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Antibiotic residues in final effluents of European wastewater treatment plants and their impact on the aquatic environment

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ABSTRACT

A comprehensive monitoring of a broad set of antibiotics in the final effluent of wastewater treatment plants (WWTPs) of 7 European countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, and Norway) was carried out in two consecutive years (2015 and 2016). This is the first study of this kind performed at an international level. Within the 53 antibiotics monitored 17 were detected at least once in the final effluent of the WWTPs, i.e.: ciprofloxacin, ofloxacin, enrofloxacin, orbifloxacin, azithromycin, clarithromycin, sulfapyridine, sulfamethoxazole, trimethoprim, nalidixic acid, pipemidic acid, oxolinic acid, cefalexin, clindamycin, metronidazole, ampicillin, and tetracycline. The countries exhibiting the highest effluent average concentrations of antibiotics were Ireland and the southern countries Portugal and Spain, whereas the northern countries (Norway, Finland and Germany) and Cyprus exhibited lower total concentration. The antibiotic occurrence data in the final effluents were used for the assessment of their impact on the aquatic environment. Both, environmental predicted no effect concentration (PNEC-ENVs) and the PNECs based on minimal inhibitory concentrations (PNEC-MICs) were considered for the evaluation of the impact on microbial communities in aquatic systems and on the evolution of antibiotic resistance, respectively. Based on this analysis, three compounds, ciprofloxacin, azithromycin and cefalexin are proposed as markers of antibiotic pollution, as they could occasionally pose a risk to the environment. Integrated studies like this are crucial to map the impact of antibiotic pollution and to provide the basis for designing water quality and environmental risk in regular water monitoring programs.

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1. Introduction

Antibiotics are a class of pharmaceutical active compounds with high usage and consumption worldwide. By definition, an antibiotic is a chemotherapeutic agent that specifically inhibits, by cell destruction or growth inhibition, the proliferation of bacteria (Kümmerer, 2009). According to Kümmerer (2009) over 250 different chemical substances are registered as antibiotics for human and/or animal health use globally. Based on the analysis of data from scientific literature and national and regional surveillance systems from 71 countries over the past 10 years, antibiotic use is growing steadily worldwide (30%), driven mainly by rising demand in low- and middle-income countries (Gelband et al. 2015). This increase in the use of antibiotics and the awareness about their side effects have led to increasing concern regarding their potentially detrimental effects in the environment. In particular, it is suspected that their occurrence can accelerate resistance spread in the environment (Bengtsson-Palme et al., 2018), with potential implications on human health. According to the World Health Organization (WHO), antimicrobial resistance is a major challenge to global human and animal health, food safety, and development today, with the perspective of aggravation in the upcoming years, if effective measures are not implemented (World Health Organization, 2014).

Because of the intensive use of antibiotics for human, veterinary and agriculture purposes, these compounds are continuously released into the environment from anthropogenic sources. In urban areas, wastewater treatment plants (WWTPs), when available, are among the main receptors of antibiotics, part of which persist after the treatment and can be released into various environmental compartments (Michael et al., 2013). Several studies about the presence of antibiotics in WWTPs have been conducted in the last couple of decades. Most of them have focused on a limited amount of compounds (between 2 and 33 target antibiotics). Further, studies comparing the situation in different countries are nearly absent and, in the case that a single country as China, Croatia, Sweden, Portugal, United Kingdom, and Greece is analysed, a limited number of WWTPs, varying between 2 and 19 within each country has been studied (Gao et al., 2012; Johnson et al., 2017; Kosma et al., 2014; Lindberg et al., 2005; Pereira et al., 2015; Senta et al., 2013; Wang et al., 2018; Zhang et al., 2019). Among the most comprehensive studies so far are those by Gracia-Lor et al. (2011), who monitored 26 antibiotics among other pollutants in 19 WWTPs in Spain and Birošová et al. (2014), who monitored up to 33 antibiotics in 2 WWTPs in Slovakia. This type of monitoring studies permits the evaluation of temporal and geographical trends in antibiotic occurrence. However, given the lack of standardized methodologies and other technical biases, data reported in distinct studies are poorly comparable. In this situation, transnational efforts constitute an essential contribution for the establishment of environmental protection guidelines that can be globally applied. To the authors' knowledge such international monitoring studies of antibiotics in WWTPs are not available, which is a major gap for implementing mitigation regulations/procedures. Global monitoring programs would represent an important step forward on water protection worldwide, as they would contribute to compare country-specific scenarios, to provide the basis for international action and also would allow assessing the progress achieved concerning any potential environmental protection action. In fact, the results of environmental monitoring are of fundamental importance to environmental management in general, as the drafting and prioritization of environmental policies is based on the findings of environmental monitoring (Helmer, 1994). The monitoring of antibiotic contamination is particularly relevant given its association with antibiotic resistance, known to be highly heterogeneous at a global scale (Hendriksen et al., 2019; Pärnänen et al., 2019).

The current study was hence motivated by the need for water monitoring at an international level to acquire reliable and comparable analytical data concerning antibiotics occurrence. With this aim, the final effluent of 13 WWTPs located in 7 European countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, and Norway) was sampled twice (early Spring and early Autumn) in two consecutive years (2015 and 2016) and was monitored for 53 antibiotic residues belonging to 10 different therapeutic classes. The countries were selected following a north-to-south gradient in the use of antibiotics as well as in the prevalence of antibiotic resistance in clinical settings as reported in the 2017 EARS-Net surveillance report (ECDC European Centre for Disease Prevention and Control, 2017). The specific objectives of the study were: (i) to provide an overview of the presence and concentration of antibiotics in final effluents of WWTPs located in different European countries; (ii) to assess geographical and temporal trends about the occurrence of these contaminants; (iii) to infer about the potential environmental and human health risk posed by antibiotic residues in final treated wastewater effluents; and (iv) to propose robust analytical tools and indicator compounds to be used in regular water-monitoring programs, hence making feasible the comparison of country-based studies.

2. Materials and methods

2.1. Sampling campaigns

Four sampling campaigns were carried out in early Spring (March 2015 and 2016) and early Autumn (October 2015 and September 2016), suggested to correspond to the highest and the lowest peaks of antibiotic consumption, respectively (Caucci et al., 2016; Sun et al., 2012). In each of the 4 sampling campaigns, 24-h composite samples were collected in 3 consecutive days (Tuesday, Wednesday, and Thursday) at the outlet of 13 urban WWTPs belonging to 7 European countries (Portugal, Spain, Cyprus, Ireland, Germany, Finland, and Norway) (Fig. 1, Table S1). Two WWTPs were monitored in each country except in Spain and Norway, where a single WWTP was sampled. In Portugal, due to a problem in the WWTP PT1 in the sampling campaign of March 2016, a third WWTP (PT3), located in the same region, was sampled instead. General characteristics of the selected WWTPs are gathered in Table S2. For every campaign, data concerning weather conditions were collected for the 3 days of sampling and the day before (Table S3).

