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Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse

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ABSTRACT

The occurrence of chemical and biological contaminants of emerging concern (CECs) was investigated in treated wastewater intended for reuse in agriculture. An agarose hydrogel diffusion-based passive sampler was exposed to the outlet of a wastewater treatment plant (WWTP) located in Cyprus, which is equipped with membrane bioreactor (MBR). Passive samplers in triplicate were exposed according to a time-series exposure plan with maximum exposure duration of 28 days. Composite flow-proportional wastewater samples were collected in parallel with the passive sampling exposure plan and were processed by solid phase extraction using HORIZON SPE-DEX 4790 and the same sorbent material (Oasis HLB) as in the passive sampler. The analysis of passive samplers and wastewater samples enabled (i) the field-scale calibration of the passive sampler prototype by the calculation of *in situ* sampling rates of target substances, and (ii) the investigation of *in silico* predicted transformation products of the four most ecotoxicologically hazardous antibiotics (azithromycin, clarithromycin, erythromycin, ofloxacin). Additionally, the wastewater samples were subjected to the analysis of seven preselected antibiotic resistant genes (ARGs) and one mobile resistant element (*int1*). All extracts were analyzed for chemicals in a single batch using a highly sensitive method for pharmaceuticals, antibiotics and illicit drugs by liquid chromatography tandem MS/MS (LC-QQQ) and for various other target compounds (2316 compounds in total) by liquid chromatography high-resolution mass spectrometry (LC-HRMS). 279 CECs and all investigated ARGs (except for $bla_{\text{CTX-M}-32}$) were detected, highlighting potential chemical and biological hazards related to wastewater reuse practices. 16 CECs were prioritized following ecotoxicological risk assessment, whereas *sul1* and the mobile resistant element (*int1)* showed the highest abundance. Comprehensive monitoring efforts using novel sampling methods such as passive sampling, wide-scope target screening and molecular analysis are required to assure safe application of wastewater reuse and avoid spread and crop uptake of potentially hazardous chemicals.

1. Introduction

Wastewater reuse is an indispensable and essential practice due to the water shortage and the increasing world population, requiring high amounts of food and water resources. Regions inhabited by more than 25% of the world's population already are in the situation that water demand exceeds supply (United Nations, 2018). In response to the increasing problem of water shortage, the reuse of treated urban wastewater is considered the most suitable and reliable alternative for sustainable water management (Marteleira et al., 2014) and agricultural development (Massoud et al., 2019). Although reuse is accompanied by a number of benefits, and major advances have been made with respect to producing safe treated effluents for reuse (e.g. successful removal of nutrients, metals, chemical oxygen demand down to low levels), several important questions are still unanswered and barriers exist regarding the safe and sustainable reuse practices (Fatta-

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Kassinos et al., 2011; Rizzo et al., 2013; Kümmerer et al., 2018; Piña et al., 2018). The major concerns that currently exist, relate to the adverse effects of chemical (Turner et al., 2019) and biological factors (Corno et al., 2019) such as contaminants of emerging concern (CEC) and antibiotic resistance genes (ARGs), respectively. To reveal the risks associated with wastewater reuse and take the appropriate protection actions, novel monitoring methods and advanced analytical instrumentation must be applied (Brack et al., 2017).

Passive sampling has been recognized as a promising and cost-effective monitoring method to achieve chemical characterization of water samples, for which future application should be pursued according to the Environmental Quality Standards Directive (EQS) issued under the European Union's Water Framework Directive (WFD) (European Commission, 2013). This recommendation derives from the fact that CECs are often present at trace but toxic concentration levels in the environment. Their low concentration (as low as few pg L^{-1}) makes them difficult to detect with traditional sampling and sample preparation protocols (Moschet et al., 2014). Passive sampling offers the detection of bioavailable CECs in ultra-trace concentration levels. The freely dissolved contaminants (indicated by the term “bioavailable”) are the contaminants that can be uptaken by organisms. Additionally, passive sampling is practical, offers a representative pollution overview, does not require transport of big volume samples to the laboratory and can be easily integrated into a variety of monitoring programs (Vrana et al., 2016; Ahrens et al., 2018).

Passive sampling can be integrated in monitoring treated wastewater from wastewater treatment plants (WWTPs) equipped with conventional or advanced treatments (Rizzo et al., 2019). In case of advanced treatments such as ozonation, not only the parent compounds but also their transformation products (TPs) may persist in the treated wastewater (Beretsou et al., 2016). It is of utmost importance to have a holistic overview of the chemical pollution in effluent wastewater intended for reuse, perform risk assessment to prioritize the CECs that can potentially harm the environment, be introduced in the trophic chain and ultimately affect human health (Cerqueira et al., 2019; Christou et al., 2019). Holistic overview can be achieved with powerful sampling methods when combined with high throughput monitoring such as non-target screening methods (Alygizakis et al., 2019b; Hollender et al., 2019) and molecular methods for measuring biological material (Cacace et al., 2019).

The objective of our study was to apply a combined chemical analytical approach of passive sampling, wide-scope target screening together with the ARGs testing and detect and quantify the chemicals and biological CECs in wastewater intended for agricultural reuse. To reveal the potential hazards from wastewater reuse, a combination of the various experimental techniques and of a battery of novel analytical and bioanalytical methods is required for the assessment of wastewater effluent quality. In addition, a simplified prioritization scheme was applied with the aim to prioritize the compounds of interest and find the ones that pose the highest risk for the environment and human health. Finally, our study tests a novel agarose hydrogel diffusion-based passive sampler prototype for chemical monitoring as a cost-effective and efficient way of monitoring effluent wastewater.

