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Impact of log(Kow) Value on the Extraction of Antibiotics from River Sediments with Pressurized Liquid Extraction

Amélie Chabilan [1], Nicolette Landwehr [1], Harald Horn [1,2,*] and Ewa Borowska [1]

[1] Engler-Bunte-Institut, Water Chemistry and Water Technology, Karlsruhe Institute of Technology, Engler-Bunte-Ring 9, 76131 Karlsruhe, Germany
[2] DVGW-Research Center at the Engler-Bunte-Institut, Water Chemistry and Water Technology, Karlsruhe Institute of Technology, Engler-Bunte-Ring 9, 76131 Karlsruhe, Germany
* Correspondence: harald.horn@kit.edu; Tel.: +49-721-608-42580; Fax: +49-721-608-46497

Abstract: The quantification of antibiotics (ABs) in sediments is an analytical challenge, but at the same time, it is indispensable to understand the fate of ABs in aquatic systems such as rivers. The aim of this study was to develop a comprehensive method to determine 19 ABs classified as macrolides, sulfonamides, fluoroquinolones, tetracyclines, clindamycin and trimethoprim in river sediments, using a combination of pressurized liquid extraction and solid phase extraction with the separation and detection with liquid chromatography coupled with mass spectrometry. Our results showed that the physical-chemical properties (e.g., log(Kow) value) of the analytes affected the extraction efficiency. Therefore, we propose to order ABs based on their log(Kow) values instead of traditional classification (macrolides, sulfonamides etc.) to select a suitable extraction solvent. ABs with log(Kow) values below zero (mainly fluoroquinolones and tetracyclines) were difficult to extract with all of the tested protocols compared to ABs with a log(Kow) larger than zero. After comparing different extraction protocols for ABs from solid and sediments, we concluded that recoveries in the range of 0.8 to 64.8% could be achieved for ABs with a log(Kow) value larger than zero using a mixture of acetonitrile and 50 mM phosphoric acid (50/50, *v*/*v*) in two extraction cycles at 100 °C.

Keywords: antibiotics; river sediment; pressurized liquid extraction; solid phase extraction; aquatic environment; method development; liquid chromatography coupled with mass spectrometry

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1. Introduction

Antibiotics (ABs) are applied against bacterial infections in human and veterinary medicine and represent one of the most important classes of pharmaceuticals. After administration, ABs are excreted via urine and feces up to 90% [1] and together with wastewater are directed to the wastewater treatment plant. The conventional wastewater treatment plants are not designed to remove ABs, and the efficiency of AB removal varies greatly, from 36 to 79% [2]. This also makes WWTPs the main point source of human-use ABs in an aquatic environment [3]. The problem is even more pronounced in WWTPs receiving hospital wastewater, which carries a large load of antibiotics [4,5]. As a result, their residuals are continuously discharged into receiving waters such as rivers [6,7]. Concentrations of ABs in municipal wastewater are typically in the low µg/L range, whereas in the receiving waters in the low to high ng/L range [7,8]. Veterinary antibiotics used in agriculture can be introduced into the aquatic environment through run off from fields fertilized with animal manure [9,10]. Overall, ABs are detected in all types of aquatic environments [11] including groundwater [12].

As reported by multiple authors, the ABs belonging to fluoroquinolones and tetracyclines tend to adsorb to a solid matrix [9,13–15]. This makes ABs prone to staying in sludge (0.9 to 425.5 µg/kg wet weight or 0.1 to 8.5 µg/kg dry matter [16,17]), sediments (1.4 to 2339 µg/kg dry weight [8,18–20]), and soil (1.0 to 198.7 µg/kg [21,22]) in the indicated concentration range. Cooler and more reducing conditions in the sediments result in

lower biodegradation rates for fluoroquinolones, macrolides, and sulfonamides [23]. Most antibiotics have limited biodegradability under aerobic conditions [8]. The presence of ABs in aquatic environments can have some adverse effects. It has been already proven that ABs occurrent in rivers may hinder the growth of algae and also some benthic invertebrates [19,24]. However, there is still much discussion on the role of antibiotics at the minimal selective concentrations or minimal inhibitory concentration on the spread of antibiotics resistance genes (ARGs) [25].

Considering the strong sorption properties and limited biodegradability of different AB classes as well as their continuous discharge to the aquatic environment, the water phase and sediments require in-depth investigation. Despite this clear need to measure ABs in river sediments, it is still a challenging task due to the complex interactions between the ABs and the solid matrix [26].

Furthermore, river sediments are impacted by different environmental and hydrological factors such as riverine shear, biofilm grow, turbidity, and altering weather conditions [27]. These factors can affect the sampling of the river sediment and are difficult to control during sampling. Sample preparation including the extraction of AB from sediments can be conducted under controlled laboratory conditions. Consequently, we decided to focus on the optimization of the extraction protocol as one element in the analytical method. Optimization was necessary due to the complex sediment matrix and concentrations of ABs in the low μg/kg range (below 1 up to 25 μg/kg [28,28–31]). These boundary conditions require a multistep and time-consuming analytical procedure, very often including several extraction and purification techniques [32–35].

Among the extraction procedures for antibiotics and pharmaceuticals dedicated for solid samples reported in the literature, we can find accelerated solvent extraction (ASE), known also as pressurized liquid extraction (PLE) [36–38], ultrasonic-assisted extraction [36,39–41], QuEChERS extraction (i.e., quick easy cheap effective rugged and safe) [42,43], and microwave-based techniques [44,45]. The first two seem to have received the most attention.

The advantage of PLE is a stronger extraction power than standard ultrasonication as well as the high degree of automation. This contributes to the higher sample throughput [37], reduced extraction times, low solvent consumption, and in the case of many ABs, also higher recoveries [46]. Extracts have to be subsequently purified to remove the residual interferences coming from the matrix, which can decrease the efficiency of the method. It seems that solid phase-extraction is the most suitable technique due to its high efficiency and low quantity of the solvent used [30,39].

Although there are some studies concerning the extraction of ABs from soil with PLE [17,21,37,38,47] and from sludge [16,17,37], the methods available for sediments including at least three AB classes are rather scarce [29,34,35]. The three PLE methods of Li et al. 2012 [34], Gibs et al. (2013) [29], and Kerrigan et al. 2018 [35] included fluoroquinolones (64.7–132.2%, 40.0–63.3%, 3–59%, respectively), macrolides (63.4–100.5%, 68.0–104.1%, 85–360%, respectively), sulfonamides (96.8–132.3%, 100.2–106.9%, 70–224%, respectively), and tetracyclines (53.3–102.9%,1–122%, respectively). Depending on the methods and AB class, the recovery range was wide. The method of Senta et al. (2021) [36] was only tested for macrolides (recovery range 54–99%). Vazquez-Roig et al. (2020) [32] developed a method for three fluoroquinolones (ciprofloxacin, norfloxacin, ofloxcin), two tetracyclines (oxytetracyline, tetracycline), sulfamethoxazole, and trimethoprim with a recovery range of 50 to 104%. The methods of Silva et al. (2011) [33] included only three macrolides (clarithromycin, erythromycin, roxithromycin, 68–149%), two sulfonamides (sulfadiazine, sulfamethazine, 45.7%), and trimethoprim (97.2%). In the reviewed methods, the recovery range for fluoroquinolones and tetracyclines were broad.

