Integrated antibiotic resistance (AR) surveillance is one of the objectives of the World Health Organization global action plan on antimicrobial resistance. Urban wastewater treatment plants (UWTPs) are among the most important receptors and sources of environmental AR. On the basis of the consistent observation of an increasing north-to-south clinical AR prevalence in Europe, this study compared the influent and final effluent of 12 UWTPs located in seven countries (Portugal, Spain, Ireland, Cyprus, Germany, Finland, and Norway). Using highly parallel quantitative polymerase chain reaction, we analyzed 229 resistance genes and 25 mobile genetic elements. This first trans-Europe surveillance showed that UWTP AR profiles mirror the AR gradient observed in clinics. Antibiotic use, environmental temperature, and UWTP size were important factors related with resistance persistence and spread in the environment. These results highlight the need to implement regular surveillance and control measures, which may need to be appropriate for the geographic regions.

INTRODUCTION

Antibiotic-resistant bacteria (ARB) can survive the inhibitory action of one or more antibiotics. These ARB reduce the success of infectious disease treatment, which results in important societal and economic costs to human well-being and health (1). ARB and their resistance genes are emerging and spreading globally among people, food, animals, plants, and the environment (soil, water, and air) (2, 3). The global action plan on antimicrobial resistance proposed by the World Health Organization (WHO) aims at combating antibiotic resistance at a global scale and across every domain of interface with humans, where the environment is implicitly included (1, 4). Surveillance and the assessment of control measures and the identification of geographical or temporal trends of antibiotic resistance distribution have been consistently shown to be crucial to understanding the impact of antibiotic resistance on human health (1, 2). However, although clinical surveillance efforts are now capable of providing insightful information on antibiotic resistance distribution (1, 5), there is a dearth of data concerning antibiotic resistance in the environment.

Urban wastewater treatment plants (UWTPs) have been recognized as one of the most important routes for propagation of antibiotic resistance from humans to the environment (e.g., fresh water, soil) (2). The sewage entering the UWTPs combines the excreta and residues produced in the served area. Therefore, it is expected that the UWTP influents mirror, at least in part, traits of the microbiome of the human population served (2, 6, 7), including the presence of ARB, resistance genes, and associated mobile genetic elements.

This study aimed at launching the first European antibiotic resistance surveillance in UWTPs, in accordance with objective 2 of the WHO global action plan on antimicrobial resistance: “Strengthen the knowledge and evidence base through surveillance and research” (4). The challenges of implementing reliable environmental antibiotic resistance surveillance efforts have been recognized, as the procedures recommended for humans, animals, or food products are not applicable to environmental samples (2). Nevertheless, the clinical antibiotic resistance surveillance data stand as an important reference to assess the status of antibiotic resistance in different regions and environmental compartments. Therefore, the current study was designed on the basis of the major trends identified by the European Antimicrobial Resistance Surveillance Network (EARS-Net) currently promoted by the European Centre for Disease Prevention and Control.
Control (ECDC). This surveillance integrates data from 30 European countries concerning invasive blood and cerebrospinal fluid isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Streptococcus pneumoniae*, *Staphylococcus aureus*, and enterococci (5). The 2017 EARS-Net surveillance report (5) highlights that, as in previous years, for the period of 2013–2016, “a north-to-south and a west-to-east gradient is evident in Europe,” with a general increase of antibiotic resistance prevalence over this gradient, particularly for the Gram-negative bacteria surveyed. Considering this information, our study included seven countries distributed over the EARS-Net conceptual gradient represented by Portugal, Spain, Cyprus, Ireland, Germany, Norway, and Finland. The relative abundance of different genes was compared in raw influent and final effluent of 12 UWTPs (because of restrictions in sampling authorization, influent samples were not available from Ireland; table S1). Our goal was to obtain an initial overview of the antibiotic resistance status; regular surveillance protocols implemented locally may support robust comparative analyses, where bias associated to sporadic events or regional features can be identified.