2. Material and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) of LC-MS grade were purchased from Merck (Darmstadt, Germany), Oasis-HLB disks were purchased from Labicom (Olomouc, Czechia), RC syringe filters (4 mm diameter, 0.2 μm pore size) from Phenomenex (USA) and formic acid (FA) 99% were obtainedfrom Sigma-Aldrich, Fluka (Buchs, Switzerland). Distilled water was provided by a Milli-Q apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Passive samplers were deployed in a protective stainless-steel cage and wastewater samples were stored in precleaned glass high-density polyethylene (HDPE) bottles. Compound names of target substances analyzed by LC-ESI-QTOF and LC-ESI-MS/MS (LC-QQQ), structural information (SMILES, StdInChI, StdInChIKey), molecular formula, monoisotopic mass, CAS number and connection with databases (PubChem, ChemSpider, USEPA Dashboard) can be found in lists UATHTARGETS and UOATARGPHARMA at NORMAN Suspect List Exchange website, respectively (NORMAN, 2020). In total, 2316 compounds were screened in the samples and details on the method can be found elsewhere (Gago-Ferrero et al., 2020). Most of the reference standards were provided by Bruker Daltonics (675 pesticides), the Swiss Federal Institute of Aquatic Science and Technology (275 compounds of various classes) or donated by the Doping Control Laboratory of the Olympic Sports Center of Athens “Spiros Louis” (142 illicit and new designer drugs). The remaining standards were purchased by Merck (Chalkidona, Greece), LGC Standards (Athens, Greece) and Alfa-Aesar (Voula, Athens, Greece). Most of the stock standard solutions were prepared in MeOH, while ACN and water were also used. Mixes of standard solutions were prepared at final concentration of 1 μg mL^{-1} in MeOH and used for spiking. Chemicals and reagents for molecular methods are provided in Section 2.8.

2.2. Study area and sampling - description of the wastewater treatment plant

The investigated WWTP is located in Cyprus and is equipped with primary sedimentation and membrane bioreactor (MBR). It receives daily 2200 m^3 of municipal wastewater serving a population equivalent of 55,000. MBR is currently widely accepted as an alternative key technology to the conventional activated sludge (CAS) treatment utilized in WWTPs, for the recovery of highly clarified wastewater effluent. The samplers and the water samples were collected from the outflow of the WWTP (final effluent). Monthly averaged chemical and physical parameters characterizing the effluent wastewater (biochemical oxygen demand, chemical oxygen demand, total suspended solids, pH, total phosphorus, total Kjeldahl nitrogen, ammonia nitrogen, conductivity and chlorides) for the year 2017 are provided in section S1 of the supplementary information (SI).

2.3. Passive sampler

The passive sampler consisted of two sorptive hydrogel disks containing 0.1134 g Oasis HLB sorbent with a diameter of 3.8 cm between diffusive hydrogel disks strengthened by nylon mesh netting and with a diameter of 5.5 cm. This two-sided design has a sampling area of 22.7 cm^2. The applied surface area to sorbent mass ratio was 200 cm^2 g^{-1}. The gel disks were all 1 mm thick and were held together by two stainless steel rings with outer and inner diameters, respectively matching those of the gel layers. 1 mm thick PTFE spacer copying the steel rings shape was inserted between the diffusive layers, around the sorptive gels. The whole system was held together by three stainless steel bolts and nuts. A more detailed description of the sampler construction is provided in section S2 of SI and in the respective publication (Urík and Vrana, 2019).

2.4. Collection of water samples and deployment of passive samplers

Hydrogel passive samplers were deployed according to the schedule presented in Fig. 1 from 9 November 2017 until 4 December 2017 at the outlet of the plant. All passive samplers were exposed in triplicate, which means that each box in Fig. 1 represents three samplers. Simultaneously to the deployment of the passive samplers, daily 24 h flow-proportional composite effluent wastewater samples were provided by the WWTP operators. Daily samples were mixed and homogenized to form weekly composite samples (red boxes as shown in Fig. 1). Wastewater samples and passive samplers were kept frozen

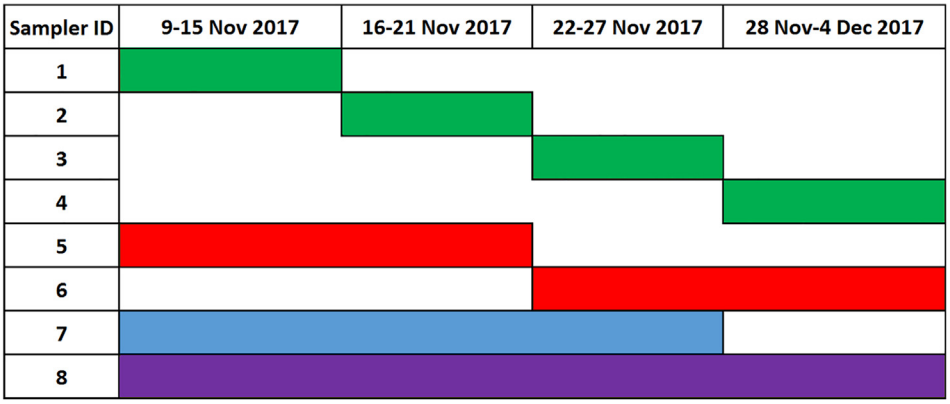

Fig. 1. Passive sampling deployment schedule. Each box represents deployment period of three passive samplers (triplicate). Green color indicates one-week exposure, red color indicates two weeks exposure, blue color indicates three weeks and purple color indicates exposure throughout the sampling period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(-20 °C) during storage and transportation, following the same procedure as in previous sampling campaigns (Alygizakis et al., 2019a). Both sorbents from passive samplers and effluent wastewater samples were processed immediately at the end of the sampling campaign.

2.5. Extraction of passive samplers and effluent wastewater samples

After the exposure, deployed samplers were disassembled, the separated sorbent gel material was spiked with internal standards (Table S5A, SI) at a final vial concentration of 50 ng L^{-1} and extracted by addition of 20 mL MeOH and overnight shaking on an orbital shaker at 60 rpm. Extraction procedure was repeated with another extra 20 mL methanol.

Organic contaminants were extracted using automatic SPE extraction with HORIZON SPE-DEX 4790, equipped with a 47 mm disk holder Atlantic HLB-M Disk (USA). Exposure of the sampler for one day resulted in absorption of compounds contained in approximately 100 mL depending on the compound physicochemical properties and the matrix (Urík and Vrana, 2019). According to this information, four weekly-composite samples (600 mL each sample) were spiked with internal standards and were preconcentrated to 500 μL. Extraction program of HORIZON SPE-DEX disks can be found in section S3 of SI. These four samples simulated the samplers exposed for 6 days (red boxes in Fig. 1). Extraction recoveries at two concentration levels, limit of detection (LOD), repeatability and matrix effect for the detected compounds can be found in Table S5B of SI.