To select the suitable PLE conditions, the physical-chemical properties of the AB and the composition of the solid matrix should be considered. The distribution of ABs between solid and liquid matrix (sediment/water system) can be viewed as partitioning processes between the aqueous phase (water) and an organic phase (e.g., n-octanol) [27].

The corresponding value is the log(Kow) value. The matrix composition depends on the sample location, which can have a lower or higher clay, silt, or sand content. It has already been reported that the adsorption of fluoroquinolones and tetracycline is higher on clay rich minerals [9,15,48,49]. Depending on the composition, different interactions with ABs are possible. Therefore, PLE methods cannot be transferred easily to another type of solid matrix.

The aim of this study was to elaborate a method for the quantification of four AB classes (fluoroquinolones, macrolides, sulfonamides, tetracyclines) as well as trimethoprim and clindamycin in river sediments. According to our knowledge, there has been no study encompassing all of these AB classes in one protocol. ABs from the same class can have different physical-chemical properties. Hence, we proposed to order ABs after their log(Kow) value to study the influence of different extraction parameters such as the type of solvent on the extraction efficiency. The method is based on pressurized liquid extraction followed by solid-phase extraction, and subsequent analysis using liquid chromatography coupled with tandem mass spectrometry. The applicability of the method was verified for sediment samples from a local river.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals were of analytical grade. The complete list of chemicals used in this study is presented in the Supplementary Materials (SM) Text S1.

2.2. Investigated Compounds

A total of 19 ABs from four classes of AB were selected: macrolides (erythromycin, erythromycin-H_2O, roxithromycin, clarithromycin), tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline), fluoroquinolones (ciprofloxacin, enrofloxacin, norfloxacin, ofloxacine), sulfonamides (sulfadiazine, sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfapyridine), clindamycin (lincosamide), and trimethoprim. For the purpose of this study, clindamycin and trimethoprim were annotated as a fifth class called 'others'. The selection of the compounds was carried out based on their usage in both human and veterinary medicine as well as to cover the most typical AB classes detected in aquatic environments [23,35,36,50,51]. Furthermore, erythromycin, clarithromycin, and ciprofloxacin have been included on the European Watch List since 2018 [52]. The basic properties of the selected ABs are collected in Table S1 of the Supplementary Materials.

2.3. Preparation of Stock Solutions

A mixture of all of the investigated ABs dissolved in MeOH and water (50/50, *v*/*v*) to a concentration of 10 mg/L of each compound served as the stock solution. Another stock solution in MeOH was prepared for deuterated derivatives in the same manner. For the method development and quantification with an external calibration, working solutions were used that were prepared by a dilution in a mixture of MeOH and pure water (pH 2).

2.4. Sampling and Preparation of Sediment Samples

Sediments used for the method development were collected from a local medium-sized mountain river in the south of Germany in the summer of 2020. This river is also a receiver of wastewater discharge from the municipal wastewater treatment plant with a population equivalent (p.e.) of 875,000 (for more details see [53]). To minimize the risk of the existing contamination of sediments with AB, the sediment samples were sampled at one location (simple) upstream from the discharge of the wastewater treatment plant. The sediment was sampled with a metal shovel into the glass bottles. The exact coordinates of the sampling point were 49°03′48″ N, 8°20′36″ E. After sampling, the sediment was transported immediately to the laboratory in cooling box protected from the sun and stored in the dark until further processing.

The tested sediment had the following characteristics: pH 7.2, total inorganic carbon 0.53%, total organic carbon 8.06%, total nitrogen 0.61%, 21.5% clay; 48.9% silt; 29.6% sand. The applied methods are described in Supplementary Materials Text S2. Further information about the composition of the sediments are in Table S2a to d.

For method development and method validation, sediment samples taken upstream from the discharge of the wastewater treatment plant were dried at 100 °C, passed through a 500 µm sieve (test sieve, Retsch GmbH, Haan, Germany), and stored in amber glass bottles at room temperature.

The applicability of the method was verified using the sediment samples from the same river, but downstream from the WWTP discharge (for more detail see [53]), where the contamination with AB was expected. Samples were taken with a self-made cylindrical sediment core sampler (Device model, manufacturer, city, country) (PVC tubes with a diameter of 2 cm, [53]). The freeze-dried sediments were homogenized in a porcelain mortar and sieved (500 µm) and extracted by PLE protocol G (Table 1).

Table 1. The characteristics and parameters of the PLE protocols used in the study.

Protocol	A [a]	B [b]	C [c]	D [a,d]	E [e]		F [a,e]		G [a]
Extraction steps	One	One	One	One	Two consecutives		Two consecutives		One
					1	2	1	2	
Organic solvent	ACN	MeOH/ ACN	MeOH	MeOH + 0.2% NH_4OH	EtOAc/ ACE	ACE	ACE	ACN	ACN
Aqueous solvent	50 mM H_3PO_4 pH 2	0.2 M CA pH 4.5	50 mM phosphate buffer pH 7	50 mM H_3PO_4 pH 2	-	1% H_3PO_4	1% H_3PO_4	50 mM H_3PO_4 pH 2	50 mM H_3PO_4 pH 2
Solvent ratio (%)	50/50	40/40/20	50/50	50/50	66/33	66/33	66/33	50/50	50/50
Cycles	6	2	2	6	2	2	2	6	2
Temperature (°C)	100	80	100	100	80	80	80	100	100
Static time (min)	15	10	5	15	5	5	5	15	10
Rinse volume (%)	150	100	150	150	60	60	60	150	150
Purge time (s)	300	40	100	300	120	120	120	300	300

Protocols were adapted from [a] Golet et al. (2002) [37], [b] Salvia et al. (2015) [17], [c] Kerrigan et al. (2018) [35], [d] Senta et al. (2021) [36], [e] Chiaia-Hernandez et al. (2017) [54], ACE—acetone, ACN—acetonitrile, CA—citric acid, EtOAc—ethyl acetate, MeOH—methanol.

2.5. Pressurized Liquid Extraction (PLE)

PLE was performed using an accelerated solvent extractor Dionex™ ASE™ 350 (Thermo Scientific™, Dreieich, Germany). For method development, the sediment (1–5 g) was spiked to a concentration of 10 µg/kg with the ABs mixture in methanol/water (50/50, *v*/*v*) and left to equilibrate overnight. The following day, spiked sediment was mixed with 5 g of sand (Thermo Fisher Scientific Ottawa Sand or Merck Sea sand) in a mortar. The mixture was transferred to a 10 mL stainless steel extraction cell containing a cellulose filter (Dionex™ASE™150/350 stainless steel extraction cell, Thermo Fisher Scientific, Dreieich, Germany) and a thin layer of sand. The cell was filled up with Ottawa sand and a second cellulose filter was placed at the top. Various solvent mixtures were used with different extraction programs. The tested protocols are depicted in Table 1. Pressure was fixed at 1500 psi, which is equal to 103 bar or 10.34 MPa. A preheating time of 5 min was applied in all protocols. The PLE extracts were collected in a 60 mL glass vial. For protocols C, E, and F, the organic phase/solvent was subsequently removed from the extract using a rotary evaporator (Laborota 4001, Heidolph, Schwabach, Germany) or a gentle stream of nitrogen. The extracts were diluted and processed using SPE.

