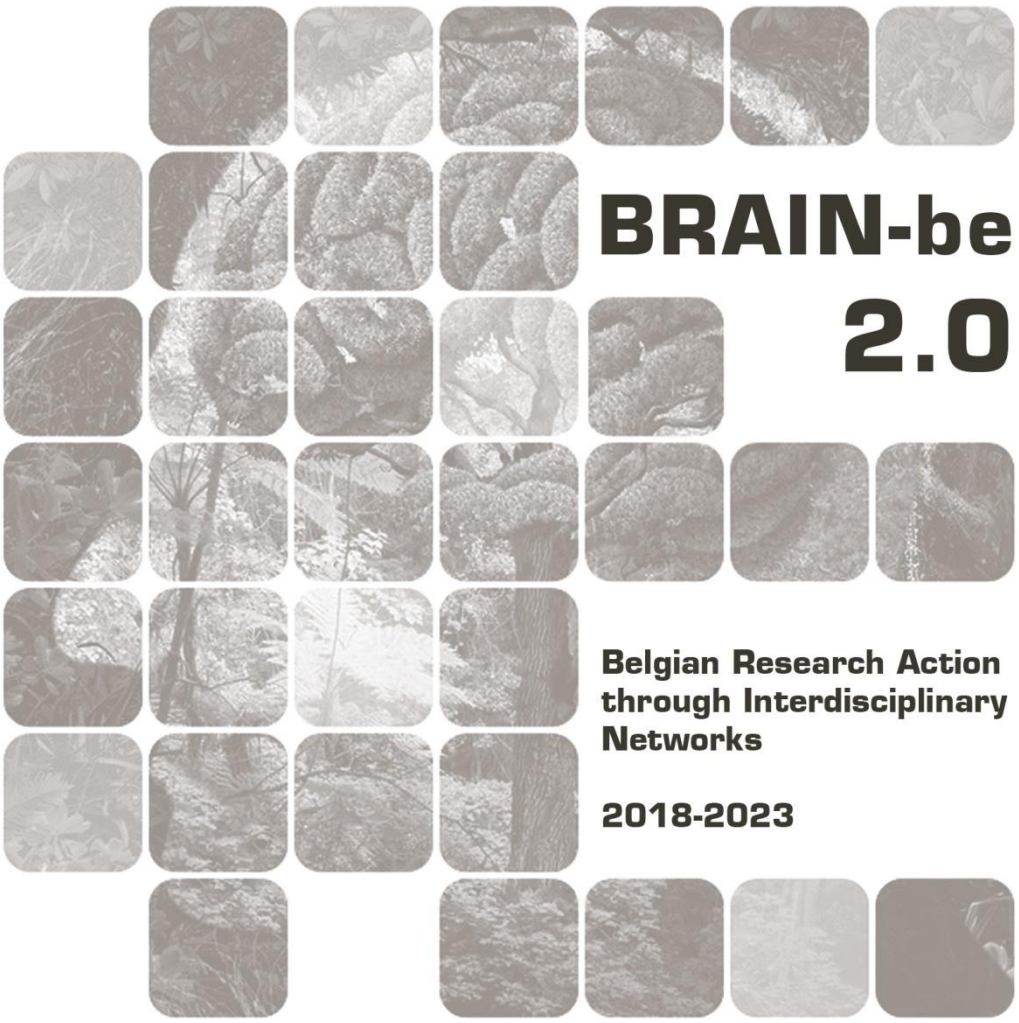

PARRTAE

FINAL REPORT

Tuts Laurens (ILVO, UGent) - Heyndrickx Marc (ILVO, UGent) - Becue Ilse (ILVO)- Rasschaert Geertrui (ILVO)

Pillar 1: Challenges and knowledge of the living and non-living world

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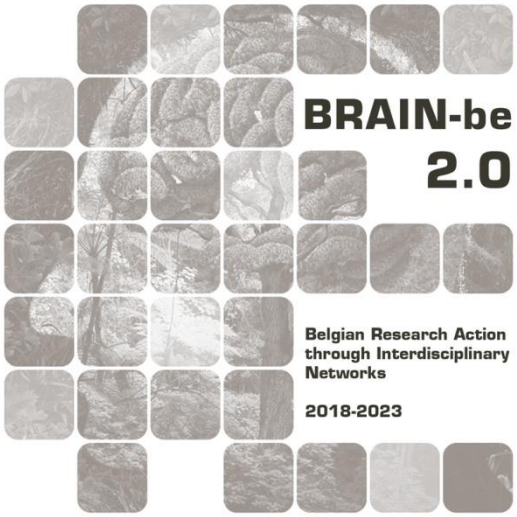

Aquatic Pollutants

PARRTAE

Probing Antibiotic Residues and Resistance Transfer in Aquatic Environments

Contract - B2/21E/P1/PARRTAE

FINAL REPORT

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1. ABSTRACT

Antibiotic resistance is a critical global health problem, driven by the use of antibiotics in health care and agriculture, which leads to environmental contamination with antibiotic residues and resistant bacteria. These contaminants, even at low levels, can drive resistance through selective pressure and horizontal gene transfer, posing risks to human, animal and environmental health systems, as the framework of "One Health" emphasizes. Aquatic environments are particularly vulnerable, receiving contaminants from wastewater, agriculture, aquaculture and industrial activities, yet research on antibiotic resistance in these settings is limited, especially beyond wastewater treatment plant discharges. To address gaps in surveillance, novel UHPLC-MS/MS methods were developed for a comprehensive analysis of antibiotic residues in water and sediments. By integrating the study of antibiotic residues and resistance, this research contributes to understanding the environmental transmission of resistance and informs policy development. Current environmental monitoring frameworks remain inadequate, highlighting the need for more comprehensive approaches to manage this growing threat.

This project investigated two major pollution hotspots. First, waterways located partly in regions with a lot of livestock agriculture in Flanders (Belgium), were studied. These waterways were chosen due to their high susceptibility to contamination from manure application. Surface and groundwater samples were collected before and after fertilization to assess antibiotic residues and resistance, focusing on *E. coli* as a fecal indicator with additional interest in the production of extended-spectrum β-lactamases. Secondly, two Belgian seaports, Nieuwpoort and Oostende, were investigated for antibiotic residues and resistance in water and sediments, analyzing organisms such as marine indicators *Shewanella* and *Vibrio* and ESBL-producing *E. coli*.

In freshwater, antibiotic residues (ABRs) were detected in 78% of the surface water samples, with 25 different residues identified, most frequently lincomycin and sulfonamides. Concentrations ranged from 0.01 µg/L to 8.83 µg/L. *E. coli* were present in 94-98% of the surface water samples, while suspected extended-spectrum β-lactamase (ESBL)-producing E. coli were detected in 26%, averaging 1% of total *E. coli*. Groundwater samples showed much lower bacterial counts and no ESBL-producing *E. coli.* In surface water, resistance to sulfamethoxazole in *E. coli* increased from 20% to 48% after fertilization. ESBL-producing *E. coli* showed higher resistance to non-β-lactam antibiotics compared to generic *E. coli.* The Predicted No Effect Concentrations (PNECs) for resistance selection were exceeded five times in freshwater (lincomycin and sulfadiazine), highlighting the risk of resistance selection. The persistent presence of sulfonamides and rising sulfamethoxazole resistance indicate the impact of veterinary antibiotic use on aquatic systems. In the marine environment, sediments contained higher concentrations of ABRs, up to 25 µg/kg, consisting mainly of quinolones and macrolides, while sulfonamides dominated in water samples. This co-occurred with higher resistance rates for sediments which showed higher resistance rates for quinolones among suspected ESBL-producing *E. coli*. *Shewanella* and *Vibrio* species were widespread. *Shewanella* showed resistance to colistin and one isolate was resistant to meropenem. Resistance in *Vibrio* was similar, but further testing is needed to draw more definitive conclusions. The work of this project demonstrates a pressing need for comprehensive environmental surveillance of antibiotics and resistance. Ultimately, these insights can inform policy frameworks to mitigate antibiotic resistance in environmental contexts.

Keywords: Antibiotic resistance, antibiotic residues, One Health, water, *E. coli*

2. INTRODUCTION

The PARRTAE project aimed to study bacteria, antibiotic resistance genes (ARGs) and antibiotic residues in European water courses. Samples were collected from diverse aquatic environments, with high and low suspected loads of antibiotic residues. These samples were investigated for the presence of antibiotic residues by means of mass-spectrometry (LC-MS/MS) and for the presence of antibiotic resistance genes using certain indicator organisms such as *Escherichia coli, Shewanella and Vibrio.*

This international project has been a collaboration between the University of Gothenburg and other partners: including Norwegian Institutet of Science and Technology (NTNU), Karolinska Institutet (KI), Universidad de las Palmas de Gran Canaria (ULPGC) and the Swedish University of Agricultural Sciences (SLU). This final report only describes the Belgian contribution by ILVO, funded by BELSPO. The position of ILVO's contribution to the entire project is clarified in the Final Report submitted to the Cofund AquaticPollutants.

3. STATE OF THE ART AND OBJECTIVES

Antibiotic resistance is an escalating global health crisis escalated by the over- and misuse of antibiotics in healthcare and agriculture, which introduces both antibiotic residues and resistant bacteria in the environment. Even at low concentrations, these residues can promote the development of antibiotic resistance by exerting selective pressure on bacteria (Murray et al., 2021; Sandegren, 2014). Resistance genes are spread between bacteria of different ecological niches and as a consequence antibiotic resistance extends national boundaries. This antibiotic resistance thread may impact both human and veterinary medicine. The interconnection of human, animal, and environmental health, described as the "One Health" approach, underscores the need to address antibiotic resistance in environmental contexts. This involves the spread of resistance genes and resistant bacteria across human, veterinary, and the environment (soil, water, and food). Antibiotic-resistant bacteria can reach aquatic systems through various pathways (wastewater, agricultural and aquaculture practices, and recreational or industrial activities). Research on the co-occurrence of antibiotic residues and resistance in aquatic environments is limited and primarily centers on wastewater treatment plant discharges (Li et al., 2010; Berglund et al., 2015). Several reports establish knowledge gaps that can be explored (Bengtsson-Palme et al., 2023; European Commission, 2017; United Nations Environment Programme, 2023).

This project aimed to provide evidence on the presence of antibiotic residues and antibiotic resistance in the aquatic environment of several European countries (Belgium, Gran Canaria, Sweden and Norway). Specifically for Belgium, two important primary contamination hotspots were chosen to study. The first area examined involved contamination associated with the application of manure in agricultural fields, acknowledged as areas of interest in AMR research and monitoring by BELMAP (2023) and the European Commission (2017). This is especially concerning in regions like Flanders in Belgium, where intensive livestock farming and population density increase the introduction of antibiotics into natural ecosystems, enhancing the risk of environmental contamination and further spread of antibiotic resistance genes. Studies have suggested that applying manure to soil may elevate antibiotic resistance levels in those soils (Huygens et al., 2022; Zhang et al., 2022). This study examines whether manure application similarly affects antibiotic resistance in nearby aquatic environments. The initial sampling round targeted agricultural waterways, selecting fifty surface water and fifty groundwater sites within the Manure Action Plan monitoring network in Flanders, given their strong connection to agricultural pollution (Vlaamse Overheid, 2019). Surface water locations were sampled both before and after fertilization periods to identify any changes in antibiotic contamination and resistance levels. *Escherichia coli,* a fecal indicator, was used as a study organism. Next to their function as indicator, the World Health Organization acknowledges *Enterobacterales* resistant to 3rd and 4th generation cephalosporins and carbapenems as priority pathogens (World Health Organization, 2024). A second risk zone involved the contamination of marine harbors. These can be considered as an interconnection point between humans, animals and the environment. In that study, we investigated the presence of antibiotic residues in both waters and sediments, along with antibiotic resistance in indicator organisms, including ESBL-producing *E. coli*, *Shewanella*, and *Vibrio*, collected from two major Belgian seaports, Nieuwpoort and Oostende.

Although policies like the EU Water Framework Directive aim to monitor some antibiotics in aquatic environments, comprehensive knowledge on a wide range of antibiotics is lacking as only 4 antibiotics are monitored annually. Furthermore, because the current Directive does not monitor a specific residue for more than 4 years, data on the persistence of antibiotic residues in the aquatic environment

is limited. Additionally, existing research methods have not been comprehensive in the number of antibiotic classes included (Balzer et al., 2016; Burke et al., 2016). Therefore, a new UHPLC-MS/MS method for water analysis was developed. Furthermore, since antibiotic residues (and resistant bacteria) can accumulate in sediments (Maghsodian et al., 2022), a new method was developed. These provide cost-effective, fast methods that can be incorporated into surveillance programs. Currently, there are no environmental standards similar to those in clinical and agricultural contexts (Bengtsson-Palme et al., 2023). By simultaneously focusing on antibiotic residues and resistance, particularly in high-risk zones, this project seeks to enhance our understanding of the environmental transmission of antibiotic resistance and support the development of informed policies to mitigate this pressing issue.