In both sample preparation methods, the extracts were evaporated under a gentle nitrogen stream until dryness, reconstituted to final volume 500 μL (50% water, 50% methanol) and filtered through 0.2 μm RC syringe filter. All extracts were analyzed using a UHPLC-ESI-QQQ system for target screening of pharmaceuticals, antibiotics and drugs of abuse (Thomaidis et al., 2016) and a UHPLC-ESI-QTOF-MS system which enabled wide-scope target screening (Gago-Ferrero et al., 2020) and suspect screening for TPs of antibiotics. Quality assurance and quality control is described in section S5 of SI.

2.6. Instrumental analysis

Instrumental analysis was performed with a Thermo UHPLC Accela system connected to a TSQ Quantum Access triple quadrupole mass spectrometer from Thermo Electron Corporation (San Jose, CA, USA) equipped with an electrospray ionization source (Thermo IonMAX) in both ionization modes. Chromatographic separation was achieved on an Atlantis T3 C18 column (100 mm × 2.1 mm, 3 μm) from Waters (Milford, MS, USA). The mobile phase, the gradient elution program and the ESI parameters are presented in Table S4A (SI). Identification and quantification were performed under multiple reaction monitoring (MRM) mode, recording two transitions between the precursor ion and the two most abundant product ions for each target compound according to the guidelines of EU (European Commission, 2002). Quantification was based on standard addition and isotopic dilution when possible. MRM transitions for each compound were optimized by infusion of standard solutions (at concentration of 1 mg L^{-1}). The optimized ionization mode, fragmentation voltages, collision energies and retention time for all compounds are summarized in list S56 "UOAT-ARGPHARMA" at the website of NORMAN network (NORMAN, 2020). The highly-sensitive LC-MS/MS method was used for the determination of pharmaceuticals, drugs of abuse and antipsychotic drugs, since it allows lower detection limits when comparing to the LC-ESI-QTOF method.

UHPLC-ESI-QTOF analysis was performed using a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), coupled to the QTOF mass analyzer Maxis Impact by Bruker (Bremen, Germany). Chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 μm) supplied by Thermo Fisher Scientific and preceded by a guard column of the same packaging material. Gradient program, ESI parameters and mobile phases are summarized in Table S4B of SI. Target analytes are summarized in list S21 "UATHTARGETS" at the website of NORMAN network (NORMAN, 2020). A compound was successfully detected if the mass error of the molecular ion was below 2 mDa, retention time deviation was below 0.30 min and at least a qualifier fragment ion was detected.

2.7. Screening of TPs of prioritized antibiotics

Screening of TPs in environmental samples is a time-consuming task. Therefore, a data-driven investigation of TPs for four antibiotics (azithromycin, clarithromycin, erythromycin, ofloxacin) was performed. The concentration of these four antibiotics exceed the ecotoxicological threshold (more details at Section 3.1) and thus were chosen to be investigated for their TPs. All compounds except for ofloxacin are also included in the updated EU Watch List (European Commission, 2018), which contains substances known to potentially have environmental implications and occurrence data has been requested by the EU member states, so that these compounds can potentially become priority substances. An in-house suspect database was developed, based on (i) *in silico* prediction softwares i.e. the Eawag-Biocatalysis/Biodegradation Database Pathway Prediction System (Eawag-BBD/PPS) and the MetabolitePredict software by Bruker Daltonics (Bremen, Germany), and (ii) pharmacokinetic literature for known metabolites and TPs of the selected antibiotics that have already been identified in relevant biotransformation and advanced wastewater treatment studies (Terzic et al., 2018; Senta et al., 2019).

The wastewater samples were screened in full scan (positive ESI ionization mode) for the detection of plausible metabolites and TPs from the suspect database. The criteria used for the reduction of features and tentative identification were the following: peak

area ≥ 2,000 counts, intensity threshold ≥ 500 counts, mass accuracy ± 5 ppm/2 mDa on the monoisotopic peaks and satisfactory isotopic pattern fit (≤100 mSigma). A second analysis was performed in data-dependent acquisition (termed AutoMS for Bruker) to acquire HRMS/MS spectra for preselected masses of interest of potential TPs. Spectra time was shortened to 0.25 s. Ramp collision energy was applied based on the mass and the charge state of preselected masses of interest to acquire high-quality HRMS/MS spectra. The level of confidence for the identification of the detected compounds is described with levels 1 to 5, where level 1 corresponds to confirmed structures (reference standard is available), level 2 to probable structure with diagnostic evidence, level 3 to tentative candidate(s), level 4 to unequivocal molecular formulas, and level 5 to exact mass(es) of interest (Schymanski et al., 2014).

2.8. qPCR

Aliquots of selected wastewater samples were filtrated in duplicates and stored in freezer (-20°C), until they were processed with the DNeasy PowerWater Kit (Qiagen, Germany), according to manufacturer instructions. Quality and quantity of the extracted DNA were assessed using NanoDrop™ (ThermoFisher, Germany). The genes were quantified using real-time PCR. The selected quantified genes in this study were the following: *qnrS* (protein family that protects DNA gyrase from the inhibition of quinolones), bla_{TEM} (class A β-lactamase), *sul1* (sulfonamide resistant dihydropteroate synthase), $bla_{CTX\text{-}M-32}$ (class A β-lactamase, cephalosporinase), *ermB* (rRNA adenine N-6-methyltransferase, which confers resistance to erythromycin) $bla_{OXA\text{-}58}$ (class D β-lactamase, carbapemenase), *tetM* (ribosomal protection protein, which protects ribosome from the translation inhibition of tetracycline), *intl1* (class I integrase; this gene is associated with horizontal gene transfer and environmental pollution), and the gene 16S rRNA, which is an indicator for the total bacterial abundance.

For the qPCR reactions, the Luna Universal qPCR Master Mix (New England Biosciences, Germany) was used. The reactions were performed with MasterCycler RealPlex (Eppendorf, Germany). Each extraction was analyzed in duplicate for each gene. Two-steps thermal cycling conditions were employed, along with an initial step of denaturation for 10 min at 95 °C and a final step of a melting curve, to assess the specificity of the reaction products. Regarding the genes 16S rRNA, *sul1*, *qnrS*, and bla_{TEM}, the conditions of the reaction cycles were the following: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. For the genes *intl1* and $bla_{CTX\text{-}M-32}$ the reaction cycles were the following: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 58.5 °C for 60 s. The reaction cycles for the gene *ermB* were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 59 °C for 30 s. Lastly, for the gene *tetM* the reaction cycles were: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 55 °C for 60 s. The final concentration of the primers in the reaction was 0.25 μM for the genes *sul1*, *qnrS*, bla_{TEM} and *intl1*. As for the genes *tetM*, the final concentration of each primer was 0.20 μM and for the genes 16S rRNA and $bla_{CTX\text{-}M-32}$ the concentration of the primers was 0.5 μM (in the reaction). The protocols for the gene *tetM* included the addition of 0.1 mg mL^{-1} of bovine serum albumin in the final reaction solution. The template concentration was set to 4–20 ng of DNA per well.

