10.79
			173.88		36	
			229.40		26	
neospiramycin	ESI+	350.26	142.12	20	14	6.6
			160.12		12	
			173.98		15	
spiramycin	ESI+	843.42	101.00	44	44	8.02
			142.02		34	
			174.06		38	
tilmicosin	ESI+	869.47	88.00	60	68	8.77
			132.01		48	
			174.00		44	
tulathromycin metabolite	ESI+	578.26	116.07	40	25	6.5
			158.09		25	
			421.14		25	
tylosin a	ESI+	916.58	174.05	47	37	10.08
			772.08		27	
tylvalosin	ESI+	1042.48	109.02	66	48	11.56
			173.92		48	
			814.20		32	
roxithromycin (IS)	ESI+	837.37	158.01	30	32	10.71
lincosamides						
lincomycin	ESI+	407.14	82.87	30	62	6.43
			126.04		30	
			359.11		18	
pirlimycin	ESI+	411.07	112.05	28	26	8.39
			363.05		16	
clindamycin (IS)	ESI+	425.08	126.10	32	26	8.68
(fluoro)quinolones						
cinoxacin	ESI+	262.9	130.99	22	38	8.56
			160.96		32	
			188.99		28	
ciprofloxacin	ESI+	331.97	203.07	26	36	6.9
			230.96		38	
			245.01		24	
danofloxacin	ESI+	357.99	82.02	15	42	7.07
			96.05		24	

			340.08		26	
difloxacin	ESI+	400.04	278.95	28	36	7.5
			284.93		32	
			298.97		28	
enoxacin	ESI+	321.03	205.67	28	28	6.68
			231.86		34	
			256.96		16	
enrofloxacin	ESI+	360.17	72.07	30	34	7.18
			203.08		40	
			245.10		27	
flumequine	ESI+	262.00	125.92	22	46	10.4
			173.96		40	
			202.00		30	
marbofloxacin	ESI+	362.98	72.07	24	20	6.58
			72.46		26	
			320.05		17	
nalidixic acid	ESI+	232.86	104.00	18	40	10.11
			130.93		34	
			158.95		30	
norfloxacin	ESI+	319.97	204.91	24	28	6.79
			233.05		25	
			256.01		20	
ofloxacin	ESI+	362.14	261.03	28	28	6.78
			318.03		20	
oxolinic acid	ESI+	261.9	130.00	20	40	9.01
			159.91		38	
			160.34		32	
sarafloxacin	ESI+	386.08	284.96	28	26	7.47
			299.01		28	
			322.02		22	
piperacillin (IS)	ESI+	540	181.94	20	26	10.07
amphenicols						
chloramphenicol	ESI-	325.85	151.85	20	14	8.93
			193.91		12	
			256.90		12	
florphenicol	ESI-	355.78	118.95	20	30	8.16
			184.87		22	
			218.87		10	
florphenicol-amine	ESI-	248.02	129.92	30	20	0.99
			206.76		15	
			230.01		12	
thiamphenicol	ESI-	353.85	184.88	24	18	7.08
			239.96		15	
			289.89		12	
chloramphenicol-D5 (IS)	ESI-	325.85	156.85	22	18	8.89
pleuromutulins						
tiamulin	ESI+	494.23	73.04	26	62	9.91

			119.01		38	
			192.02		20	
valnemulin	ESI+	565.26	163.86	20	30	10.82
			262.06		18	
			285.04		28	
clindamycin	ESI+	425.08	126.10	32	26	8.68
diaminopyrimidines						
trimethoprim	ESI+	290.98	122.99	36	22	6.62
			230.01		22	
			260.96		24	
trimethoprim-D9 (IS)	ESI+	300.04	263.98	36	26	6.55
ansamycins						
rifaximin	ESI+	786.21	150.91	35	35	12.78
			754.13		25	
roxithromycin (IS)	ESI+	837.37	158.01	30	32	10.71

2.1.4. Quantification

Residues are quantified using the relative peak area of an analyte (peak area / peak area internal standard) and corresponding calibration curves. Due to differences in sensitivity between the substances included in this multiresidue method, a distinction is made between quantification ranges. Therefore, two separate calibrations are used. Blank (surface) water samples, in which no residues have previously been detected, are spiked with concentrations in two sequences (2nd sequence between brackets): 0.01 µg/L (0.1 µg/L) – 0.05 µg/L (1 µg/L) – 0.1 µg/L (5 µg/L) – 1 µg/L (10 µg/L) – 5 µg/L (20 µg/L) – 10 µg/L (30 µg/L) – 20 µg/L (40 µg/L). Substances belonging to group A are quantifiable between 0.01 µg/L and 20 µg/L, group B on the other hand follows a calibration curve between 0.1 µg/L and 40 µg/L. Concentrations in these calibration curves are selected based on previously detected concentrations in relevant environmental waters. It was expected that similar locations would be investigated (Flanders Environment Agency, 2018; Rasschaert et al., 2022).