4. METHODOLOGY

4.1. STUDY AREA

4.1.1. Freshwater

All freshwater samples, including surface water and groundwater, were located in agricultural areas in Flanders. For that purpose, Manure Action Plan (MAP) locations derived from the implementation of the EU Nitrate Directive (Vlaamse Overheid, 2019) were sampled (**Figure 1**). These represent catchments for agricultural runoff, with no influence from sewage or industrial runoff. Of the existing MAP sites, sites in the *IJzer* and Northern *Maas* basins have been identified as the most polluted areas (Flanders Environment Agency, 2024). In total, fifty locations were chosen; 35 in the *IJzer* basin and 15 in the Northern *Maas* basin. The Flanders Environmental Agency took grab samples in March 2022 (spring) and a repetition (49 out of 50) occurred in September-November 2022 (fall) intending to detect changes as a consequence of the fertilization period. In Flanders, in general, manure can only be applied on arable fields from February until August (Vlaamse Overheid, 2019). In this way, the effect of this period on the presence of antibiotic residues and antibiotic resistance can be observed between sampling before the start of the fertilization period (spring) and after the fertilization period (fall). Nearby (< 1 – 4 km distance) groundwater samples (n = 50) were selected and investigated. Surface water sampling was conducted by VMM, while groundwater sampling was carried out by Eurofins.

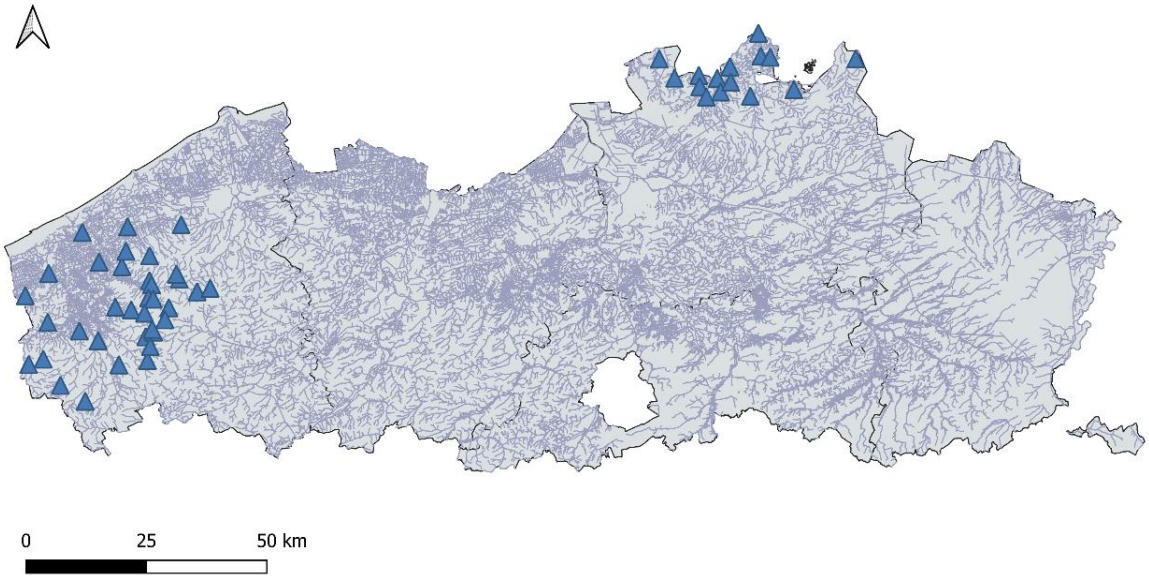

Figure 1: Hydrological map of Flanders indicating the sampled locations in Flanders (Belgium): 35 locations in West Flanders around the *Yser*-Basin and 15 around the *Meuse*-Basin in Antwerp. Both regions are associated with high intensive livestock production. Made in Geopunt4Qgis.

4.1.2. Marine environment

In June 2023, marine sampling was carried out in the ports of Oostende (10 locations) and Nieuwpoort (9 locations), chosen by the diversity in activities (industrial, urban, recreational and agricultural, due to the proximity to the *Yser* in Nieuwpoort). Marine harbors are acknowledged as hubs of interaction between humans, animals and the environment. Both water and sediment samples were taken, in collaboration with ILVO Marine and Flanders Marine Institute (VLIZ). Grab water samples were taken

at surface level. Sediment samples were taken with a Van Veen grab sampler, sampling the upper layer of the ground.

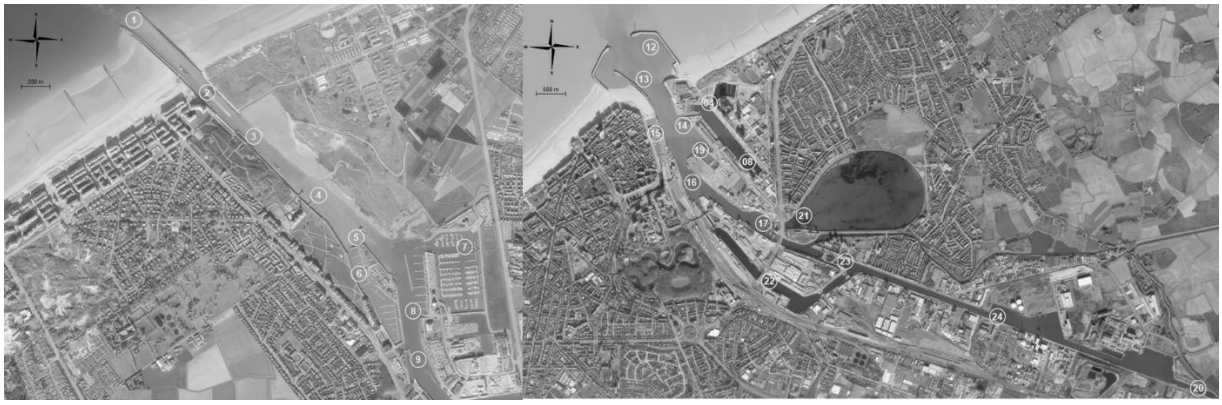

Figure 2: Sampling sites in seaports of Nieuwpoort (left) and Oostende (right). Oostende was sampled twice, with additional locations 22, 23, and 24 included in the second round to sample nearby the effluent of the wastewater treatment plant.

4.2. UHPLC-MS/MS METHOD FOR QUANTIFICATION OF ANTIBIOTICS IN WATER SAMPLES

(Adapted from Tuts et al. (2024)

A method was developed and optimized to quantify 78 antibiotic residues from 10 different classes**.** In short, after thawing the water samples, to 100 mL water of each sample, 3 mL Na_2EDTA (0.1 M) was added before adjusting the pH to 3 with HCl (3.5 M). Internal standards (cefotaxime, ceftiofur-d_3, clindamycin, methacycline, piperacillin, roxithromycin, sulfadimethoxine $^{13}C_6$, threo-chloramphenicol-d_5 and trimethoprim-d_9) were added. OASIS HLB (6 cc, 500 mL) (Waters) solid-phase extraction columns were used for the clean-up of the samples. Columns were conditioned with 5 mL MeOH and 5 mL HPLC water. After 100 mL of the sample has passed through the column, the column was washed with 5 mL HPLC water and dried. The retained antibiotics were eluted with 5 mL MeOH and the obtained extract was evaporated under N_2 at 40°C until dryness. The residues wee then resolved in 1 mL of reconstitution liquid (50:25:25 v/v H2O:ACN:MeOH + 0.05% AA) and filtered through a 0.22 µM filter. Residues were quantified with a calibration curve. Due to differences in sensitivity between the substances within this multi-residue method, there was a distinction in quantification ranges. Therefore, two separate calibrations were used. Blank water samples were spiked with concentrations in two sequences (2^{nd} sequence between brackets): 0.01 ppb (0.1 ppb) – 0.05 ppb (1 ppb) – 0.1 ppb (5 ppb) – 1 ppb (10 ppb) – 5 ppb (20 ppb) – 10 ppb (30 ppb) – 20 ppb (40 ppb). Substances belonging to group A were quantifiable between 0.01 ppb and 20 ppb, group B on the other hand followed a calibration curve between 0.1 ppb and 40 ppb.

Separation was performed in an ACQUITY UPLC H-class (Waters) system over a reversed-phase ACQUITY UHPLC BEH C18-column (2.1 × 150 mm; 1.7 µm, 100 Å) (Waters). The elution followed 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 (45 ◦C). No solvent B was used for the first 2.45 min, followed by a linear increase of solvent B to 95 % from min 2.45–14.45. This was held for 4.5 min and re-equilibration of the gradient at 0% of solvent B was maintained from 18.95 to 23 min. This UPLC H-class system was 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 for internal standards. Ions were generated in positive mode (ESI +) or negative mode (ESI -) with optimized cone voltage and collision energy. As many antibiotics were analysed in a single run, prior screening is required, meaning the transition of the precursor ion to only one fragmentation ion was followed. To prevent peak distortion, 1 µL (ESI +) or 10 µL (ESI -) of the extracted sample was injected. If a signal was obtained, the sample was reinjected and at least 2 fragmentation ions were followed for confirmation. Only if this requirement was met, the analyte could be identified. The data generated was processed using MassLynx software version 4.2 (Waters).

This method was subsequently validated according to the Commission Implementing Regulation (EU) 2021/808. Our method dealt with environmental samples, but due to the lack of a specific legislation, this method was validated according to the European Regulation 2021/808, dealing with the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals. The limit of detection (LOD) and linearity (R^2) were determined using at least three series of the previously described calibration curve. The LOD was calculated as 3 times the standard error of the y-intercept of the regression, divided by the slope. The recovery, repeatability (RSD_r) and intra- laboratory reproducibility (RSD_R) were determined using 3 sets of 6 repetition points at 3 concentration levels, depending on the group to which the residue belongs (Group A or Group B). Stability issues were addressed by testing the degradation of water samples for 14 days at 2 storage conditions (4 °C and -18 °C).

4.3. UHPLC-MS/MS METHOD FOR QUANTIFICATION OF ANTIBIOTICS IN SEDIMENT SAMPLES

Sediment samples were lyophilized, sieved (<250 µm) and 2 g of the sample was extracted according to Huygens et al. (2022). Hydration of the spiked samples was performed using 5 mL McIlvaine-EDTA buffer (pH 4). The McIlvaine-EDTA buffer was prepared by adding 5 mL 1M citrate solution, 28 mL 0.2M Na_2HPO_4-solution and 7,44 g $Na_2EDTA.2H_2O$ to 100 mL high-performance liquid chromatography (HPLC) grade H_2O. Next, tubes were placed on the shaker for 20 minutes at 250 rotations per minute (rpm) to homogenize the mixture. Then, 5 mL of the freshly prepared 0.125% (v/v) trifluoroacetic acid (TFA) in acetonitrile (MeCN) (125 µL TFA in 100 mL MeCN) was added and the tubes were placed on the shaker for 10 min at 250 rpm and centrifuged for 15 min at 3500 g. Phase separation was obtained between the liquid and solid phase, with the supernatant, containing the desired analytes, transferred to a graduated test tube. This extraction was performed twice and the resulting supernatants were combined. Following this extraction, a new clean-up was developed. QuEChERS is a technique commonly used to analyze pesticides in various matrices (Lehotay et al., 2010) and has already been used for other environmental samples (He et al., 2018; Rashid et al., 2020). In short, dispersive solid-phase extraction is performed with absorbents. When applying this approach to sediments, the best results were obtained by adding 1400 mg K_2HPO_4, 500 mg C18 and 100 mg PSA and shaking for 10 minutes at 250 rpm, followed by centrifugation (15 min. x 3500 g). This creates a phase separation between organic and aqueous phases. Subsequently, the organic phase or supernatant was transferred into a graduated test tube and fully dried under N_2 gas at 40°C and finally, redissolved in 1 mL of a mixture of the mobile phases (H_2O/MeCN/MeOH 50/25/25 + 0.05% acetic acid). The redissolved extract was then filtered over a 0.22 µm polyvinylidene difluoride (PVDF) membrane filter, into a vial. UHPLC-MS/MS conditions followed Tuts et al. (2024).