The quantification of all genes was based on the standard curve of a plasmid containing the amplified region of each gene. To quantify the genes *tetM* and $bla_{OXA\text{-}58}$, *E. coli* strains were transformed with the vector pTZ57R/T, containing the inserted PCR amplicon. To quantify the gene *ermB*, we used a pGEM vector with the inserted plasmid. For the rest of the genes, the standard was the pNORM plasmid (Rocha et al., 2018; Cacace et al., 2019). Every plasmid was extracted with MiniPrep (Qiagen, Germany), according to the manufacturer instructions. The extracted DNA was quantified with Nanodrop (ThermoFisher Scientific, Germany) and preserved in aliquots at −20 °C. The aliquots were used once and disposed after one cycle of thawing. Plasmid aliquots were renewed, when they were exceeding one month of storage. The limit of quantification (LOQ) for the genes varied for the tested genes. 16S rRNA showed the highest LOQ (10^4 copies μL^{-1}) in the reaction, followed by the genes *sul1*, *intl1*, bla_{TEM}, *tetM*, $bla_{OXA\text{-}58}$, where the LOQ was 100–10 copies μL^{-1}. For clinically related genes (*qnrS*, $bla_{CTX\text{-}M-32}$), the LOQ was usually 10 copies μL^{-1} (40 copies per reaction). However, in a few runs the LOQ was 1 copy μL^{-1} (4 copies per reaction). The LOD was set at 3 copies per reaction (0.75 copies μL^{-1}). The standard curves exhibited efficiency 0.90–1.10 and $R^2 \geq 0.990$. In addition, samples with products with unspecific melting curves were considered as negative (below LOQ). Inhibition test was assessed by performing reactions for a gene that was detected in very low concentration in our samples ($bla_{CTX\text{-}M-32}$) and spiking 4 μL of a stock solution, which contained 10^6 copies μL^{-1} of the pNORM plasmid standard, along with 4 μL of DNA template, in the usual 20 μL reaction volume per well. No PCR inhibition was detected in the samples.

The absolute abundance was calculated in copies L^{-1} and the relative abundance in gene copies to 16S rRNA copies. Prior to data analysis, the relative and absolute abundance values were log-transformed. R-packages ggplot2 (Whickam, 2020), and ggpubr (v. 0.2.3) (Kassambara, 2020) were used for the generation of graphical representations.

3. Results and discussion

3.1. Prioritization of targeted compounds

Two hundred and seventy-nine (279) CECs were detected in the collected wastewater samples (Table S6 in SI) at concentration levels ranging from 0.09 ng L^{-1} (for Deacetyl-Diltiaze and N-Desmethyl-Tramadol) to 1279 ng L^{-1} (for Lidocaine). To enable the prioritization of the detected CECs based on their ecotoxicological properties, their concentration levels (derived from solid phase extraction using HORIZON SPE-DEX 4790) were benchmarked against their provisional no-effect concentration (PNEC) thresholds, which were extracted from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/) according to the following priority: Environmental Quality Standards (EQS) thresholds of legal documents, followed by experimental PNECs and *in silico* predicted PNECs (Aalizadeh et al., 2017).

The comparison of the concentration levels with PNEC enabled the calculation of the frequency of PNEC exceedance (FoE) as the percentage of samples for which exceedance was observed and the calculation of extent of exceedance (EoE), which is a normalized metric expressing how many times the observed concentrations were higher than the PNEC threshold. The linear combination of FoE, EoE and frequency of appearance (FoA) of the substances in the collected samples resulted in the risk score. Sixteen (16) compounds with risk score more than one were prioritized (Table 1).

The list of prioritized substances is dominated by antibiotics (4 compounds), antihypertensive drugs (3 compounds), antipsychotic drugs (3 compounds) and nonsteroidal anti-inflammatory drugs (NSAIDs) (3 compounds). It is worth highlighting that the prioritized compounds may be also relevant for other effluent water quality monitoring. Most of the prioritized substances are known to persist in treated wastewater and have been prioritized in studies that took place in other European WWTPs (Tousova et al., 2017). The antihypertensive compounds candesartan and telmisartan received the highest score and were prioritized first and second, respectively. Occurrence of telmisartan is alarming because of its remarkable persistency in wastewater (Alygizakis et al., 2019a) and in highly diluted seawater samples from the Black Sea (Slobodnik et al., 2016). For both compounds (candesartan and telmisartan), predicted PNEC (P-PNEC) was used due to lack of experimental PNECs in the database, indicating that verification of PNEC is required to draw definite conclusions. The same remark applies for the rest of the prioritized substances for which P-PNEC was used (galaxolidone, lorazepam, medazpam, meclofenamic acid and

Table 1
List of the prioritized compounds, their maximum observed concentration (n = 4), the provisional no-effect concentration (PNEC) thresholds used and their reference. Additionally, the table represents the frequency of appearance (FoA), the frequency of exceedance (FoE), the extent of PNEC exceedance (EoE) and the risk score for the prioritized compounds.