2.6. Clean-up of PLE Extracts by Solid-Phase Extraction (SPE)

Tandem SPE was performed: (1) to remove the interferences from the PLE extracts, and (2) to concentrate the analytes in the samples. Optimization of the SPE protocol reported

by [35,55] was performed as a preliminary study (results not shown). The recoveries for the final SPE protocol are summarized in Table S10. The PLE extracts were transferred to 250 mL flasks. Ethylenediaminetetraacetic acid (EDTA) was added to a concentration of 0.5 g/L as a chelating agent for multivalent cations (Ca^{2+}, Mg^{2+}). Multivalent cations form complexes with tetracyclines and prevent the interaction of tetracyclines with the sorbent of the SPE. Flasks were filled up with ultrapure water adjusted to pH 2 (with HCl 32%) and covered with aluminum foil to prevent the photodegradation of ABs.

For tandem SPE, a combination of a hydrophilic-lipophilic balance reversed phase sorbent (Oasis® HLB, 500 mg, 6 mL, Waters, Milford, USA) and a mixed-mode cation exchange sorbent (Oasis® MCX, 150 mg, 6 mL, Waters, Milford, USA) was used. All the details concerning the solvents and their volumes, applied in the individual steps of the SPE (conditioning, washing, and elution), are collected in Table S3. SPE cartridges were preconditioned separately. For the tandem configuration, the MCX cartridge was connected to the vacuum rack and filled with ultrapure water of pH 2. The HLB cartridge was attached to the top. Large volume tubing and SPE adapters were used to introduce the sample on the cartridges. Samples were loaded on the cartridges with a flow rate of 5 mL/min. After loading, the cartridges were dissembled and washed separately. To remove the rest of the solvent, cartridges were dried under a stream of N_2. Elution was performed in the following steps: (1) The HLB cartridge was filled with 2.5 mL of methanol; (2) the MCX cartridge was attached on top of the HLB and filled with another 2.5 mL of methanol; (3) 5 mL of acetonitrile was added to MCX-HLB to pass through both cartridges; and (4) MCX was detached from HLB and eluted separately with 2.5 mL of 5% NH_4OH in methanol. The eluate was collected in a graduated 15 mL glass test tube. Afterward, the solvents were evaporated to the volume of 1 mL under a stream of N_2. Before the quantification, 50 µL of the extract was diluted in 950 µL ultrapure water acidified to pH 2.

2.7. Analysis of Antibiotics by Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

The concentrations of ABs in the extracts were determined with an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 Triple Quadrupole LC/MS system. ABs were separated on an Agilent ZORBAX Eclipse Plus C-18 column (50 × 2.1 mm, 1.8 µm particle size) using HPLC water and acetonitrile, both acidified with formic acid to the concentration of 0.05% as mobile phases. Details of the quantification method used in the study is shown in the Supplementary Materials (Tables S4–S6). The quantification was achieved with an external calibration curve in the range of 10 to 1500 ng/kg. The R^2 coefficient was 0.990 or higher.

2.8. Quality Control

To assure the high quality of analytical procedure, several quality control measures were applied. In every PLE run, a blank sample containing only Ottawa sand (no sediment) assisted in the complete extraction protocol to exclude the contamination during the procedure. To evaluate the initial concentration of ABs in the sediment, a matrix blank (1–5 g of non-spiked sediment) was extracted in every PLE run. During every SPE, a blank sample containing only water (pH 2) was extracted together with extracts from PLE to monitor the potential contamination during sample enrichment. During LC-MS/MS measurement, quality control samples (standards of 500 ng/L of each of investigated ABs) and solvent blanks (HPLC water) were inserted in the sequence to assess the potential contamination and/or carry-over during the chromatographic run and the stability of the instrument. Random standard samples in the sequence were measured in duplicate to monitor reproducibility.

To control the performance of the instrument as well as to mitigate the matrix effect of the extracts, the internal standards (IS) were added to the samples before the separation on the chromatographic column. The list of IS used for the individual analytes can be found in SM Table S7. The limit of detection (LOD) for LC-MS/MS was set as the lowest point

of the calibration curve with a signal-to-noise larger than 3. The limit of quantification (LOQ) was the lowest calibration standard (in general 10 ng/kg) with a signal-to-noise ratio of minimum 10. The LOQ range was 5–250 ng/L (Table S7). All of the blank samples showed an AB concentration below LOQ, indicating no contamination during the sample pretreatment and analysis. The relative standard deviation (RSD) of the quality control samples and duplicate injections were <10%, which indicates good repeatability of analysis. During method development labeled compounds were used as surrogates (spiked to the sediment before extraction). The results of the preliminary studies (data not shown) confirmed the same behavior of the labeled and non-labeled compounds in the samples. Because of the strong matrix effects, we decided to use the label compounds as the internal standard (added before the LC-MS/MS measurement) for the method validation.

2.9. Determination of the Recovery

The efficiency of each method was evaluated using spiked sediment (triplicates). Regarding the method development, we used the real river sediment that could potentially be exposed to ABs, calculated the relative recovery (RR) by subtracting the concentration of the non-spiked sediment sample (c_{sample}) from the spiked sediment sample ($c_{spiked\ sample}$), and divided this value through the spiked concentration ($c_{spiked,theory}$), according to Equation (1):

$$RR\ [\%] = \frac{c_{spiked\ sample} - c_{sample}}{c_{spiked,theory}} \cdot 100\% \quad (1)$$

The RR was determined for all of the analytes of interest above-mentioned as well as for the surrogates. For every class of ABs, one surrogate standard was proposed with the structure similar to the target compounds. During the measurement of the environmental samples, based on the relative recovery of individual surrogates, the concentrations of the targets were corrected. The following surrogates were applied: (i) sulfachloropyridazine for sulfonamides, trimethoprim, and clindamycin; (ii) meclocycline for tetracyclines [55]; (iii) clinafloxacin for fluoroquinolones [35]; and (iv) oleandomycin-triacetate for macrolides [55]. The tested methods were evaluated based on the calculated recovery range. The acceptable range was set as 45 to 125%.

2.10. Method Validation

The protocol that provided the most satisfactory results was validated. The validation included the determination of the range of linearity, method limit of detection (MLOD), method limit of quantification (MLOQ), method accuracy, precision, and selectivity. The selection of the validation parameter was based on parameters determined in other method validations [32,36,38].