Subsequently, this method was validated according to Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021), with procedures as described in the previous year report. The linearity (R^2), recovery, repeatability (RSDr), intra-laboratory reproducibility (RSDR) and limits of

detection are determined for every class included in the multi-residue method (n = 57). Sixteen β-lactam, 10 (fluoro)quinolones, 19 sulfonamides, 2 lincosamides, 3 amphenicols, 4 tetracyclines, 2 pleuromutulines and trimethoprim were included. Concentrations were validated at 1, 10, 30 µg/kg or 10, 30, 50 µg/kg depending on the specific residue. Validation guidelines of EU regulation 2021/808 were followed, meaning the limit of detection, linearity (R^2), recovery, repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) were determined.

4.4. DETECTION OF *E. COLI* AND EXTENDED SPECTRUM B-LACTAMASE PRODUCING *E. COLI*

4.4.1. Isolation

4.4.1.1. Water

4.4.1.1.1. Surface water

A 500 mL freshwater sample was homogenized by shaking the bottle thoroughly or by placing the bottle on a shaker. From this water sample, three successive dilutions were analyzed from 10^0 to 10^{-2}, or from 10^{-1} to 10^{-3}. In bottles filled with 900 mL sterile Ringer (1/40) (Oxoid), 100 mL of the suspension of the lowest dilution was added and mixed. This procedure was carried out until all the desired dilutions were achieved. Membrane filtration was performed using a filtration device with a pump. Flamed tubes were fitted with 0.45 µm sterile CN membrane filters (VWR) by means of tweezers and used to filter 100 mL. The original sample (100 mL of 10^0) was filtered in duplicate (100 mL, 100 mL) and also 100 mL of each dilution (10^{-1}, 10^{-2}) was filtered. One filter of each dilution was placed on Rapid *E. coli* 2 for water testing (Bio-Rad) (with supplement (No. 3555298)), whilst avoiding air bubbles between membrane and soil, and incubated at 37°C for 21 to 24 hours, to determine the number of *E. coli*. This medium distinguished *E. coli* from other coliforms. Testing for ESBL-producing *E. coli* was performed by placing a filter (10^0) on Brilliance ESBL (Oxoid) plates, incubated at 37°C for 24 hours. ESBL-producing *E. coli* were indicated by the formation of purple colonies. A maximum of four *E. coli* colonies on plates of the highest possible dilution were picked for purification on Rapid *E. coli* 2 (without supplement). Colonies were re-streaked on MacConkey No.3 and incubated at 37°C for 24h. Purified colonies were picked to make isolates in Brain Hart Infusion Broth (Oxoid) + 15% glycerol. A swab of coliforms on Rapid *E. coli* 2 for water testing was also performed and stored in BHI + 15% glycerol for later use. Isolates were stored at -20°C.

4.4.1.1.2. Groundwater

Groundwater samples followed a modified procedure. A 500 mL groundwater sample was homogenized by shaking the bottle thoroughly or by placing the bottle on a shaker. Very turbid samples, containing plenty of residual large particles, could be 1/100 diluted with Ringer (1/40). 100 mL was membrane-filtered using a filtration device connected to a vacuum-pump pump. Sterile tubes fitted with 0.45 µm sterile filters, were used. The filter was placed on Rapid *E. coli* 2 for water testing (with supplement), whilst avoiding air bubbles between membrane and soil, and incubated at 37°C for 21 to 24 hours, to determine the number of *E. coli* and coliforms. At the same time 100 mL of the initial sample was enriched in Lauryl Tryptose Broth (Oxoid) (1:1). If there was no growth on the filter on Rapid *E. coli* 2, after 24 h incubation of the enrichment at 30°C, 10 µL of the enrichment was plated on Rapid *E. coli* 2 for water testing and incubated at 37°C for 24 hours. Testing for ESBL-producing *E. coli* was performed by inoculating 10 µL of the enrichment on *Brilliance*™-plates and incubating at 37°C for 24 hours. ESBL-producing *E. coli* were indicated by the formation of purple colonies. Maximum four *E. coli* colonies on plates of the highest possible dilution were picked for purification on Rapid *E. coli* 2 (without supplement). Colonies were re-streaked on MacConkey No.3 and incubated at 37°C for 24h.

Red/pink colonies were confirmed as *E. coli.* Purified colonies were picked to make isolates in Brain Hart Infusion Broth (Oxoid) + 15% glycerol. A swab of coliforms on Rapid *E. coli* 2 for water testing was also performed and stored in BHI + 15% glycerol for later use. Isolates were stored at -20°C.

4.4.1.1.3. Marine water

In order to detect suspected ESBL-producing *E. coli*, 100 mL of a water sample was enriched with Lauryl Tryptose Broth (1:1) and incubated at 30°C for 24 h. Next, 100 µL enrichment was streaked on *Brilliance*™ ESBL medium to isolate presumed ESBL-producing *E. coli* after 24 h at 30 °C. Another purification check involving MacConkey no. 4 agar was the last step before the storage of isolates (2 per sample) in Brain Heart Infusion Broth + 15% glycerol.

4.4.1.2. Sediment

Twenty-five g of sediment was enriched with Lauryl Tryptose Broth (1:1) and incubated at 30 °C for 24 h after 1 min of vigorous shaking with a stomacher. Subsequently, 100 µL of this enrichment was plated onto *Brilliance* ESBL at 30 °C for 24 h. Positive colonies were purified on MacConkey no. 4 (incubation at 30 °C for 24 h).

4.5. DETECTION OF *SHEWANELLA* SPP. AND *VIBRIO* SPP.

4.5.1. Isolation

4.5.1.1. Water

100 mL of the mixed water sample was filtered 2 times (together with possible dilutions in phosphate buffered saline) over 0.45 µm sterile CN filters (Millipore) with a vacuum-pumping filtration device. One filter was incubated on Lingby Iron agar (Oxoid) (*Shewanella)*, supplemented with cysteine, and another on thio-sulfate-citrate-bile salts-sucrose (TCBS) agar (Merck) (*Vibrio*) at 28 °C for 24 to 48 h. At the same time, 100 µL was directly plated onto each respective plate and incubated under the same conditions. At the end, four colonies were picked for further purification on Lingby Iron agar and TCBS, from the highest possible dilution(s) (e.g. 3 colonies from (10^{-2}) and 1 colony from (10^{-1})). Isolates were stored in Marine Broth (Oxoid) + 15% glycerol.

Only for *Vibrio*, the repeated sampling of 2024, incubation was also performed at 37 °C for 24 hours to isolate more possible pathogenic strains.

4.5.1.2. Sediment

The sediment sample (25 g) was enriched with phosphate-buffered saline and shaken for 15 minutes. After 10 minutes without shaking, 1 mL of supernatant was collected and diluted in phosphate buffer saline till 10-3. From every dilution, 100 µL was streaked on Lingby Iron Agar containing cysteine (*Shewanella*) and TCBS (*Vibrio*), incubated at 28 °C for 24 h. After purification on Marine agar, isolates were stored at – 20 °C.

Only for *Vibrio*, in the repeated sampling of 2024, incubation was also performed at 37 °C for 24 hours to isolate more possible pathogenic strains.

4.5.2. Confirmation

The identification of *Vibrio* and *Shewanella* has been optimized using a matrix-assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF) (Bruker). Two approaches were executed. The first method, the indirect method, consisted of applying 1 µL cell to a spot on the MALDI-TOF plate and adding 1 µL Bruker Matrix (HCCA) after drying. The target was then ready to be identified by

comparison with the Bruker database. The Bruker Bacterial Test Standard (BTS), containing an extract of *Escherichia coli* DH5 alpha, was used as a control. According to the manufacturer's recommendations, a score greater than 2 was considered reliable for species identification, scores between 1.7 and 1.99 were considered reliable at the genus level only, and scores < 1.7 are considered unreliable. If the result was unreliable, a second approach (extraction method) was tried. Single colonies were transferred to 300 µL ELGA (HPLC) water. Following vortexing, 900 µL of cold ethanol was added, followed by centrifugation at 14000 g for 2 minutes. The supernatant was poured off, followed by a short spin to 14000 g. The supernatant was pipetted off and the pellet was allowed to dry. 25 µL of 70% formic acid and 25 µL of acetic acid were added to the pellet and mixed carefully. The supernatant (1 µL) was applied to a spot on the MALDI-TOF plate and dried, then 1 µL of HCCA was added and dried again.

4.6. ANTIBIOTIC SUSCEPTIBILITY TESTING

4.6.1. *E. coli*

The antibiotic resistance profile was established based on minimum inhibitory concentrations (MICs) and could be determined using the commercially available 96 wells-EUVSEC-plates (Thermofisher) for *E. coli*. The antibiotic susceptibility of *E. coli* (EUVSEC 3) was tested for the following antibiotics: amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim (**Table I**). Specifically, for ESBL-producing *E. coli* EUVSEC 2 plates were used, testing the resistance to the antibiotics: cefepime, cefotaxime (+ clavulanic acid), cefotoxin, ceftazidime (+ clavulanic acid), ertapenem, imipenem, meropenem and temocillin. The Sensititre (ThermoFisher) system could determine at which concentration of antibiotics the tested isolate will no longer grow (minimum inhibitory concentration). Based on (EUCAST) guidelines, isolates were resistant or susceptible, by comparing these obtained MIC values with defined cut-off values (ECOFF). In that way, an antibiotic resistance profile could be established.

Table I: MIC-range (µg/mL) and ECOFF for *E. coli* (µg/mL) per antibiotic as used with EUVSEC 2 and EUVSEC 3.

Antibiotic (EUVSEC 3)	Concentration (µg/mL)	ECOFF (µg/mL)	Antibiotic (EUVSEC 2)	Concentration (µg/mL)	ECOFF (µg/mL)
ampicillin	1 – 32	8	cefepime	0.06 – 8	0.125
azithromycin	2 – 64	16	cefotaxime	0.25 – 64	0.25
cefotaxime	0.25 - 4	0.25	cefotaxime / clavulanic acid	0.06/4 – 64/8	0.25
ceftazidime	0.5 – 8	1	cefoxitin	0.5 – 64	16
chloramphenicol	8 – 64	16	ceftazidime	0.25 – 32	1
ciprofloxacin	0.015 – 8	0.064	ceftazidime / clavulanic acid	0.12/4 – 128/4	1
colistin	1 – 16	2	ertapenem	0.015 – 2	0.03
gentamicin	0.5 – 16	2	imipenem	0.12 – 16	0.5
meropenem	0.03 – 16	0.06	meropenem	0.03 – 4	0.06
nalidixic acid	4 – 64	8	temocillin	0.5 - 128	16
sulfamethoxazole	8 – 512	64			
tetracycline	2 – 32	8			
tigecycline	0.25 – 8	0.5			
trimethoprim	0.25 - 16	2			

4.6.2. Shewanella

Shewanella isolates were grown on Mueller-Hinton agar first at 28 °C and subsequently at 35 °C for 24 hours in preparation for inoculation. The antibiotic susceptibility was tested using microbroth dilution by means of EUVSEC 3 (Thermofisher) for the following antibiotics: amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim, incubated at 35 °C for 18 h – 24 h following guidelines for *other non-Enterobacteriaceae* (CLSI, 2020). ATCC 25922 (*E. coli*) acted as a reference organism (CLSI, 2020). Epidemiological cut-off values were established based on acquired distributions during testing (**Table II**). Resistance to an antibiotic was observed if the MIC value was higher than the corresponding cut-off value.

Table II: MIC-range (µg/mL) and ECOFF (µg/mL) for *Shewanella* and *Vibrio* per antibiotic as used with EUVSEC.

Antibiotic (EUVSEC 3)	Concentration (µg/mL)	ECOFF *Shewanella* (µg/mL)	ECOFF *Vibrio* (µg/mL)
amikacin	4 - 128	4	16
ampicillin	1 – 32	8	16
azithromycin	2 – 64	4	2
cefotaxime	0.25 - 4	0.5	0.25
ceftazidime	0.5 – 8	1	1
chloramphenicol	8 – 64	8	32
ciprofloxacin	0.015 – 8	0.06	0.25
colistin	1 – 16	4	4
gentamicin	0.5 – 16	1	4
meropenem	0.03 – 16	2	0.25
nalidixic acid	4 – 64	4	4
sulfamethoxazole	8 – 512	64	16
tetracycline	2 – 32	2	2
tigecycline	0.25 – 8	1	0.25
trimethoprim	0.25 - 16	4	1

4.6.3. *Vibrio*

Vibrio isolates were initially cultured on Mueller-Hinton agar with 2% NaCl at 28 °C, followed by incubation at 35 °C for 24 hours. Antibiotic susceptibility was assessed via microbroth dilution using EUVSEC 3 (ThermoFisher) for a range of antibiotics: amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim. Incubation occurred at 35 °C for 18–24 hours, following guidelines for *Vibrio* spp. (CLSI, 2015). ATCC 25922 (*E. coli*) acted as a reference organism (CLSI, 2020). Epidemiological cut-off values were subsequently established based on acquired distributions during testing (**Table II**). Resistance to an antibiotic was observed if the MIC value was higher than the corresponding cut-off value.

