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Title: **Antibiotic resistance in wastewater, does the context matter? Poland and Portugal as a case study**

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ABSTRACT

Antibiotic resistance has been considered a major human health threat that may endanger the success of medicine. Recent studies have unveiled worldwide asymmetries of antibiotic resistance occurrence, being factors as diverse as climate, socioeconomic, or antibiotic use possible drivers of such asymmetric distribution. In Europe, where clinical antibiotic resistance is surveyed for more than 20 years, the European Centre for Disease Prevention and Control (ECDC) consistently describes an increasing gradient from North-to-South and from West-to-East. This observation motivated the current perspective paper aiming to qualitatively compare two countries located at the extreme latitude of Europe and also at distant longitude - Poland in the Central-East region and Portugal in the South-West. Both countries have been among those with the highest prevalence of antibiotic resistance in clinical settings, although as it is discussed, climate, socioeconomic factors, and antibiotic use are different. In general, in Poland higher antibiotic consumption and resistance prevalence is observed, mainly at the community level, when compared to Portugal. However, in Portugal, treated wastewater may hold identical or slightly higher resistance loads. Based on these observations, it is discussed how different factors may influence the abundance of antibiotics, antibiotic resistant bacteria, and genes in wastewater before and after treatment.

Keywords: Urban resistome, wastewater treatment plants, antibiotic resistant bacteria, antibiotic resistance genes

1. INTRODUCTION

Antibiotic resistance has become a serious threat to human health, mainly through opportunistic infections in patients under health care due to any other pathology (Fair and Tor 2014). However, antibiotic resistance is not a strict health care issue, as it is spread among healthy people, domestic and farm animals, wildlife, and the natural environments, in particular water and soil (Hernando-Amado et al. 2019; McEwen and Collignon 2018). The implications of the wide dissemination and unavoidable contact with antibiotic resistant bacteria (ARB) depend largely on the complex context where it occurs, from the socioeconomic and climate conditions to other factors still poorly understood (e.g. the effects of pollution or the ecology and biodiversity of distinct world regions). This is nicely discussed and evidenced in Collignon et al. (2018), who investigated the potential association of anthropological and socioeconomic factors. The study involved data of prevalence of third-generation cephalosporin (e.g. ceftazidime, cefotaxime), fluoroquinolone, and carbapenem resistance in *Escherichia coli* and in *Klebsiella* spp., for which was also used the prevalence of methicillin-resistance in *Staphylococcus aureus*. The data was collected from scientific literature, the database ResistanceMap (https://resistancemap.cddep.org/), and the antimicrobial resistance report published by the World Health Organization (WHO) in 2014 from 73 to 103 countries (depending on data availability) distributed over the five continents. Important variables that Collignon et al. (2018) concluded to be related to resistance included temperature and socio-economic factors such as governance, gross domestic product, infrastructures, education, and health-care expenditure. Indeed, the database ResistanceMap, which combines information from distinct data sources (EARS-Net, GLASS, CHINET, among others) offers a worldwide overview of the contrasts on the clinical prevalence of antibiotic resistance in some important human pathogens. In general, Russia and Asian countries, a few African countries for which data is available, and some Latin American countries are those with the highest prevalence values (% of resistant invasive isolates), followed by Europe, North America, and Oceania (https://resistancemap.cddep.org/ accessed January 7, 2021). Despite the important gaps that may be noticed for some world regions, existing clinical surveys offer an outlook of the geographic trends of antibiotic resistance prevalence. This situation contrasts with the current knowledge of antibiotic resistance occurrence in the environment, typically unsystematic and scarce. The most studied environmental compartment is wastewater, and the metagenomics analysis of raw wastewater has been proposed as a method of community surveillance (Aarestrup and Woolhouse 2020). This approach was used to analyse raw wastewater collected in 79 sites in 60 countries. The pattern of antibiotic resistance was similar to that described above for clinical isolates, with a clear separation between Europe, North America, and Oceania, with lower antibiotic resistance gene relative abundance than Africa, Asia, and South America (Hendriksen et al. 2019). Although Europe is in the low-prevalence group, it is quite asymmetric in terms of resistance prevalence. Consistently, in clinical settings, a general increase of antibiotic resistance prevalence is observed from North-to-South and from West-to-East, particularly for the Gram-negative bacteria (ECDC 2020). This same pattern was observed in raw and treated wastewater from seven countries across this gradient (Pärnänen et al. 2019). In this study, the resistance determinants measured by polymerase chain reaction (PCR) presented higher relative abundance in samples from Southern countries, in both raw wastewater and final effluents. These values were not significantly correlated with the concentration of antibiotic residues detected in the same samples, although, in general, these presented higher concentrations in the Southern than in Northern countries (Rodriguez-Mozaz et al. 2020). The authors concluded that, besides the antibiotic use, the temperature which is higher in Southern countries might also explain the described gradients of antibiotic resistance (Pärnänen et al. 2019). Indeed, different publications have been showing that higher temperature may be a driver for antibiotic resistance increase (MacFadden et al. 2018; McGough et al. 2020; Pärnänen et al. 2019).

The previous considerations and findings were major motivations to proceed with a literature and database-supported study comparing two European countries, Poland (Central-East Europe) and Portugal (South-West Europe), with contrasting climate conditions and placed among those with the highest resistance prevalence in clinical settings (ECDC 2020). In a study analysing the attributable deaths and disability-adjusted life-years (DALYs) caused by infections with ARB in European Union (EU) and European Economic Area (EEA), modelling analysis positioned both Poland (position 8) and Portugal (position 4) in the group of nine countries with a worse situation than the EU/EEA average, with about two times higher burden of ARB infections in Portugal than in Poland (24 021 vs. 41 069 reported cases, 1 158 vs. 2 218 attributable deaths), and also higher DALYs per 100 000 population (~240 vs.~190) (Cassini et al. 2019). This preliminary overview drove the definition of the objectives of the current analysis: 1) compare both countries regarding socioeconomic and climate conditions, antibiotic use in animal farming and human medicine, and prevalence of resistance in clinical pathogens; 2) compare the occurrence of ARB, antibiotic resistance genes (ARGs) and antibiotic residues in both countries, and infer about the fitness of these biological contaminants in wastewater.

2. COMPARISON of SOCIOECONOMIC, GEOGRAPHIC, and CLIMATE CONDITIONS

Poland and Portugal are located at latitude extremes of Europe, Central-East and Southern-West, respectively, having Poland an area approximately three times larger than Portugal (Table 1). The same is observed in terms of total population (~38.4 vs. ~10.3 million, respectively in Poland and Portugal), making the average population density of the two countries similar (123 vs. 111 inhabitants/km^2) (Table1).

The different geographic locations influence the climate and weather conditions, as average temperature, global solar radiation, and precipitation (Table 1). According to the Köppen-Geiger climate classification (http://koeppen-geiger.vu-wien.ac.at), Poland has a humid continental climate and Portugal has a Mediterranean climate. Depending on the region, insolation in Portugal can be more than twice as intensive as in Poland, with the highest insolation levels observed in the south of Portugal. While the average temperatures in the warmest months may be similar in both countries, the average winter temperatures differ in 12 ^{o}C, with negative values in Poland (-3.7 ^{o}C) and above 3.0 ^{o}C in Portugal. The local minimum temperature has been used to predict the survival of bacteria in specific environments, being higher temperatures responsible for enhanced survival or proliferation of bacteria (including pathogens and antibiotic resistant) (MacFadden et al. 2018). Also precipitation, which may occur in the form of rain or snow, presents higher average annual values in Poland than in Portugal (Table 1). Nevertheless, the highest precipitation periods occur in summer in Poland and in the winter in Portugal (Table 1).

Precipitation is one of the factors that may directly influence the volume of wastewater entering urban wastewater treatment plants (UWWTPs), when combined sewers are used, as is common in urbanized areas. This additional flow of water, mainly stormwater, may decrease the residence time of wastewater in the treatment tanks, therefore affecting the efficiency of the treatment, raising the risks that increased loads of ARB and ARGs are released to the environment (Eramo et al. 2017; Honda et al. 2020). Moreover, stormwater may be a source of contaminants such as heavy metals or microorganisms (Barbosa et al. 2012). Previous studies have shown that precipitation may influence the load of pathogenic bacteria and ARGs abundance, suggesting the relevance of this parameter to accurately monitor ARB/ARGs in aquatic environments (Ahmed et al. 2018; Di Cesare et al. 2017). Hence, the level of precipitation and the time of the year when it is more intense, may contribute to explain differences in the regional or seasonal pattern of antibiotic resistance occurrence. In turn, in UWWTPs with open tanks, solar radiation may also influence the treatment, contributing both to the photodegradation of chemical compounds such as antibiotic residues or bacteria photoinactivation (Rizzo et al. 2012). Besides, solar radiation may facilitate the application of advanced oxidation processes (AOPs), reducing the costs of advanced treatment processes (Marcelino et al. 2015). It can be argued that the relatively high temperatures observed in Mediterranean countries, such as Portugal, may contribute to improving removal efficiencies of the biological treatments (Zouboulis and Tolkou 2015). In both analysed countries, treated wastewater is mainly released into rivers (EEA 2021). UWWTPs are most often built near a river downstream of served populations and may represent a source of chemical and microbial pollution of/in the receiving water body. The temperature of river water can affect microbial growth, whereas ultraviolet (UV) radiation might limit the growth of some microorganisms (Mishra et al. 2019). The impact that the discharge of treated wastewater may have on freshwater may be estimated considering the dilution factor per country - the ratio between the volume of freshwater available and the domestic wastewater discharge (Keller et al. 2014). Although Poland has larger basin areas and longer rivers than Portugal, the estimated dilution factor for Poland (38.71) is 1.5 times lower than the one estimated for Portugal (61.23) (Table 1). This may contribute to the lower percentage of bathing waters classified with excellent or good quality (measured based on the monitoring of *E. coli* and intestinal enterococci) - ~90% compared to the 99% reported for Portugal (EEA 2020b). The higher number of coastal bathing sites in Portugal also contributes to these numbers, as coastal bathing sites are generally better than inland bathing sites (EEA 2020b). However, in some cases, wastewater discharges may be also contributing to the degradation of the bathing water quality. A well reported case was the Arturówek ponds located in the Northern area of Łódź, in Poland (EEA 2020a).