MLOD and MLOQ were calculated after Equations (2) and (3) from Chiaia-Hernandez et al. [56] based on the limit of detection or quantification of the instrument, taking into consideration the matrix factor (see Equation (4)) and a factor for sample preparation (dilution and concentration factor).

$$MLOD = \frac{LOD}{\text{matrix factor}} \times \text{factor sample preparation} \quad (2)$$

$$MLOQ = \frac{LOQ}{\text{matrix factor}} \times \text{factor sample preparation} \quad (3)$$

For the matrix factor, see Equation (5). The factor sample preparation included the different dilution or concentration factors of the sample preparation. The overall method's accuracy and precision were determined by recovery experiments of five replicates (sediments) spiked at 50 µg/kg and 100 µg/kg ABs and surrogates because no reference material was available. Spiking experiments were performed as described in Section 2.5 with PLE protocol G (see Table 1). The method accuracy was calculated as the extraction

recovery for the PLE-SPE processes according to Equation (1). The method precision, here expressed as repeatability, was calculated as the relative standard deviation (RSD) and coefficient of variation (CV) (see Equation (4)) of the calculated recoveries from five spike sediment samples.

$$\text{CV} = \frac{\text{standard deviation (recovery)}}{\text{average recovery}} \times 100\% \quad (4)$$

The selectivity of the method is determined by the matrix effect. The matrix effect was assessed by comparing the area of the target compound and surrogates spiked into the final PLE-SPE extract ($\text{area}_{\text{spiked extract}}$) with the area of the target compound and surrogates in nano pure water used as the calibration standard of the same concentration ($\text{area}_{\text{calibration standard}}$), considering the area of the target compound already present in the matrix blank ($\text{area}_{\text{matrix blank}}$) (see Equation (5)).

$$\text{Matrix factor} = \frac{\text{area}_{\text{spiked extract}} - \text{area}_{\text{matrix blank}}}{\text{area}_{\text{calibration standard}}} \quad (5)$$

3. Results and Discussion

3.1. Initial Screening of PLE Methods

We started our study by testing some of the already reported methods in the literature [17,35,37]. Our main extraction technique was pressurized liquid extraction (PLE), which satisfactory efficiency has already been reported for a few AB classes such as macrolides [36], sulfonamides [29], and to lesser extent, also tetracyclines [17] and fluoroquinolones [37]. However, according to our knowledge, there has been no study encompassing all of these AB classes in one protocol.

Recoveries for the four different protocols A, B, C, and D (conditions in Table 1), adapted after [17,35–37], are shown in Figures S1 and S2. As illustrated in Figures S1 and S2, there is no clear relation between the AB class and extraction parameters. This fact can be explained by differences in the physical-chemical properties (e.g., log(Kow) value) of the compounds. For better visualization, we reorganized the selected ABs into four groups depending on their log(Kow) value (below −1, between −1 and 0, between 0 and 1, and larger than 1). Figure 1 presents the four log(Kow) groups and the ABs that belong to these groups. The studied ABs covered the range of log(Kow) values from −1.37 to 3.16 (see Table S8). A positive log(Kow) value indicates a lipophilic/hydrophobic and nonpolar compound. A low and negative log(Kow) represents a hydrophilic and polar compound. Seven ABs had a log(Kow) smaller than zero and 11 ABs were larger than zero. The majority of the ABs belonged to the lipophilic/hydrophobic and nonpolar compounds. Tetracyclines were only found in groups 1 and 2. Fluoroquinolones were distributed among groups 1 to 3. The macrolides all belonged to group 4 and sulfonamides were spread among groups 2 and 4.

Figure 2 shows the recoveries for the different ABs regrouped according to their log(Kow) values (groups 1 to 4). The letters (A to D) related to the tested protocols in Table 1. The results for protocols E and F are discussed in Section 3.3 and protocol G in Section 3.4 for the method validation. For log(Kow) group 1, the recoveries achieved with the protocols A, B, and D were in the range of 4.0 to 28.3%. After extraction, according to protocol C, none of the ABs of this group were detected in the extracts. Overall, none of the protocols reached the defined recovery range of 45–125% for AB from group 1. For log(Kow) group 2, consisting mainly of tetracyclines, protocol A achieved recoveries in the range of 12.9 to 81.3% followed by protocol B (5.4 to 40.6%) and protocol D (3.1 to 24.3%). For protocol C, only sulfadiazine was quantified in the extract (32.2%). The median of protocols B and D was below the minimum satisfactory recovery of 45% (7.8 and 15.0% respectively, Figure 2).

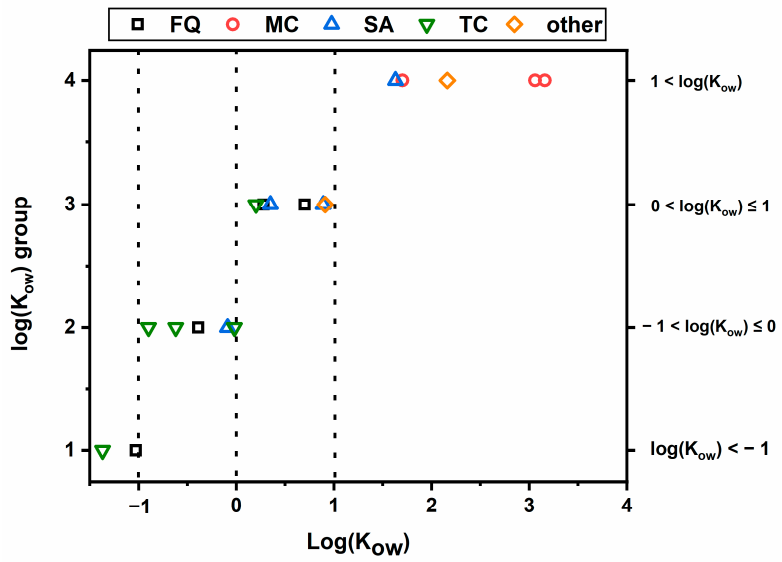

Figure 1. The ABs regrouped into four groups after their log(Kow) values (*y*-axis). Four fluoroquinolones (FQ), three macrolides (MC), five sulfonamides (SA), four tetracyclines (TC), and others (clindamycin and trimethoprim). For erythromycin-H_2O, the value of log(Kow) was not available.