4.7. EXTENDED-SPECTRUM B-LACTAMASE CONFIRMATION

ESBL-producing *E. coli* are often associated with *bla*CTX-M, *bla*TEM, and *bla*SHV genes. A molecular typing method based on Monstein et al. (2007) was used to determine the presence of these genes. Lysates were prepared by incubating a single colony in 100 µL H_2O at 90 °C for 17 minutes, followed by centrifugation at 10000 g for 1 minute. The PCR master mix included Taq

polymerase (2U), 1x Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, primers, and deionized H_2O. Multiplex PCR was conducted following the conditions specified by (Monstein et al., 2007).

4.8. ENTEROTOXIGENIC IDENTIFICATION

To screen marine ESBL-*E. coli* isolates for enterotoxigenic properties, a PCR was carried out for the presence of LTIa, LTIb, STIa, STII en VT2 genes, according to Botteldoorn et al. (2003).The PCR master mix included Taq polymerase (1.5 U), 1 x Buffer, 1.5 mM $MgCl_2$, 0.25% Tween, 0.005% Gelatine, 100 µM dNTPs, primers and deionized H_2O. PCR conditions followed Botteldoorn et al. (2003).

5. SCIENTIFIC RESULTS AND RECOMMENDATIONS

5.1. VALIDATION OF UHPLC-MS/MS METHODS

5.1.1. Validation of UHPLC-MS/MS method for water analysis (adapted from (Tuts et al., 2024))

5.1.1.1. Identification

The use of UHPLC-MS/MS in this study meets requirements by providing 5 identification points (European Commission, 2021). Additionally, all ions must have a signal/noise (S/N) ratio above 3, and the analyte ion ratio must align with matrix-fortified standards within a ±40% relative deviation. This multi-residue method identifies 78 veterinary antibiotics in aquatic matrices, marking it as one of the most comprehensive UHPLC-MS/MS methods to date (Balzer et al., 2016; Burke et al., 2016; Kivits et al., 2018). While primarily for veterinary antibiotics, it also partially overlaps with antibiotics used in human medicine, enabling analysis of urban wastewater and hospital effluents. A limitation is that aminoglycosides are excluded due to the need for separate cleanup and different chromatographic conditions, which would add cost and time (Huygens et al., 2021).

5.1.1.2. Trueness by Recovery

To evaluate trueness, linearity was tested using a model (y = ax + b) with a 1/X weighting function, requiring $R^2 > 0.99$ for most substances (**Table A1, A2**). This threshold was met for nearly all, except benzylpenicillin, cefadroxil, cefquinome, rifaximin, and lincomycin, which had $R^2 > 0.95$. Residuals are acceptable across analytes. Calibration curves were also created using blank seawater samples, yielding similar correlation coefficients, indicating suitability for various aquatic environments, including surface water, groundwater, and marine samples. Determined recoveries (%) meet Commission Regulation (EU) 2021/808 standards, except for sarafloxacin and tiamulin, which cannot be quantified at certain levels using this method.

5.1.1.3. Sensitivity

Detection limits ranged between 0.01 and 1 µg/L, comparable to similar studies (Goessens et al., 2020; Zhou et al., 2012). Compromises in detection limits were made to create a comprehensive multiresidue method. Using a smaller sample volume (100 mL) limits the detection limit but streamlines the process, reducing analysis time by 4.5–7 hours. Variations in LODs depended on chemical structure; β-lactams have higher LODs due to degradation during acidification.

5.1.1.4. Precision: Repeatability and Reproducibility

Most residues were quantified with acceptable variation coefficients for repeatability and reproducibility for mass fractions between 10–120 µg/L (25%) and <10 µg/L (30%) (**Table A1, A2**). Benzyl-penicillin, cefquinome, ciprofloxacin, erythromycin and sarafloxacin can only be semi-quantified.

5.1.1.5. Specificity

The method was able to distinguish compounds to prevent false positives and negatives. Tests injecting single compounds revealed minor interference: cefradin with cefalexine, sulfamonomethoxine with sulfamethoxypyridazine, norfloxacine with enoxacine, and chlortetracycline with tetracycline, each at low percentages. In conclusion, no significant specificity issues were anticipated.

5.1.1.6. Requirements for Chromatographic Separation

EU Regulation 2021/808 standards for chromatographic conditions were met. Analytes' retention times are within ±0.1 min of a matrix-fortified standard, and relative retention times differ by no more than 1%, meeting the required criteria.

5.1.1.7. Stability

Stability was tested by storing spiked water samples at 4 °C and -18 °C, showing that β-lactams, macrolides, and ansamycins degrade significantly under both conditions. Ampicillin, for example, loses 50% stability in just four days at 4 °C. Pleuromutilins, (fluoro)quinolones, and tetracyclines also degrade more quickly at 4 °C. On the other hand, phenicols, lincosamides, and trimethoprim remain stable. To minimize degradation, samples should be stored at -18 °C and analyzed within two weeks, as temperature strongly impacts antibiotic stability (Tuts et al., 2024).

5.1.2. Validation of UHPLC-MS/MS method for sediment analysis

5.1.2.1. Identification

In sediment matrices, a high number of different antibiotics (n = 57) can be identified with this multiresidue method, the majority of which can be quantified (**Table A3**). Penicillins and cephalosporins are generally only semi-quantifiable. Although lower LODs could be achieved in other studies (Chung et al., 2017; da Silva et al., 2020; Meng et al., 2017; Rashid et al., 2020), this method is the result of a compromise between the number of residues included and the sensitivity, making this the most comprehensive to our knowledge.

5.1.2.2. Trueness by Recovery

To evaluate trueness, linearity was tested using the same model (y = ax + b) with a 1/X weighting function, requiring $R^2 > 0.99$ for most substances (**Table A3**). This threshold was met for nearly all, except amoxicillin, ampicillin, cloxacillin, cefalonium, cefoperazone and cefquinome, which had $R^2 > 0.95$ and nafcillin ($R^2 = 0.94$). Residuals are acceptable across analytes. Determined recoveries (%) meet Commission Regulation (EU) 2021/808 standards in most cases for 2 validated concentrations, except for norfloxacin and ofloxacin (only 1 concentration fully validated) and cefadroxil, cefalexin, cefazoline and nalidixic acid (at no concentration fully validated), which can only be semi-quantified.

5.1.2.3. Sensitivity

Detection limits ranged between 0.1 and 40 µg/kg, comparable to the research of Siedlewicz et al., (2016). Compromises in detection limit were made to create a comprehensive multiresidue method (**Table A3**). Variations in LODs between antibiotic residues depended on chemical structure.

5.1.2.4. Precision: Repeatability and Reproducibility

Most residues could be quantified with acceptable variation coefficients for repeatability and reproducibility for mass fractions between 10–120 µg/kg (25%) and <10 µg/kg (30%) (**Table A3**). β-lactams and quinolones can only be semi-quantified.

5.1.2.5. Specificity

The same residue characteristics apply to both aquatic and sediment matrices (**5.1.1.5**).

5.1.2.6. Requirements for Chromatographic Separation

The same UHPLC-MS/MS characteristics apply to both aquatic and sediment matrices (**5.1.1.6**).

5.2. QUANTIFICATION OF ANTIBIOTIC RESIDUES

5.2.1. Freshwater

Antibiotic residues (ABRs) were detected in surface water using UHPLC-MS/MS. In spring, at least one antibiotic was found in 78% of samples, decreasing to 45% in fall. Eight sites showed no ABRs in either period. Overall, 25 different ABRs were identified, with up to 12 in a single sample. Lincomycin (8.83 µg/L), sulfamethoxazole (1.60 µg/L), and sulfadiazine (0.75 µg/L) were detected at the highest concentrations, while lincomycin, sulfadiazine, sulfamethazine, and trimethoprim showed the highest detection frequencies. The class of sulfonamides was most common, with 12 detected antibiotics, followed by smaller occasional detection of tetracyclines, phenicols, macrolides, (fluoro)quinolones, and β-lactams. Antibiotic residue concentrations ranged from 0.01 µg/L to 8.83 µg/L, with a median of 0.01 µg/L. No significant differences were found between samples from the *IJzer* and *Maas* basins, or between samples taken before (spring) and after fertilization (fall), in terms of both ABR amounts and concentrations ($p > 0.05$).

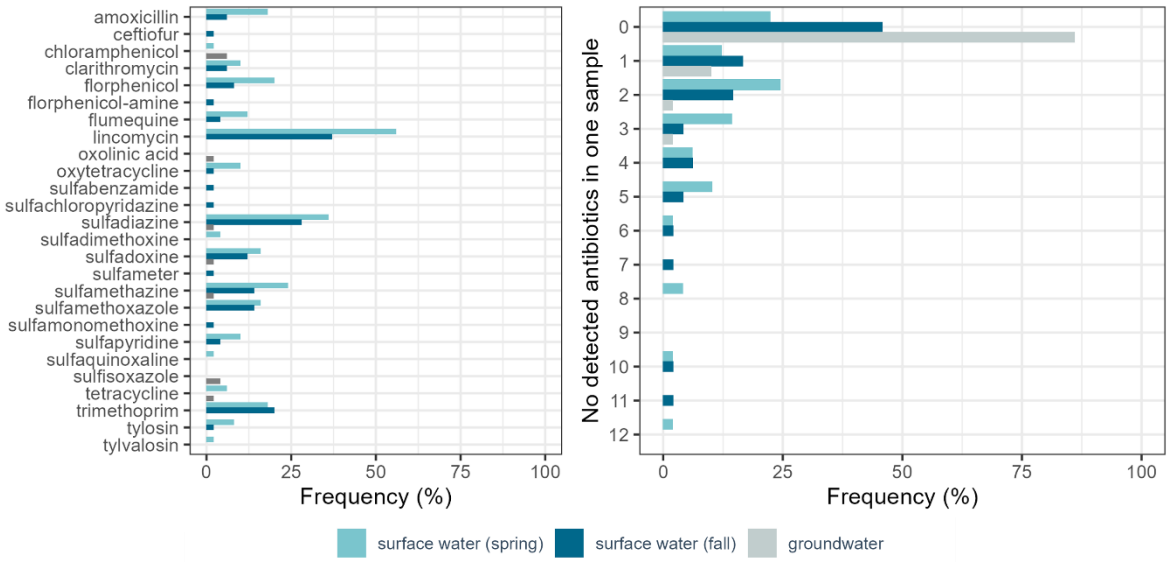

Figure 3: Frequency (%) of occurrence of an antibiotic residue and frequency (%) of number of antibiotic residues detected in surface water (spring; n = 50), surface water (fall; n = 49) and groundwater (n = 50).