Although the gross domestic product per capita based on purchasing power parity (GDP per capita, PPP) is almost the same in Poland and Portugal (33 739.40 vs. 33 131.41, respectively), the investment in healthcare expenditure relative to GDP (%) is lower in Poland than in Portugal (Table 1). A negative and significant correlation between GDP per capita and antibiotic resistance prevalence has been demonstrated at the global level (Savoldi et al. 2020). In general, countries with high GDP per capita have improved conditions to prevent infections, which explains such correlation (Collignon et al. 2018), although it can be argued that higher antibiotic consumption, might contribute to the antibiotic resistance propagation, in the absence of adequate sanitary or health care conditions (Collignon and Beggs 2019). In Portugal, the business sector seems to be more active, with a higher birth rate of new companies, mainly dedicated to services (~76%). In Poland, companies are more evenly distributed between the services (57.4%) and industrial (40.2%) sectors (Table 1). The different types of companies present in the two countries may have a reflex in the type of residues that are being produced and discharged into UWWTPs and water bodies. The impact of some industries as sources of environmental contamination (e.g. heavy metals and antibiotics), may drive or accelerate the dissemination and possible diversification of antibiotic resistance in aquatic environments (Hubeny et al. 2021).

The indicators described in this section related to geography, demographics, climate, as well as to the economic status of Poland and Portugal are directly or indirectly connected to the volume of wastewater collected by sewage systems, and their contamination by chemical and microbial pollutants.

3. URBAN WASTEWATER TREATMENT PLANTS (UWWTPs)

Globally, a significant part of the human population lives in urban areas and an increasing percentage of the population is connected to the municipal sewage system. Therefore, it is assumed that UWWTP influents reflect the microbiome of the local human population, including the presence of ARB, ARGs, and mobile genetic elements (MGEs) (Hendriksen et al. 2019; Pärnänen et al. 2019). UWWTPs commonly employ several operations, including mechanical, biological, and chemical processes. Comparing urban wastewater agglomerations larger than 2 000 population equivalent (p.e.) in 2016, Poland had 1674 and Portugal 472 (Figure 1), which generated a total load of 36 $x10^6$ m^3 and 12 $x10^6$ m^3 (EEA 2021), respectively. These values are in good agreement with the population size in both countries, as it is also confirmed by Eurostat data (Eurostat 2020) that indicates that 73.6% and 85.0% of the population is connected to UWWTP in Poland and Portugal, respectively. Conventional wastewater treatment may include distinct successive stages: primary, secondary, and tertiary (more stringent) treatment (Quach-Cu et al. 2018). The percentage and number of UWWTPs (for agglomerations ≥ 2 000 p.e.) using different stages of treatment in Poland and Portugal is presented in Figure 1. According to the data available for the year 2016, in both countries most of the UWWTPs (97-98%) had at least secondary wastewater treatment system (Figure 1). Only a small fraction of UWWTPs relied exclusively on primary treatment (Figure 1), a method that is mostly used as a pre-treatment for further secondary and more stringent treatments. As the sole treatment process, primary treatment systems are usually applied in small plants with low daily processing values. Preliminary treatment is not expected to eliminate microorganisms in the liquid stream, and the reduction of viral, bacterial, and protozoan pathogens has been described to reach 0 to 1 log units after conventional primary sedimentation. It makes UWWTPs based solely on this treatment potential sources for the spread of antibiotic resistance (Marín et al. 2015; Oakley 2018; Szostkova et al. 2012).

Although the biggest share of the UWWTPs in both countries operates with a secondary treatment (~57-61%) (Figure 1), according to the latest EEA data (EEA 2021) this type of treatment serves 14.0% and 46.7% of the population in Poland and Portugal, respectively. Worldwide, the most common biological treatment processes include: the activated sludge (AS), aerobic sequencing batch reactor (SBR), membrane biological reactor (MBR), biological filter (BF), and upflow anaerobic sludge blanket (UASB) (Wang et al. 2020; Yuan et al. 2016). In Poland, the largest UWWTP (Czajka, Warsaw) with a dimension of 2 100 000 p.e. operates with an AS-based technology. The Portuguese largest UWWTPs are located in Lisbon and Loures, as Alcântara (756 000 p.e.) and Frielas (700 000 p.e.) UWWTP, operating with biological treatment carried out by BF using BIOSTYR® technology and modified AS process (Nereda® technology), respectively (ADP 2018; MPWIK 2021). On average, during biological treatment, the abundance of ARB and ARGs is efficiently reduced by 2 log-units (Wang et al. 2020). When comparing different methods of treatment: SBR and MBR processes significantly reduce ARB abundance, with log values ranging between 2.70-3.13 and 2.80-3.54, respectively, followed by AS system of 1.76-2.06 log (Yuan et al. 2016). BF and UASB are assumed to have lower reduction levels. AS and SBR also demonstrate significant potential on ARGs reduction, with log values ranging 2.36-4.24 and 1.66-3.56, respectively

(Yuan et al. 2016). BF resulted in a lower reduction of ARGs abundance (0.58-1.18 log-units). According to the same study (Yuan et al. 2016), MBR and UASB are the least effective in ARGs removal from wastewater. The AS treatment, the most common worldwide, has been suggested as being more effective than BF in removing ARB and ARGs (ARB log reduction: 1.76-2.06 and 2.36-4.24, respectively; ARGs log reduction: 0.87-1.23 and 0.58-1.18, respectively) (Yuan et al. 2016).

More stringent treatment, meaning post-secondary treatment, is applied to wastewater discharged in areas covering 76% of EU territory (EuropeanCommission 2017). In Poland and Portugal, more stringent treatment is used to treat wastewater from 59.5% and 38.0% of the population, respectively (EEA 2021). This type of treatment is utilized in ~36% of the UWWTPs in Poland and ~41% in Portugal (Figure 1). More stringent treatment often includes disinfection, an important barrier to reduce the release of ARB and ARGs into the environment (Guo et al. 2013; Rizzo et al. 2020). However, in Poland, permanent disinfection of treated wastewater in properly operated UWWTP (mechanical and biological) is not used. According to Polish law on collective water supply and collective sewage disposal (Michałkiewicz et al. 2011; PolishLaw 2005), only wastewater discharged from hospitals of infectious diseases, and those with such wards, and from blood donation stations must be disinfected before being discharged to municipal wastewater systems. Authorities' recommendations regarding the pre-treatment of hospital effluents are not in place in Portugal. Nevertheless, in Portugal, disinfection is used in numerous UWWTPs, mostly in coastal regions and during the summer season. For example, six of the 15 largest Portuguese UWWTPs use UV disinfection process (Guia, Alcântara, Frielas, Barreiro/Moita, Ave, and Chelas treatment plants), sometimes supplemented by sand filtration (Guia, Alcântara, and Chelas) (UWWTD 2016). As countries belonging to European Commission, Portugal and

Poland are under the Council Directive 91/271/EEC (EuropeanComission 2001) concerning urban wastewater treatment that was adopted on 21 May 1991 and that was under Online Public Consultation till July 2021. This Directive does not include any microbiological or specific chemical pollutant as surrogates to assess treatment quality, although each country is free to impose more restrictive criteria. This Directive aimed to minimize the impacts in the receiving environment, considering the type and load of anthropogenic substances, particles and microorganisms that might be a threat for the environment and humans 30 years ago. Both countries, Poland and Portugal, follow the European Directive.