2.1.5. Validation

Due to a lack of specific guidelines, a validation procedure is adopted, derived from Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021), which normally deals with the method performance of the analysis of pharmacologically active substances in food-producing animals. Using matrix-matched samples, the linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined three times by using the 8 points in the calibration range. To illustrate the versatility of this approach, calibration curves have also been established using blank seawater samples. Linearity is checked by evaluating R^2 coefficients and residuals, calculated with TargetLynx (Waters) software. The limit of detection is determined as 3 times the standard deviation of the y-intercept of the regression line divided by the slope. The limit of quantification is subsequently calculated as 10 times the standard error of the y-intercept of the regression line divided by the slope. The recovery, repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) are measured with 3 separate analyses of 6 repetition points at 3 different concentration levels. Since there is no available certified reference material, recoveries by trueness are calculated as the ratio between the detected average result and the actual spiked concentrations. Quality Control (QC) samples are put in place at 0.1, 1 and 10 µg/L for group A and at 1, 10 and 30 µg/L for group B at the same time by spiking blank surface water samples. Specificity is investigated by separately injecting all standards at 1 ng/µL in ammonium acetate (0.2 M). Possible non-target peaks are checked for elution at or near target components to exclude different components and metabolites interfering with each other.

2.1.6. Stability during storage

Stability issues are addressed by fortifying blank surface water to a concentration of 1 µg/L (group A) and 10 µg/L (group B). For each preservation condition: 1) storage at 4°C (refrigerator) and 2) storage in a freezer (-18 °C), the stability is examined after 4, 7, 11 and 14 days. Concentrations are determined by quantification with a calibration curve. Stability (%) is determined as the recovery after a specific storage time (t = 4, 7, 11, 14 days) compared to the calibration range made at t = 0 days. The experiment is repeated in fourfold (duplicates were

analyzed at 2 different times). Statistical analysis is performed in R using a non-parametric Kruskal-Wallis one-way ANOVA on ranks, assuming that the antibiotics in one class can be grouped.

2.1. Study area and sample collection

For validation and quantification purposes, a nearby stream in Melle, Belgium, was chosen as the blank matrix. Repeated analyses made sure no residues were present above the limits of detection. A sample was taken in Oostende, Belgium to test the suitability for the marine environment. The validated method is put to the test in the determination of antibiotic residue contamination in groundwater. For this purpose, Manure Action Plan (MAP) locations, following the implementation of the nitrate directive (91/676/CEE), were selected as representatives for regions with intensive livestock production in Flanders (Belgium) (**Error! Reference source not found.**). Fifty groundwater locations are chosen in close proximity to those MAP locations in the *Yser*-Basin in West-Flanders (35 locations) and the Northern part of Antwerp, the *Meuse*-Basin (15 locations) during the period August-December 2022, and sampled by Eurofins, according to ISO 17025. Samples were taken at depths between 1.80 m – 7.45 m, depending on available filters and the water level at the moment of sampling, because the months of August until October 2022 were drier than usual, based on meteorological data (not shown), obtained from a 5 km interpolated grid, provided by the Belgian Royal Meteorological Institute (RMI). This resulted in an extended period of sampling until December for groundwater that were dried-up and could not be sampled previously. At each site, 1 L of water was collected, transported in refrigerated conditions and frozen at -18 °C until analysis by means of UHPLC-MS/MS. Field parameters: temperature (°C), pH, electrical conductivity (µS/cm) and dissolved oxygen concentration (mg/L), are measured each time at the moment of sampling.