All participants collected the samples on the same dates and adopted a common protocol for sampling and sample processing (Gros et al., 2013). Amber glass bottles pre-rinsed with ultrapure water were used to collect ca. 200–250 mL wastewater from each of the WWTPs, and transported at 4 °C to the laboratory for further sample pre-

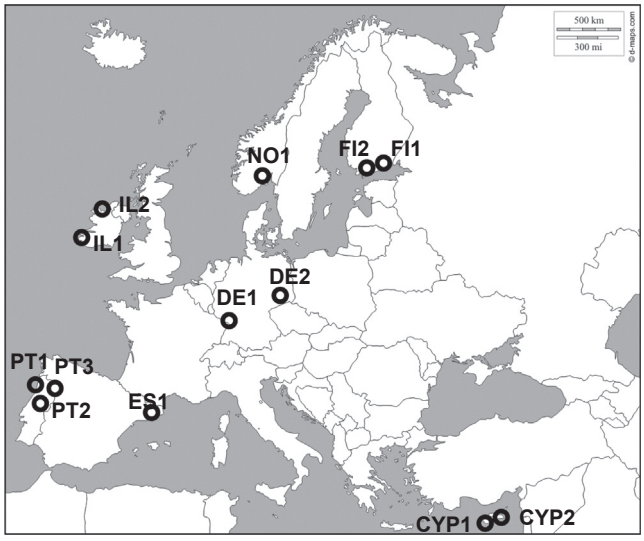

Fig. 1. Map of the 13 European wastewater treatment plants (WWTPs) from where effluent wastewater was sampled. Portugal (PT), Spain (ES), Cyprus (CYP), Ireland (IL), Germany (DE), Finland (FI), Norway (NO).

treatment.

2.2. Reagents and chemicals

Fifty-three antibiotics, distributed in 10 classes, were monitored: fluoroquinolones (n = 8), quinolones (n = 4), penicillins (n = 5), cephalosporins (n = 6), macrolides (n = 6), tetracyclines (n = 4), lincosamides (n = 2), sulfonamides (n = 15), a dihydrofolate reductase inhibitor (n = 1), and nitroimidazole antibiotics (n = 2) (Table S4). All antibiotic standards were of high purity grade (> 90%) and purchased from Sigma–Aldrich: tetracycline, chlortetracycline, oxytetracycline, and lincomycin were purchased as hydrochloride salts; oxacillin, cefazolin, cefotaxime, and cefapirin were acquired as sodium salts; penicillin V and penicillin G as potassium salts; amoxicillin and ampicillin were purchased as trihydrate salts; tylosin and doxycycline were acquired as tartrate and hyclate salts, respectively. Isotopically labelled compounds, used as internal standards, were: ofloxacin-d3, ciprofloxacin-d8 (as hydrochloride hydrate salt), erythromycin-N,N-dimethyl ^{13}C, ampicillin-^{15}N, and ronidazole-d3, purchased from Sigma–Aldrich, and azithromycin-d3, sulfamethoxazole-d4, and lincomycin-d3, from Toronto Research Chemicals (Ontario, Canada). Sulfadimethoxine-d6 and sulfadoxine-d3, used as surrogate standards, were purchased from Sigma–Aldrich.

Individual stock standard solutions, as well as isotopically labelled internal standard and surrogate standard solutions, were prepared at a concentration of 1000 mg/L. After preparation, the solutions were stored at −20 °C (Gros et al., 2013). Standard solutions containing the antibiotic mixtures were prepared in methanol/water (50:50, v/v) just before the analysis; by mixing appropriate amounts of the intermediate standard solutions. Separate mixtures of isotopically labelled internal standards were prepared in methanol, except ampicillin-^{15}N, which was diluted in acetonitrile/water (50:50, v/v). Further diluted solutions were prepared in a methanol/HPLC water mixture (50:50, v/v).

Glass fibre filters (1 μm) and PVDF filters (0.45 μm) from Whatman (UK) were used for filtration of the samples. OASIS Hydrophilic-Lipophilic-Balanced (HLB) cartridges (60 mg, 3 mL) from Waters Corporation (Milford, MA, U.S.A.) were used for solid phase extraction.

HPLC grade methanol, acetonitrile, formic acid 98% and water (LiChrosolv) were purchased by Merck (Darmstadt, Germany). Ammonium hydroxide, hydrochloric acid 37% and the nitrogen for drying were purchased by Abelló Linde S.A. (Valencia, Spain).

2.3. Analytical procedure

2.3.1. Sample pre-treatment

Each sampling day, water was filtered by 1 μm glass fibre filter followed by 0.45 μm PVDF filter. An appropriate volume of 0.1 M Na_2EDTA solution was added to the filtered water to get a final concentration of 0.1% (g solute/g solution), and the pH adjusted to 2.5 with hydrochloric acid 0.1 M. Samples were further processed: 50 μL of surrogates mix was added to each of the 50 mL samples. Solid phase extraction (SPE) was performed according to Gros et al. (2013). Briefly, Oasis HLB (60 mg, 3 mL) cartridges were first conditioned with 2 × 3 mL methanol followed by 2 × 3 mL water (HPLC grade, and pH adjusted to 2.5 with HCl 0.1 M). Then 50 mL of filtered effluent were loaded onto the SPE cartridge samples at approximately 1 mL/min, approximately, under vacuum conditions. Each cartridge was cleaned with 2 × 3 mL HPLC water (under gravity conditions) and dried under vacuum conditions for 10–15 min. The cartridges were protected with parafilm and preserved at −20 °C before shipment with dry ice to the reference analytical laboratory, where the samples were stored at −20 °C until analysis (< 2 weeks). For analyses, the samples were eluted from the cartridge with 6 mL of ultra-pure methanol, evaporated under N_2 stream near dryness, and reconstituted with 1 mL of mixture of methanol and water (50:50, v/v). Finally, 10 μL of a standard 1 ng/mL mixture containing all isotopically labelled standards was added to the extract as internal standards. For an accurate quantification, concentrations were calculated by internal calibration using the isotope-labelled standards. Recovery values of the extraction method were calculated in each occasion (each sampling campaign) and used to correct the quantification values obtained using these calibration curves. To determine the recoveries, wastewater effluent was spiked in triplicate with a standard mixture containing all antibiotics at a final concentration of 10 ng/L. Recoveries were determined by comparing the initial concentrations after spiking with the concentrations obtained after the whole SPE procedure. Method detection limits (MDL) and method quantification limits (MQL) were also determined in each sampling campaign and for each antibiotic as the minimum detectable amount of compound with a signal-to-noise of 3 and 10, respectively. The analytical quality parameters MDL, MQL and recovery values (%) can be found in Table S4. The MDL achieved ranged from 5.31 ng/L up to 19.21 ng/L, whereas MQLs ranged from 17.72 to 46.91 ng/L. Concerning the extraction methodology, recoveries achieved for all target compounds ranged between 60 and 120%.

2.3.2. Chemical analysis

The chemical analysis was carried out with an ultra-performance liquid chromatography system (UPLC) using an Acquity HSS T3 column (50 mm × 2.1 mm i.d., 1.7 μm particle size), both from Waters Corporation (Mildford, MA, USA). The volume of sample injected was 5 μL. The UPLC system was coupled to a mass spectrometer hybrid quadrupole-ion trap (UPLC-5500 QTRAP, Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V electrospray ionization source working in positive ionization mode. Two multiple reaction monitoring (MRM) transitions per compound were recorded by using the Scheduled MRM™ algorithm (see Gros et al., 2013 for details). All data were acquired and processed using Analyst 1.6.3 software.