In different world regions, the reuse of UWWTPs effluents has been encouraged as a method of water protection, although concerns regarding the spread of antibiotic resistance have been raised (Hong et al. 2018; Sorinolu et al. 2020; Zammit et al. 2020). In May 2020, the European Commission launched a new regulation (2020/741) on the minimum requirements for water reuse that defines the criteria for safe water reuse in agriculture irrigation (EU 2020). The microbiological parameters include microorganisms of faecal origin and do not specifically address the antibiotic resistance issue. In Poland, the use of treated wastewater for agriculture is legally permitted (EuropeanCommission 2016; PolishLaw 2017). However the practice is not applied and the reuse of treated wastewater for irrigation in agriculture has not been reported (EuropeanCommission 2016; Lemitor 2019). In Portugal, around 1% of the treated wastewater was reused in 2019 (> 78 million m^3/year) for agriculture and golf course irrigation, industry, or urban uses (ERSAR 2021; Rebelo et al. 2020).

4. ANTIBIOTICS CONSUMPTION

Antibiotics have been broadly used for years in humans, veterinary, livestock, and agricultural purposes. The high loads of antibiotic residues released in the environment represent an important fraction of contaminants of emerging concern (CECs), increasingly detected at low concentrations in wastewater, surface, ground, and drinking waters, as well as in soils and sediments (Booth et al. 2020; Rodriguez-Mozaz et al. 2020). Over the almost 80 years of antibiotic use, antibiotic residues, ARB, and ARGs have accumulated and spread in the environment, constituting a triad of presumably interlinked CECs. The use of antibiotics has been associated with resistance development, as numerous studies have demonstrated (Caron and Mousa 2010; Llor and Bjerrum 2014). This knowledge laid the foundations to encourage and recommend stewardship in antibiotic use, adopted by different European countries in the first decades of the 20th century (Klein et al. 2018).

Poland is among the European countries with the highest antibiotic consumption (community and hospital sector combined), reaching values of 23.6 defined daily dose (DDD) per 1 000 inhabitants per day in the year 2019, when the maximum was 34.1 in Greece (ESAC-Net 2020). In the same year, Portugal had an average consumption rate of 19.3 DDD per 1 000 inhabitants, close to the average level in EU/EEA countries. The average total consumption of antibacterials for systemic use (ATC group J01) in the EU/EEA was 19.4 DDD per 1 000 inhabitants, ranging from 9.5 to 34.1 (ESAC-Net 2020). Poland and Portugal had managed differently the antibiotic stewardship recommendations, as the compound annual growth rate (CAGR) of total antibiotic consumption reveals. Between the years 2010 and 2019, the CAGR values were calculated to be 2.4% for Poland, corresponding to an increase of use, and -0.4 % for Portugal, meaning a slight decrease. These trends were similar both in the community (primary care sector) and in the hospital sectors in each country (Figure 2). In the community sector, during the same period, Poland had a continuous increase (average 20.9 DDD per 1 000 inhabitants), while in Portugal had a slight decrease (average 17.6 DDD per 1 000 inhabitants), which followed the EU/EEA trend (average 19.0 DDD per 1 000 inhabitants). In the hospital sector, Poland and Portugal were below the average value for EU/EEA (1.8 DDD per 1 000 inhabitants, range 0.8 to 2.5), with values of 1.40 – 1.42 DDD per 1 000 inhabitants.

Beta-lactam antibiotics are the most used in the community and hospital sectors in EU/EEA, including Poland and Portugal (Figure 3). In the community, the second most used class of antibiotics was macrolides, lincosamides and streptogramins (MLS), followed by tetracyclines in Poland, as well as in EU/EAA, contrasting with Portugal where the third class of most used antibiotics was quinolones. In the hospital sector, MLS was the second most used class of antibiotics in Portugal, contrasting with Poland where this position was occupied by quinolones, as well as in EU/EAA (Figure 3).

Although antibiotics are mainly used in human health care, the prophylactic use of antibiotics in food animal production (e.g. poultry, swine, and cattle) takes the largest proportion of antibiotic consumption (Vaz-Moreira et al. 2019). Confirming this general European trend, data referring to the year 2014 showed that both in Poland and Portugal were used about double of antibiotics in animals, compared to humans, specifically 578 vs. 263 and 190 vs. 76, respectively (ECDC/EFSA/EMA 2017). Just a very small fraction (< 1%) of the antibiotics used for animals are consumed by companion animals (Figure S1a).

The latest ESVAC (European Surveillance of Veterinary Antimicrobial Consumption) report published in 2020, showed that sales of antibiotics for use in animals in Europe fell by more than 34% between 2011 and 2018 (EMA 2020). Contrary to the European trend, in Poland, from 2011 to 2018, it was registered an increase of 33% in overall sales (mg/population correction unit (PCU)) of veterinary antimicrobial agents (EMA 2020). In Portugal, in the same period, the overall sales (mg/PCU) increased 15%. The patterns of sales (mg/PCU) in Poland and Portugal were, in general similar, characterized by the predominance of tetracyclines, beta-lactams, and MLS (Figure S1b). However, the existing differences may be influenced by the type of animal population, production systems, and prescription guidelines or habits in both countries (EMA 2020).

5. ANTIBIOTIC RESISTANCE in HOSPITAL and PRIMARY CARE ISOLATES

In 2019, 30 EU/EEA countries participated in the European Antimicrobial Resistance Surveillance Network (EARS-Net) with routine antimicrobial susceptibility testing (AST) data from invasive (blood or cerebrospinal fluid) isolates under surveillance: *Escherichia coli* (Figure 4a), *Klebsiella pneumoniae* (Figure 4b), *Pseudomonas aeruginosa* (Figure 4c), *Acinetobacter* species (Figure 4d), *Streptococcus pneumoniae*, *Staphylococcus aureus* (Figure 4e), *Enterococcus faecalis* and *Enterococcus faecium* (*Enterococcus* species, Figure 4f) (ESAC-Net 2020). Portugal reported an estimated population coverage of 97% with high sample representativeness (ECDC 2020). Poland is one of the few countries reporting medium geographical coverage or hospital data representativeness, with a comparatively low population coverage (17%) of EARS-Net contributing laboratories and hospitals. This is an important limitation to the comparison between countries, with unknown implications in the reliability of data analysis and interpretation. However, it is suggested that in general, higher percentage of resistance was observed in Poland than in Portugal and EU/EEA (population-weighted mean percentage) (Figure 4).

In the group of *E. coli* isolates, the highest resistance prevalence values (%) were reported for aminopenicillins (61.6% in Poland and 58.5% in Portugal) and fluoroquinolones (33.0% in Poland and 26.5% in Portugal), followed by resistance to third-generation cephalosporins and aminoglycosides (Figure 4a). Combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides in *K. pneumoniae* isolates was reported at a higher percentage for both Poland (45.0%) and Portugal (26.5%) countries than the EU/EEA average values (Figure 4b). *P. aeruginosa*, with high rates of fluoroquinolone resistance, presented higher carbapenem-resistance values than *E. coli* or *K. pneumoniae,* and a higher percentage in Poland (24.4%) than in Portugal (17.8%). *Acinetobacter* species displayed the major differences in resistance prevalence between Poland and Portugal (39.9% for carbapenem and 59.4% for fluoroquinolones). In this bacterial genus, the percentage of fluoroquinolone resistance reported was 85.5% in Poland and 26.1% in Portugal, while for carbapenems it was 71.0% in Poland and 31.1% in Portugal (Figure 4d). In Portugal, the percentage of methicillin-resistant *Staphylococcus aureus* (MRSA) was 34.8%, a result of a significantly decreasing trend between 2015 and 2019 (ECDC 2020). In the same period, the MRSA prevalence reported by Poland ranged 15-16% (ECDC 2020). Since 2016, vancomycin resistance prevalence in *Enterococcus* species (*E. faecalis* and *E. faecium*) was low in Portugal, while it increased in Poland from 17.7% in 2015 to 44.0% in 2019 (ECDC 2020). High-level gentamicin resistance in *E. faecium* decreased over 10 years of surveillance in both countries, from 64.9% to 46.3% in Poland and 53.3% to 21.8% in Portugal. This resistance phenotype also decreased in Portugal in *E. faecalis* for the period 2015-2019 (ECDC 2020).

6. ANTIBIOTICS IN WASTEWATER

Wastewater has been considered an important mirror of the community use of antibiotics and antibiotic resistance status (Hendriksen et al. 2019; Pärnänen et al. 2019; Rodriguez-Mozaz et al. 2020). This section and the following focus on this aspect. Antibiotics are usually only partly metabolized in the human body, being excreted in urine and feces as active compounds, with the possibility of reaching the municipal wastewater system in this form.

Current UWWTP technologies, usually based on AS process, have been developed and successfully applied to control the dissemination of organic matter and nutrients (mainly nitrogen and phosphorus). Antibiotics may be refractory to biodegradation in conventional UWWTPs (Michael et al. 2013; Zhang and Li 2011).