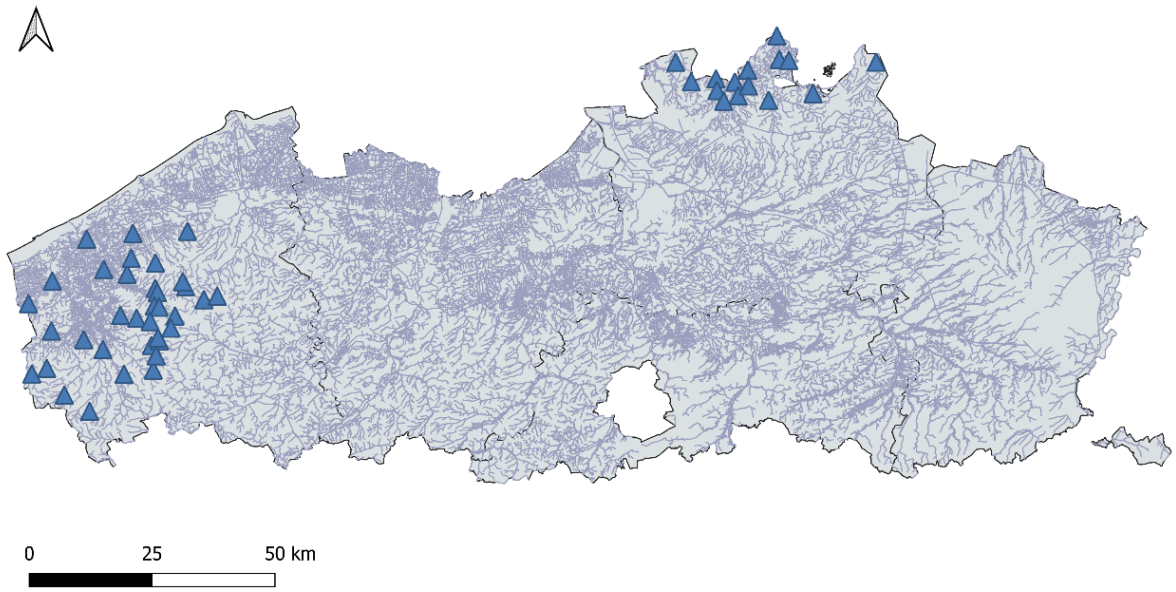

Figure 1: Hydrological map of Flanders indicating the sampled locations in Flanders (Belgium): 35 locations in West-Flanders around the *Yser*-Basin and 15 around the *Meuse*-Basin in Antwerp. Both regions are associated with high intensive livestock production. Made in Geopunt4Qgis. ***(IN COLOR)***

3. Results and discussion

3.1. Validation study

3.1.1. Identification

Identification is based on relative retention times. For confirmatory purposes of identifying an antibiotic substance, a minimum of 4 identification points is required. For prohibited residues, 5 identification points are demanded. The use of UHPLC-MS/MS in this research fulfills these requirements since it provides 5 identification points (European Commission, 2021). Besides, the signal/noise (S/N) ratio of all ions must be greater than 3. The ion ratio of the analyte to be confirmed should correspond to that of the matrix-fortified standards at comparable concentrations and measured under the same conditions, within ± 40 % relative deviation. The method resulted in a multiresidue analysis of 78 different veterinary antibiotics, which is, to our knowledge, the most extensive UHPLC-MS/MS method for the quantification in aquatic matrices, including groundwater, yet (Balzer et al., 2016; Burke et al., 2016; Kivits et al., 2018). This method can be used for the identification of veterinary antibiotics, which is the main purpose,

but the partial overlap with human medicine makes it possible to analyze urban wastewater and hospital effluents as well, regarding antibiotic use in human medicine. One limitation, however, is that aminoglycosides are not represented in this method due to separate sample clean-up and different chromatographic requirements (Huygens et al., 2021), which would increase costs and analysis time.

3.1.2. Trueness by recovery

In order to assess trueness, repeatability and reproducibility, linearity was examined. A linear model ($y = ax + b$) with a corresponding 1/X weighting function (x = concentration) is followed. A linearity criterium ($R^2 > 0.99$) as an average over 3 series is met for almost all substances, except for benzylpenicillin, cefadroxil, cefquinome, rifaximin and lincomycin ($R^2 > 0.95$) (**Table S1** and **Table S2**). Residuals are accepted for all analytes. To illustrate the versatility of this approach, calibration curves have also been established using blank seawater samples. Similar correlation coefficients were calculated (data not shown). It can be assumed that this method is suitable for the quantification of antibiotic residues in diverse aquatic environments, such as: surface water, groundwater and marine samples. **Table S1** and **Table S2** show the determined recoveries (%). Requirements, as laid out in Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021) are fulfilled, except for some substances (sarafloxacin and tiamulin) which are excluded from being quantified at that level with this method. For mass fractions not greater than 1 µg/kg (validation level 0.01 µg/L), deviations between -50% and +20% are allowed. Between 1 µg/kg and 10 µg/kg (validation levels 1 µg/L and 10 µg/L), these variations in recovery can range between -30% and +20%. Higher than 10 µg/kg, so in this case validation level 30 µg/L, recoveries may vary between -20% and +20%.