Compound	Maximum concentration (ng L^{-1})	PNEC (ng L^{-1})	Reference PNEC	FoA	FoE	EoE	Risk Score
Candesartan	96	3.1	P-PNEC	1.00	1.00	1.00	3.00
Telmisartan	1086	42	P-PNEC	1.00	1.00	0.88	2.88
4-OH-E1 (4-Hydroxyestrone)	90	3.6	EQS-proposal	1.00	1.00	0.87	2.87
Ofloxacin	335	21	PNEC exp. Aquire 80421	1.00	1.00	0.49	2.49
Azithromycin	262	19	EQS-proposal	1.00	1.00	0.40	2.40
Venlafaxine	415	38	EQS-proposal	1.00	1.00	0.29	2.29
Galaxolidone	900	101	P-PNEC	1.00	1.00	0.27	2.27
Ibuprofen	57	10	EQS chronic water	1.00	1.00	0.16	2.16
Lorazepam	334	96	P-PNEC	1.00	1.00	0.09	2.09
Medazepam	512	206	P-PNEC	1.00	1.00	0.04	2.04
Carbamazepine	86	50	PNEC chronic Aquire 152195	1.00	1.00	0.02	2.02
Clarithromycin	166	120	EQS-proposal	1.00	0.50	0.01	1.51
Diclofenac	56	50	EQS-proposal	1.00	0.50	0.00	1.50
Atorvastatin epoxide	11	10	P-PNEC	1.00	0.50	0.00	1.50
Erythromycin	244	200	EQS-proposal	1.00	0.25	0.00	1.25
Meclofenamic Acid	98	97	P-PNEC	1.00	0.25	0.00	1.25

atorvastatin epoxide). Overall, *in silico* predicted PNECs and experimental PNECs are not expected to deviate more than one order of magnitude for compounds within the applicability domains of the models. However, experimental confirmation of PNEC remains a necessity. High concentration levels of the antiepileptic drug carbamazepine and the NSAIDs ibuprofen and diclofenac were observed despite the advanced treatment applied at the WWTP. Their high concentration levels, however, were expected since some of them are so persistent that have been proposed as tracers of anthropogenic activity and wastewater pollution (Lara-Martín et al., 2014; Cantwell et al., 2017; Cui et al., 2019). Four out of the 16 substances that were prioritized (25% of the prioritized list) were antibiotics (ofloxacin, azithromycin, clarithromycin and erythromycin), which is of concern because of their hazardous properties to exert effects at low concentrations (Lara-Martín et al., 2014), and because antibiotics may trigger unwanted effects such as antibiotic resistance (Manaia et al., 2018).

3.2. Assessment of results acquired from passive sampling

3.2.1. Stability of wastewater composition during passive sampling exposures

All the results of the analysis acquired by passive sampling are given in section S7 of SI. The Table S7A represents the compound name, structure as InChIKey, uptake graph, R_s and $\log K_{ow}$. An indicative uptake graph of an integratively sampled substance (pentobarbital) is also presented in Fig. 3. Four subsequent 7-day exposures of triplicate passive samplers allowed to assess the variability of concentration in the wastewater during the entire sampling period. For that purpose, relative standard deviation (RSD) was calculated from the analyte amounts detected in the triplicate samplers that were deployed for four subsequent 7-day periods. Based on our previous findings, we assumed that the sorbent uptake capacity is high enough for all compounds to be sampled integratively at least for the 7-day period (Urík and Vrana, 2019). The observed variability included both the repeatability of passive sampling and its analysis, as well as the variability of water composition due to changes in WWTP operation regime. The repeatability of triplicate passive samplers was very good, with a median RSD of 5%. In general, the observed overall variability of 7-day passive sampling data was low, with a median RSD of 15%. Only five compounds, which were detected close to their LOQ in passive samplers (diazepam, EDDP, fentanyl, imipramine and valsartan) exhibited variability higher than 43% (75th percentile + 1.5 × inner quartile range of data). The observed variability for fentanyl was partially caused by a bad repeatability (RSD = 33%) of passive sampling. For the remaining four compounds, a fluctuation or decreasing trend could be observed in 7-day passive sampling data over the 28 days.

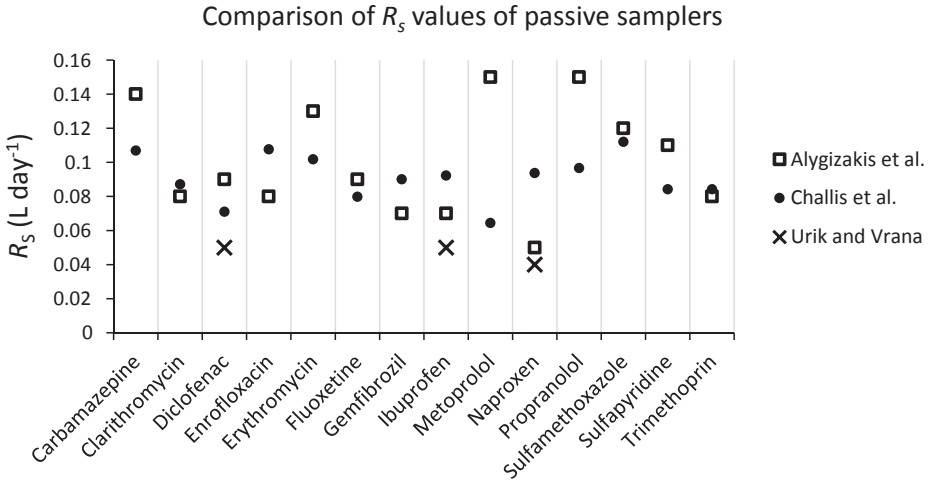

Fig. 2. Comparison of R_s values of compounds common for our study (square) and Challis et al., 2016, corrected for the same sampling area (circle), as well as values from Urík and Vrana, 2019 (cross).