According to ESAC-Net data, in Poland higher consumption of antibacterials was noted than in Portugal, especially at the community (primary care sector) level (Figure 2 and Figure 3). This tendency is however not mirrored in the concentration of antimicrobials observed in wastewater, as a higher concentration of antimicrobial agents was detected in Portuguese than in Polish wastewater systems (Table 2). However, the data being compared result from sporadic sampling events and possible influencing variables (e.g. season, share of hospitals and nursing/residential care facilities, retention time in the sewer network, wastewater treatment systems and sewage sludge management, among others) are not being considered. Other reasons limit a systematic comparison, mainly the fact that the measurement of antimicrobial agents concentration in wastewater is mainly performed as academic research and the data is scattered and rare. For example, data of beta-lactams quantification in wastewater is available only for Portugal (Table 2). Even when data are available for both countries, factors such as the number of studies/UWWTPs that were sampled, the type of sample that was collected (grab or composite), or the season in which samples were collected may have interfered with the results. Langas et al. (2019), observed high seasonal variability in the quantity of some targeted antibiotics in the raw wastewater of 4 Polish UWWTPs. In general, the concentration of antimicrobials in the raw wastewater was lower in summer than in winter (e.g. azithromycin - up to 0.216 µg/L and up to 24.145 µg/L; clarithromycin - up to 2.166 µg/L and up to 7.294 µg/L; sulfamethoxazole - up to 0.387 µg/L and up to 2.020 µg/L, respectively) (Langas et al. 2019). This variability can be explained by the increasing consumption of these antibiotics due to a higher incidence of respiratory tract infections in the winter (Lange et al. 2020).

In many cases, antimicrobial agents were detected in the UWWTP influent, both from Poland and Portugal, in concentrations higher than the predicted no effect concentrations (PNECs) for resistance selection, as was proposed by Bengtsson-Palme and Larsson (2016). A similar tendency was also noted for treated wastewater since conventional UWWTPs based on secondary treatment (which account for over 50% of all facilities; Figure 1) remove antimicrobial agents with different efficiency (Table 2). It is also important to note that some antimicrobials (e.g. penicillins) are degraded easier than e.g. fluoroquinolones or tetracyclines, which may accumulate in treatment sludges or in the water body receiving the treated wastewater. In Poland, tetracycline, which use is mainly in animals as reported above, was detected in treated wastewater in concentrations up to 0.240 µg/L, while in Portugal it varied from undetected to 2.420 µg/L.

The results obtained for macrolides in raw wastewater indirectly confirmed the growing use of the newer generation (i.e., broad-spectrum) of antimicrobials in Poland, such as the new class of macrolides (azithromycin and clarithromycin, if compared with erythromycin) (Table 2). However, such tendency was not confirmed in Portugal, where in the UWWTP influent was mainly detected erythromycin (up to 2.300 µg/L). It can be partly explained by the difference in medication structure in both countries, as well as the possibility to buy antimicrobials over the counter. It was estimated, for example, that in a UWWTP serving a municipality of about 571 350 residents (average flow 93 000 m^3/d; average load 750 000 p.e.) the mean load of azithromycin in the UWWTP inlet might reach 265 kg per year, and be reduced to 76.1 kg per year in the outlet (Langas et al. 2019). Azithromycin is a re-positioned macrolide antibiotic, active against both Gram-positive and -negative bacteria. However, the unique chemical architecture of this and other macrolides makes that in addition to the antibacterial effect they may also have an antiviral effect. Thus, the potential to treat or prevent viral co-infection (including new influenza A(H1N1), Zika, and possibly others) (effectiveness against SARS-CoV-2 is under debate) (Sterenczak et al. 2020; Tran et al. 2019), might increase their usage.

From the above, it can be concluded that constant discharge of treated wastewater constitutes an important load of pharmaceuticals introduced into the receiving water bodies, which relevant environmental burden must be assessed and mitigated whenever possible. Although there is a need to monitor some antimicrobials (e.g. amoxicillin, ciprofloxacin, and macrolides: erythromycin, clarithromycin, and azithromycin) and other micropollutants, such as azole compounds, at a European level (Decision_2020/1161 2020), there are no requirements regarding the extent at which they must be removed from wastewater. Efforts to remove micropollutants from wastewaters have been under discussion and an example of implementation and milestone definition is illustrated by Switzerland, where in 2016 was launched the legal basis for an additional (fourth) step of wastewater treatment. The goal is that by 2040 treatment processes are capable of reducing in 80% (primary clarified wastewater vs. final effluent) different surrogate substances (minimum 6 out of 12; clarithromycin - representing antibiotics; amisulpride, citalopram, venlafaxine - antidepressants; irbesartan, hydrochlorothiazide - antihypertensives; diclofenac - anti-inflammatories; metoprolol - beta blockers; carbamazepine, candesartan - tranquillisers; and benzotriazole, mecoprop – other substances). Special concern was given to populated regions, where surface water resources serve as a source of potable water as well as treated wastewater receivers have limited dilution capacity. The feasibility of upgrading (selected) UWWTPs to more advanced treatment, capable to eliminate a broad range of micropollutants at reasonable costs (in fit-for purpose manner) deserves more investigation.

7. ARB and ARGs ABUNDANCE in WASTEWATER

UWWTPs are recognized reservoirs of antibiotic resistance, where it is expected to find a high relative abundance of ARB and ARGs. Culture-dependent methods are commonly used to study the abundance of ARB, while ARGs quantification is frequently based on real-time PCR (Manaia et al. 2018). These have been the methods used for screening antibiotic resistance in raw and treated wastewater in Poland and Portugal. Because both are targeted methods and the experiments were designed independently, the comparison of data may be biased by the monitoring of distinct bacterial groups and ARGs (Tables S1 and S2). As noted before (Berendonk et al. 2015), the inexistence of recommendations about monitoring targets and methods is a major limitation to assess impacts and control risks due to environmental antibiotic resistance. However, the volume of studies on the subject suggests that some inter-study comparisons provide interesting insights (Krzeminski et al. 2019; Manaia et al. 2016). Hence, even considering that data from Portugal frequently refers to the broad group of enterobacteria, while Poland data refer to coliforms or *Escherichia coli*, it is possible to infer major patterns in both countries (Table S1).

An interesting observation is that despite the variations observed over distinct sites or sampling campaigns, Polish UWWTPs seem to achieve higher reductions of cultivable ARB. In both countries, total heterotrophic ARB were generally above 4 log-units colony forming units (CFU)/mL in raw wastewater, being the treatment processes responsible for reductions of 2-3 log-units in Poland and of 1-2 log-units in Portugal (Table S1, Figure 5a). Although loads of amoxicillin resistant bacteria were higher in Poland influents, due to the higher removal rates, after treatment the abundance of these culturable bacteria was identical in both countries (3-5 log CFU/mL). Enterobacteria presented higher counts in Portuguese wastewater, both before and after the treatment. However, these results may have been biased by the fact that coliforms and *E. coli*, measured in Poland, are subgroups of enterobacteria (Table S1). Marano et al. (2020) used a harmonized methodology to enumerate cefotaxime resistant coliforms in the influent and effluent of 57 UWWTPs, located in Europe, Asia, Africa, Australia, and North America (a total of 22 countries), including Poland and Portugal. In that study, Portugal presented lower counts of cefotaxime resistant coliforms in raw wastewater (1 log-unit lower) than Poland, although also lower removal efficiencies (removing 1-2 log-units, while in Poland were removed 1-3 log-units). Enterococci presented counts ranging 3-4 log CFU/mL in the influents of both countries, although higher loads in the final effluents in Portugal (1-2 log-units CFU/mL in Poland vs. 1-3 log-units CFU/mL in Portugal). In contrast, vancomycin resistant enterococci presented higher counts in influent and effluent samples in Poland (1-2 log-units above the values observed for Portugal) (Table S1, Figure 5a). This observation agrees with what was observed at the clinical level (Figure 4F), with a higher percentage of vancomycin resistant enterococci in Poland than in Portugal (17.7% in Poland vs. 3.0% in Portugal). The relationship between clinical and wastewater vancomycin resistant enterococci in Poland was suggested by Sadowy and Luczkiewicz (2014). These authors found isolates belonging to the nosocomial HiRECC (high-risk enterococcal clonal complex) in raw and treated wastewater as well as in the receiving coastal area of Gdansk Bay, Poland, and had evidence that suggested the ability of those clones to survive in the environment. These observations are aligned with the perspective that the urban community resistome is mirrored in their sewage (Aarestrup and Woolhouse 2020; Pärnänen et al. 2019). In turn, the contrast observed between Poland and Portugal suggests that local conditions may shape the fate of clinical bacteria thriving in the environment, as has been discussed about the ecology of enterococci from different geographic regions (Blanch et al. 2003).

Because most wastewater bacteria are non-culturable, the measurement of genes brings additional and relevant information about treatment efficiency. Genes conferring resistance to beta-lactams, tetracyclines, aminoglycosides, quinolones, sulfonamides, and integrase genes associated with integrons were among the most frequently monitored in Poland and Portugal (Table S2). Wastewater treatment unequivocally contributes to reducing the absolute gene abundance (expressed per volume of wastewater), although if a specific gene suffers lower removal rates than the average of bacteria, its relative abundance (expressed per 16S rRNA gene) may increase, suggesting the enrichment of that gene (Makowska 2019; Makowska et al. 2016; Narciso-da-Rocha et al. 2018; Narciso-da-Rocha et al. 2014; Osinska et al. 2020; Rocha et al. 2019; Zielinski et al. 2020).