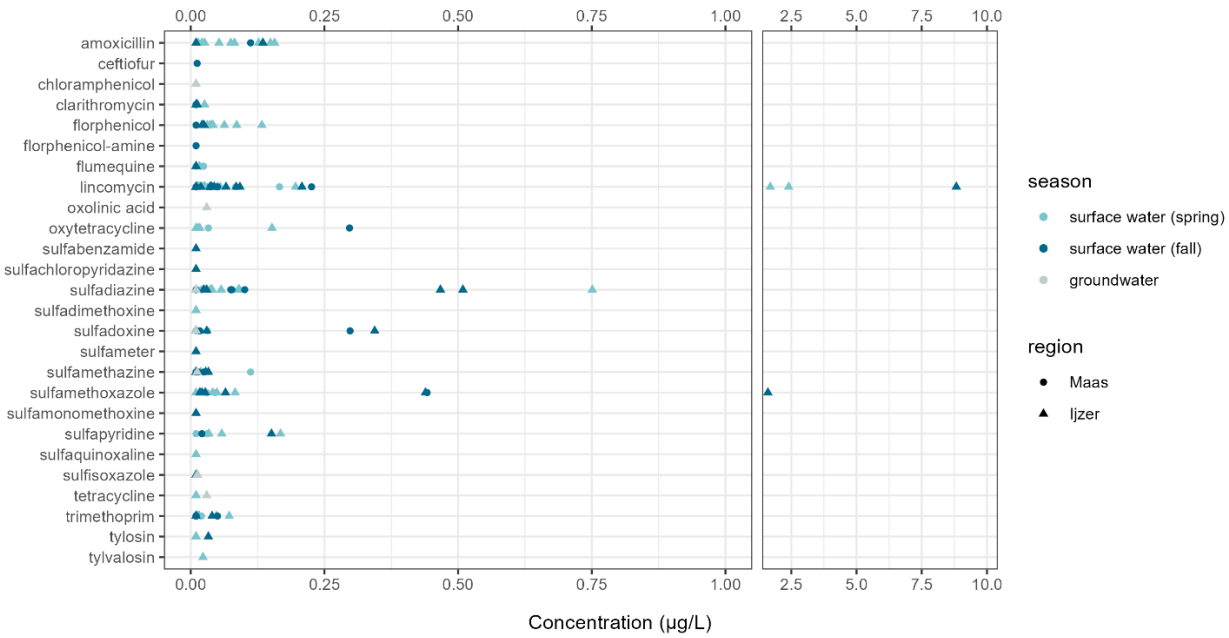

Figure 4: Concentrations (µg/L) of antibiotic residues in surface water (n = 99) and groundwater (n = 50)

5.2.2. Marine environment

Antibiotic concentrations in water samples were low, comparable to median concentrations in surface water. Sediments, on the other hand, contained antibiotics at higher concentrations, with a maximum of 25 µg/kg. Next to the concentration of these residues, water and sediment samples also differed between antibiotic class. In general, the antibiotics accumulating in sediments are quinolones and macrolides. In water, sulfonamides (sulfamethoxazole) are again dominant. Possible pathways for the discharge of antibiotic residues were identified as wastewater treatment and hospital effluents in combination with harbour-related activities (marina, recreation).

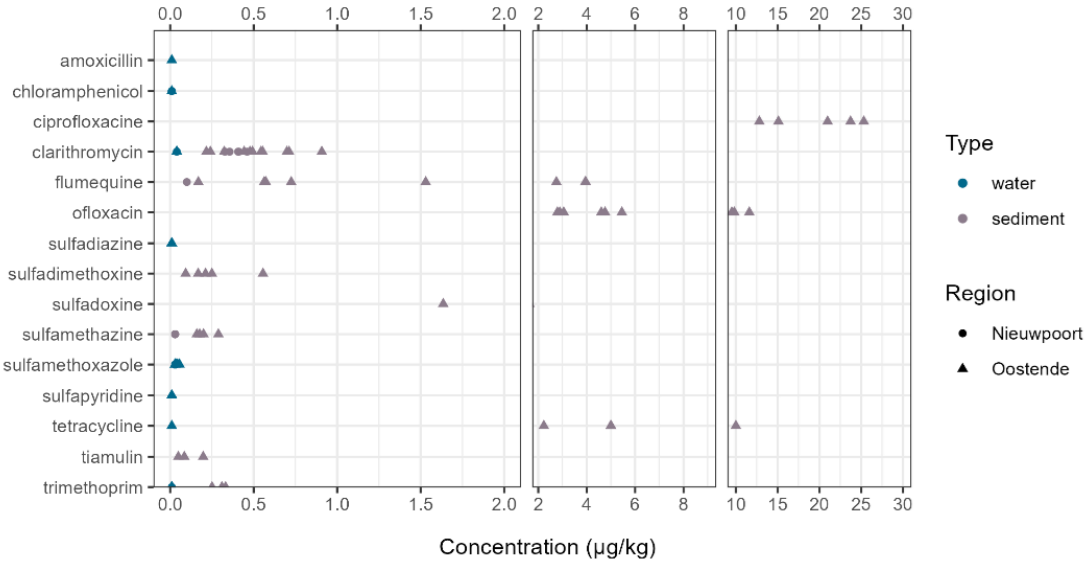

Figure 5: Concentration of antibiotic residues in water (n = 9 in Nieuwpoort and n = 23 in Oostende) and sediment samples (n = 9 in Nieuwpoort) and n = 22 in Oostende).

Table III: Frequency of detection (%) of antibiotic residues in water and sediment samples

Antibiotic	***Frequency (%)***	
	Water	Sediment
amoxicillin	4	0
chloramphenicol	12	0
ciprofloxacin	0	20
clarithromycin	36	64
flumequine	0	36
ofloxacin	0	36
sulfadiazine	32	0
sulfadimethoxine	0	20
sulfadoxine	0	4
sulfamethazine	0	36
sulfamethoxazole	76	0
sulfapyridine	36	0
tetracycline	4	20
tiamulin	0	12
trimethoprim	48	12

5.3. DETECTION OF INDICATOR ORGANISMS

5.3.1. Freshwater

E. coli was detected in surface water samples 94% (pre-fertilization) and 98% (post-fertilization) of the time, with 94 isolates in spring and 92 in fall. Suspected ESBL-producing *E. coli* were found in 26% of samples across both periods, averaging 1% of total E. coli (0%-17% range), with 69 isolates. Other coliforms were present in every sample and most abundant after fertilization ($p < 0.05$). In groundwater, E. coli appeared in 24% of groundwater samples, but suspected ESBL-producing *E. coli* were absent, even after enrichment. Other coliforms were found in 46 of 50 samples in higher quantities. *E. coli*, other coliforms, and suspected ESBL-producing *E. coli* were significantly lower in groundwater than in surface water.

5.3.2. Marine environment

5.3.2.1. Extended-spectrum β-lactamase producing *E. coli*

Vibrio were widespread, both in water and sediment samples. Also *Shewanella* isolates were often recovered. Suspected ESBL-producing *E. coli* were detected in varying degrees, but generally to a lesser extent (**Figure 5)**.

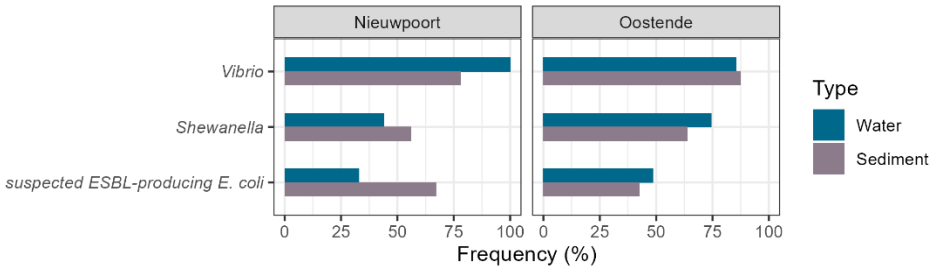

Figure 6: Frequency of detection (%) of *Vibrio*, *Shewanella* and suspected ESBL-producing *E. coli* in water- and sediment samples from Nieuwpoort and Oostende (total of 2023 and 2024).

5.3.2.2. *Shewanella*

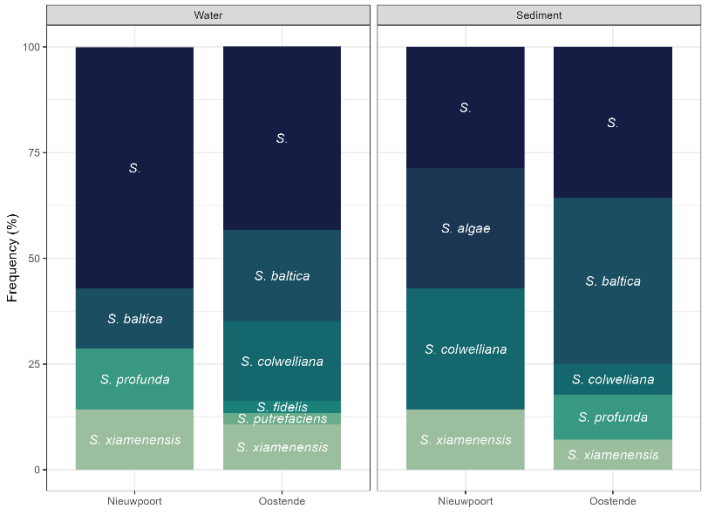

Figure 7: Identified *Shewanella* strains isolated from water (7 in Nieuwpoort, 37 in Oostende) and sediments (7 in Nieuwpoort, 28 in Oostende).

S. baltica, *S. colwelliana,* and *S. xiamenensis* were widespread in all seaports and sample types. Most of the isolates were identified as non-harmful bacteria. Part of the collection can only be identified on a genus level, due to the limitations of the Bruker library. Occasionally, the opportunistic pathogens *S. algae* and *S. putrefaciens* were detected. In total, 79 strains were collected.

5.3.2.3. *Vibrio*

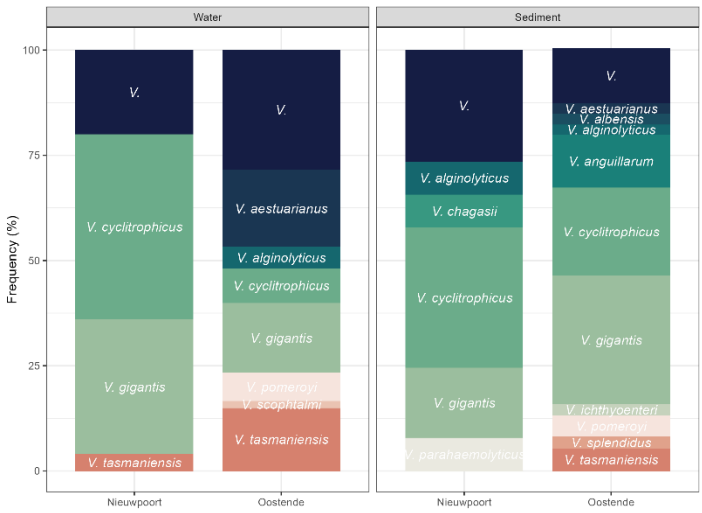

Figure 8: Identified *Vibrio* strains isolated from water (25 in Nieuwpoort, 60 in Oostende) and sediments (12 in Nieuwpoort and 37 in Oostende).

V. cyclitrophicus and *V. gigantis* were detected the most frequently. Some of the isolated *Vibrio*'s are considered opportunistic pathogens, including: *V. alginolyticus* and *V. parahaemolyticus.* A total of 134 strains were identified and stored.

5.4. SUSCEPTIBILITY TO ANTIBIOTICS

5.4.1. Freshwater

5.4.1.1. E. coli

Minimum inhibitory concentrations for *E. coli* from surface water were assessed for various antibiotics during two sampling periods. In spring, 68% of E. coli isolates were sensitive to all antibiotics, decreasing to 45% in fall. Resistance was most prevalent for sulfamethoxazole, rising from 20% in spring to 48% in fall, followed by resistance to ampicillin, tetracycline, and trimethoprim (**Table IV**). A significant increase in sulfamethoxazole resistance occurred after fertilization. Multidrug resistance (resistance to more than three antibiotic classes) remained consistent at 6%.

5.4.1.2. Extended-spectrum β-lactamase producing *E. coli*

Suspected ESBL-producing *E. coli* additionally showed higher resistance to most non-β-lactam antibiotics compared to generic *E. coli,* except for azithromycin, colistin, and tigecycline. Additional testing on these isolates revealed occasional resistance to cefotaxime + clavulanic acid, cefoxitin, ceftazidime + clavulanic acid, and ertapenem, with consistent resistance observed for cefotaxime, cefepime, and ceftazidime (**Table IV)**.

Table IV: Antibiotic resistance frequencies (%) (EUVSEC 3 and EUVSEC 2) in the *E. coli* population out of surface water, sampled in spring and fall. Suspected ESBL-producing E. coli are additionally tested with EUVSEC 2. Only suspected ESBL-producing *E. coli* isolates from both water and sediment in the marine environment were tested. ND: not determined.