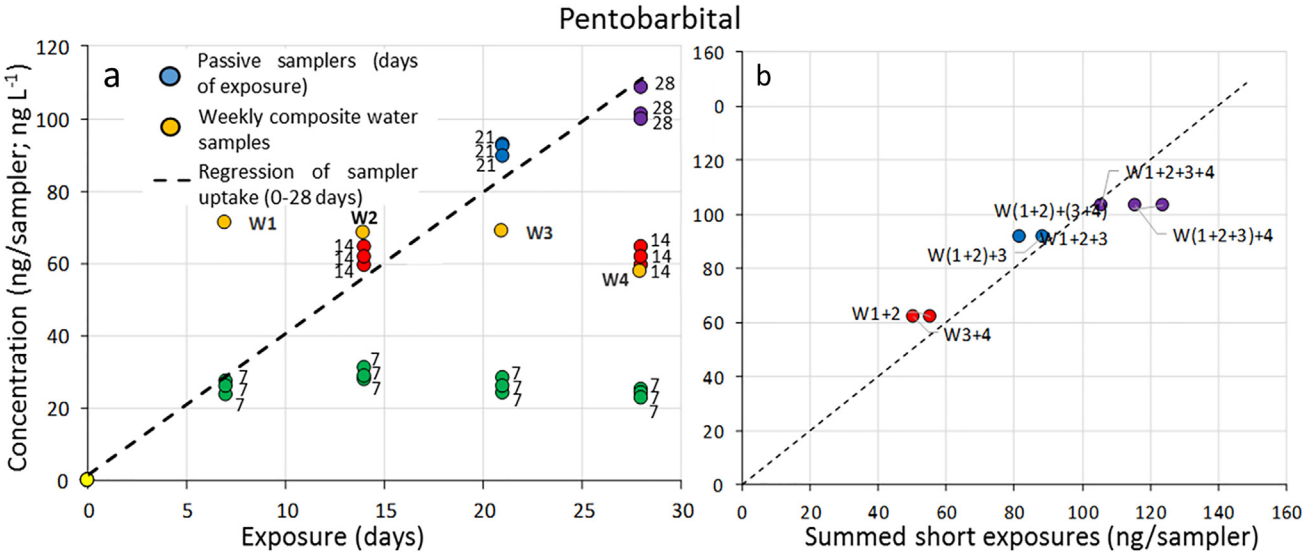

Fig. 3. a. Uptake of a compound from water to passive samplers (ng sampler^{-1}) after 0, 7, 14, 21 and 28 days of exposure. The exposure duration in days is indicated by colour of the dot (as shown also in Fig. 1) and by the label mark next to the dot. The dashed line shows linear regression of compound uptake to samplers, deployed at the same time, after 0, 7, 14, 21 and 28 days, as related to their exposure time period. Yellow dots labeled W1-W4 show concentrations in four weekly composite water samples (ng L^{-1}). b. Check of integrative uptake: comparing summed compound uptake to samplers (ng sampler^{-1}) during several short versus a concurrent longer exposure covering the same time period. Data labels indicate how the short exposures (in weeks) are summed up, e.g. W(1 + 2) + 3 means sum of one 14-day exposure in the first two weeks of deployment W(1 + 2) plus a 7-day exposure in the third week (+3). The sum (on x-axis) is compared with a single concurrent 3-week exposure on y-axis. The dashed line indicates unity (y = x). The sampler uptake is integrative if the points are close to the unity line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.2. Calculation of in situ sampling rates

The relatively constant exposure conditions over the entire passive sampler deployment simplified further interpretation of the passive sampling data and allowed to estimate *in situ* substance-specific sampling rates R_S, equivalent to the compound specific apparent water volume extracted during the exposure time (t). For R_S calculation, compound amounts accumulated in sampler from 7, 14, 21 and 28 day exposures starting at the first day, were fitted by linear regression as a function of exposure time (Fig. 3). For most of the compounds exceeding LOQ, a linear uptake was observed during the entire exposure period, which suggests integrative uptake. Only for 16 compounds the coefficient of determination R^2 of the linear regression was lower than 0.86. Among those, a linear uptake could be observed up to 21 days for atenolol, caffeine, cefaclor, ciprofloxacin, clobazam, difloxacillin, hydrochlorothiazide, ketoprofen and theophylline, followed by a level off or decrease of passive sampling concentrations after 28 days of exposure. The remaining compounds including ampicillin, cortisone, flunitrazepam, MDA, methamphetamine, norephedrine and sulfaguanidine (SGN) were likely sampled integratively and the uptake was linear. However, data from the first 14 days of exposure were below LOQ, which resulted in a relatively poor correlation. Low concentrations of the later 7 compounds with the exception of cortisone in sampled water were confirmed also below their respective LOQs in composite water samples.

In the next step, *in situ* R_s was calculated by dividing the slope of the linear uptake curve by the average concentration of compounds in the four composite water samples collected during passive sampling exposure ($\bar{C_w}$).

$$R_s = slope/\bar{C_w}$$

The calculation was performed for 245 compounds, for which the exposure conditions were constant over the exposure period, concentrations in water samples were above their LOQs, and the uptake was linear over the entire exposure period. R_S values ranged from 47 to 300 mL d^{-1}, with a median value of 87 mL d^{-1}. 50% of the values were within the range between 70 and 117 mL d^{-1}. Extremely high R_S values exceeding 188 mL d^{-1} were calculated for 11 compounds including benzoylecgonine, citalopram, diazepam, gabapentine, mirtazapine, normirtazapine, norfentanyl, norbuprenorphine, ronidazol, sarafloxacin, and tetrazepam. Most of the 11 compounds are present in aqueous solution at neutral pH as cations, zwitterions or neutral molecules. Since for most compounds integrative (linear in case of constant aqueous concentration) uptake was observed up to one month of exposure, it can be assumed that the sampler uptake capacity was high enough to act as an infinite sink of the investigated compounds up to 28 days of exposure. In such case, uptake rate should not be related to the sorbent properties, but only to the mass transfer rate from water to sorbent. Thus, there is no expectation of any causal relationship between R_S and compound affinity to the adsorbent in passive samplers. Indeed, there was no correlation between R_s and compound hydrophobicity, expressed by log K_{ow} (Fig. S7B in SI).

Elevated R_S of some compounds were likely related to an enhanced hydrogel permeability, caused by a weak reversible compound sorption of those compounds to diffusive agarose hydrogel. From theory, hydrogel behaves as a stagnant layer of water and if it is thick enough, the compound uptake is controlled by diffusion in the hydrogel according to the equation: $R_S = k \times A$, where k is the overall mass transfer coefficient of substance (m s^{-1}) in the hydrogel and A (m^2) is the sampler surface area. In absence of compound sorption in the hydrogel, $k = D_g/\delta_g$ where D_g is the diffusion coefficient in hydrogel and δ_g is the thickness of the hydrogel layer. The values of diffusion coefficient in gel are similar to those in water (Urík et al., 2020). However, when a compound has some affinity to the hydrogel, the term k changes to $k = D_g \times K_{gw} \times \delta_g$, where K_{gw} is the gel/water distribution coefficient. In case of a constant δ_g, the magnitude of k is determined by the product $D_g \times K_{gw}$, which is the hydrogel permeability. When a compound reversibly sorbs to hydrogel, K_{gw} is higher than 1, but D_g decreases. If the permeability is higher than D_w, it should be manifested by an increase in R_s. Since agarose has a polar molecular structure, compound sorption should be driven mainly by dipol-dipol or ion-dipol interactions between molecules and hydroxyl agarose polymer. We observed increased R_S for compounds that are neutral, zwitterions or cations. However, we did not analyse the compound concentrations in diffusive hydrogel layers that were discarded during sampler processing. Thus,

the outlined hypothesis needs to be tested in future studies.