The data compiled in Table S2 shows that treatment promoted reductions of total bacteria, measured based on the 16S rRNA gene quantification, of up to 1 log-unit (gene copy/mL) in both countries. This suggests that the culturable bacterial groups examined are more efficiently removed during wastewater treatment (2-3 log-units for Poland and 1-2 log-units for Portugal) than total bacteria, and/or that DNA-based methods may overestimate inactive bacteria (Tables S1 and S2, Figures 5a and 5b). Contrary to what was observed for ARB, the removal of ARGs was in the same order of magnitude in both countries (frequently 1-2 log-units per volume of water). Probably as a result of the different endurance of the host bacterial groups, treatment may lead to distinct patterns of variation in different genes. While in general there was a decrease in the abundance (per volume), the relative abundance (normalized per 16S rRNA) did not vary after treatment, although in some occasions it decreased or increased (Table S2, Figure 5c). This general pattern is expected since ARGs hosts and total bacteria are, in principle, removed at the same rate, with some exceptions for specific bacterial groups that are more or less extensively removed during treatment. Different studies have explored these dynamics by monitoring ARGs and the composition and structure of the wastewater bacterial communities in parallel (Fernandes et al. 2019; Grehs et al. 2019; Lira et al. 2020; Narciso-da-Rocha et al. 2018). Another important driver to explain the variable behavior of ARGs during wastewater treatment may be the accumulation of the bacterial ARGs hosts in activated sludges (Meng et al. 2020).

Concerning beta-lactamase genes (bla_{TEM}, bla_{OXA}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{VIM}), bla_{TEM} and bla_{OXA} were the most abundant in the wastewater of both countries. Although the abundance of those two genes varied over wastewater samples in Poland (Figure 5b), in general decreased 1-2 log-units (per volume) after the treatment, while the relative abundance was fairly stable. In Portugal, the decrease in bla_{TEM} reached 3 log-units, higher than of the 16S rRNA gene, as was reflected also in the decrease of the gene relative abundance. The opposite was observed with bla_{OXA} that might have been occasionally enriched after treatment (Figure 5b,c). Also, the genes $bla_{CTX\text{-}M}$ and bla_{VIM} were not efficiently removed from Portuguese wastewater. In Poland, the removal of the genes $bla_{CTX\text{-}M}$, and bla_{VIM} was higher, although only occasionally with implications on the reduction of the relative abundance (Figure 5c). The gene bla_{SHV} presented higher removal in Portuguese than in Polish UWWTPs. The differences observed for the removal of these ARGs, often associated with enterobacteria, may be connected to the ecology of bacteria, and the higher stability of some bacterial groups (heterotrophs or enterobacteria) in Portugal than in Poland. These effects may need to be considered when additional control measures are designed.

The abundance of the tetracycline resistance gene *tetM* in raw wastewater from Poland ranged 2-6 log-units/mL, with reductions of 1-3 log-units after the treatment. Only one study quantified *tetM* in Portugal, being quantified 5 log-units/mL of treated wastewater, the highest value detected in equivalent samples from Poland (Figure 5b). The fluoroquinolone resistance gene *qnrS* occurred in similar abundance in Polish and Portuguese raw wastewater, being suggested a more efficient removal in Portugal, reaching 3 log-units (per volume) vs. 1 log-unit observed in Poland (Figure 5b). The sulfonamide resistance genes *sul1* and *sul2,* and the integrase *intI1* gene were observed to be weakly removed by the wastewater treatments used in both countries, with maximum removal of 1 log-unit in the genes abundance and no change in the relative abundance (Table S2, Figure 5b,c). The abundance of *sul1* and *intI1* was observed to be in the same order of magnitude in Poland and Portugal, although *sul2* was more abundant in both Portuguese influents and effluents.

8. DOES THE CONTEXT MATTER?

One of the problems of dealing with the monitoring of dynamic and complex systems as UWWTPs is the high variability due to external and often not measurable variables. The values of ARB and ARGs abundance presented in this study reflect such variability that may be due to the properties of the wastewater, the treatment processes, analytical methodological options, season of sampling campaigns, among others. Studies conducted with multiple UWWTPs have highlighted that a vast array of factors may influence both the resistance in the raw wastewater and the treatment efficiency. If these differences have implications on the final impact that UWWTPs have in the receiving environment or if they can be explained based on measurable and controllable variables is still a major question (An et al. 2018; Limayem et al. 2019). One of the outcomes of the data compilation herein presented is the evidence that a holistic vision is necessary to better understand and control antibiotic resistance dissemination. It is not only important to measure the abundance of ARB and ARGs that are being released by the UWWTPs to the environment, but also to understand their ecology in a broader context, as well as the context where it occurs and the respective permissiveness to ARB dissemination. This knowledge may provide useful guidelines for optimizing the treatment systems regarding antibiotic resistance control. The characterization of the antibiotic resistance genotypes of some bacterial isolates recovered from wastewater samples has been performed both in Poland and Portugal (Table S3). Environmental *E. coli* and *Aeromonas* spp. are important harbours of a high diversity of ARGs, namely conferring resistance to beta-lactams and quinolones, and some studies have shown that some of those strains can survive treatment, even when UV or ozone disinfection is used (Dolejska et al. 2011; Tavares et al. 2020; Varela et al. 2015; Varela et al. 2016). *Enterococcus faecium, Klebsiella pneumoniae*, and *Enterobacter* spp. are other important vehicles of antibiotic resistance dissemination in wastewater (Table S3). Although the characterization of isolates is very important for better assessing the association of ARGs to their hosts, it has the limitation of requiring cultivation disregarding the non-cultivable bacteria fraction, which are the majority in environmental samples (Manaia et al. 2018). The use of culture-independent approaches surpasses these limitation and can be applied using non-targeted methods to analyse the wastewater resistome. The use of correlation and clustering analysis allowed to infer for Portuguese wastewater which taxonomic groups are potential harbours of ARGs. For example, Fernandes et al. (2019) concluded that members of the families *Aeromonadaceae*, *Campylobacteraceae*, *Veillonellaceae*, [*Weeksellaceae*], and *Porphyromonadaceae* were positively correlated with the genes $bla_{CTX\text{–M}}$, $bla_{OXA\text{-A}}$, bla_{SHV}, and *intI1*, while *Intrasporangiaceae* were negatively correlated. Narciso-da-Rocha et al. (2018) also observed that the families *Campylobacteraceae*, *Comamonadaceae*, *Aeromonadaceae*, *Moraxellaceae*, and *Bacteroidaceae*, highly abundant in raw wastewater samples, strongly correlated with the genes *qnrS*, $bla_{CTX\text{-M}}$, $bla_{OXA\text{-A}}$, bla_{TEM}, bla_{SHV}, *sul1*, *sul2*, and *intI1*.

9. CONCLUSIONS/ FINAL REMARKS

Poland and Portugal have similar demographic characteristics with different climatic conditions, resulting from the respective geographic location. Poland holds a strong industrial sector, while in Portugal services, mainly tourism, are pillars of the economy. Inevitably, this profile generates a distinct pattern and load of pollution in both countries. Unfortunately, these data are not available. While in Poland the consumption of antibiotics is higher, mainly at the community level, it is also in this country that health expenditure is lower, although legislation recommends dedicated treatment of hospital effluents generated at infectious diseases wards and blood donation stations. The fact that Poland has a poor adherence to clinical ARB surveillance (17% population coverage) and a later start of reporting antibiotic consumption explains why resistance trends are not yet to decrease as it is apparent in Portugal. This is both a cause and a consequence of a lower awareness for the problem of resistance in Poland than in Portugal, among the community and the authorities. Although Poland has a National Antibiotic Protection Program (http://antybiotyki.edu.pl/) in place since 2004. In Portugal, the national plan to fight antimicrobial resistance with the major objective of reducing the consumption of antibiotics (https://www.dgs.pt/programa-nacional-de-controlo-da-infeccao) is in place since 2010 and is now producing the first results. After some years of a critical situation of clinical antibiotic resistance, Portugal is now tracing a corrective path characterized by the decrease of antibiotic use and also by the reduction of some pathogens, as MRSA. However, these measures may have a lower impact than in other world regions since wastewater loads of antibiotic resistance and removal rates due to treatment are in general low in this country. While wastewater resistome has been considered a good sensor of the resistance occurrence in a region (Aarestrup and Woolhouse 2020; Pärnänen et al. 2019), the treatment efficiency will be crucial to determine the impacts of community and health-care related resistance on the environment. UWWTPs are major barriers to protect the environment. Even if the situation improves at the clinical level, efforts are still needed to avoid the dissemination to the environment and, eventually, back to humans. In a European study, the comparatively lower capacity of UWWTPs to remove ARGs in Southern countries (where Portugal was included) compared with Northern countries was demonstrated (Pärnänen et al. 2019), reinforcing the need for holistic characterization and interventions. Southern countries have higher temperatures, more favourable for human and animal commensal survival and proliferation in the environment, explaining positive correlations between antibiotic resistance and temperature (McGough et al. 2020). This trend was confirmed here mainly for culturable ARB. This also suggests that wastewater treatment may need to be customized for each socioeconomic and climate context. The load of resistance and microbial community composition of the sewage, the type of treatment implemented, the degree of general pollution, the organic matter load, and other factors such as temperature may dictate the efficiency of each treatment process. The duration of aeration/anoxic cycles, the addition of flocculation agents, the extension or reduction of hydraulic residence time, or the composition of treating activated sludge biomass may need to be adjusted to specific treatment scenarios, which are nowadays mainly conducted as black boxes in what concerns antibiotic resistance removal.