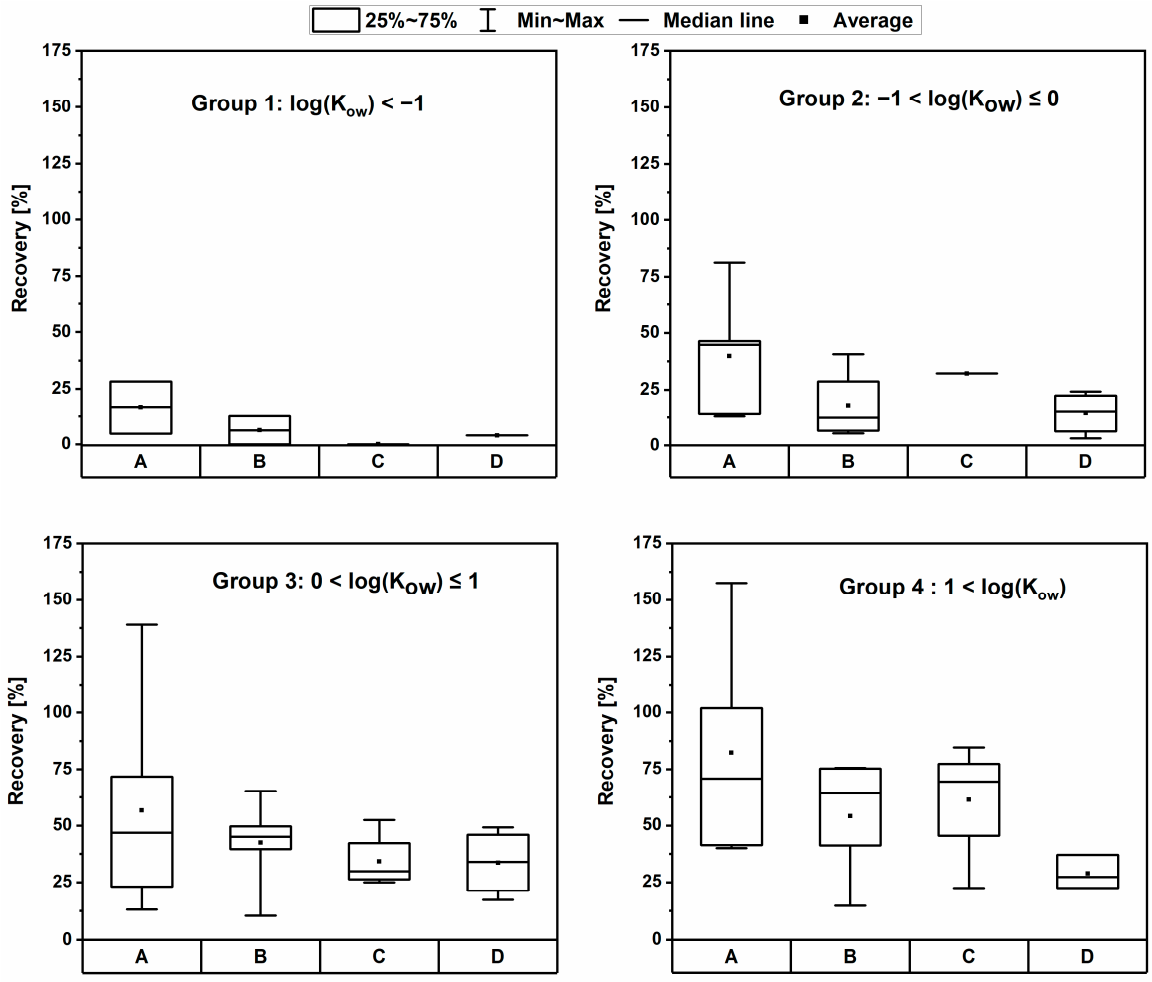

Figure 2. The boxplots for the recoveries of protocols A to D. ABs were regrouped according to their log(Kow) values in the four groups. The recoveries for protocols E and F are presented in Figure 3 and for protocol G in Table 2.

Table 2. An overview of the parameters of the method validation regroup after the log(Kow) groups for 50 and 100 µg/kg. For each concentration, five replicates were extracted and analyzed.

Group No. Log(Kow) Range	1 $x \leq -1$	2 $-1 < x \leq 0$	3 $0 < x \leq 1$	4 $1 < x$
Recovery [%]	1.1–4.2	0.2–26.3	0.8–64.8	13.7–48.8
Coefficient of variation (CV) [%]	16–32	4–80	7–61	14–48
Standard deviation	0.2–1.3	0.9–5.1	0.1–12.8	2.4–10.4
Matrix effect	0.42–0.83	0.08–0.83	0.04–0.29	0.03–0.66

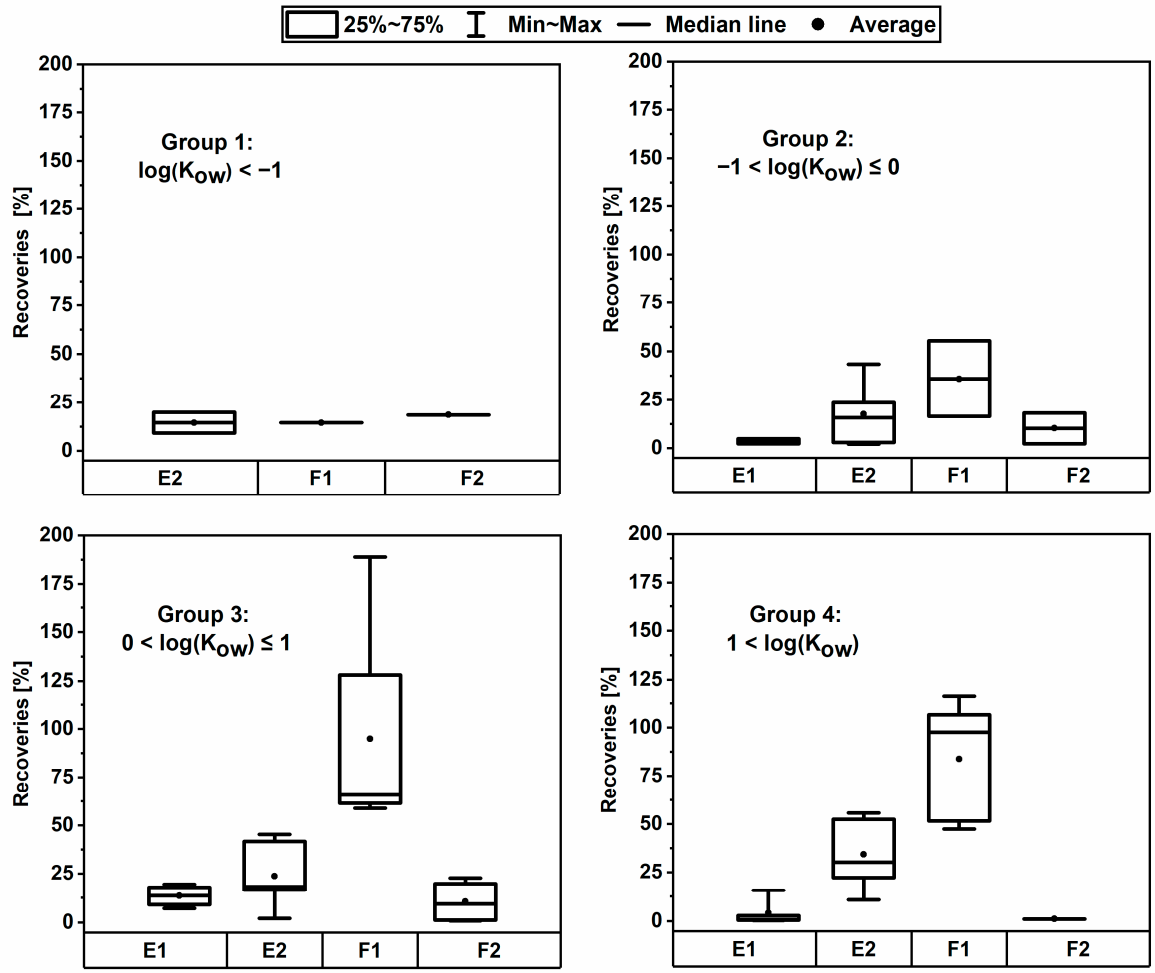

Figure 3. The boxplots for the recoveries of protocols E and F with two consecutive steps indicated as 1 and 2. ABs were regrouped after their log(Kow) values in the four groups.