2.4. Environmental risk assessment (ERA)

The environmental risk associated with antibiotic residues released with the WWTP effluents was assessed using risk quotient (RQ). Risk quotients are indices based on empirical data for quantification of the environmental risk of chemicals and involve the comparison of the environmental concentrations of pollutants with the concentrations at which adverse effects on target organisms are expected (Isidori et al., 2005). Risk quotients were calculated according to the European Community guidelines (EC TGD, 2003), using Eq. (1):

$$RQ = (PEC)/(PNEC) \quad (1)$$

where PEC is the “Predicted Environmental Concentration” for each antibiotic and PNEC is its “Predicted No Effect Concentration”. Calculations of PEC were based on the antibiotic levels detected in WWTP effluent of the 13 WWTPs considered in this work (Table 1 for average values and Table S5 for complete raw data). In the present study, it was assumed that the final effluents of the WWTPs are discharged in freshwater ecosystems so that the impact of the antibiotic presence in wastewater in the different scenarios in the 7 countries can be compared. Therefore, PEC was calculated by applying a dilution factor (the ratio between the volume of freshwater available and the domestic sewage discharge) to the corresponding occurrence values following the approach by Keller et al. (2014) using Eq. (2):

$$PEC = (MC)/(DF) \quad (2)$$

where MC is the “Measured Concentration” in the wastewater effluents for each antibiotic and WWTP and DF is the “National annual median dilution factor” calculated for each country by Keller et al. (2014). The paper reports the calculated DF for domestic effluents for approximately 100 countries. These DF values were used by the same authors as surrogates to compare risk levels caused by chemical exposure between countries. The DF for the 7 countries of our study were extracted from this report and can be found in Table S6.

Two PNEC values were considered following the approach by Tell et al. (2019): environmental PNECs (PNEC-ENVs) and the PNECs based on minimum inhibitory concentrations (PNEC-MICs). PNEC-ENVs were calculated based on the environmental toxicity data collected from industrial and literature sources by Tell et al. (2019). Preference was given to data generated following the OECD (Organization for Economic Cooperation and Development) guidelines and from cyanobacteria, as they are considered more sensitive to antibiotics than other organisms (Le Page et al., 2017). PNEC-MICs values were developed by Bengtsson-Palme and Larsson (2016) based on MIC data from the EUCAST (European Committee on Antimicrobial Susceptibility Testing database). As recommended by Tell et al. (2019), the lowest of the two PNEC values was used for the environmental risk assessment in this work in order to be protective with ecological resources, and also to lower the pressure for the evolution, selection and maintenance of antimicrobial resistance in the environment. PNEC-ENV and PNEC-MIC extracted from the communication by Tell et al. (2019) for those antibiotics detected in our study can be found in Table S7.

2.5. Statistical analysis and data treatment

The variations between sampling campaigns for the same WWTP and between different WWTPs were analysed in order to obtain the temporal and geographical variations, respectively. The mean values comparison was performed using the analysis of variance (ANOVA) with the post hoc Tuckey's test ($p < 0.05$) using the IBM SPSS Statistics v26. To perform the data analysis, and avoid missing values, the results below the method quantification limits (MQL) were assumed as half of the value of the MQL of the respective sampling campaign (see Table S5), and in the case of results below method detection limit (MDL) it was used the value of zero.

Annual human consumption of antibacterials for systemic use (ATC group J01) in the community (primary care sector) as defined daily dose (DDD) per 1000 inhabitants was extracted from the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) for years 2015 and 2016 for each of the 7 countries (Fig. 4).

3. Results and discussion

3.1. Occurrence of antibiotic residues in treated wastewaters in different European countries

Fifty-three antibiotics belonging to 10 different chemical classes were monitored in the final effluents collected in wastewater treatment plants located in different European countries. Targeted antibiotics were selected based on their human and veterinary usage worldwide, as well as their occurrence and ubiquity in the aquatic environment (Gros et al., 2013; ECDC EFSA EMA, 2017. Among the 53 antibiotic compounds analysed in wastewater effluents (Table S4), 17 were detected in at least one of the 13 urban WWTPs studied, located in the 7 different countries (Table 1). The concentration of these 17 antibiotics (ciprofloxacin, ofloxacin, enrofloxacin, orbifloxacin, azithromycin, clarithromycin, sulfapyridine, sulfamethoxazole, trimethoprim, nalidixic acid, pipemidic acid, oxolinic acid, cefalexin, clindamycin, metronidazole, ampicillin, and tetracycline) in the studied WWTP are shown in Table S5 and represented in Fig. S1. The highest antibiotic concentrations observed were for fluoroquinolones: up to 1435.5 ng/L of ciprofloxacin in Portugal, and 613.0 ng/L of ofloxacin in Cyprus (Table S5). Of the four fluoroquinolones detected, two (enrofloxacin and orbifloxacin) are used in veterinary medicine (Table S4) and were only occasionally detected in samples from Spain and Cyprus, respectively (Fig. S1). The macrolides azithromycin and clarithromycin were observed in all countries presenting their maximal concentrations of 1577.3 ng/L and 346.8 ng/L, respectively in WWTP effluents from Portugal. In the case of azithromycin, the concentrations observed in the Portuguese WWTP PT2 samples were significantly higher ($p < 0.05$) than those observed in the WWTP effluent samples from Cyprus, Finland and Norway (Fig. S1). Of the 15 sulfonamide antibiotics screened, only sulfapyridine and sulfamethoxazole were detected in the final effluents, with maximal values of 583.6 ng/L in Norway and 220.9 ng/L in Cyprus, respectively. Considering average values, the Spanish WWTP effluent samples presented significantly higher ($p < 0.05$) concentrations of sulfamethoxazole than most of the other samples (Fig. S1). The dihydrofolate reductase inhibitor trimethoprim was detected in all the WWTPs, in almost all the sampling campaigns. Trimethoprim is commonly used in combination with sulfamethoxazole, as cotrimoxazole (Batt et al., 2006). However, the ratio between the concentrations of these two compounds was not consistent with the ratio used in clinical treatment (1:5; trimethoprim:sulfamethoxazole) for any of the samples from the different countries. The concentration of SMX in influent wastewater has been reported as four times higher than that of trimethoprim in several studies (Perez et al., 2005; Jelic et al., 2015). However, trimethoprim biodegradation in CAS commonly is not as efficient as that for SMX (Perez et al., 2005; Verlicchi et al., 2012) and therefore trimethoprim concentration was even higher than that for SMX in most of the final effluents monitored in this study (Table 1). Three of the quinolone antibiotics measured (i.e. nalidixic acid, oxolinic acid, and pipemidic acid) were detected in at least one sample. The levels of pipemidic acid presented no significant variation among WWTP effluent samples. The highest concentration was observed in Portugal (117.6 ng/L) in WWTP PT1, although this compound was detected just in the first sampling campaign. Nalidixic acid and oxolinic acid were only detected in some of the sampling campaigns in Ireland, in samples from both WWTPs in the case of nalidixic acid or just in WWTP IL2 samples for oxolinic acid. In addition, ampicillin was also only quantified in the Irish samples collected in Spring (although detected below the limit of quantification in Autumn 2015), with the maximum concentration observed being 231.1 ng/L (Fig. S1). Other antibiotics presented maximum concentrations in different countries – cefalexin in Finland (1047.8 ng/L), clindamycin in Spain (194.5 ng/L), metronidazole in Ireland (230.0 ng/L), and tetracycline in Portugal (613.6 ng/L) (Table S5) indicating the need of studying several antibiotics to get a clear picture of antibiotic pollution. The concentration values measured in our study (ranging between ng to μg/L) are in agreement with those found in final effluents of other European WWTP, as reported by other authors (Johnson et al., 2017; Lindberg et al., 2005; Senta et al., 2013; Gracia-Lor et al., 2011; Birošová et al., 2014) and in particular by Carvalho and Santos in their review on the occurrence of antibiotics in wastewater and different environmental matrices (Carvalho and Santos 2016) and, most recently, in the report by the European Commission Joint Research Center (JRC) (Sanseverino et al., 2018) where concentrations in WWTP effluents for the majority of antibiotics were between 0.1 and 1 μg/L. Nevertheless, some differences were identified among the examined samples in the present study. The countries exhibiting the highest average effluent concentrations were Ireland and the southern countries Portugal and Spain. In contrast, Cyprus, where tertiary treatment or membrane bioreactors are used, and northern countries exhibited lower total concentrations of antibiotics (Fig. 3).