This study based on two countries aims also to demonstrate that, despite the numerous limitations, there is nowadays a plethora of antibiotic resistance information available worldwide that can be crossed with socioeconomic and climate data, allowing the establishment of informing principles to control antibiotic resistance.

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Declaration of interest

None to declare.

REFERENCES

Aarestrup, F.M. and Woolhouse, M.E. (2020). Using sewage for surveillance of antimicrobial resistance. *Science* 367, 630-632.

ADP (2018) *Águas de Portugal: Alcântara WWTP "The Water Factory"* Retreived from http://www.danube-water-program.org/media/Events/2018/WB_REgulators_Study_Tour/201801012_-_Visita__Alcantara.pdf

Ahmed, W., Zhang, Q., Lobos, A., Senkbeil, J., Sadowsky, M.J., Harwood, V.J., Saeidi, N., Marinoni, O. and Ishii, S. (2018). Precipitation influences pathogenic bacteria and antibiotic resistance gene abundance in storm drain outfalls in coastal sub-tropical waters. *Environment International* 116, 308-318.

An, X.-L., Su, J.-Q., Li, B., Ouyang, W.-Y., Zhao, Y., Chen, Q.-L., Cui, L., Chen, H., Gillings, M.R. and Zhang, T. (2018). Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR. *Environment International* 117, 146-153.

Barbosa, A.E., Fernandes, J.N. and David, L.M. (2012). Key issues for sustainable urban stormwater management. *Water Research* 46, 6787-6798.

Bengtsson-Palme, J. and Larsson, D.G. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environment International* 86, 140-149.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F. and Martinez, J.L. (2015). Tackling antibiotic resistance: the environmental framework. *Nature Reviews Microbiology* 13, 310-317.

Blanch, A.R., Caplin, J.L., Iversen, A., Kuhn, I., Manero, A., Taylor, H.D. and Vilanova, X. (2003). Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *Journal of Applied Microbiology* 94, 994-1002.

Booth, A., Aga, D.S. and Wester, A.L. (2020). Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. *Environment International* 141, 105796.

Caron, W.P. and Mousa, S.A. (2010). Prevention strategies for antimicrobial resistance: a systematic review of the literature. *Infection and Drug Resistance* 3, 25-33.

Cassini, A., Hogberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., Ouakrim, D.A., Oliveira, T.C., Struelens, M.J., Suetens, C., Monnet, D.L. and Burden of, A.M.R.C.G.

(2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infectious Diseases* 19, 56-66.

Collignon, P. and Beggs, J.J. (2019). Socioeconomic enablers for contagion: factors impelling the antimicrobial resistance epidemic. *Antibiotics* 8, 86.

Collignon, P., Beggs, J.J., Walsh, T.R., Gandra, S. and Laxminarayan, R. (2018). Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. *Lancet Planet Health* 2, e398-e405.

de Jesus Gaffney, V., Cardoso, V.V., Cardoso, E., Teixeira, A.P., Martins, J., Benoliel, M.J. and Almeida, C.M.M. (2017). Occurrence and behaviour of pharmaceutical compounds in a Portuguese wastewater treatment plant: Removal efficiency through conventional treatment processes. *Environmental Science and Pollution Research* 24, 14717-14734.

Decision_2020/1161 (2020). Commission Implementing Decision (EU) 2020/1161 of 4 August 2020 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council.

Di Cesare, A., Eckert, E.M., Rogora, M. and Corno, G. (2017). Rainfall increases the abundance of antibiotic resistance genes within a riverine microbial community. *Environmental Pollution* 226, 473-478.

Dolejska, M., Frolkova, P., Florek, M., Jamborova, I., Purgertova, M., Kutilova, I., Cizek, A., Guenther, S. and Literak, I. (2011). CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella* spp. isolates in municipal wastewater treatment plant effluents. *Journal of Antimicrobial Chemotherapy* 66, 2784-2790.

ECDC (2020). European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2019. *Stockolm*.

ECDC/EFSA/EMA (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals: Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA journal* 15, e04872.

EEA, E.E.A. (2020a). Bathing water management in Europe: Successes and challenges.

EEA, E.E.A. (2020b). European bathing water quality in 2019.

EEA, E.E.A. (2021) *Urban waste water treatment in Europe.* . Retreived from https://www.eea.europa.eu/

EMA (2020). Sales of veterinary antimicrobial agents in 31 European countries in 2018. Trends from 2010 to 2018. Tenth ESVAC report.

Eramo, A., Delos Reyes, H. and Fahrenfeld, N.L. (2017). Partitioning of antibiotic resistance genes and fecal indicators varies intra and inter-storm during combined sewer overflows. *Frontiers in Microbiology* 8, 2024.

ERSAR (2021). Relatório Anual dos Serviços de Águas e Resíduos em Portugal (RASARP) - Caracterização do sector de águas e resíduos

ESAC-Net (2020) *ESAC-Net, interactive database.* Retreived from https://ecdc.europa.eu/en/antimicrobial-consumption/surveillance-and-disease-data/database

EU (2020). Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse.

EuropeanComission (2001). Council Directive 91/271/EEC - Collective Supply of Water and Collective Waste Disposal. *Journal of Laws 2001 no. 72 item 747*.

EuropeanCommission (2016). EU-level instruments on water reuse Final report to support the Commission's Impact Assessment. Prepared by Amec Foster Wheeler Environment & Infrastructure UK Ltd, IEEP, ACTeon, IMDEA and NTUA.

EuropeanCommission (2017). Report from the commission to the European Parliament, the council, the European Economic and Social Committee and the Committee of the regions. Brussels.

Eurostat (2020). Population connected to urban wastewater collecting and treatment systems, by treatment level. Urban waste water collection and treatment in Europe, 2017. .

Fair, R.J. and Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspectives in medicinal chemistry* 6, PMC. S14459.

Fernandes, T., Vaz-Moreira, I. and Manaia, C.M. (2019). Neighbor urban wastewater treatment plants display distinct profiles of bacterial community and antibiotic resistance genes. *Environmental Science and Pollution Research International* 26, 11269-11278.

Giebułtowicz, J., Nałęcz-Jawecki, G., Harnisz, M., Kucharski, D., Korzeniewska, E. and Płaza, G. (2020). Environmental risk and risk of resistance selection due to antimicrobials' occurrence in two polish wastewater treatment plants and receiving surface water. *Molecules* 25, 1470.

Grehs, B.W., Lopes, A.R., Moreira, N.F., Fernandes, T., Linton, M.A., Silva, A.M., Manaia, C.M., Carissimi, E. and Nunes, O.C. (2019). Removal of microorganisms and antibiotic resistance genes from treated urban wastewater: A comparison between aluminium sulphate and tannin coagulants. *Water Research* 166, 115056.

Guo, M.T., Yuan, Q.B. and Yang, J. (2013). Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater. *Chemosphere* 93, 2864-2868.

Harnisz, M., Korzeniewska, E. and Gołaś, I. (2015). The impact of a freshwater fish farm on the community of tetracycline-resistant bacteria and the structure of tetracycline resistance genes in river water. *Chemosphere* 128, 134-141.

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Roder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Global Sewage Surveillance project, c., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N. and Aarestrup, F.M. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature communications* 10, 1124.

Hernando-Amado, S., Coque, T.M., Baquero, F. and Martinez, J.L. (2019). Defining and combating antibiotic resistance from One Health and Global Health perspectives. *Nature Microbiology* 4, 1432-1442.

Honda, R., Tachi, C., Yasuda, K., Hirata, T., Noguchi, M., Hara-Yamamura, H., Yamamoto-Ikemoto, R. and Watanabe, T. (2020). Estimated discharge of antibiotic-resistant bacteria from combined sewer overflows of urban sewage system. *npj Clean Water* 3, 15.

Hong, P.-Y., Julian, T.R., Pype, M.-L., Jiang, S.C., Nelson, K.L., Graham, D., Pruden, A. and Manaia, C.M. (2018). Reusing treated wastewater: consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes. *Water* 10, 244.

Hubeny, J., Harnisz, M., Korzeniewska, E., Buta, M., Zieliński, W., Rolbiecki, D., Giebułtowicz, J., Nałęcz-Jawecki, G. and Płaza, G. (2021). Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. *PLoS One* 16, e0252691.