The recoveries of protocols A, B, C, and D for log(Kow) group 3 were in the range of 13.0 to 139.1%, 10.5 to 65.4%, 24.8 to 52.4% and 17.2 to 49.3%, respectively. For protocols A and B, the median reached the defined minimal satisfactory recovery of 45% (46.9 and 45.1%, respectively). Instead, for protocols C and D, the median was below 45% (28.9 and 34.0%, respectively). Compared to groups 1 and 2, group 3 yielded higher recoveries for all four protocols.

The log(Kow) group 4 achieved higher recoveries for protocols A, B, and C (40.1–157.5%, 14.8–75.7%, 22.4–84.7%, respectively) than in group 3. Protocol D yielded lower recoveries (22.5 to 37.1%) for group 4. For group 4, the median recovery of protocols A to C was in the range of 45 to 125% (70.9, 64.3, and 69.5%, respectively, Figure 2).

3.2. Influence of log(Kow), pK_A, and pH on the Recovery

Protocols A and D had the same extraction parameters except for the extraction solvent. We compared the results and showed the influence of the log(Kow) value on recovery. In protocol A, acetonitrile, and in protocol D, methanol with 0.2% NH_4OH, were used as organic solvents. Protocol A showed higher recoveries for all four log(Kow) groups than protocol D. The difference can be explained by the nature of the organic solvents. Acetonitrile is a polar aprotic solvent with a log(Kow) of −0.34 and methanol is a polar protic solvent with a log(Kow) of −0.77. The majority of the ABs belonged to groups 2 or 3 with log(Kow) between −1 and 1 (11 out of 19 ABs). Acetonitrile is more hydrophobic than methanol and therefore extracted the ABs from groups 2 and 3 more efficiently. In protocol C, methanol was also applied as the extraction solvent. Lower recoveries for log(Kow) groups 2 and 3 were observed for protocol C (32.3 and 24.9 to 52.4%) compared to protocol A (12.9 to 81.3% and 13.0 to 139.1%). In protocol B, acetonitrile and methanol in the same ratio served as the extraction solvent. Recoveries for log(Kow) groups 1 to 3 were higher (12.6%, 5.4 to 40.6% and 10.5 to 65.4%) than the ones achieved by extraction according to protocol C (32.2%, 24.9 to 52.4%), but lower than the recoveries of extraction according to protocol A (4.7 to 28.3, 12.9 to 46.4%, and 13.0 to 139.1%). For log(Kow) group 4, protocols C (24.9 to 52.4%) and D (17.2 to 49.3%) yielded similar recovery ranges. These results depicted the influence of the log(Kow) value on the extraction efficiency. For sulfonamides (mainly group 3), Ding et al. showed that higher recoveries were achieved with acetonitrile/water mixture (*v*/*v* 5:5) (56–63%) than for the methanol/water mixture (*v*/*v* 5:5) (48–59%) used for the extraction of ABs from biosolids [57]. The tetracyclines belong to groups 1 and 2. Both groups showed low recoveries (3.2 to 40.6%) for protocols B to D. The methanol ammonium solution in method D had a pH of 6.5. Popova et al. showed for the aqueous methanol solution at pH 6.5, the lowest recoveries for tetracyclines [47].

Recoveries lower than 45% for log(Kow) groups 1 and 2 with protocols A to D show that the extraction of these ABs is an analytical challenge. To these groups belonged all the tetracyclines and two fluoroquinolones. Both ABs classes had a zwitterionic character as indicated by the pKa values in the range of 5.97–8.3 and 3.3–9.7 for fluoroquinolones and tetracyclines, respectively (see Table S1 in the Supplementary Materials). At pH in the range of $pK_{a1} < pH < pK_{a2}$, the fluoroquinolones and tetracyclines had two charged sides (one positive, one negative), resulting in an overall neutral molecule. This is the case for the typical pH range of 6–8 for the soils and sediments used in this study (pH 7.2). Therefore, fluoroquinolones and tetracyclines can interact with cationic and anionic sites in the soils/sediments [15]. For fluoroquinolones, electrostatic interactions, next to hydrophobic partitioning, play a significant role in the sorption process [9]. Because of their zwitterionic character, they can form stable complexes with cations such as magnesium, calcium, and aluminum. The mechanism for sorption on clay minerals involves cation bridging, electrostatic, and hydrogen bond interactions. In addition, the characteristics of the sediments such as cation exchange capacity and organic content affect the sorption [58]. The sorption affinity is expressed by the sorption coefficient K_d either for sediments or soil. For fluoroquinolones, the K_d values for sediment (marine and fresh water) were in the rage of 30–18620 L/kg [9]. The K_d values for oxytetracycline in the freshwater and marine sediment ranged from 290 to 490 L/kg [14] and those for tetracyclines in sandy and clay loam ranged from 417 to 11,908 L/kg [59]. For erythromycin and sulfonamides, the Kd values were given in a lower range (0.6–7.4 L/kg, [59]). The comparison of the Kd values indicated a higher sorption affinity for fluoroquinolones and tetracyclines compared to macrolides and sulfonamides. This could be a possible explanation for the low recoveries of log(Kow) groups 1 and 2.

Apart from the organic solvent used for extraction, the pH of the extraction solvent also has an influence on the recovery of the analytes. Protocol A was adapted after Golet et al. [37], who studied the extraction of ciprofloxacin and norfloxacin and achieved recoveries for both compounds of 97.6 and 94.4%, respectively. Golet et al. explained higher recoveries for ciprofloxacin and norfloxacin at lower pH with an electrostatic repulsion

between the protonated anionic sites of the fluoroquinolones and sewage sludge surface. Another point addressed by Golet et al. was increasing the solubility at low or high pH and a limited solubility at neutral pH related to the zwitterionic form [37]. We observed a similar trend. The pH values of the aqueous phase in the tested methods from A to C were in the range of 2–7. For neutral pH, we did not detect any fluoroquinolones (log(Kow) group 1 and 2). As for fluoroquinolones, tetracyclines form complexes with divalent metal ions (e.g., calcium and magnesium). Popova et al. investigated the influence of pH 6.5, 8, and 10 on the extraction. A higher recovery was achieved at pH 10 than at pH 6.5. At pH 8 and 10, the negative charge prevails and a reduction in ionic interaction is possible [47].