			Surface water		Marine environment	
Antibiotic	**Resistance frequency (%) generic *E. coli***		**Resistance frequency (%) of suspected ESBL-producing *E. coli* (%)**		**Resistance frequency (%) of suspected ESBL-producing *E. coli* (%)**	
	spring (n = 94)	**fall (n = 92)**	**spring (n = 38)**	**fall (n = 30)**	**water (n = 29)**	**Sediment (n = 30)**
EUVSEC 3						
amikacin	ND	ND	ND	ND	0	0
ampicillin	13	13	100	100	100	100
azithromycin	1	1	13	0	24	7
cefotaxime	1	1	100	100	100	100
ceftazidime	1	1	89	78	76	73
chloramphenicol	3	4	8	25	21	20
ciprofloxacin	3	3	45	47	59	73
colistin	0	2	11	0	0	0
gentamicin	0	0	21	22	10	3
meropenem	0	0	0	0	10	3
nalidixic acid	1	2	24	28	21	53
sulfamethoxazole	20	48	53	69	62	60
tetracycline	9	9	45	66	28	33
tigecycline	0	0	0	0	0	0
trimethoprim	7	8	39	50	41	41
EUVSEC 2						
cefepime	ND	ND	100	100	100	100
cefotaxime	ND	ND	100	100	100	100
cefotaxime + clavulanic acid	ND	ND	5	0	3	0
cefoxitin	ND	ND	3	3	3	0
ceftazidime	ND	ND	92	90	79	80
ceftazidime + clavulanic acid	ND	ND	5	0	3	0
ertapenem	ND	ND	18	10	10	13
imipenem	ND	ND	0	0	0	0
meropenem	ND	ND	0	0	7	3
temocillin	ND	ND	0	0	0	0

5.4.2. Marine environment

5.4.2.1. Extended-spectrum β-lactamase producing *E. coli*

The resistance in suspected ESBL-producing *E. coli* isolated from marine environments was comparable to those isolated from freshwater (**Table IV)**. Seventy % of tested isolates were multidrug-resistant, meaning that these were resistant to more than 3 different antibiotic classes (**Figure 9**). Among those classes, the remarkable resistance to the critical class of carbapenems (meropenem and ertapenem) was noted. Furthermore, higher resistance to quinolones such as ciprofloxacin and nalidixic acid was observed in sediment samples than in water samples. Additionally, high resistance to azithromycin in water samples is reported, similar to research in urban settings (Niang et al., 2023). The presence of resistance to mainly human antibiotics (azithromycin, ciprofloxacin) in the sampling locations closest to the effluent from the waste-water treatment plant in Ostend indicates a possible impact of this effluent.

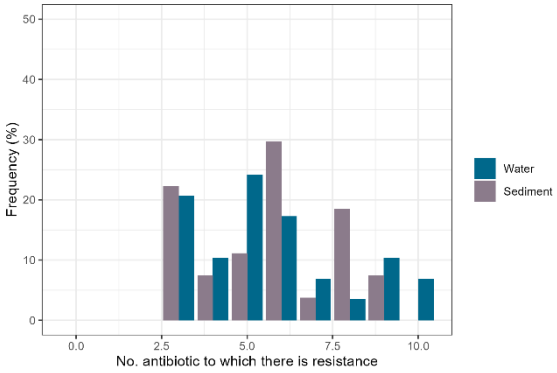

Figure 9: Amount of antibiotics to which ESBL *E. coli* from marine environments were resistant (n = 59).

5.4.2.2. *Shewanella*

A more modest extent of resistance to antibiotics was observed within *Shewanella* (**Figure 10**). Used as an antibiotic of last resort, the observed resistance to the antibiotic colistin may be intrinsic to the *Shewanella* genome and is unlikely to be the result of selection in the presence of antibiotics.(Mondal et al., 2024; Zhang et al., 2019). One isolate showed resistance to meropenem.

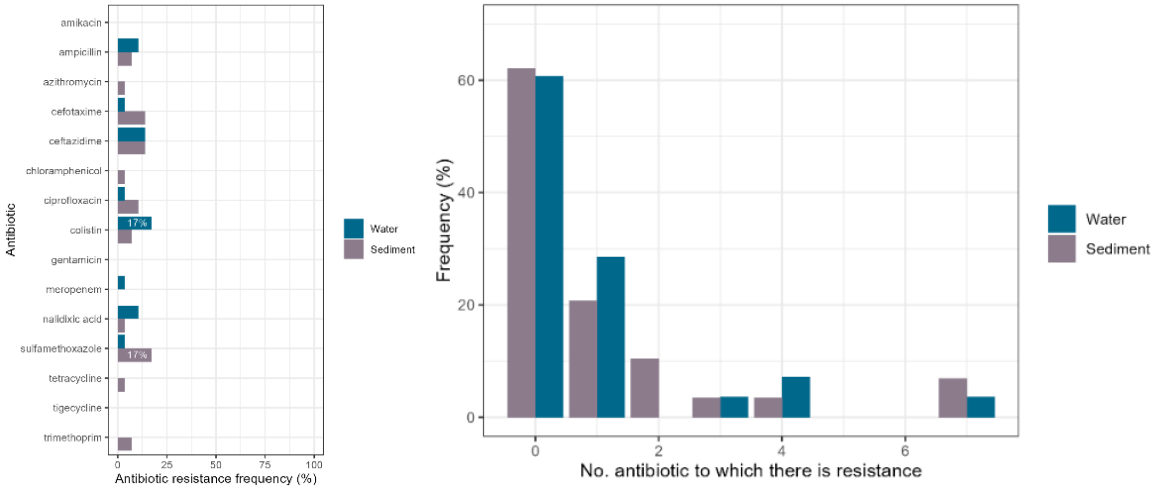

Figure 10: Left: resistance (%) to antibiotics covered by testing with EUVSEC 3 of *Shewanella*. Right: Amount of antibiotics to which ESBL *E. coli* were resistant (n = 58).

5.4.2.3. *Vibrio*

Resistance within tested *Vibrio* isolates is low (**Figure 11**). However, only a few isolates could be tested. Furter tests would be needed to provide thorough insights in the prevalence of antibiotic resistance.

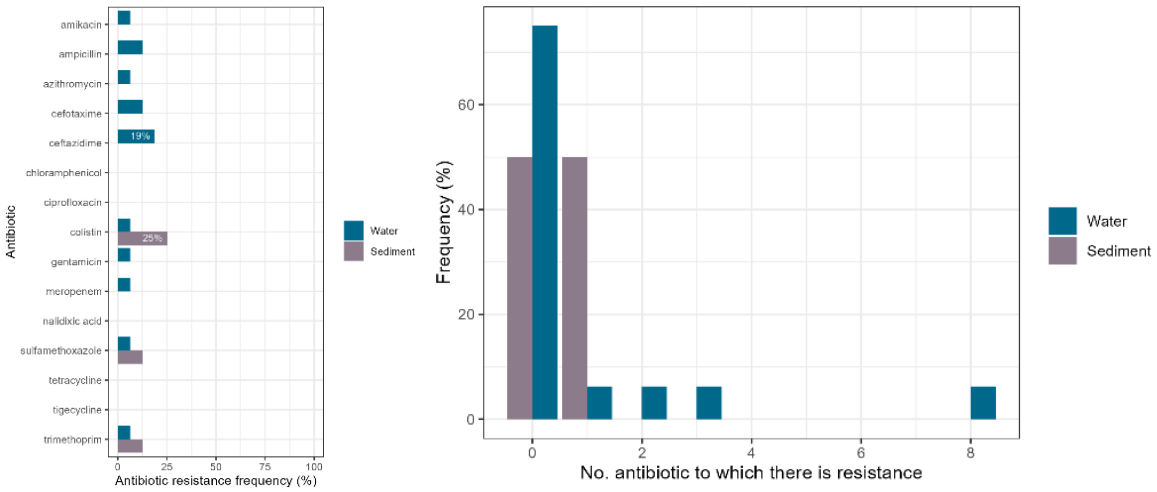

Figure 11: Left: resistance (%) to antibiotics covered by testing with EUVSEC 3 of *Vibrio*. Right: Amount of antibiotics to which ESBL *E. coli* were resistant (n = 24).

5.5. PCR-GENOTYPING

5.5.1. ESBL-confirmation

5.5.1.1. Freshwater

To confirm suspected ESBL-producing *E. coli*, PCR-genotyping was performed. Evaluated isolates (n = 69) were positive for blaCTX-M and blaTEM (48%) together, blaCTX-M (38%), blaTEM (10%) or both blaTEM and blaSHV (1%) meaning that 86% is confirmed to have ESBL properties due to the presence of the CTX-M enzyme. DNA sequencing should be used to screen for mutations in the genes encoding for TEM and SHV to classify these isolates as ESBL-producing. Two ESBL-isolates weren't positive for any of the genes tested. Whole genome sequencing on a selection of ESBL-strains will result in a comprehensive of view of resistance mechanisms.

5.5.1.2. Marine environment

Among the isolates tested (n = 59), 56% was positive for only CTX-M, 29% for both CTX-M and TEM, 2% for CTX-M and SHV and 2% for CTX-M, SHV and TEM. Additionally, 8% carried only the *TEM* gene, while two isolates lacked any of the targeted genes. These findings confirm that 88% of the isolates are ESBL-producing *E. coli*. Whole genome sequencing is recommended for the remaining strains to confirm ESBL properties or identify other resistance mechanisms.

5.5.2. ETEC-identification

Only a subset of isolates was tested. All ESBL *E. coli* strains that were isolated during the marine sampling round of 2023 were screened. Strains from one location tested positive for the LTIb gene, whilst isolates from another location contained both LTIa and LTIb genes. Whole genome sequencing on a selection of ESBL-strains can provide more insights in the presence of virulence genes in the sampled areas.

5.6. CO-OCCURRENCE OF ANTIBIOTIC RESIDUES AND ANTIBIOTIC RESISTANCE

There are indications that even concentrations below MIC, described as minimum selective concentrations, can also lead to antibiotic resistance (Sandegren, 2014). To test whether these concentrations can lead to antibiotic resistance, Predicted No-Effect Concentrations (PNEC), specific for antibiotic resistance and based on the lowest minimum inhibitory concentrations, are applied and compared to measured concentrations. PNECs from Bengtsson-Palme and Larsson (2016) are used for all antibiotics, sulfadiazine excluded. As no calculations were made for this residue, other research of (Menz et al., 2019) was consulted. PNECs were exceeded 5 times (twice in spring, 3 times in fall) in freshwater. Twice for lincomycin (detected concentrations 8.8 µg/L and 2.4 µg/L) and three times for sulfadiazine (detected concentrations 0.75, 0.47, 0.51 µg/L). In these cases, based on the PNECs, the presence of antibiotic residues could theoretically lead to resistance selection. Additionally, it was revealed that both a widespread presence of sulfonamides and a high rate of resistance to sulfamethoxazole suggest a possible connection. Moreover, since the sampling campaign in the fall (after fertilization) shows an increase in this specific resistance (Wilcoxon signed rank test, $p < 0.05$), it can be implied that the use of antibiotics in veterinary medicine, has an impact on the aquatic environment. In the Belgian seaports, it was observed that the accumulation of quinolones in sediments coincides with higher resistance rates to those same antibiotics.

5.7. COLLABORATIVE RESULTS

Extensive collaboration was performed between ILVO and the project partners regarding the harmonization of methods for bacteriological studies. Besides this, ILVO was responsible for the analysis of antibiotic residues. Water samples from NTNU (Norway, n = 20, wastewater, sewage and marine water), KI (Sweden, n = 14, wastewater, freshwater, marine water) and ULPGC (Gran Canaria, n = 20, wastewater, marine water) were screened for antibiotic residues with UHPLC-MS/MS. An overview of all detected antibiotic residues (**Figure 12**) highlights the presence of different types of antibiotic residues in water and sediment (only analyzed in Belgian samples). Wastewater and sediment samples were the most contaminated. The highest concentrations and frequencies were found for ciprofloxacin, sulfamethoxazole and lincomycin. Excluding Belgian samples, predicted no-effect concentrations were exceeded for amoxicillin, cefazoline, ciprofloxacin, clarithromycin, ofloxacin, phenoxymethylpenicillin, sulfamethoxazole, tetracycline and trimethoprim in wastewater samples (Bengtsson-Palme and Larsson, 2016). This means that there could be selection for antibiotic resistance in several samples.

In addition, NTNU samples were analyzed for the presence of antibiotic residues and their metabolites as part of a study on a recirculating aquaculture system where florfenicol was supplemented to the feed.

Furthermore, All *E. coli* isolates (resulting from the sampling in 2022) that showed resistance to one or more of the following antibiotics: ciprofloxacin, tetracycline, cefotaxime, streptomycin and trimethoprim were sent to GU for conjugation assays. The transfer of conjugative plasmids from these donors occurred at a frequency of 39%. Further collaboration with coordinator Åsa Sjöling at GU continued in the context of an FWO-funded research stay abroad for PhD student Laurens Tuts. During this stay, whole genome sequences of 50 interesting ESBL-*E. coli* strains were investigated for the

presence of antibiotic resistance genes, virulence factors, plasmid groups and phylogeny. The results will be published in a joint paper.