Temporal variations of antibiotic concentrations (grouped based on their antibiotic class) were evaluated based on the comparison of data collected in two different seasons (early Spring and early Autumn) and in two consecutive years. The comparison of the two years of sampling (2015 *vs* 2016) revealed significant differences in the cumulative values of antibiotics concentration only in Cyprus, with significantly ($p = 0.006$) higher concentrations in 2016 (Fig. S2(a) and (c)). When evaluating the antibiotic presence by season, significant variation in cumulative antibiotic concentrations was only observed for some countries (Fig. 2): Antibiotic concentrations in early Spring (March 2015 and 2016) were significantly higher than those in early Autumn (September 2015 and October 2016) in samples from Spain, Ireland and Finland WWTPs (Fig. 2). These findings are in line with the seasonality

Table 1
Occurrence values (ng/L) for the 17 antibiotics detected in the samples from the 13 WWTPs considered in this study: Portugal (PT), Spain (ES), Cyprus (CYP), Ireland (IL), Germany (DE), Finland (FI), and Norway (NO). Concentrations (ng/L) are calculated as the average from the 4 sampling campaigns or 3 sampling campaigns in the case of PT1 and 1 sampling campaign in the case of PT3. Occurrence values for each day and sampling campaign can be found in Table S5.

Chemical group	Antibiotic	PT1	PT2	PT3	ES1	CYP1	CYP2	IL1	IL2	DE1	DE2	FI1	FI2	NO1
Cephalosporins	Cefalexin	38.4	37.0	< MDL	65.2	66.3	65.0	66.4	87.6	< MDL	< MDL	203.3	308.0	60.7
Dihydrofolate reductase inhibitors	Trimethoprim	69.1	146.3	190.6	102.8	74.2	44.0	141.3	121.0	15.2	105.0	182.0	186.7	119.7
Fluoroquinolones	Ciprofloxacin	457.3	584.9	231.4	200.3	316.8	252.3	259.8	234.0	43.8	230.6	38.4	43.2	159.2
	Enrofloxacin	< MDL	< MDL	< MDL	69.4	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
	Ofloxacin	89.7	184.9	132.8	142.3	305.1	196.7	65.4	39.9	< MDL	66.5	20.0	22.8	27.1
	Orbifloxacin	< MDL	< MDL	< MDL	< MDL	6.7	6.5	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
Lincosamides	Clindamycin	8.5	86.6	31.5	101.4	6.5	27.8	42.5	59.1	< MDL	110.7	88.8	94.2	97.1
Macrolides	Azithromycin	361.8	597.5	178.9	299.5	48.0	45.2	266.7	260.8	126.2	290.4	129.3	130.7	149.7
	Clarithromycin	74.2	118.7	313.2	112.0	< MDL	11.9	204.4	189.4	76.5	123.4	4.5	4.8	20.8
Nitroimidazole antibiotics	Metronidazole	< MDL	< MDL	< MDL	76.1	19.6	< MDL	88.6	78.2	7.6	20.3	20.1	41.9	93.2
Penicillins	Ampicillin	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	99.4	68.1	< MDL	< MDL	< MDL	< MDL	< MDL
Quinolones	Nalidixic Acid	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	50.3	25.3	< MDL	< MDL	< MDL	< MDL	< MDL
	Oxolinic Acid	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	5.3	< MDL	< MDL	< MDL	< MDL	< MDL
	Pipemidic Acid	15.0	10.8	20.1	30.1	10.1	15.2	18.2	4.4	11.8	< MDL	4.8	3.2	7.5
Sulfonamides	Sulfamethoxazole	30.2	7.1	< MDL	123.4	13.3	68.5	53.0	44.0	22.9	34.9	< MDL	< MDL	48.6
	Sulfapyridine	84.5	4.7	48.8	63.9	8.7	48.7	95.5	93.6	22.7	112.0	89.2	98.8	184.0
Tetracyclines	Tetracycline	231.2	165.7	147.5	< MDL	36.9	24.5	141.0	194.2	< MDL	15.4	70.6	16.8	179.2

< MDL Values below method detection limit.

of antibiotic prescriptions reported in other publications (Caucci et al., 2016; Sun et al., 2012). However, the inverse was observed in one of the Cyprus WWTPs (CYP1), with higher concentrations of antibiotics in early autumn than in early spring, whereas no clear seasonal trend was observed for Portugal, Germany or Norway. The analysis of each antibiotic class did not show clear seasonal patterns. For instance, macrolides, one of the antibiotic classes detected at the highest concentrations in effluent samples in this study, did not exhibit significant differences in the sampling campaigns. On the contrary, they did display high seasonality in their consumption in some countries such as Switzerland, with a high peak in winter as reported in other studies (Coutu et al., 2013). Macrolides are usually prescribed to treat respiratory tract infections in patients living in the community, and in the case of clarithromycin specifically to treat lung infections (Tanaka et al., 2002). However, significant variation between Spring and Autumn was observed for certain antibiotics in certain effluents collected from the same WWTP: cefalexin (CYP1, IL1 and IL2), ofloxacin (FI1), ciprofloxacin (FI2), enrofloxacin (ES1), pipemidic acid (ES1 and IL1), and ampicillin (IL1) (Fig. S1). In summary, there are antibiotic- and country-specific trends in the concentration of antibiotics found in wastewater effluents. These findings did not corroborate the hypothesis that higher concentration should be detected overall in early Spring (end of winter) than in early Autumn (or late Summer). The quantification of antibiotics concentration in wastewater before the treatment would probably better reflect the antibiotics consumption. The treatment processes reduce antibiotic levels to different extents depending not only on the type of treatment applied in each case (Table S2), but also on other conditions, including physical-chemical properties of the pollutant, climate variations as for example the temperature, which influences the efficiency of the biological processes as better removal is obtained at temperatures of 15–20 °C compared to below 10 °C (Krzeminski et al., 2019; Ramin et al., 2018; Vieno et al., 2005). In conclusion, a higher sampling frequency (at least monthly) and the analysis of the raw influent is recommended to bring further insight about the relationship between antibiotic consumption versus wastewater antibiotic occurrence.