3.1.3. Sensitivity

Limits of detection range between 0.01 and 1 µg/L (**Table S1** and **Table S2**), depending on the substance and are similar to those found in other studies (Goessens et al., 2020; Zhou et al.,

2012). González-Gaya et al., (2018) achieved lower limits of detection, but only considered 3 antibiotics in their method (González-Gaya et al., 2018). The same explanation goes for other studies, with an additional possible cause for lower LODs at higher sampling volumes (1 L and 1.5 L) (Borecka et al., 2013; Zhou et al., 2012). The limited sample volume (100 mL) during our method enables a more efficient and less time-consuming process, with reductions in analysis time between 4.5 h – 7 h. A UHPLC-HRMS approach obtained similar LODs for most antibiotic classes, whereby differences can be attributed to other mass spectrometric properties and the use tap water as a blank matrix (Goessens et al., 2020). LODs vary depending on the chemical structure of antibiotic residues. The relatively higher limits of detection for β-lactam antibiotics are based on degradation during acidification of the water samples (Goessens et al., 2020; Gros et al., 2013). Overall, compromises had to be made to achieve a multiresidue method. Obtained LODs in this study vary depending on the antibiotic class following differences in the chemical structure. It has been reported that better results for β-lactam are possible when samples were acidified below 3.5 before extraction (Gros et al., 2013; Grujić et al., 2009). To achieve the best overall multiresidue method, compromises had to be made.

3.1.4. Precision: repeatability and reproducibility

Under diverse conditions, quantitative measurements should remain consistent. Acceptable variation coefficients for repeatability and intra-laboratory reproducibility are fixed at 25% and 30% for the respective mass fractions ranges 10-120 µg/L and <10 µg/L. The conditions are met for almost all residues at all levels, except for some substances (marked x in **Table S1** and **Table S2**) which are excluded from being quantified at that level with this method. Additionally, lacking a sufficient number of repetitions (indicated by (-) in **Table S1** and **Table S2)** it can be concluded that benzyl-penicillin, cefquinome, ciprofloxacin, difloxacin, erythromycin, ofloxacin, spiramycin, sulfaguanidine, tiamulin, tilmicosin and tulathromycin metabolite can only be semi-quantified. Comparing the obtained results and taking into account the number of analytes and sample volume, this method shows to be an excellent way of quantifying a diverse number of

antibiotic residues in water (Borecka et al., 2013; Gbylik-Sikorska et al., 2015; Mirzaei et al., 2017).

3.1.5. Specificity

This method has to be able to distinguish between different compounds to avoid false-positive and false-negative results. By injecting solely 1 compound at each time, it was noted that: cefradin gives a 6.2 % response for cefalexine, sulfamonomethoxine results in a 10.5 % increase of sulfamethoxypyridazine, norfloxacine results increases enoxacine (4.4 %) and chlortetracycline interferes with tetracycline (5.8 %). No other significant responses were observed. This evaluation by Wille et al. did not encounter any specificity problems, but the antibiotics that interfered in this study were not included in their research method (Wille et al., 2010). Goessens et al. didn't report interference of chlortetracycline with tetracycline, which can be attributed to the differences between HRMS and MS/MS (Goessens et al., 2020). Taking this into account in the analysis, no profound difficulties are expected.

3.1.6. Requirements for chromatographic separation

As laid out in Commission Implementing Regulation 2021/808 (European Commission, 2021), criteria on chromatographic conditions have to be fulfilled. In this method, the retention time of all analytes of interest falls within a tolerance of ± 0.1 min, compared to a matrix-fortified standard in the calibration. Besides, the ratio of the retention time of analytes to that of the internal standards (relative retention time), has a maximum deviation of 1%.

3.1.7. Stability during storage

Antibiotics are unstable when degradation results in a recovery below 85%, according to Commission Implementation Regulation (EU) 2021/808 (European Commission, 2021). However, in order to make comparisons with the results obtained by Llorca et al., (2014), the same criterion of a recovery above 80% is used to confirm stability (Llorca et al., 2014). For all