A subset of *Shewanella* isolates was sent to KI for further collaboration and testing. Additionally, a subset of *Aeromonas* isolates was shipped to NTNU since these proved to be dominant in different environment sampled by the partners. Results are expected to be delivered after the end of this project.

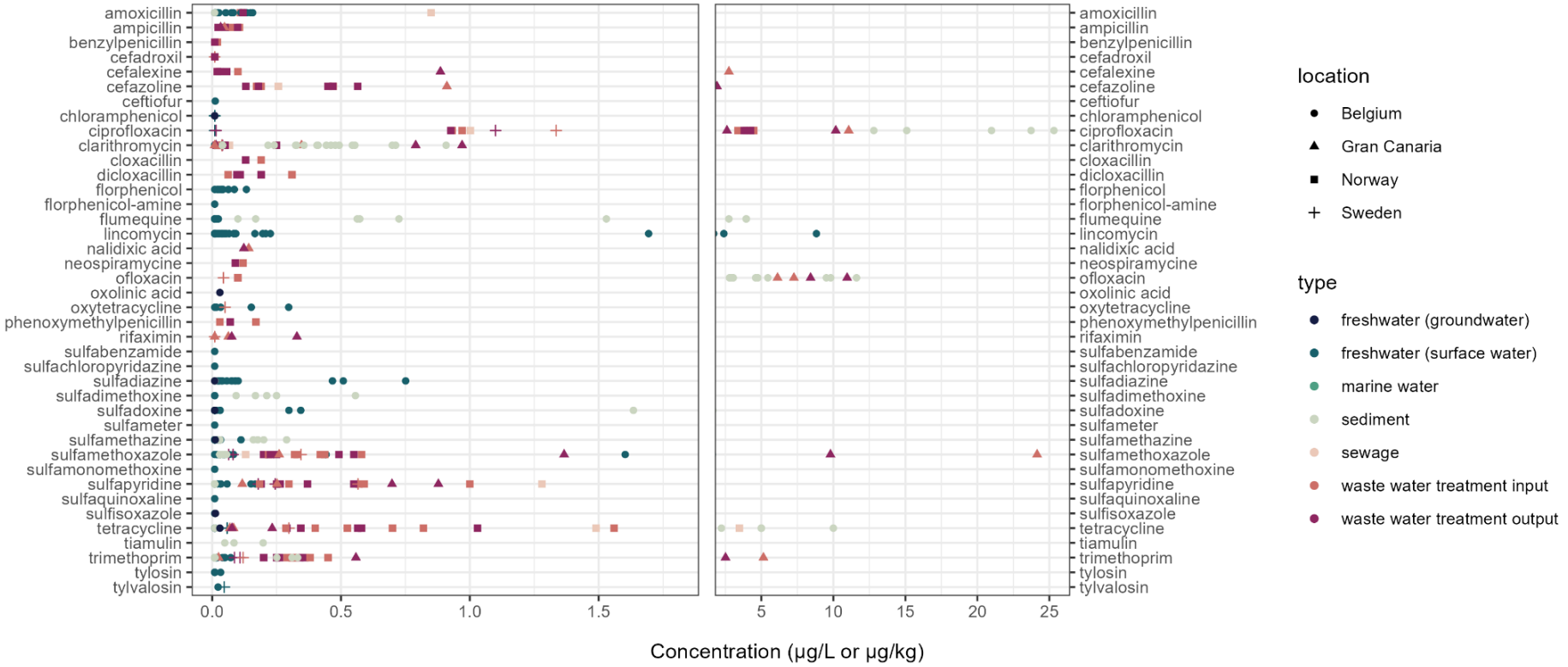

Figure 12: Antibiotic residues in samples from Belgium, Gran Canaria, Norway and Sweden

5.8. RECOMMENDATIONS

Policies addressing antibiotic residues in aquatic environments include the Watch List under the Water Framework Directive (2000/60/EC), the Directive on Environmental Quality Standards in the Field of Water Policy (2008/105/EC), the Strategic Approach to Pharmaceuticals in the Environment, and the EU Water Innovation Strategy (EUROPEAN COMMISSION, 2019). Currently, the Watch List monitors only a limited number of antibiotics in aquatic systems, specifically sulfamethoxazole, trimethoprim, clindamycin, and ofloxacin. Antibiotics may be removed from or added to the Watch List every few years, making it challenging to track long-term trends.

This study highlights the need to expand the monitoring scope to include more antibiotic residues. For example, lincomycin and sulfonamides were frequently detected and, in some cases, exceeded predicted no-effect concentrations (Bengtsson-Palme and Larsson, 2016). Future updates to the Water Framework Directive and the Environmental Quality Standards could benefit from incorporating these residues and others of concern into monitoring programs.

Currently, these legislative frameworks primarily focus on ecological toxicity when addressing antibiotic residues in the environment. However, additional monitoring of antibiotic resistance is strongly recommended. A comprehensive monitoring network would provide robust data to support evidence-based policymaking.

Furthermore, the findings of this study emphasize that sediments act as reservoirs for both antibiotic residues and resistant bacteria, underlining the importance of including sediment analysis in environmental monitoring programs. Integrating sediment studies with water analyses offers a more holistic understanding of resistance dynamics in aquatic ecosystems.

Finally, the study demonstrates that *E. coli* can serve as a valuable indicator for antimicrobial resistance (AMR) surveillance beyond animal-related settings, effectively detecting resistance to critical antibiotics such as colistin, third- and fourth-generation cephalosporins, and carbapenems. Complementing this approach with culture-independent methods, such as (q)PCR and whole-genome sequencing, could provide deeper insights into resistance mechanisms and improve surveillance strategies.

6. DISSEMINATION AND VALORISATION

6.1. STAKEHOLDER ENGAGEMENT

The resulting data was provided to VMM (Flanders Environment Agency) for inclusion in their databases, as they are responsible for water sampling under the EU Nitrate Directive. Additionally, certain sampling points were part of the EU Watch List surveillance. Since the applied LC-MS/MS method could quantify more antibiotic residues than required for surveillance, the results from the PARRTAE project are complementary to the routine analysis of the VMM. The results were communicated to VMM, and a presentation was also given at VMM headquarters (*Communications*). They were also listed as co-authors in the publications of Tuts L. (*Publications*).

AMCRA was informed of the results obtained during the PARRTAE project (poster AMCRA study day, *Communications*). AMCRA is the Knowledge center on antibiotic use and resistance in animals in Belgium.

As antibiotic use in veterinary medicine is high, an effort was made to increase awareness among veterinarians about the presence of antibiotic residues and resistance in the environment including the water. Several presentations (*Communication*) were given for veterinarians in the context of:

- World Association of Veterinary Food Hygienists (WAVFH vzw)
- Flemish Society for Veterinary Epidemiology & Economics (VEE)
- Academy for Veterinary Science (AcVetMed)

The BELMAP report, Belgium's "One Health" report on antibiotic use and resistance, is an initiative by the Belgian Federal Public Service Public Health, Food Chain Safety and Environment. The report aims to summarize the results and trends of existing monitoring programs in humans, food-producing animals, the food chain, and the environment. It seeks to identify potential gaps and formulate general recommendations to improve monitoring. The PARRTAE project and the first results were mentioned in the reports of 2022 and 2023.

Representatives of the FPS Public Health, Food Chain Safety and Environment were invited to the follow-up committee where the progress was discussed and the results were presented.

6.2. COMMUNICATIONS

6.2.1. 2022

- Tuts, L., Rasschaert, G., Daeseleire, E., Heyndrickx, M. & Becue, I. Antibiotic Residues in aquatic environments: validation of a UHPLC-MS/MS method, EURORESIDUE IX may 2022: Veterinary residue control, the beginning of a new era (poster)

6.2.2. 2023

- Rasschaert G. - One health in de praktijk: antibioticaresiduen en resistentie in de landbouwomgeving, AcVetMed, 11/05/2023, Ghent (presentation)
- Tuts, L., Heyndrickx, M., Becue, I., Boon, N., De Maesschalck, P., Eppinger, R. & Rasschaert, G., 2023. Antibiotic resistance in *E. coli* isolated from surface and ground water in areas with intensive livestock farming. Antimicrobial resistance: challenge for public and animal health, June 2023, Brussels, Belgium (poster)
- Tuts L. - Antibiotic residues and resistance in environmental surface- and groundwater, associated with intensive farming, VMM symposium 2023, Leuven, Belgium (presentation)
- Tuts, L., Heyndrickx, M., Becue, I., De Maesschalck, P., Eppinger, R. & Rasschaert, G. Antibiotic Resistance in E. coli isolated from surface and ground water in areas with intensive livestock farming. 27th Conference on Food Microbiology, oct. 2023, Brussels, Belgium (poster)
- Rasschaert G. – *Antibiotic resistance in a One Health perspective: from animal to the plant, VEE symposium* "A Glimpse into the Future: Animals, Health, and the Environment", 26/10/2023, Brussels, Belgium (presentation)
- Rasschaert G. - Antibiotic residues and resistance in a One Health perspective: from animal to the plant, WAVFH, 14/12/2023, Belgium (presentation)

6.2.3. 2024

- Rasschaert, G. - Antibiotic resistance in a One Health perspective: from animal to the plant OHID 2024 international conference dedicated to "One Health", 28/06/2024, Lille, France (presentation)
- Tuts, L., Heyndrickx, M., Becue, I., Boon, N., & Rasschaert, G. (2024). From Pollution to Resistance: Antibiotics in the waters of North Sea Seaports. AquaticPollutants Final Conference: For a healthy aquatic environment. 22/10/2024, Frankfurt (Germany) (poster)

6.3. PUBLICATIONS

6.3.1. Published

- Tuts, L., Rasschaert, G., Heyndrickx, M., Boon, N., Eppinger, R., & Becue, I. (2024). Detection of antibiotic residues in groundwater with a validated multiresidue UHPLC-MS/MS quantification method. Chemosphere, 352, 141455. https://doi.org/10.1016/j.chemosphere.2024.14145

6.3.2. In preparation

- Tuts L., Heyndrickx M., Becue I., Boon N., De Maesschalck P, Rasschaert G. (in preparation). Dissemination of antibiotic residues and resistance in surface water and groundwater after fertilization period.

- Tuts L., Becue I., Boon N., Heyndrickx M., Rasschaert G. Antibiotic pollution in the marine environment and its effect on antibiotic resistance in ESBL *E. coli¸ Shewanella & Vibrio*.

7. ACKNOWLEDGEMENTS

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9. ANNEXES

Table A1: Linearity (R2), recovery (REC), repeatability (RSDr), intra-laboratory reproducibility (RSDR) and limits of detection (LOD) and quantification (LOQ) of the validated UHPLC-MS/MS method for water analysis (Group A). (*): not based on calibration curve, but on confirmed observations. (x): excluded to be quantified at that level with this method, since requirements according to EU regulation 2021/808 are not fulfilled (-): not enough repetitive series were obtained.