3.2. Human antibiotic consumption and antibiotic occurrence in wastewater effluents

Consumption of antibiotics varies among countries and it could be hypothesized that these differences are still noticeable in treated wastewater effluents. To test this hypothesis, annual human consumption of antibacterials for systemic use (see Section 2.5) was compared with actual concentrations found in wastewater effluents for each of the 7 countries in the two years monitoring study (Fig. 4). Based on the ECDC reports, Cyprus was the country with the highest consumption of antibiotics followed by Ireland, Spain, Portugal, Finland, Norway, and Germany (Fig. 4b). As described in the previous section, Ireland, Spain and Portugal were the countries exhibiting the highest total antibiotic concentrations whereas Norway, Finland, and Germany showed lower concentrations (Fig. 4a). Therefore, higher antibiotic consumption rates were, in general, in agreement with higher total antibiotic concentrations in the WWTP effluent samples for Ireland, Portugal, and Spain. In contrast, lower antibiotic consumption rates correlated with lower total antibiotic concentrations in the WWTP effluent samples from Finland, Norway, and Germany. Curiously, Cyprus, with the highest antibiotic consumption rates, exhibited one of the lowest total concentrations in final effluent samples (Fig. S2). This may be due to either an overestimation of Cyprus antibiotic consumption, since it was the only country for which ESAC-net data on consumption was also including hospital sector, or/and to the advanced wastewater treatment used in the Cyprus WWTPs analysed; i.e. sand filtration + chlorination as additional tertiary treatment to CAS (CYP1) and MBR treatment with ultrafiltration membrane (CYP2) (Table S2). The search for potential correlations between antibiotic consumption and its occurrence in final effluent is influenced by different factors, in particular antibiotic metabolism in the human body, the chemical and biological stability of the antibiotics during wastewater treatment and the specific wastewater treatment process and operational conditions of each WWTP (Jelic et al., 2015; Michael et al., 2013). Therefore, if community-based epidemiological studies or health and/or well-being assessments based on wastewater are the aims, the analysis of raw (influent) wastewater is preferred (Gracia-Lor et al., 2017). In contrast, the measurement of the range and concentration of antibiotics in final effluent permits the assessment of the impact of antibiotics use in the environment, when sanitation systems that comply with legislative recommendations are implemented. This was the aim of the present study.

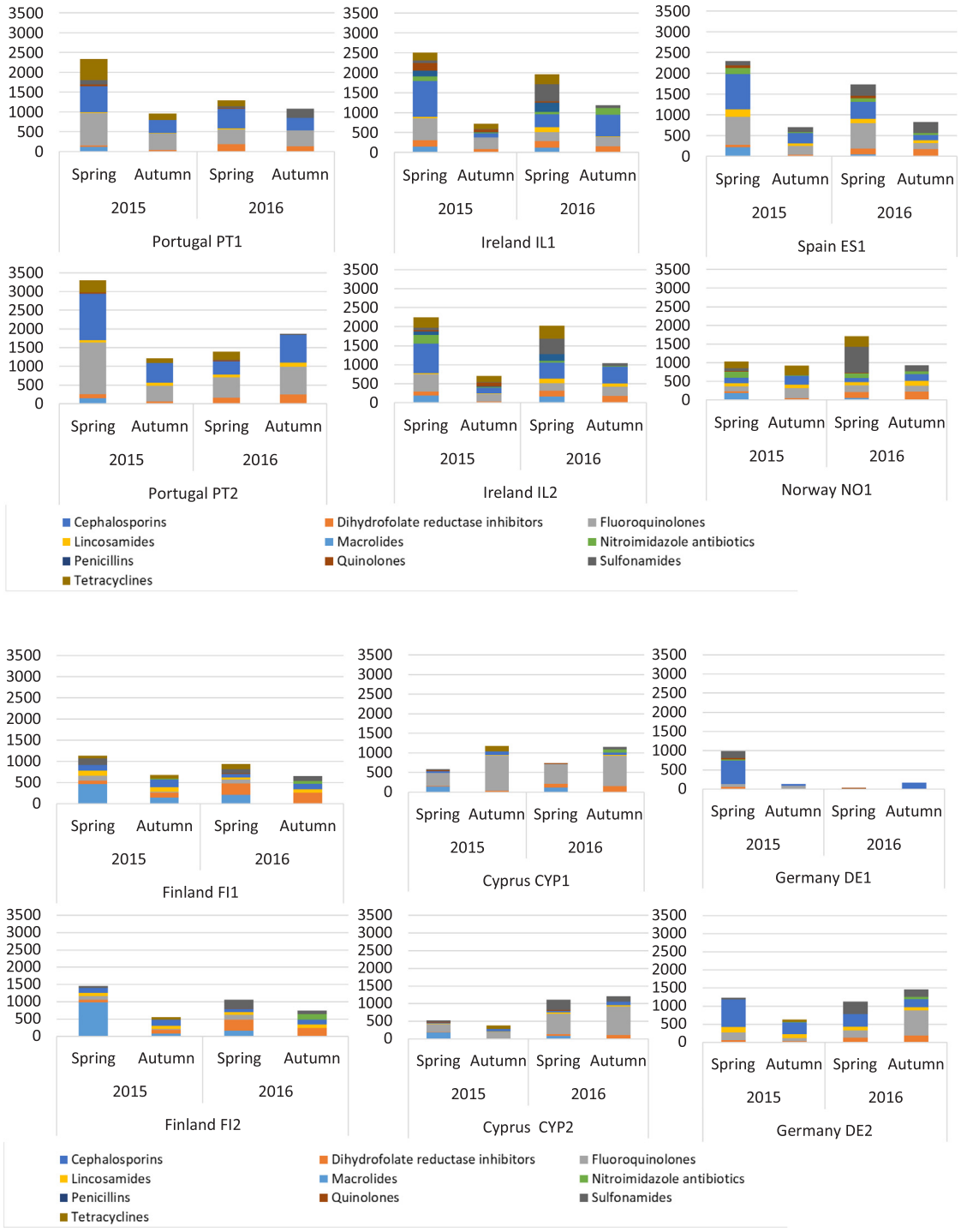

Fig. 2. Cumulative antibiotic concentrations (ng/L), grouped by chemical family, in the 4 sampling campaigns (Spring and Autumn, 2015, and Spring and Autumn, 2016) in each of the 13 WWTPs. Each band represents the sum of single antibiotics belonging to a chemical family; and each single antibiotic concentration is calculated as the average concentration of the 3 consecutive sampling days. a, b, c and d indicate significant differences ($p < 0.05$) between sampling campaigns of the same WWTP.

3.3. Antibiotic residues in final effluents of wastewater treatment plants located in different European countries and their impact on the aquatic environment"

Taking advantage of the dataset of antibiotics measured in the effluent samples from 13 WWTPs, an environmental risk assessment (ERA) for the different European countries was performed. For this assessment, it was assumed that final effluents were discharged in freshwater systems, a condition necessary to compare the potential impact of the wastewater effluent of WWTPs located in different European countries. The Environmental risk of antibiotics was assessed by evaluation of the Risk Quotient (RQ), calculated as the ratio between the Predicted Environmental Concentration (PEC) of each antibiotic in the aquatic environment and the Predicted No Effect Concentration (PNEC) (Section 2.4). Average values from the four sampling campaigns (Table 1) were used to calculate PEC, whereas in the case of PNEC values, the antibiotic-specific approach by Tell et al. (2019) was implemented (see Section 2.4). PNEC is the concentration below which a