classes, the recovery (%) is plotted as a function of time, distinguishing between the two different storage conditions (4°C and -18°C) and the type of antibiotic (**Figure 2, Figure 3, Figure S1 and Figure S2**). The mean and standard deviations of 4 replicates are plotted. Displayed confidence intervals (a first-order degradation is assumed) are merely used as a way to visualize the data transparently. Significantly different ($p < 0.05$) results indicate that there are differences in recovery between the different times, implying changes between the start and the end of the experiment. Together with the illustrated degradation behavior, it is possible to draw some conclusions. The degradation of antibiotics in water samples during a two-week simulation study is highly dependent on chemical properties. Moreover, different storage conditions (temperature) can lead to more rapid destruction. The group of β-lactams (penicillins and cephalosporins), macrolides and ansamycins (rifaximin) degrade under both temperature conditions ($p < 0.05$). Some antibiotics reach their half-life after only two weeks. For instance, ampicillin is 50% degraded after 4 days of storage at 4°C. Pleuromutilins, (fluoro)quinolones and tetracyclines are more susceptible to stability issues when stored at 4 °C ($p < 0.05$). Phenicols are stable, except for florphenicol-amine. Lincosamides and trimethoprim are considered stable over two weeks. Sulfonamides also display differences in stability between both storage conditions, but since the degradation line of 80% has not been crossed, no such precautions need to be taken specifically for sulfonamides. However, in general, since it must be assumed that the type of residue present in a sample is not known, samples should be preserved at -18 °C. Keeping a sample at -18 °C will result in reduced degradation. This is in line with previous observations (Cha and Carlson, 2019; Llorca et al., 2014; Loftin et al., 2008). Sulfonamides, trimethoprim and lincosamides are among the most stable classes. (Fluoro)quinolones, tetracyclines and macrolides proved to be unstable (<80%) (Esther et al., 2004; Gbylik-Sikorska et al., 2015; Llorca et al., 2014; Loftin et al., 2008). The rapid degradation of β-lactam antibiotics has been demonstrated in previous studies (Mitchell et al., 2014; Pérez-Parada et al., 2011). Besides, tetracyclines were more stable in this experiment, than previously reported by Llorca et al., (2014). These differences can be explained by the influence of pH and

biodegradation because our stability study was carried out on real, blank samples, not sterile or HPLC pure water. These aspects, as well as photodegradation, are not controlled specifically considered in this experiment. It can be decided that water samples need to be stored as cool as possible and analyzed within two weeks.