Antibiotic	R^2	REC (%)			RSD_r (%)			RSD_R (%)			LOD	LOQ
		0.1	1	10	0.1	10	10	1	10	30	[µg/L]	[µg/L]
		[µg/L]			[µg/L]			[µg/L]				
amoxicillin	1.00	94	96	99	9	7	8	11	14	7	0.05	0.17
ampicillin	1.00	96	97	96	8	5	5	8	19	7	0.01	0.03
benzylpenicillin	0.96	91	111	98	15	21	10	33	26	28	0.03	0.1
cefadroxil	0.98	106	93	100	5	5	6	8	5	8	0.02	0.07
cefalexin	1.00	95	97	103	9	6	6	7	10	10	0.01	0.03
cefalonium	1.00	93	100	100	11	7	6	9	12	10	0.01	0.03
cefapirin	1.00	103	103	101	9	5	5	8	6	7	0.03	0.1
cefazolin	1.00	97	113	102	10	10	7	9	21	11	0.04	0.13
cefoperazone	0.99	114	118	112	20	13	7	22	23	22	0.01 *	0.03
cefquinome	0.96	103	89	95	35	19	5	14	8	7	0.01 *	0.03
ceftiofur	1.00	97	103	100	7	7	5	6	9	7	0.03	0.1
cefradin	1.00	97	94	102	11	6	4	9	8	10	0.04	0.13
chloramphenicol	1.00	95	100	99	6	5	4	9	9	4	0.01	0.03
chlortetracycline	1.00	96	91	89	10	8	7	11	9	9	0.08	0.27
cinoxacin	1.00	94	92	99	9	6	5	14	9	6	0.01	0.03
clarithromycin	1.00	103	99	100	5	6	4	4	6	4	0.04	0.13
cloxacillin	1.00	94	93	92	10	8	6	16	25	10	0.03	0.1
dapson	0.99	100	89	100	8	7	7	7	10	7	0.05	0.17
dicloxacillin	1.00	106	93	93	12	6	8	17	26	12	0.05	0.17
erythromycin	0.99	94	107	93	43	18	13	68	18	19	0.08	0.27
florphenicol	1.00	102	92	99	9	4	5	12	11	8	0.02	0.07
florphenicol-amine	1.00	109	110	113	25	25	13	25	25	16	0.02	0.07
flumequine	1.00	91	95	94	9	5	8	6	10	6	0.01	0.03
josamycin	1.00	102	99	103	7	7	4	6	7	5	0.01	0.03

lincomycin	0.96	108	119	112	12	7	5	14	10	8	0.01	0.03
nafcillin	0.99	101	100	84	8	22	9	20	12	8	0.02	0.07
nalidixic acid	1.00	95	95	96	11	5	6	15	19	8	0.01	0.03
nesopiramycin	1.00	95	96	99	14	9	6	28	11	7	0.01	0.03
oxacillin	0.99	102	94	91	11	8	7	13	20	7	0.01	0.03
oxolinic acid	1.00	89	98	97	8	5	7	19	15	7	0.03	0.1
oxytetracycline	0.99	96	98	93	10	5	5	12	15	10	0.04	0.13
phenoxymethylpenicillin	1.00	95	91	92	9	8	6	15	25	8	0.02	0.07
pirlimycin	1.00	100	95	99	10	5	4	11	7	7	0.02	0.07
rifaximin	0.98	101	90	97	8	8	9	8	13	10	0.01	0.03
sarafloxacin	0.99	129	105	106	6	8	10	34	19	10	0.10 *	0.3
sulfabenzamide	1.00	98	101	98	8	6	5	11	15	4	0.01	0.03
sulfacetamide	1.00	93	106	103	17	10	7	19	12	8	0.10 *	0.3
sulfachloropyridazine	1.00	103	99	102	8	7	5	10	11	6	0.01	0.03
sulfaclozine	1.00	99	89	96	9	7	5	11	10	5	0.02	0.07
sulfadiazine	0.99	100	106	96	11	6	5	12	10	6	0.03	0.1
sulfadimethoxine	1.00	98	95	99	8	7	5	9	7	12	0.03	0.1
sulfadoxine	1.00	100	100	103	8	7	5	10	10	6	0.01	0.03
sulfamerazine	0.99	91	109	100	14	6	6	15	10	7	0.10	0.33
sulfameter	1.00	102	98	101	9	7	5	9	11	5	0.01	0.03
sulfamethazine	1.00	106	99	99	8	7	7	8	9	8	0.01	0.03
sulfamethizole	1.00	99	100	100	9	6	5	9	8	5	0.01	0.03
sulfamethoxazole	1.00	98	94	99	9	5	6	9	8	6	0.03	0.1
sulfamethoxypyridazine	0.99	98	99	103	9	7	6	15	11	7	0.04	0.13
sulfamonomethoxine	1.00	102	96	103	9	7	6	9	9	6	0.01	0.03
sulfaphenazole	1.00	102	99	101	10	6	6	10	7	6	0.01	0.03
sulfapyridine	1.00	104	93	98	10	6	5	13	6	7	0.01	0.03
sulfaquinoxaline	1.00	100	95	95	9	8	8	9	7	7	0.01	0.03
sulfathiazole	1.00	95	93	99	9	7	6	13	10	6	0.05	0.17
sulfisoxazole	1.00	100	97	101	7	5	6	8	7	7	0.03	0.1
tetracycline	1.00	91	97	92	11	9	5	10	14	6	0.01	0.03

tiamulin	0.99	-	109	97	-	5	15	-	10	28	0.10 *	0.3
trimethoprim	1.00	103	103	98	7	6	5	8	9	6	0.01	0.03
tylosin	1.00	91	103	99	18	11	5	17	14	7	0.02	0.07
tylvalosin	1.00	97	82	99	14	14	5	17	13	5	0.03	0.1
valnemulin	1.00	103	95	98	10	10	3	14	10	3	0.011	0.04

Table A2: Linearity (R^2), recovery (REC), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and limits of detection (LOD) and quantification (LOQ) of the validated UHPLC-MS/MS method for water analysis (Group B). (*): not based on calibration curve, but on confirmed observations. (x): excluded to be quantified at that level with this method, since requirements according to EU regulation 2021/808 are not fulfilled (-): not enough repetitive series were obtained.

Antibiotic	R^2	REC (%)			RSD_r (%)			RSD_R (%)			LOD	LOQ
		1	10	30	1	10	30	1	10	30	[µg/L]	[µg/L]
		[µg/L]			[µg/L]			[µg/L]				
cefacetrile	0.96	103	115	106	16	7	8	19	10	9	1.00 *	3.33
cefuroxime	0.98	100	108	108	18	16	5	11	25	6	0.10 *	0.33
ciprofloxacin	0.97	120	115	101	10	8	5	48	8	-	0.10 *	0.33
danofloxacin	0.97	117	106	100	2	10	10	64	28	18	1.00 *	3.33
desacetyl-cefapirin	1.00	99	105	110	7	5	5	9	5	11	0.10 *	0.33
difloxacin	0.97	120	86	89	1	21	6	-	22	11	0.10 *	0.33
doxycycline	1.00	96	95	101	23	7	5	23	7	6	0.10 *	0.33
enoxacin	0,99	104	94	101	6	3	4	59	16	25	0.10 *	0.33
enrofloxacin	0.95	115	116	99	1	11	12	117	22	18	1.00 *	3.33
marbofloxacin	0.98	10	106	102	3	14	4	58	13	3	1.00 *	3.33
norfloxacin	0.98	115	93	101	18	5	4	55	20	9	0.10 *	0.33
ofloxacin	0.97	95	91	96	8	18	13	-	36	21	0.10 *	0.33
spiramycin	0.96	-	97	98	-	6	19	-	19	18	0.10 *	0.33
sulfaguanidine	0.97	123	94	92	4	4	6	-	16	6	0.10 *	0.33
sulfisomidine	0.98	106	91	95	11	7	5	12	15	7	0.10 *	0.33
thiamphenicol	1.00	88	94	99	8	6	4	13	16	8	0.05	0.17
tilmicosin	0.98	-	105	98	-	14	27	-	24	27	1.00 *	3.33
tulathromycin metabolite	0.98	-	75	92	-	9	14	-	15	18	1.00 *	3.33

Table A3: Linearity (R^2), recovery (REC), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and limits of detection (LOD) and quantification (LOQ) of the validated UHPLC-MS/MS method for sediment analysis. (*): not based on calibration curve, but on confirmed observations. (-): The number of repetition series in compliance with EU Regulation 2021/808 is insufficient.

ANTIBIOTIC	R^2	RECOVERY (%)				REPEATABILITY (%)				REPRODUCIBILITY (%)				
		1 µg/kg	*10 µg/kg*	*30 µg/kg*	*50 µg/kg*	*1 µg/kg*	*10 µg/kg*	*30 µg/kg*	*50 µg/kg*	*1 µg/kg*	*10 µg/kg*	*30 µg/kg*	*50 µg/kg*	LOD (µg/kg)
amoxicillin	0.95	-	-	72	86	-	-		15	-	-	-	-	20
ampicillin	0.99	-	-	69	-	-	-	7	-	-	-	-	-	7
benzylpenicillin	1	-	111	101	-	-	14	12	-	-	-	-	-	10
phenoxymethylpenicillin	1	-	113	104	-	-	7	9	-	-	-	-	-	3
cefadroxil	1	-	-	-	-	-	4	12	-	-	16	11	-	0.6
cefapirin	1	-	83	97	-	-	5	-	-	-	6	-	-	5
cefalexin	1	-	-	-	-	-	-	14	-	-	-	14	-	1
cefalonium	0.97	-	85	107	-	-	19	-	-	-	17	-	-	6
cefazolin	1	-	-	-	-	-	-	-	6	-	-	-	-	5
cefoperazone	0.97	-	-	102	87	-	-	-	-	-	-	-	-	25
cefquinome	0.96	-	-	95	84	-	-	-	16	-	-	-	-	40
ceftiofur	1	-	81	82	-	-	-	23	-	-	-	-	-	5
cephradine	1	-	111	114	-	-	25	-	-	-	-	-	-	1
cinoxacin	1	-	93	95	-	-	7	10	11	-	-	-	-	1
ciprofloxacin	1	-	-	92	101	-	-	9	15	-	-	-	-	5 (*)
enrofloxacin	1	-	95	99	-	-	7	5	-	-	-	-	-	3
flumequine	1	114	94	-	-	8	5	-	-	-	-	-	-	1
nalidixic acid	1	-	-	-	-	21	11	-	-	-	-	-	-	1
norfloxacin	1	-	-	-	82	-	-	-	14	-	-	-	-	25
ofloxacin	1	-	95	-	-	-	2	-	-	-	-	-	-	1
oxolinic acid	1	71	80	-	-	-	-	-	-	-	-	-	-	1
sarafloxacin	1	-	63	93	-	-	18	9	-	-	-	-	-	5

lincomycin	1	77	97	-	-	-	8	-	-	26	7	-	-	1
pirlimycin	1	110	98	-	-	8	6	-	-	6	6	-	-	1
clarithromycin	1	93	91	-	-	8	4	-	-	-	-	-	-	1
erythromycin	1	104	98	-	-	12	7	-	-	17	11	-	-	1
josamycin	1	101	98	-	-	7	5	-	-	9	8	-	-	1
tylosin A	1	108	97	-	-	9	5	-	-	7	7	-	-	1
tylvalosin	1	109	101	-	-	8	7	-	-	9	8	-	-	1
chloramphenicol	1	101	101	-	-	8	5	-	-	10	6	-	-	1
florphenicol	1	100	100	-	-	7	4	-	-	6	5	-	-	1
thiamphenicol	1	107	102	-	-	11	4	-	-	10	6	-	-	1
tiamulin	1	94	99	-	-	12	7	-	-	12	11	-	-	1
valnemulin	1	84	101	-	-	10	11	-	-	28	10	-	-	1
dapsone	1	98	100	-	-	8	6	-	-	8	8	-	-	0.1
sulfabenzamide	1	101	101	-	-	6	7	-	-	4	6	-	-	0.1
sulfa-chloropyridazine	1	100	98	-	-	7	5	-	-	6	10	-	-	0.1
sulfaclozine	1	106	99	-	-	9	6	-	-	9	7	-	-	0.5
sulfadimethoxine	1	99	100	-	-	7	5	-	-	7	6	-	-	0.1
sulfadoxine	1	100	100	-	-	11	5	-	-	13	7	-	-	0.1
sulfadiazine	1	97	97	-	-	9	7	-	-	8	9	-	-	0.1
sulfamethazine	1	99	97	-	-	10	5	-	-	9	7	-	-	0.1
sulfamethizole	1	99	99	-	-	8	6	-	-	9	7	-	-	0.1
sulfamerazine	1	104	100	-	-	12	5	-	-	11	7	-	-	0.5
sulfameter	1	101	100	-	-	12	6	-	-	11	9	-	-	0.5
sulfamethoxazole	1	96	99	-	-	8	6	-	-	13	7	-	-	0.1
sulfa-methoxypyridazine	1	109	100	-	-	7	6	-	-	9	8	-	-	0.5
sulfa-monomethoxine	1	79	97	-	-	17	7	-	-	29	10	-	-	0.5
sulfaphenazole	1	99	100	-	-	14	7	-	-	11	6	-	-	0.5
sulfapyridine	1	100	98	-	-	15	7	-	-	13	8	-	-	0.5

sulfaquinoxazoline	1	99	99	-	-	9	6	-	-	10	6	-	-	0.1
sulfathiazole	1	99	98	-	-	9	6	-	-	12	8	-	-	0.1
sulfisoxazole	1	98	95	-	-	9	6	-	-	8	6	-	-	0.5
chlortetracycline	1	-	83	98	-	-	13	11	-	-	8	15	-	5
doxycycline	1	-	-	95	99	-	-	9	12	-	-	8	13	20
oxytetracycline	1	-	94	89	-	-	9	14	-	-	8	15	-	5
tetracycline	1	-	90	91	-	-	9	14	-	-	7	15	-	5
trimethoprim	1	100	102	-	-	8	6	-	-	8	7	-	-	1