Several compounds investigated in this study were also previously examined by Urík and Vrana using the same sampler design in the laboratory conditions (Urík and Vrana, 2019). The R_S values were obtained with reasonable precision of the fit ($R^2 > 0.8$) for diclofenac, ibuprofen and naproxen (0.09, 0.07 and 0.05 L day^{-1}, respectively). For all compounds, the *in situ* R_S values were slightly higher than those derived from laboratory condition (0.05, 0.05 and 0.04 L day^{-1}, respectively).

The R_S values can also be compared to the values obtained using the typical o-DGT design, if they are corrected by the factor representing the difference in sampling area or gel thickness. Common compounds can be found in the study of Challis et al. and the results are well comparable (Challis et al., 2016). Even though R_s values of some compounds differ up to the factor of 2.3 (metoprolol), we observed no systematic trend in the differences (see Fig. 2).

3.3. Tentative identification of TPs for prioritized antibiotics

Screening of the MBR effluents for the metabolite and TPs of the selected antibiotics yielded 64 hits by applying the criteria for the reduction of features regarding peak area, intensity, mass accuracy, isotopic pattern and identical chromatographic retention time. High-quality data dependent spectra were acquired for 36 out of 64 masses of interest and their spectra were compared with (i) the HRMS/MS spectra uploaded in European MassBank (https://massbank.eu), and (ii) the identified metabolites and TPs of the selected antibiotics from relevant biotransformation and advanced wastewater treatment studies (Terzic et al., 2018; Senta et al., 2019). Seven suspected compounds (not belonging to the 279 CECs detected by target screening) were tentatively identified in positive ionization mode fulfilling all criteria (mass accuracy < 2 mDa, satisfactory isotopic fit < 100 mSigma and plausible HRMS/MS fragmentation). Table S8 summarizes the tentatively identified suspected TPs corresponding to the selected parent antibiotic compounds along with their theoretical and experimental monoisotopic mass of the precursor ions ($[M + H]^+$), the molecular formula, the retention time, the reached identification levels and the proposed structures.

Among the identified suspects, TP434 and TP592 belong to the group of azithromycin TPs with an intact macrolactone ring, which have been previously reported in the literature (Terzic et al., 2018) and were formed by the removal of one or both sugar units. TP392 has *m/z* value lower than 434, which suggested that the (bio)transformation may have included opening and modification of the macrolactone ring of azithromycin. TP765 was formed either by N-oxidation of desosamine sugar moiety or by oxidation of the hydroxy group. The mass spectral evidence showed that the detected TP766 belongs to the group of clarithromycin TPs and was formed by enzymatic hydrolysis of the macrolactone ring. Moreover, TP764 could be identified either as 14-OH clarithromycin or as clarithromycin-N-oxide. All tentatively identified TPs have been previously reported in the literature (Terzic et al., 2018; Senta et al., 2019).

Although the signal of the TPs was sufficient to acquire HRMS/MS spectra of high quality, the observed signals were lower than the corresponding parent compounds. Therefore, concentration of TPs is not expected to exceed the concentration of the parent compounds and the respective ecotoxicological thresholds. However, it is worth highlighting that the potential synergistic effects of the parent drugs and their TPs cannot be excluded (Beretsou et al., 2016). The fact that PNEC exceedance was observed for the parent antibiotics and the co-occurrence of their TPs which retain their antimicrobial part of the compound intact raises concerns about their effects including the spread of antibiotic resistance. TPs significantly contribute to the complexity and toxicity of the chemical mixtures formed in the environment and therefore it is important to create workflows and methods such as the one presented in our study for effective screening of TPs in environmental samples. However, routine monitoring of TPs in WWTPs would require high expertise of the WWTPs' operators and increase of the monitoring costs and time. This, can possibly be tackled in the future due to automatization and software developments provided together with mass spectrometers and use of a bioassay battery comprising multiple endpoints that can provide very useful information and serve as a powerful tool for decision-making that can greatly benefit environmental monitoring efforts and regulatory bodies.

3.4. ARGs abundance and correlation with antibiotics

In addition to the analysis of antibiotics and their TPs, samples were subsjected to qPCR analysis for seven ARGs and one mobile resistant element. Almost all tested ARGs (except for $bla_{\text{CTX-M-32}}$) were detected in the treated wastewater at varying concentration levels. The selection of ARGs was based on previous reports (Alygizakis et al., 2019a; Paulus et al., 2019) and by experience gained in the context of NORMAN network (Manaia et al., 2018; Cacace et al., 2019), the European network NEREUS COST Action ES1403 (Fatta-Kassinos et al., 2015) and the ANSWERITN project (H2020-MSCA-ITN-2015/675530). The absolute and relative abundances of ARGs in the investigated samples are presented in section 9 of SI.

The results showed that the most prevalent gene was the gene 16S rRNA, which is something to be expected, since it is the indicator of the absolute bacterial abundance. It was followed by the genes *sul1* (resistance against sulfonamides) and *intl1* ($\sim 10^5$ copies L^{-1}), which has been previously suggested as indicator for ARG pollution (Gillings et al., 2015). Both genes were detected at lower abundance than other European effluents, while their relative abundance to 16S rRNA was similar to previous studies (Cacace et al., 2019; Pärnänen et al., 2019). These two genes are usually associated together, where *sul1* is the part of the genes that are found in several *int1* gene cassettes (Rafraf et al., 2016).

The genes *ermB*, *tetM* and *qnrS* were detected at concentration $\sim 10^4$ copies L^{-1}, while the genes $bla_{\text{OXA-58}}$ and bla_{TEM} were detected in lower concentration (10^3 copies L^{-1}). The abundance order of the ARGs matched with the findings from our previous study (Alygizakis et al., 2019a) or other studies (Cacace et al., 2019), indicating the persistence of ARGs despite the application of advanced treatment technology applied in the monitored WWTP. Furthermore, a recent study associated the relative abundance of ARGs with the profiles of resistant isolates in the different countries (Pärnänen et al., 2019).