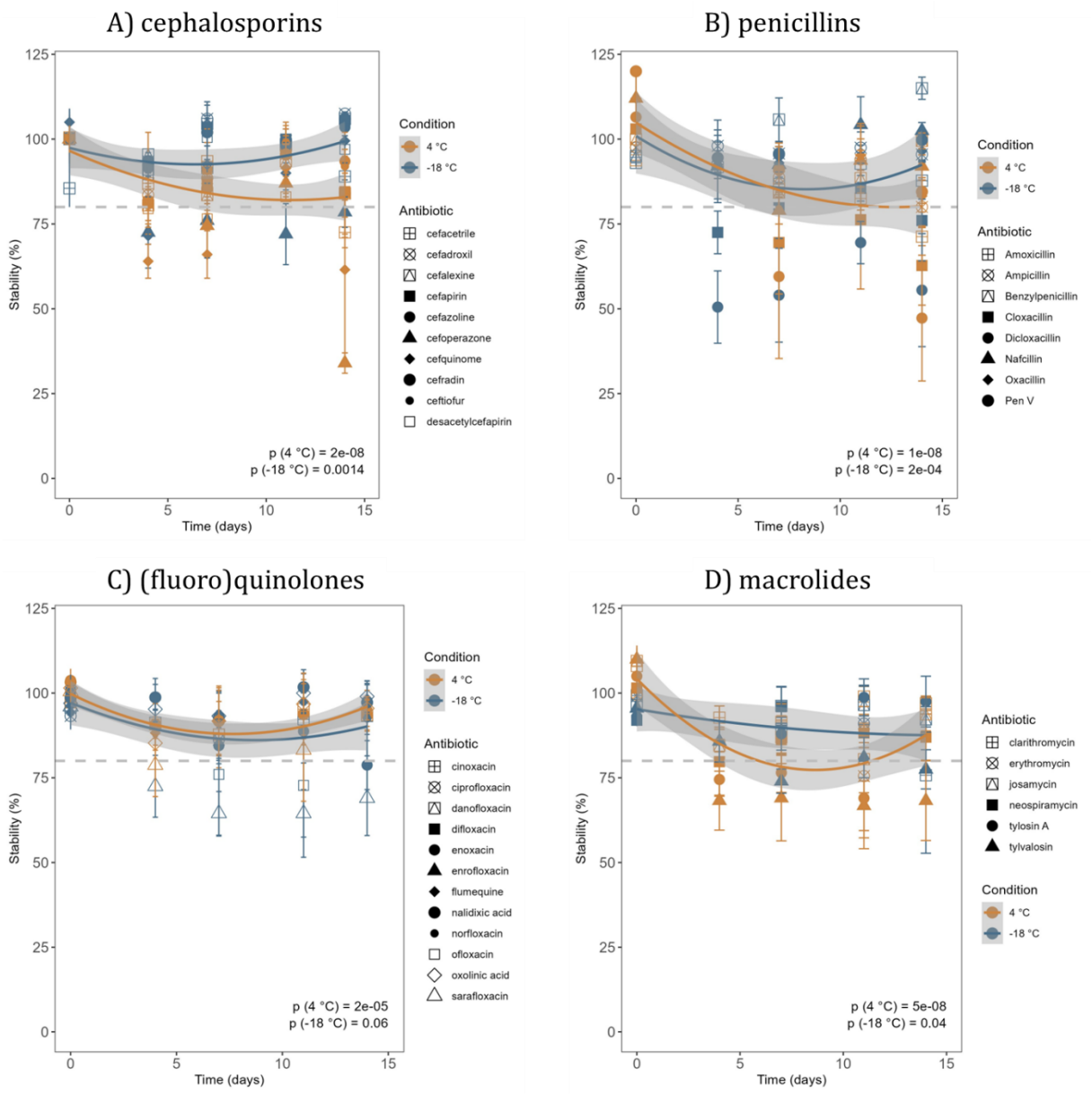

Figure 2: Stability (%) progression of β-lactam antibiotics: A) cephalosporins and B) penicillins and C) (fluoro)quinolones and D) macrolides over time in function of storage temperature (4 °C and -18 °C). If identification criteria were not sufficiently met, the data point is not included in the graph. A 1st-order degradation (95% confidence interval) is plotted as the best fit for the data. Statistical p-values are calculated after Kruskal-Wallis one-way ANOVA analysis on Ranks, where $p < 0.05$ indicates that data points differ in function of the time. The dotted line represents the stability criterium of 80 %. ***(IN COLOR)***