Our results showed that acetonitrile with a log(Kow) value of −0.34 is a suitable extraction solvent for the selected ABs in contrast to methanol (Log(Kow) of −0.77). The log(Kow) value can therefore be an easily applicable criterion for the evaluation of PLE extraction methods including different AB classes. The aqueous phase of acidic pH (protocol A and B) led to higher recoveries than the solutions of neutral pH (protocol C) for log(Kow) groups 1 to 3. The comparison between the recoveries of protocols A, B, C, and D for log(Kow) groups 1 and 2 pointed out that they are difficult to extract. The interaction of the analytes with the matrix was stronger than for log(Kow) groups 3 and 4. For log(Kow) groups 2 to 4, protocol A provided satisfactory recoveries (12.9–81.3%, 13.0–139.1%, 40.1–157.5%, respectively). However, only for log(Kow) groups 3 and 4 did the recoveries fall in the satisfactory range of 45 to 125% when protocol A was used.

3.3. Recovery with Two Consecutive Extraction Steps

As shown before, the ABs varied in their chemical properties (e.g., in their pKa range or log(K_{ow}) values). Each log(Kow) group would need different extraction conditions (e.g., solvent, pH). To overcome this problem, two PLE protocols can be consecutively applied [54,60]. We tested the method of Chiaia-Hernandez [54] (protocol E) and combined two of the previously tested protocols E and A (based on [37]) to produce a new protocol F. Compared to the previous protocols, protocols E and F consisted of two consecutive extraction steps, and for method development, we collected the extracts from each PLE protocol separately (E1, E2, and F1, F2). In Figure 3, the recoveries for protocols E and F are depicted. In protocol E1, two polar aprotic solvents, namely ethyl acetate (66%) with a log(Kow) of 0.73 and acetone (33%) with a log(Kow) of −0.24 were used. In protocol E2, water as a polar protic solvent (33%) and acetone (66%) were applied. The conditions of protocol F1 were the same as in protocol E2. In protocol F2, acetonitrile and water at pH 2 were used as extraction solvent.

ABs from log(Kow) groups 1 and 2 (log(Kow) below zero) were either not detected or the recovery was below 10% for E1 (see Figure 3). The execution of protocol E2 resulted in recoveries in the range of 2–43%, 2–45% and 11–56% for log(Kow) groups 2, 3, and 4, respectively. Therefore, protocol E2 could be a suitable option for the extraction of the selected ABs. Protocol F1 yielded recoveries in the range of 17–55%, 59–189%, and 47–116% for log(Kow) groups 2 to 4, respectively. Protocol F2 achieved recoveries in the range of 1–23% for log(Kow) groups 1 to 4. When comparing F1 and F2, protocol F1 revealed higher recoveries than F2 for all log(Kow) groups. The ABs were already extracted with protocol F1, and the recoveries of protocol F2 were much lower than for protocol A (5–139%, protocol F2 represent the parameter from protocol A). The fluoroquinolones were extracted only by protocol F2 (belonging to log(Kow) groups 1 to 3) with a recovery of 17–22% (similar to recoveries with protocol A, 5–23%). For protocol F1, the signal-to-noise ratio was below 10. When comparing the different log(Kow) groups, for protocol E2 and F1, the recovery efficiency increased from group 1 (9–20%) to group 4 (11–166%). This trend could already be observed for protocol A (see Figures 2 and 3). Comparing the recoveries from protocols A and F1 for ABs from groups 3 and 4 (macrolides and most of the sulfonamides), they were in a similar range of 23–139% and 40–158% for protocol A and 59–189% and 47–116% for protocol F1. However, for log(Kow) groups 1 and 2, we were only able to quantify three

out of seven ABs, with recoveries of 15–55% with protocol F1. For protocol A, we could quantify all seven ABs (5–81%) including all fluoroquinolones.

In general, ABs belonging to log(Kow) groups 3 and 4 were extracted from the sediments with higher efficiencies. The possible explanation for such differences would be that neither macrolides nor sulfonamides have a zwitterionic structure, therefore, the formation of complexes with cations in the sediments does not occur. As a result, the interaction between the sediment matrix and macrolides or sulfonamides is by principal weaker than for fluoroquinolones and tetracyclines.

We concluded that the combination of polar aprotic organic solvents (E1) is not suitable for the extraction of the selected ABs. We recommend using a combination of a polar aprotic solvent such as acetone or acetonitrile and a protic solvent such as acidified water (E2 or A). The application of the protocol F1 rather than F2 to the extracted ABs with different chemical-physical properties did not improve the overall recovery of the method.

3.4. Method Validation

Overall, protocol A provided the most satisfactory results and therefore it was validated as protocol G. The validation parameters, namely the method limit of detection (MLOD), method limit of quantification (MLOQ), accuracy (expressed as recovery, see Section 2.10), intraday precision (also called repeatability, expressed as standard derivation), and selectivity (expressed as matrix effect) are presented in Table S9. The range of linearity was determined with a calibration curve from 10 to 1500 ng/kg. The R^2 was 0.990 or higher. The MLOD ranged from 0.1 to 13.6 µg/kg (Table S9). Kerrigan et al. [35] reported a LOD of 0.15 to 8.1 µg/kg for their PLE method, Vazquez-Roig et al. [32] determined MLOD in the range of 0.3 to 6.8 µg/kg, and Silva et al. obtained 0.06 to 3.1 µg/kg [38].

The MLOQ varied between 0.1 and 27.1 µg/kg (Table S9). Kerrigan et al. [35] determined the LOQ for their PLE method in the range of 0.32 to 22.4 µg/kg and Vazquez-Roig et al. [32] gave a MLOQ in the range of 0.9 to 23 µg/kg. Silva et al. reported MLOQ in the range of 0.2 to 10.2 µg/kg [38]. Our MLOQ was in a similar range and indicated that the method is suitable to determine the selected AB in the low µg/kg-range.

The recovery for the log(K_{ow}) group below −1 (group 1) was between 1 and 4%. For the log(K_{ow}) group between −1 and 0 (group 2), the recovery ranged from 0.2 to 26.3%. The log(Kow) groups 1 and 2 are composed of fluoroquinolones and tetracyclines. These AB classes are difficult to extract, as discussed before. Low recovery could be related to their interaction with the matrix. For the log(K_{ow}) group between 0 and 1 (group 3), the recovery varied from 0.8 to 64.8%. Ciprofloxacin showed the lowest recovery (0.8%) of this group and trimethoprim had the highest recovery (64.8%). The group with log(K_{ow}) larger than 1 (group 4) achieved recoveries from 13.7 to 48.8% (values see Tables 2 and S9 for detailed information about each AB). The overall method accuracy was in an acceptable range for ABs from log(Kow) groups 3 and 4. The defined recovery range from 45 to 125% was only achieved for trimethoprim and clindamycin (44.9 to 64.8%).