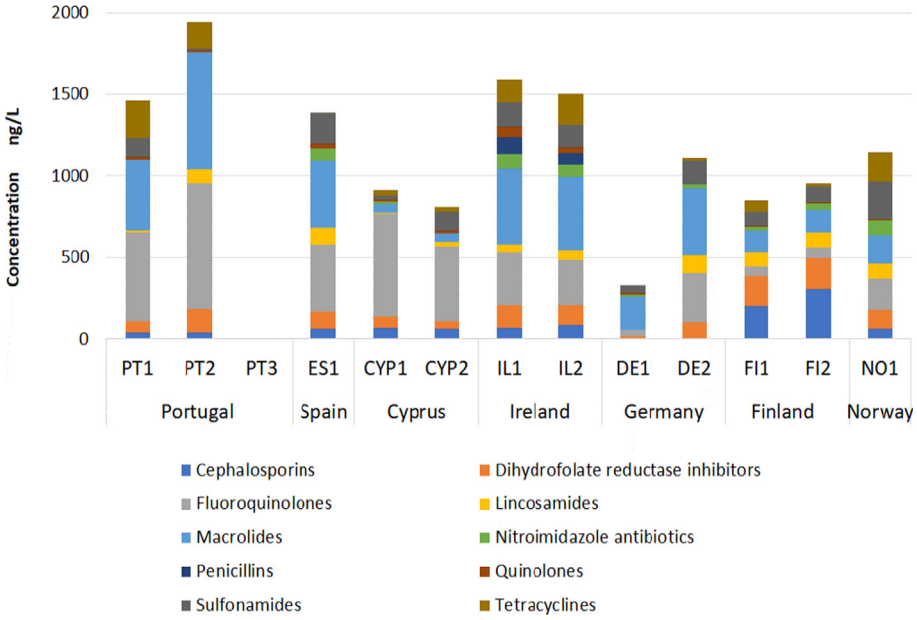

Fig. 3. Antibiotic concentrations profile (ng/L) in the 13 WWTPs under study. Each column represents one WWTP and each of the bars in the column represents the average concentration from the 4 sampling campaigns (March 2015, October 2015, March 2016 and September 2016) for all the antibiotics belonging to a chemical family, except PT1 (average of March 2015, October 2015 and September 2016). PT3 (just one sampling campaign in Spring 2016) is omitted.

chemical will likely have no adverse effect in an ecosystem. Among other pharmaceuticals and emerging pollutants, antibiotics are of particular concern since they are explicitly designed to have an effect on microorganisms and therefore, they are prone to impact microbial communities in aquatic systems. A detrimental effect of antibiotics on natural microbial communities could be the disappearance or inhibition of some microbial groups involved in key ecosystem functions by bactericidal and bacteriostatic effects (Grenni et al., 2018). However, low concentrations of antibiotics, below the so-called "minimum inhibitory concentration" (MIC), might also act as a selective force on some microbial populations, which can develop resistance (Andersson and Hughes 2014). Having this in mind and with the aim of performing an integrative environmental risk assessment we followed the recommendation by Tell et al. (2019) and took into account both, the environmental PNECs (PNEC-ENVs) and the PNECs based on MIC (PNEC-MICs): the lowest of the two values of PNEC-ENV or PNEC-MIC for each antibiotic was chosen for the environmental risk assessment (Table S7). Three categories according to the RQ value can be defined using commonly used risk ranking criterion (Verlicchi et al., 2012; EC TGD, 2003):

- RQ $\leq$ 0.1: Low environmental risk;
- 0.1 $<$ RQ $\leq$ 1: Moderate environmental risk;
- RQ $>$ 1: High environmental risk.

The RQ determined for 14 of the 17 antibiotics resulted in low environmental risk. The three exceptions were the cephalosporin cefalexin, the fluoroquinolone ciprofloxacin and the macrolide azithromycin, whose RQ exceeded the threshold of 0.1 (up to 0.14, 0.90 and 0.58 respectively) (Table 2). According to the results of this study, these 3 antibiotics should be considered as posing a moderate environmental risk in water bodies in Portugal, Spain, Cyprus, and

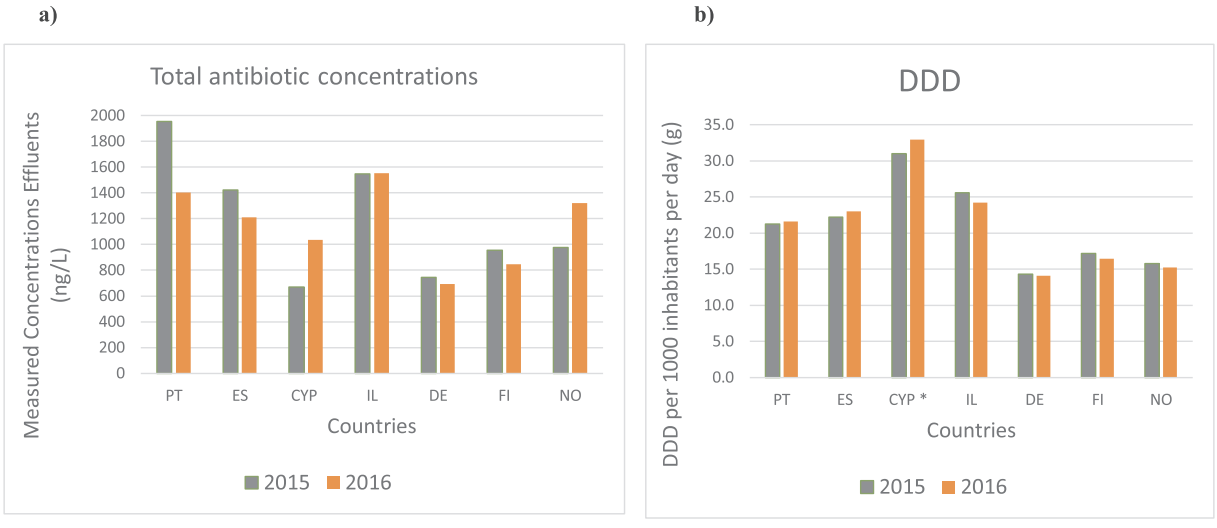

Fig. 4. Comparison of (a) the total antibiotic concentration (excluding veterinary antibiotics enrofloxacin and orbifloxacin) measured in each country (ng/L). Values for each year calculated as the average of antibiotic concentrations in early Spring and early Autumn sampling campaigns; and (b) the defined national daily dose values (DDD) per 1000 inhabitants per day, for antibiotics consumed by the community (primary sector) (g). Values extracted from the "Annual Epidemiological Report for 2016" from the European Centre for Disease Prevention and Control (ECDC) (https://ecdc.europa.eu/en/publications-data/antimicrobial-consumption-annual-epidemiological-report-2016. Countries: PT, Portugal; ES, Spain; CYP, Cyprus; IL, Ireland; DE, Germany; FI, Finland; NO, Norway. * For Cyprus data on consumption in hospital sector is also included. Specific information about the antibiotic types in each year and country is shown in Fig. S2.

Table 2
Risk Quotients (RQs) in freshwater calculated for the average concentration (4 sampling campaigns) for the 17 antibiotics detected in the samples from the 13 WWTPs. RQ are calculated using Eqs. (1) and (2) (see Section 2.4). Environmental Risk: RQ ≤ 0.1: Low; 0.1 < RQ ≤ 1: Moderate; RQ > 1: High. Values in bold correspond to RQ > 0.1, moderate or high risk. Portugal (PT), Spain (ES), Cyprus (CYP), Ireland (IL), Germany (DE), Finland (FI), Norway (NO).