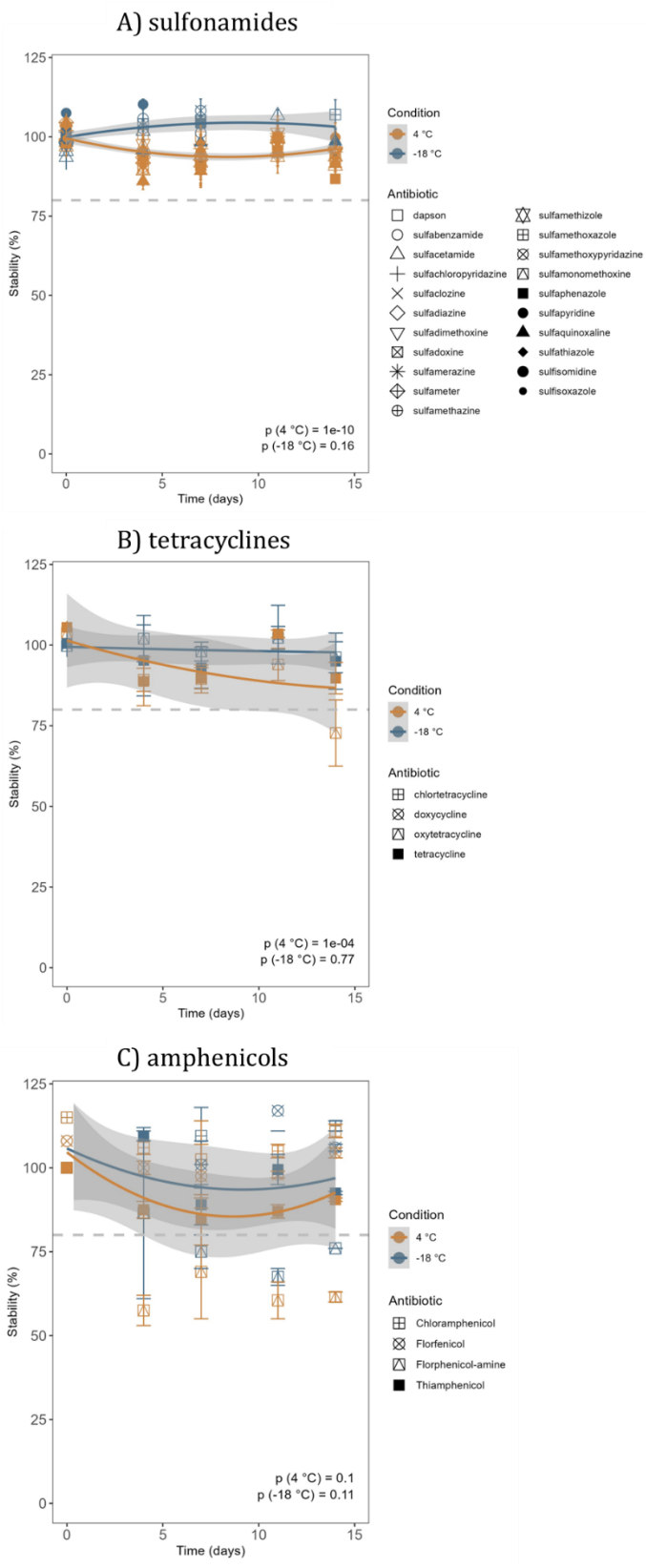

Figure 3: Stability (%) progression of A) sulfonamides, B) tetracyclines and C) amphenicols over time in function of storage temperature (4 °C and -18 °C). If identification criteria were not sufficiently met, the data point is not included in the graph. A 1st-order degradation (95% confidence interval) is plotted as the best fit for the data. Statistical p-values are calculated after Kruskal-Wallis one-way ANOVA analysis on

Ranks, where $p < 0.05$ indicates that data points differ in function of the time. The dotted line represents the stability criterium of 80 %. ***(IN COLOR)***

3.2. Detection and quantification of antibiotic residues (ABRs) in groundwater samples

Antibiotic concentrations were overall, as expected, low. In only 7 (out of 50) groundwater locations, ABRs were detected at concentrations between < LOD – 0.03 µg/L **(Table 4)**. Traces (detections below the LOD) are regarded as a confirmed presence of the antibiotic if previously described identification criteria are sufficiently met **(Figure S3)**. Samples can vary in biological and chemical composition which can result in lower concentrations than the obtained limit of detection during the validation procedure. Out of these 7 samples, only 2 contained more than 1 ABR. Two ABRs (2%) or at most 3 (2%) distinctive ABRs were detected in an individual sample. In total 7 ABRs from 4 classes were identified; chloramphenicol, oxolinic acid, sulfadiazine, sulfadoxine, sulfamethazine, sulfisoxazole and tetracycline.

Table 2: Reported concentrations (µg/L) of ABRs in samples groundwater (n = 50). Antibiotic residues reported more than once (chloramphenicol, sulfamethazine) are present at the same concentration in each sample.

Substance	Concentration (µg/L)	Frequency (%) (n = 50)
chloramphenicol	< LOD	8%
oxolinic acid	< LOD	2%
sulfadiazine	< LOD	2%
sulfadoxine	0,010	2%
sulfamethazine	0,012	4%
sulfisoxazole	0,013	2%
tetracycline	0.03	2%

Other comparable studies show largely similar results (Balzer et al., 2016; Boy-Roura et al., 2018; Burke et al., 2016; Flanders Environment Agency, 2018; Kivits et al., 2018; Szekeres et al., 2018). In contrast to studies carried out regarding antibiotics in surface water (Burke et al., 2016; Danner et al., 2019; Gbylik-Sikorska et al., 2015; Siedlewicz et al., 2018), the occurrence of antibiotic residues is more modest in groundwater. Sulfonamides are, as a group, most repeatedly observed, because their properties allow a high mobility to water and this is

394 combined with a high use in Flanders. Tetracyclines, often registered as an antibiotic (BelVet-
395 SAC, 2022), are only once detected at a low concentration. One reason for infrequent detection
396 may be that with high sorption coefficients and resulting lower mobility, this group is less
397 known to be present in the aquatic matrices (groundwater and surface water), considering the
398 higher retention to soil (Huygens et al., 2022). Another reason may be the complexation with
399 calcium and magnesium ions (Kümmerer, 2009). Lastly, oxolinic acid is detected in one sample
400 and has, to our knowledge, never before been detected in groundwater, where the influence of
401 veterinary medicine was indisputable (MAP). Other fluoroquinolones are absent in the studied
402 groundwater, possibly due to (photo)degradation and adsorption to organic materials (Golet et
403 al., 2003). The presence of chloramphenicol is remarkable, considering the unauthorized use of
404 this antibiotic for food-producing animals since 2010 (EFSA, 2018), but not unique (Kivits et al.,
405 2018). Several reasons are indicated as possible causes for this presence in groundwater
406 samples, including: natural occurrence (Berendsen et al., 2010) and permitted use in human
407 medicine and for treatments of infections in non-food producing animals (EFSA, 2018),
408 accompanied by a high persistence in the environment (Mitchell et al., 2015). Analytical errors
409 and contamination during lab work are ruled out by analyzing samples at different times
410 (separate days, including repetitions) and since blank samples were all negative. All other
411 detected antibiotics are believed to be strictly related to veterinary use, since all wells are
412 chosen close to Manure Action Plan locations in areas with intensive livestock production, away
413 from urban influences such as wastewater. β-lactam antibiotics are not detected, although
414 penicillins are most often registered for veterinary use (BelVet-SAC, 2022). Degradation by
415 hydrolysis of β-lactam antibiotics, as supported by the data on stability, results in the absence of
416 this group in groundwater. Macrolides are, unsurprisingly, not detected as a result of the low
417 water solubility, low stability, higher sorption properties and limited use (Felis et al., 2020).
418 Trimethoprim, previously frequently detected in groundwater (Burke et al., 2016), is not
419 reported in this study. It is possible that the limit of detection with our method doesn't allow to
420 detect similar minor concentrations (< 0.01 µg/L). The findings from a similar study in 2018