Keller, V.D., Williams, R.J., Lofthouse, C. and Johnson, A.C. (2014). Worldwide estimation of river concentrations of any chemical originating from sewage-treatment plants using dilution factors. *Environmental Toxicology and Chemistry* 33, 447-452.

Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H. and Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences* 115, E3463-E3470.

Krzeminski, P., Tomei, M.C., Karaolia, P., Langenhoff, A., Almeida, C.M.R., Felis, E., Gritten, F., Andersen, H.R., Fernandes, T. and Manaia, C.M. (2019). Performance of secondary wastewater treatment methods for the removal of contaminants of emerging concern implicated in crop uptake and antibiotic resistance spread: A review. *Science of the Total Environment* 648, 1052-1081.

Langas, V., Garnaga-Budrė, G., Björklund, E., Svahn, O., Tränckner, J., Kaiser, A. and Luczkiewicz, A. (2019) *Determination of the Regional Pharmaceutical Burden in 15 Selected WWTPs and Associated Water Bodies using Chemical Analysis, Status in four coastal regions of the South Baltic Sea; Germany, Sweden, Poland and Lithuania.* Retreived from http://www.morpheus-project.eu/downloads/

Lange, J., Kozielski, J., Bartolik, K., Kabicz, P. and Targowski, T. (2020). Analysis of the incidence of acute respiratory diseases in the paediatric population in Poland in the light of the “Health Needs Map”. *Advances in Respiratory Medicine* 88, 204-214.

Lemitor (2019). Ocena projektu rozporządzenia Parlamentu Europejskiego i Rady w sprawie minimalnych wymogów dotyczących ponownego wykorzystania wody. Wrocław.

Limayem, A., Wasson, S., Mehta, M., Pokhrel, A.R., Patil, S., Nguyen, M., Chen, J. and Nayak, B. (2019). High-throughput detection of bacterial community and its drug-resistance profiling from local reclaimed wastewater plants. *Frontiers in Cellular and Infection Microbiology* 9, 303.

Lira, F., Vaz-Moreira, I., Tamames, J., Manaia, C.M. and Martínez, J.L. (2020). Metagenomic analysis of an urban resistome before and after wastewater treatment. *Scientific Reports* 10, 1-9.

Llor, C. and Bjerrum, L. (2014). Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Ther Adv Drug Saf* 5, 229-241.

Luczkiewicz, A., Felis, E., Ziembinska, A., Gnida, A., Kotlarska, E., Olanczuk-Neyman, K. and Surmacz-Gorska, J. (2013). Resistance of *Escherichia coli* and *Enterococcus* spp. to selected antimicrobial agents present in municipal wastewater. *Journal of Water and Health* 11, 600-612.

MacFadden, D.R., McGough, S.F., Fisman, D., Santillana, M. and Brownstein, J.S. (2018). Antibiotic resistance increases with local temperature. *Nature Climate Change* 8, 510-514.

Makowska, N. (2019). Geny integraz i oporności na antybiotyki β-laktamowe i glikopeptydowe rezystomu ścieków miejskich *Faculty of Biology*: Adam Mickiewicz University in Pozna.

Makowska, N., Koczura, R. and Mokracka, J. (2016). Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere* 144, 1665-1673.

Manaia, C.M., Macedo, G., Fatta-Kassinos, D. and Nunes, O.C. (2016). Antibiotic resistance in urban aquatic environments: can it be controlled? *Applied Microbiology and Biotechnology* 100, 1543-1557.

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C. and Zammit, I. (2018). Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environment International* 115, 312-324.

Marano, R.B., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Telson, T., Corno, G. and Fatta-Kassinos, D. (2020). A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. *Environment International* 144, 106035.

Marcelino, R., Queiroz, M., Amorim, C., Leão, M. and Brites-Nóbrega, F. (2015). Solar energy for wastewater treatment: review of international technologies and their applicability in Brazil. *Environmental Science and Pollution Research* 22, 762-773.

Marín, I., Goñi, P., Lasheras, A. and Ormad, M. (2015). Efficiency of a Spanish wastewater treatment plant for removal potentially pathogens: Characterization of bacteria and protozoa along water and sludge treatment lines. *Ecological Engineering* 74, 28-32.

McEwen, S.A. and Collignon, P.J. (2018). Antimicrobial Resistance: a One Health Perspective. *Microbiology Spectrum* 6(2).

McGough, S.F., MacFadden, D.R., Hattab, M.W., Molbak, K. and Santillana, M. (2020). Rates of increase of antibiotic resistance and ambient temperature in Europe: a cross-national analysis of 28 countries between 2000 and 2016. *Euro Surveill* 25.

Meng, L., Wang, J. and Li, X. (2020). Insight into effect of high-level cephalexin on fate and driver mechanism of antibiotics resistance genes in antibiotic wastewater treatment system. *Ecotoxicology and environmental safety* 201, 110739.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C. and Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Research* 47, 957-995.

Michałkiewicz, M., Jeż-Walkowiak, J., Dymaczewski, Z. and Sozański, M.M. (2011). Wastewater disinfection. *Inżynieria Ekologiczna* 24, 38-51.

Mishra, S., Kneis, D., Berendonk, T.U. and Aubeneau, A. (2019). Optimum positioning of wastewater treatment plants in a river network: A model-based approach to minimize microbial pollution. *Science of the Total Environment* 691, 1310-1319.

MPWIK (2021) *Oczyszczalnia Ścieków „Czajka". W: Miejskie Przedsiębiorstwo Wodociągów i Kanalizacji w m.st. Warszawie.*

Narciso-da-Rocha, C., Rocha, J., Vaz-Moreira, I., Lira, F., Tamames, J., Henriques, I., Martinez, J.L. and Manaia, C.M. (2018). Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. *Environment International* 118, 179-188.

Narciso-da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C. and Manaia, C.M. (2014). bla(TEM) and vanA as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. *Journal of Global Antimicrobial Resistance* 2, 309-315.

Oakley, S. (2018). Preliminary Treatment and Primary Settling In J. B. R. a. B. Jiménez-Cisneros (ed.) *Global Water Pathogen Project.* . Michigan State University, E. Lansing, MI: UNESCO.

Osinska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestepski, S. and Konopka, I. (2020). Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. *Journal of Hazardous Materials* 381, 121221.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sorum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V.D., Walsh, F., Vaz-Moreira, I., Virta, M. and Manaia, C.M. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science Advances* 5, eaau9124.

Pena, A., Albert-Garcia, J.R., Silva, L.J., Lino, C.M. and Calatayud, J.M. (2010). Photo-induced fluorescence of magnesium derivatives of tetracycline antibiotics in wastewater samples. *Journal of Hazardous Materials* 179, 409-414.

Pereira, A.M., Silva, L.J., Lino, C.M., Meisel, L.M. and Pena, A. (2016). Assessing environmental risk of pharmaceuticals in Portugal: An approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU. *Chemosphere* 144, 2507-2515.

PolishLaw (2005). On the amendment to the Act on collective water supply and collective sewage disposal and certain other acts *Journal Of Laws No. 85, item 729 of 16 May 2005 16*.

PolishLaw (2017). Water Law. Art. 84. [Agricultural use of wastewater]].

Quach-Cu, J., Herrera-Lynch, B., Marciniak, C., Adams, S., Simmerman, A. and Reinke, R.A. (2018). The effect of primary, secondary, and tertiary wastewater treatment processes on antibiotic resistance gene (ARG) concentrations in solid and dissolved wastewater fractions. *Water* 10, 37.

Rebelo, A., Quadrado, M., Franco, A., Lacasta, N. and Machado, P. (2020). Water reuse in Portugal: New legislation trends to support the definition of water quality standards based on risk characterization. *Water Cycle* 1, 41-53.

Rizzo, L., Fiorentino, A. and Anselmo, A. (2012). Effect of solar radiation on multidrug resistant E. coli strains and antibiotic mixture photodegradation in wastewater polluted stream. *Science of the Total Environment* 427, 263-268.

Rizzo, L., Gernjak, W., Krzeminski, P., Malato, S., McArdell, C.S., Perez, J.A.S., Schaar, H. and Fatta-Kassinos, D. (2020). Best available technologies and treatment trains to address current challenges in urban wastewater reuse for irrigation of crops in EU countries. *Science of the Total Environment* 710, 136312.

Rocha, J., Fernandes, T., Riquelme, M.V., Zhu, N., Pruden, A. and Manaia, C.M. (2019). Comparison of Culture- and Quantitative PCR-Based Indicators of Antibiotic Resistance in Wastewater, Recycled Water, and Tap Water. *Int J Environ Res Public Health* 16.

Rodriguez-Mozaz, S., Vaz-Moreira, I., Della Giustina, S.V., Llorca, M., Barceló, D., Schubert, S., Berendonk, T.U., Michael-Kordatou, I., Fatta-Kassinos, D. and Martinez, J.L. (2020). Antibiotic residues in final effluents of European wastewater treatment plants and their impact on the aquatic environment. *Environment International* 140, 105733.