Chemical groups		PT1	PT2	PT3	ES1	CYP1	CYP2	IL1	IL2	DE1	DE2	FI1	FI2	NO1
Cephalosporins	Cefalexin	0.01	0.01	0.00	0.03	**0.14**	**0.14**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dihydrofolate reductase inhibitors	Trimethoprim	0.00	0.00	0.01	0.01	0.03	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Fluoroquinolones	Ciprofloxacin	**0.12**	**0.16**	**0.06**	**0.13**	**0.90**	**0.72**	0.02	0.02	0.02	**0.12**	0.00	0.00	0.00
	Enrofloxacin	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Ofloxacin	0.00	0.01	0.00	0.01	0.10	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Orbifloxacin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lincosamides	Clindamycin	0.00	0.01	0.01	0.04	0.01	0.05	0.00	0.00	0.00	0.03	0.00	0.00	0.00
Macrolides	Azithromycin	**0.30**	**0.49**	**0.15**	**0.58**	**0.41**	**0.39**	0.06	0.06	**0.20**	**0.45**	0.00	0.00	0.00
	Clarithromycin	0.02	0.02	0.06	0.05	0.00	0.03	0.01	0.01	0.03	0.05	0.00	0.00	0.00
Nitroimidazole antibiotics	Metronidazole	0.00	0.00	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Penicillins	Ampicillin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Quinolones	Nalidixic Acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Oxolinic Acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Pipemidic Acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sulfonamides	Sulfamethoxazole	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Sulfapyridine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tetracyclines	Tetracycline	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Germany. Portugal, Spain and Ireland were the countries with the highest concentrations of antibiotics encountered in the WWTP effluent samples (Fig. 3). The average concentration values for ciprofloxacin in this study ranged between 38.4 in Finland (FI2) to 584.9 in Portugal (PT2) (Table 1), whereas in previous studies levels reported were of 140 ng/L in USA (Renew and Huang, 2004), 199 ng/L in Greece (Papageorgiou et al., 2016), 211 ng/L in Slovakia (Birošová et al., 2014), 640 ng/L in Australia (Watkinson et al., 2007), or 700 ng/L in Spain (Gracia-Lor et al., 2012). Also, similar values for azithromycin (between 45.2 ng/L in CYP2 and 597.5 ng/L in PT2; table 1) were found in other countries: 50 ng/L in Czech Republic (Golovko et al., 2014), 277 ng/L in Germany (Rossmann et al., 2014), 504 ng/L in Slovakia (Birošová et al., 2014). In the case of cefalexin, found up to 308 ng/L in Finland (FI2) (table 1) in this study, it has been detected in China, 980 ng/L (Gulkowska et al., 2008), U.S.A. 2 330 ng/L (Mohapatra et al., 2016) or Australia at 3 900 ng/L (Watkinson et al., 2007). The relatively high concentrations determined for these 3 compounds in conjunction with the low dilution factors defined for Portugal, Spain and Cyprus (61, 26 and 6, respectively) place these countries in the top list of environmental risk due to antibiotic pollution emitted by final effluents of urban WWTPs, among the countries studied. The dilution factor in these countries may be relatively low (Acuña et al., 2015) as in these areas streams can be dominated by municipal and/or industrial effluent discharges, particularly in urbanized watersheds. In addition, Iberian rivers (Portugal and Spain) may be highly influenced by water scarcity in drought periods (Pereira et al., 2017). In the case of Cyprus, where rivers are inexistent, treated wastewater is reused for irrigation (agriculture and landscape), or it is infiltrated through shallow ponds reaching the aquifer (IMPEL, 2018). Contrasting with Cyprus, where risk was aggravated by the low dilution factor, rather by the high antibiotic load, in Ireland, the high dilution factor of 230 compensated the high concentrations of antibiotics found in the effluents of the WWTPs analysed. The low dilution factor defined for Germany (32) together with some remarkable high concentrations of antibiotics in the effluent samples from one WWTP, DE2, where only CAS treatment was applied (Table S2), lead to relatively high RQ for ciprofloxacin and azithromycin (0.12 and 0.45 respectively, Table 2). In contrast, in the effluent samples from WWTP DE1, where ozone treatment was applied as tertiary treatment, and low concentrations of antibiotics were found, no risk for the environment was identified. Finally, northern countries Norway and Finland, exhibited the highest dilution factor (2453 and 1702 respectively) and also the lowest levels of antibiotics, resulting in low risk for the environment (RQs < 0.1).

This picture produced for water bodies (based on the average concentration values of antibiotics in the effluent samples from WWTPs) can mask some occasional critical situations regarding antibiotic pollution. Therefore, RQs were also calculated for the highest concentration measured in all sampling campaigns in order to predict worst-case scenarios (Table S8). Fig. 5 shows the PEC values in all sampling campaigns for the antibiotics that showed the highest RQ values, i.e. the fluoroquinolone ciprofloxacin and the macrolide azithromycin. As shown in Fig. 5, in a single occasion PEC exceeded the lowest PNEC (Table S7), a situation that corresponds to RQ ≥ 1. This scenario was observed for azithromycin occurrence in the effluent samples from the WWTP in Spain in the winter campaign of 2015.

RQ values determined in other studies, based on the measured environmental concentration (MEC) instead of the PEC values, revealed low or moderate environmental risk for some trophic levels in different countries. Surface water RQ values indicated a moderate risk of adverse chronic effects (RQ > 0.1) for trimethoprim (out of 10 antibiotics measured) in a river in Sweden (Sörengård et al., 2019), whereas sulfamethoxazole and ofloxacin were reported to be the antibiotics with the highest risk to the environment out of 13 antibiotics monitored in surface water from China, with RQ values of 0.23 and 0.45, respectively (Hu et al., 2018). RQ values higher than 1 have been reported in some surface waters in China: up to 31.3 for sulfamethoxazole and 1.5 for ofloxacin (Li et al., 2012) or in the surface water of a highly urbanized area in Italy: up to 7.09 for clarithromycin and 11.33 for amoxicillin (Riva et al., 2019). Most of these studies did not consider PNEC-MIC values when assessing environmental risk but regular PNEC-ENV, which were available from the literature or by *in-silico* calculations and selected based on authors own criteria in each case and therefore, comparison cannot be considered conclusive.

Ciprofloxacin and azithromycin, both highlighted in this study as antibiotics of potential environmental concern, have also been included in the last version of the surface water Watch List (WL) under the Water Framework Directive (WFD) (Commission Implementing Decision, 2018). This Watch list proposes 15 substances that should be monitored in water by the EU Member States. Besides ciprofloxacin and azithromycin, the macrolides erythromycin and clarithromycin, and the penicillin amoxicillin are also included in this list. The inclusion of 5 antibiotics among the 15 compounds in this list is consistent with the latest "European One Health Action Plan against Antimicrobial Resistance (AMR)", which supports the use of the watch list to improve knowledge of the occurrence and spread of antimicrobials in the environment (European Commission, 2017). Other organisms as the U.S. Environmental Protection Agency (EPA), responsible for testing and regulating chemicals in drinking water, does not regulate the presence