showed a higher detection frequency (53% of samples contained at least one antibiotic) and a maximum of 3 antibiotics in one sample was reported (Flanders Environment Agency, 2018; Rasschaert et al., 2022). Besides, the focus was not only on high-risk agricultural areas, but also on a wide variety of areas, which may explain disparities. In contrast with our results, cefapirin, lincomycin and tylosin were sporadically reported, next to the frequent presence of sulfamethazine. Sulfamethazine was highly present (35%) in groundwater, albeit at very low concentrations, which are not detectable with full certainty by the method in this study. Kivits et al., (2018) detected antibiotics in groundwater, also associated with agricultural use, in comparable concentrations (0,30-18 ng/L) and type of antibiotics, most often sulfonamides (Kivits et al., 2018). However, a higher detection frequency (67%) is reported in that study and the sporadic presence of ciprofloxacin and lincomycin can be assigned to differences in analytical method performance and the single sample approach without repetitions. Besides, the dry period during our sampling period and the resulting limited flow of antibiotics with infiltrating water can additionally explain differences. At last, matrix properties influence the detection of residues as well. On-site measurements reveal temperature fluctuations between 12,50-15,40 °C, a pH range between 4.09-8.00, EC variations from 158-4374 µS/cm and there were differences in the measured dissolved oxygen concentration (mg/L) (0.50-4.65 mg/L) between the groundwater samples. Positive samples ranged in pH between 5.81 and 7.46. The pH influences the sorption of antibiotic residues to the soil. Low pH can promote sorption affinity, because cationic residues are expected to sorb to negatively charged soil organic matter (Zhang & Yuan., 2023). This can limit detection frequencies. At the same time, other studies show that breakdown by hydrolysis can be enhanced with increasing pH (Loftin et al., 2008). Additionally, residues were only detected in samples with measured oxygen concentrations below 1.71 mg/L. It has been shown that higher dissolved oxygen concentrations can benefit the degradation of chemicals (Schaider et al., 2014). Besides, a higher conductivity promotes the occurrence of residues (de Sousa et al., 2014). Because of a limited number of positive samples, no statistical association between the detection of antibiotics and groundwater characteristics

448 was made in this study. A study on antibiotic stability using water parameter ranges would be
449 necessary to improve the discussion on the possible occurrence of antibiotics in groundwater.

450

4. Conclusions

This paper investigates the extent of antibiotic contamination in groundwater. After validation of the proposed UHPLC-MS/MS method, it is possible to (semi-)quantify 78 antibiotics, belonging to 10 different antibiotic classes: β-lactams (20), sulfonamides (22), (fluoro)quinolones (13), macrolides (9), tetracyclines (4), amphenicols (4), lincosamides (2), pleuromutilins (2). diaminopyrimidines (1) and ansamycins (1). A stability study was carried out showing that especially penicillins and macrolides are most at risk for degradation and that sulfonamides, lincosamides and pleuromutilins are considered stable. Preservation at -18 °C is generally preferable to storage at 4 °C. This method was finally used to analyze 50 groundwater samples, originating from regions associated with intensive livestock production. Concentrations varied between < LOD and 0.030 µg/L and were, as expected, low. Chloramphenicol, oxolinic acid, tetracycline and sulfonamides (sulfadiazine, sulfadoxine, sulfamethazine and sulfisoxazole) were detected. This correlates with the veterinary use, chemical properties of these antibiotics and matrix characteristic. The effect of these residues on the selection of antibiotic resistance still needs to be investigated.

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6. Declaration of competing interest

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

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