Sadowy, E. and Luczkiewicz, A. (2014). Drug-resistant and hospital-associated Enterococcus faecium from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiology* 14, 1-15.

Salgado, R., Noronha, J., Oehmen, A., Carvalho, G. and Reis, M. (2010). Analysis of 65 pharmaceuticals and personal care products in 5 wastewater treatment plants in Portugal using a simplified analytical methodology. *Water Science and Technology* 62, 2862-2871.

Savoldi, A., Carrara, E. and Tacconelli, E. (2020). Gross national income and antibiotic resistance in invasive isolates: analysis of the top-ranked antibiotic-resistant bacteria on the

2017 WHO priority list—authors' response. *Journal of Antimicrobial Chemotherapy* 75, 2018-2018.

Sorinolu, A.J., Tyagi, N., Kumar, A. and Munir, M. (2020). Antibiotic Resistance Development and Human Health Risks during Wastewater Reuse and Biosolids Application in Agriculture. *Chemosphere*, 129032.

Sterenczak, K.A., Barrantes, I., Stahnke, T., Stachs, O., Fuellen, G. and Undre, N. (2020). Co-infections: testing macrolides for added benefit in patients with COVID-19. *The Lancet Microbe* 1, e313.

Szostkova, M., Vitez, T., Marecek, J. and Losak, T. (2012). Microbial contamination of screenings from wastewater treatment plants. *Polish Journal of Environmental Studies* 21.

Tavares, R.D., Tacão, M., Figueiredo, A.S., Duarte, A.S., Esposito, F., Lincopan, N., Manaia, C.M. and Henriques, I. (2020). Genotypic and phenotypic traits of blaCTX-M-carrying *Escherichia coli* strains from an UV-C-treated wastewater effluent. *Water Research* 184, 116079.

Tran, D.H., Sugamata, R., Hirose, T., Suzuki, S., Noguchi, Y., Sugawara, A., Ito, F., Yamamoto, T., Kawachi, S., Akagawa, K.S., Omura, S., Sunazuka, T., Ito, N., Mimaki, M. and Suzuki, K. (2019). Azithromycin, a 15-membered macrolide antibiotic, inhibits influenza A(H1N1)pdm09 virus infection by interfering with virus internalization process. *The Journal of Antibiotics (Tokyo)* 72, 759-768.

UWWTD (2016) *Urban Wastewater Treatment Directive Structured Implementation and Information Framework 2016.* Retreived from https://uwwtd.eu/Poland/stats

Varela, A.R., Andre, S., Nunes, O.C. and Manaia, C.M. (2014). Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. *Water Research* 54, 327-336.

Varela, A.R., Macedo, G.N., Nunes, O.C. and Manaia, C.M. (2015). Genetic characterization of fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital effluents. *FEMS Microbiology Ecology* 91.

Varela, A.R., Nunes, O.C. and Manaia, C.M. (2016). Quinolone resistant Aeromonas spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. *Science of the Total Environment* 542, 665-671.

Vaz-Moreira, I., Ferreira, C., Nunes, O.C. and Manaia, C.M. (2019). Sources of Antibiotic Resistance: Zoonotic, Human, Environment. *Antibiotic Drug Resistance*, 211-238.

Wang, J., Chu, L., Wojnárovits, L. and Takács, E. (2020). Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview. *Science of the Total Environment*, 140997.

Yuan, Q.-B., Guo, M.-T., Wei, W.-J. and Yang, J. (2016). Reductions of bacterial antibiotic resistance through five biological treatment processes treated municipal wastewater. *Environmental Science and Pollution Research* 23, 19495-19503.

Zammit, I., Marano, R.B., Vaiano, V., Cytryn, E. and Rizzo, L. (2020). Changes in antibiotic resistance gene levels in soil after irrigation with treated wastewater: a comparison between heterogeneous photocatalysis and chlorination. *Environmental science & technology* 54, 7677-7686.

Zhang, T. and Li, B. (2011). Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. *Critical Reviews in Environmental Science and Technology* 41, 951-998.

Zielinski, W., Korzeniewska, E., Harnisz, M., Hubeny, J., Buta, M. and Rolbiecki, D. (2020). The prevalence of drug-resistant and virulent *Staphylococcus* spp. in a municipal wastewater treatment plant and their spread in the environment. *Environment International* 143, 105914.

Zouboulis, A. and Tolkou, A. (2015). Effect of climate change in wastewater treatment plants: reviewing the problems and solutions *Managing water resources under climate uncertainty*, 197-220): Springer.

Tables and Figures:

Table 1: Territory and population characteristics, climate and weather conditions, and hydrological information for Poland and Portugal.

Table 2. Occurrence of antibiotics (µg/L) in wastewater influent and effluents in Poland and Portugal compared with predicted no-effect concentrations (PNEC)

(de Jesus Gaffney et al. 2017; Giebułtowicz et al. 2020; Harnisz et al. 2015; Langas et al. 2019; Luczkiewicz et al. 2013; Pena et al. 2010; Pereira et al. 2016; Rodriguez-Mozaz et al. 2020; Salgado et al. 2010; Varela et al. 2014)

Figure 1. Types of treatment reported at urban wastewater treatment plants (UWWTPs) for agglomerations ≥ 2 000 p.e. (population equivalent) in Poland (**PL**) and Portugal (**PT**). The number of UWWTPs reported per country is indicated above the bar. More stringent treatments include disinfection (chlorination, UV, or ozonisation), sand filtration, microfiltration (e.g., membrane filtration), and other types of unspecified additional treatments.

Source: The European Environment Agency (EEA). Available from: https://www.eea.europa.eu/

Figure 2. Total consumption of antibacterials for systemic use (ATC group J01) in the **community (bars)** and in the **hospital sector (lines)** for 2009–2018 (expressed as defined daily dose, DDD per 1 000 inhabitants per day) for Poland (**PL**), Portugal (**PT**) and average values for **EU/EEA** (European Union and European Economic Area) countries. No data is available for the hospital sector in Poland before 2014.

The population under surveillance was of about 38 million for Poland and about 9-10 million for Portugal.

Source: European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA, annual epidemiological report for 2020 (ECDC, 2020) and ESAC-Net *interactive database*, available from: https://ecdc.europa.eu/en/antimicrobial-consumption/surveillance-and-disease-data/database

Figure 3. Human consumption of different classes of antibacterials in the **(a)** community (primary care sector) and **(b)** hospital sector in the **year of 2019** (expressed as defined daily dose, DDD per 1 000 inhabitants) for Poland (**PL**), Portugal (**PT**) and average values for **EU/EEA** (European Union and European Economic Area) countries.

Classes of antibacterials: Tetracyclines (J01A), Beta-lactams (J01C and J01D), Sulfonamides and trimethoprim (J01E), MLS (Macrolides, lincosamides and streptogramins (J01F)), Quinolones (J01M), and Others include the sum of Amphenicols (J01B), Aminoglycosides (J01G), combinations of antibacterials (J01R) and other antibacterials (J01X).

Source: European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA, annual epidemiological report for 2020 (ECDC 2020) and ESAC-Net *interactive database*, available from: https://ecdc.europa.eu/en/antimicrobial-consumption/surveillance-and-disease-data/database

Figure 4. Percentage values of antibiotic resistance in hospital and primary care (invasive) isolates in Poland (**PL**), Portugal (**PT**) and average values in **EU/EEA** for the year **2019**. Data included most of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*), *Acinetobacter* spp., *Escherichia coli* and *Enterococcus faecalis.* Combined resistance includes: for *E. coli* and *K. pneumoniae*, resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides; for *Pseudomonas aeruginosa*, resistance to three or more antimicrobial groups among piperacillin ± tazobactam, ceftazidime, fluoroquinolones, aminoglycosides, and carbapenems; for *Acinetobacter* species, resistance to fluoroquinolones, aminoglycosides and carbapenems. EU/EEA values are not available (n/a) for all classes of antibiotics for *Enterococcus* spp.

Source: European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU/EEA, annual epidemiological report for 2019 (ECDC 2020) and ESAC-Net *interactive database*, available from: https://ecdc.europa.eu/en/antimicrobial-consumption/surveillance-and-disease-data/database

Figure 5. ARB abundance **(a)**, ARGs abundance **(b)** and relative abundance **(c)** in UWWTP influent (**I**) and effluent (**E**), in Poland (**PL**) and Portugal (**PT**). R_{AMX}, resistant to amoxicillin; R_{TET}, resistant to tetracycline; R_{CIP}, resistant to ciprofloxacin; R_{CTX}, resistant to cefotaxime; R_{VAN}, resistant to vancomycin. bla_{OXA} includes $bla_{OXA\text{-A}}$, and $bla_{OXA\text{-1}}$.

Note: Effluents from tertiary treatment are not represented in this figure; data is available in Tables S1 and S2. Most of the Polish data on *Enterobacteria* is reporting coliforms of *E. coli* counts (please see Table S1 for detail).