The results for the matrix effect are summarized in Tables 2 and S9. For all ABs, suppression was observed because the matrix factor was below one. Values close to 1 indicated a light suppression. Values close to zero suggest a strong suppression. For log(Kow) groups 3 and 4, the matrix factor ranged between 0.03 and 0.66. For fluoroquinolones and tetracyclines (groups 1 and 2), the matrix factor varied between 0.08 and 0.83. This could possibly explain the low recoveries of doxycycline, enrofloxacin, and ofloxacin, which showed a matrix factor of 0.26, 0.08, and 0.08, respectively. In general, the validated method allowed for the accurate and reliable quantification of the ABs from log(Kow) groups 3 and 4 (clindamycin, macrolides, sulfonamides, and trimethoprim).

3.5. Quantification of ABs in Environmental Samples

Applicability of the validated method was verified with three sediment samples from a local mountain stream river after the discharge of treated wastewater. The effluent of the WWTP presents an anthropic source for ABs to the aquatic environment [50]. The results

are summarized in Table 3. From 19 ABs, we quantified six ABs in a concentration range of 0.3 to 12.8 µg/kg. Erythromycin-H_2O, the transformation product of erythromycin under an acid condition (e.g., stomach), showed the highest concentration (0.8 to 12.8 µg/kg). The lowest concentration (0.3 to 1.3 µg/kg) was measured for the fluoroquinolone, ofloxacin (OFC). Antibiotics such as OFC, sulfapyridine (SPD), trimethoprim (TMP), and clindamycin (CDC) were quantified at all three sampling points. All aforementioned ABs were semi- or synthetic origin and prescribed for humans [35].

Table 3. The calculated concentration (in µg/kg) of the three environmental sediment samples. Given as range for duplicates. Ciprofloxacin (CFC), enrofloxacin (EFC), norfloxacin (NFC), ofloxacin (OFC), erythromycin (ETM), erythromycin-H2O (ETM-H2O), roxythromycin (RTM), clarithromycin (CTM), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfapyridine (SPD), chlortetracyclin (CTC), oxytetracyclin (OTC), tetracyclin (TCT), doxycyclin (DXC).

	Compounds																		
Location	CFC	EFC	NFC	OFC	CTM	ETM	ETM-H_2O	RTM	SDZ	SDM	SMZ	SMX	SPD	TMP	CDC	CTC	DCX	OTC	TCT
1	2.9–4.5	n.d.	n.d.	0.8–1.3	n.d.	<MLOD	2.8 *	n.d.	<MLOD	<MLOQ	n.d.	<MLOQ	4.2–7.0	0.8–1.5	5.5–11.0	n.d.	<MLOQ	n.d.	n.d.
2	n.d.	n.d.	n.d.	0.3–0.4	n.d.	n.d.	0.8–12.8	n.d.	<MLOD	n.d.	n.d.	<MLOQ	2.2–3.0	0.5 *	2.6–4.8	n.d.	<MLOQ	n.d.	n.d.
3	n.d.	n.d.	n.d.	0.4–0.5	n.d.	<MLOD	<MLOQ	n.d.	<MLOD	<MLOD	n.d.	n.d.	4.9–8.4	1.0 *	3.9–7.3	n.d.	<MLOQ	n.d.	n.d.

* Duplicates, if no range is indicated, one of the duplicates was either below the method limit of quantification (MLOQ) or the signal-to-noise ratio was below 10.

The developed and validated protocol G is suitable for the quantification of ABs in the low µg/kg range in sediment samples. These preliminary results confirm the presence of selected ABs in river sediments. Haenni et al. summarized the available data for ABs detected in the aquatic environment in France. The authors concluded that WWTP discharge is a major source for ABs in an aquatic environment in France. The concentrations for ciprofloxacin, sulfamethoxazole, trimethoprim, erythromycin, and oxytetracycline were in the range of below 0.1 µg/kg up to 100 µg/kg [23]. For sediments from the Minnesota and Mississippi Rivers in the USA, a study [35] determined erythromycin, ofloxacin, sulfamethazine, sulfapyridine, and trimethoprim to be the most frequently detected ABs in a concentration range of 0.03 to 6.20 µg/kg. The quantified ABs and the concentration range were comparable to our results. In principle, ABs can undergo different reactions in the aquatic environment. Aside from adsorption to a solid matrix, biodegradation, hydrolysis, and photodegradation are possible reaction pathways. For instance, the lack of tetracyclines in the studied sediments could be explained by hydrolysis, to which tetracyclines are susceptible [8].

4. Conclusions

We showed the importance of the log(Kow) values in the process of AB extraction from river sediments. ABs with log(Kow) values below zero yielded, in general, lower recoveries than ABs with log(Kow) values above zero, independently of the tested protocols. We concluded that ordering ABs based on log(Kow) values is more relevant during extraction method development than traditional classification (macrolides, sulfonamides, etc.) and facilitates the selection of the most suitable extraction solvent. We also showed that the existing PLE methods applied for solids and sludge are not transferable to sediments.

Lower recoveries for ABs with log(Kow) values below zero were related to a stronger interaction between the analytes and the matrix. A combination of aqueous solvents at acidic pH and an aprotic polar organic solvent (e.g., acetone or acetonitrile) yielded higher recoveries than protocols with a neutral aqueous solvent for ABs with log(Kow) values larger than zero (groups 3 and 4).

The optimal method for the determination of 19 ABs with a wide range of physical-chemical properties was composed of two consecutive pressurized liquid extraction cycles using mixture solvents of different polarity. We proposed acetonitrile and 50 mM phosphoric acid (50/50, v/v) at 100 °C, followed by extract purification with solid phase

extraction. With this method, we achieved the recoveries in the range of 0.3 to 1.9% for fluoroquinolones, 3.3 to 30.9% for macrolides, 48.8 to 64.8% for clindamycin and trimethoprim (others), 8.7 to 33.5% for sulfonamides, and 4.2 to 26.3% for tetracyclines.

The performed validation indicated that our method is suitable for the quantification of ABs in sediments in the low µg/kg scale (MLOQ range from 0.1 to 27.1 µg/kg). With the developed method, we determined the concentration of six ABs in the river sediments in three locations, which resulted in a concentration range of 0.3 to 12.8 µg/kg. The antibiotic with the highest concentration was erythromycin-H_2O (0.3 to 12.8 µg/kg).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14162534/s1, Figure S1: Recoveries of four different AB classes for four different protocols A, B, C, and D (see Table 1); Figure S2: Recoveries for trimethoprim and clindamycin for four different methods; Table S1: Properties of the ABs used in the study, Table S2a to d: Characterization of the sediment used in the study, Table S3: Conditions used for the tandem SPE method, Table S4: The chromatographic conditions used for the quantification of selected antibiotics, Table S5: Setting of the mass spectrometer used for the quantification of ABs; Table S6: The source parameter used for the AB quantification; Table S7: The surrogates and internal standards for protocols A to G; Table S8: The classification of ABs related to their log(Kow) values; Table S9: The parameters of the method validation; Table S10: Recoveries for the SPE protocol; Text S1: The chemicals and reagents; Text S2: The characterization of the sediment samples. References [61–64] are cited in the supplementary materials.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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