Application of risk assessment for ARGs was not feasible due to lack of established EQS. Very little is known about the occurrence of antibiotics, their TPs, ARGs and their association with the spread of antibiotic resistance. The systematic surveillance of antibiotics and antibiotic resistance determinants is considered imperative for the management of bacterial infectious diseases (WHO, 2020; JPIAMR, 2020). However, description of the dynamics of these genes over time employing long-term sampling campaigns is recommended to trace the trends of ARGs. Trend analysis of ARGs and the various antibiotic classes is presented in Fig. 4. Good correlation was observed between the trend plots of the concentration of ARGs and the concentration of the respective antibiotic classes. For example, the variation in concentration for sulfonamides was observed to follow the same trend as *sul1*, which is the ARG against sulfonamides. The same observation was valid for quinolones and the quinolone resistance gene *qnrS*, for macrolides and the macrolide resistance gene *ermB*, for tetracyclines and the tetracycline resistance gene *tetM*, for beta-lactams and the beta-lactam resistance genes *blaTEM* and *blaOXA58*. Our findings highlight that concentration of ARGs may reflect the variation in concentration for antibiotic classes. This observation can potentially be exploited for future monitoring programs.

4. Conclusions

The combined chemical analytical approach of passive sampling,

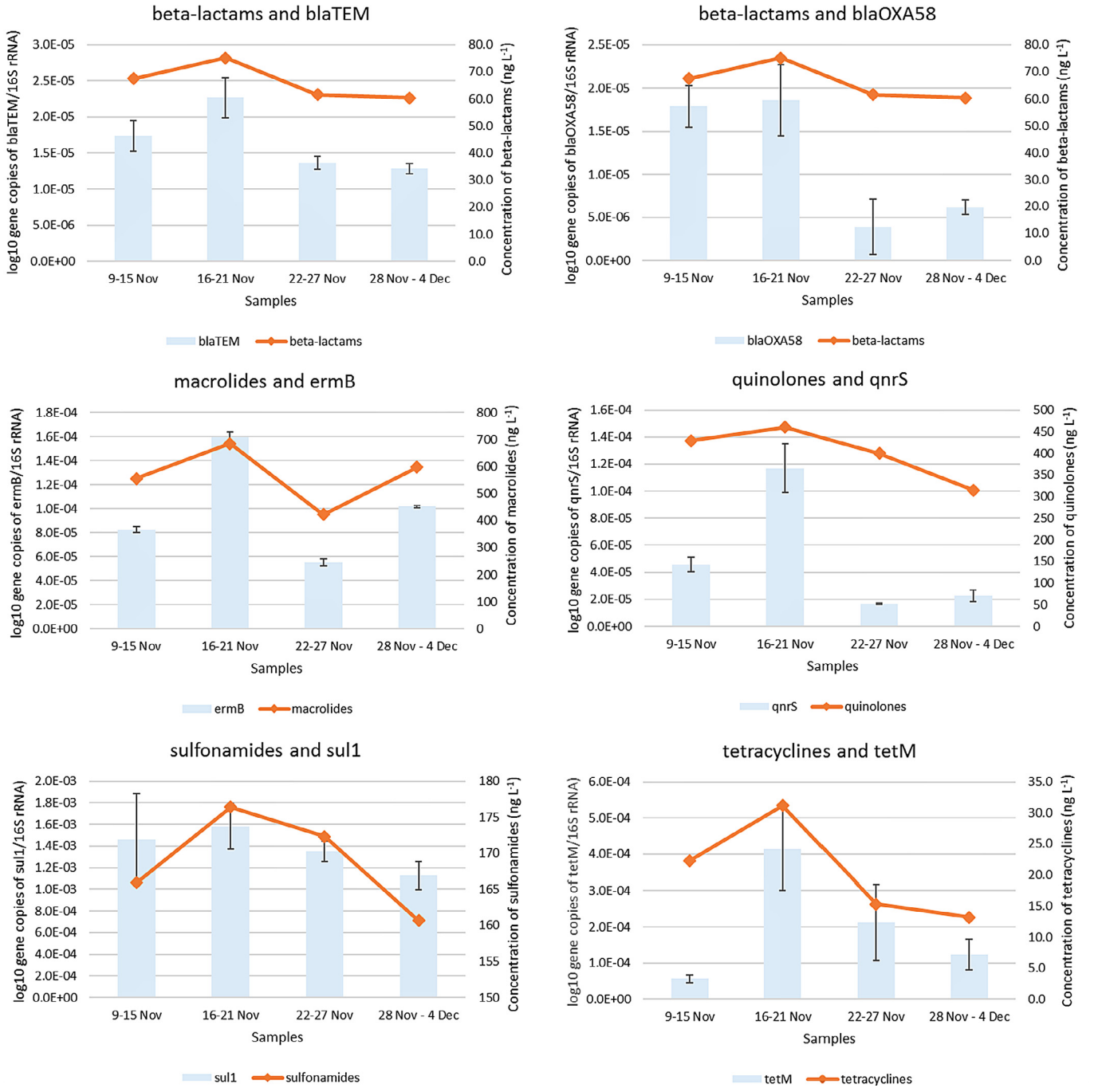

Fig. 4. Gene copies of antibiotic resistant genes (ARGs) normalized to 16S rRNA versus the sum of the concentration of their respective antibiotic class (beta-lactams versus bla_{TEM}, beta-lactams versus bla_{OXA58}, macrolides versus *ermB*, quinolones versus *qnrS*, sulfonamides versus *sul1*, tetracyclines versus *tetM*).

wide-scope target screening together with ARGs testing is a promising approach for monitoring wastewater reuse with a potential for further studies. The approach allows screening of chemical and biological threats associated to wastewater reuse, which is essential to avoid unwanted implications in wastewater reuse (e.g. uptake of CECs in plant and fruits). Our observation that gene copies of ARGs can indicate the concentration of specific antibiotic classes is worth further research and can potentially be exploited by future monitoring programs. Additionally, a simplified prioritization scheme was used to narrow down the two hundred and seventy-nine (279) CECs that were detected to 16 compounds. Among the compounds, four antibiotics (three macrolides and one fluoroquinolone) were prioritized. This fact triggered the retrospective investigation of TPs for the prioritized antibiotics. Even though this approach is right now a laborious task, it can be automatized and provide additional tools to better-understand the complex chemical mixtures in wastewater. Our approach enabled the tentative identification of seven TPs. Finally, in our study the feasibility of wastewater monitoring using a novel passive sampler prototype was evaluated. The passive sampler prototype showed integrative performance for most of the substances and revealed 35 additional compounds that would remain undetected with traditional sample preparation methods.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105597.

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