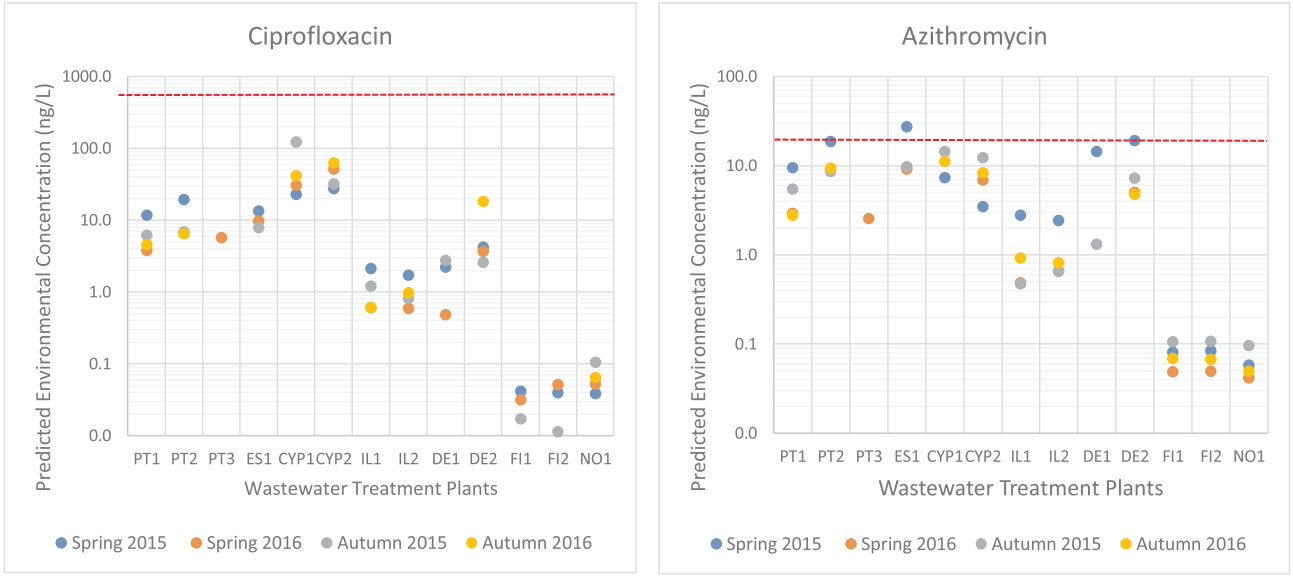

Fig. 5. Predicted Environmental Concentration (PEC) of ciprofloxacin and azithromycin (ng/L) associated with the 13 WWTPs under study in each of the 4 sampling campaigns. Dashed line corresponds to the lower PNEC (ng/L) selected based on Tell et al. 2019; i.e. 60 and 20 ng/L for ciprofloxacin and azithromycin respectively.

of any antibiotic although the macrolide erythromycin is now included on the "Drinking Water Contaminant Candidate List (CCL-4) (U.S. EPA, 2016), a list of c.a. 100 contaminants that are anticipated to occur in public water systems. The inclusion of antibiotics in the lists of different pre-regulation initiatives, is a recognition of the growing concern related to these compounds.

As it is currently not feasible to analyse all micropollutants, the selection of a set of indicator compounds in regular water-monitoring programs is recommended. In Section 3.1 the 17 antibiotics detected at least once in the sampling campaigns (among a group of 53 substances screened) were suggested as potential markers for antibiotic pollution, so that "smart monitoring" can be attained by analysing this reduced number of compounds. However, the assignment of RQs to these 17 selected contaminants detected in the effluent samples from WWTPs support a further reduction of possible indicator compounds. Based on risk assessment criteria, three compounds, ciprofloxacin, azithromycin, and cefalexin, are suggested as indicator candidates. These antibiotics had the highest RQs in a number of countries (Table 2). The use of the broader panel (17 compounds) or the short-list (3 compounds) can both be options to consider depending on the goals of the monitoring program.

Some final considerations should be kept in mind concerning the environmental risk assessment. Firstly, antibiotics enter and are present in the aquatic environment as a mixture, either of different compounds belonging to the same class of antibiotics, acting by similar mechanisms, or of different therapeutic groups, which may have synergistic or antagonistic effects, sometimes with the possibility of exerting exacerbated effects in relation to the single compounds (Backhaus and Faust 2012). In addition, it is known that body- or environment-driven transformation of parent compounds cause metabolites (or by-products) to occur in the environment. These antibiotic by-products very often retain the antibiotic activity to a certain degree and can all be found at high concentrations in the final wastewater effluent (Fatta-Kassinos et al., 2011). The monitoring of these compounds, based on chemical analytical methods or bioassays is, therefore, necessary to improve the accuracy of risk assessment due to antibiotic pollution in water bodies.

4. Conclusions

This comprehensive monitoring study of a broad set of antibiotics in several countries is the first such study performed at a European level. The study has primarily allowed us to define the current water quality status of the urban wastewater effluents in Europe. Within the 53 antibiotics analysed in the sampling campaigns, 17 were detected in treated wastewater effluents. Ciprofloxacin, azithromycin, and cefalexin were selected as markers of antibiotic pollution and are suggested to be used for widespread temporal and geographical characterization of environmental water or WWTP effluents. In addition, a north-to-south geographical gradation was observed in terms of antibiotic amounts released in the environment. Compounds with the highest loads in all countries were macrolides and fluoroquinolones. Although the WWTPs examined in this study were complying with EU legislation, antibiotic residues of at least 7–12 distinct compounds, each at concentrations ranging from 3 to 598 of ng/L, were observed to be continuously discharged in freshwater and marine ecosystems, impacting the environment and possibly contributing to antibiotic resistance evolution. Although the detected levels of antibiotics released in the environment were predicted to exhibit a moderate impact on the environment in general, antibiotics such as azithromycin and ciprofloxacin can occasionally pose a risk to the environment and antibiotic resistance development. The situation regarding environmental antibiotic pollution can be further worsened in the future in the frame of global change, with population growth, intensified agricultural and industrial activity, together with water scarcity in vulnerable areas such as southern European countries. Our study provides a framework for predicting the likelihood for selecting antibiotic resistant bacteria in water bodies containing antibiotics released from WWTPs.

CRediT authorship contribution statement

Sara Rodriguez-Mozaz: Conceptualization, Data curation, Funding acquisition, Investigation, Resources, Supervision, Writing - original draft, Writing - review & editing. **Ivone Vaz-Moreira:** Investigation, Conceptualization, Data curation, Software, Visualization, Writing - review & editing. **Saulo Varela Della Giustina:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation. **Marta Llorca:** Investigation, Data curation, Methodology, Visualization, Validation. **Damià Barceló:** Investigation, Resources. **Sara Schubert:** Investigation. **Thomas U. Berendonk:** Conceptualization, Investigation. **Irene Michael-Kordatou:** Conceptualization, Investigation, Writing - review & editing. **Despo Fatta-Kassinos:** Conceptualization, Investigation, Writing - review & editing. **Jose Luis**

Martinez: Conceptualization, Investigation, Writing - review & editing. **Christian Elpers:** Investigation, Writing - review & editing. **Isabel Henriques:** Conceptualization, Investigation, Writing - review & editing. **Thomas Jaeger:** Investigation. **Thomas Schwartz:** Conceptualization, Investigation. **Erik Paulshus:** Investigation. **Kristin O'Sullivan:** Conceptualization, Investigation. **Katariina M.M. Pärnänen:** Investigation. **Marko Virta:** Conceptualization, Investigation. **Thi Thuy Do:** Investigation. **Fiona Walsh:** Conceptualization, Investigation, Writing - review & editing. **Célia M. Manaia:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing - review & editing.

Declaration of Competing Interest

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

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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.2020.105733.

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