Metagenomics methods, based on shotgun high-throughput sequencing, have been used to analyze the presence of antibiotic resistance genes (ARGs) in ecosystems such as activated sludge, wastewater, and surface water. Whereas these studies are useful to obtain a general view of the most abundant genes, they are not effective for detecting low-abundance genes. As noted before (6), abundance does not necessarily correlate with risk, and in the case of antibiotic resistance, surveillance and risk estimation require the implementation of targeted metagenomic techniques (8), able to quantify those genes that are of relevance for human health, even when they are present in low amounts that may be nondetectable by using current metagenomics approaches. For these reasons, and also because we sought a quantitative analysis, we used a quantitative polymerase chain reaction (qPCR) array, whose appropriateness as a tool for environmental surveillance has been demonstrated in previous research (9, 10). The array targeted sequences involved in gene transfer and recombination (*n* = 25; integrase, transposase, insertion sequence, and plasmid replicon type) and antibiotic resistance, prevalent in bacterial pathogens (*n* = 229), including multidrug resistance genes (*n* = 39) (table S2). Twenty-four–hour composite samples were collected on each of three consecutive days (Tuesdays, Wednesdays, and Thursdays, in early autumn of 2015, early spring, and early autumn of 2016) at all sampling sites to mitigate biases and improve comparability. In addition, DNA was extracted using a common protocol (table S1), and the highly parallel qPCR analyses were all performed using the same equipment and operational conditions. Of the 384 qPCR array primer pairs, 289, targeting 259 genetic determinants, of which 229 were of resistance genes, 25 of mobile genetic elements, and 5 housekeeping genes, produced amplification in one or more of the analyzed samples, while 95 did not amplify (table S2 and fig. S1). The number of primers for each class of genetic determinants that produced amplification did not differ (*P* > 0.01, Mann-Whitney *U* test) among countries (data not shown). The abundance of antibiotic resistance and mobile genetic elements are, unless otherwise stated, all designated as ARGs. They were normalized to the 16S ribosomal RNA (rRNA) gene abundance within the same sample, as described in Materials and Methods. Because this is a measure of the abundance of ARGs per total bacteria, we refer to this ratio as the ARG prevalence. Multidimensional scaling was used to identify the patterns in the distribution of ARGs in the UWTPs’ wastewater (Fig. 1). This analysis indicated that the relative abundance of influent ARGs supported the formation of two distinct groups: one group comprising Portugal, Spain, and Cyprus, and the other group Germany, Norway, and Finland. Although one of the Germany influent samples clustered together with

![Fig. 1. PCoA showing the distribution of resistance and mobile genetic elements using the Bray-Curtis dissimilarity index.](image)

A) Influent water from European UWTPs. The HAC and LAC countries clustered separately (*R*² = 0.37, *P* = 0.015). The exception was for three influent samples from German UWTPs that clustered with the HAC samples (DE2 in the figure). (B) Effluent water from European UWTPs. The HAC and LAC countries did not cluster together (*R*² = 0.11, *P* = 0.181). The significance of the cluster separation between the HAC and LAC countries was calculated using adonis with 9999 permutations. The points represent individual samples in the ordination, named according to the UWTP, and latitude is indicated by the color gradient. The point size is according to the mean total antibiotic consumption in humans from 2005 to 2015 from ECDC reports consisting of the human consumption of antibacterials for systemic use (ATC group J01) in the community (primary care sector) and the hospital sector expressed as defined daily dose (DDD) per 1000 inhabitants and per day (available at https://ecdc.europa.eu/en/ antimicrobial-consumption/database/country-overview) (see also table S3). Countries: HAC—PT, Portugal; ES, Spain; CYP, Cyprus; IL, Ireland; LAC—DE, Germany; FI, Finland; NO, Norway.
the Portugal, Spain, and Cyprus influent samples, the observed ARG distribution supported the conceptual EARS-Net antibiotic resistance gradient. The relative abundance of ARGs in influents from southern countries (Portugal, Spain, and Cyprus) and northern countries (Germany, Norway, and Finland) clustered separately ($R^2 = 0.37$, $P = 0.015$) (Fig. 1A). Because the UWTP influent is strongly influenced by human excreta, its resistome can be considered a picture of the general community resistome, hereafter designated “urban resistome.” This resistome can be influenced by, among a myriad of other factors, the general antibiotic resistance prevalence and antibiotic consumption in the community (2, 5). The distribution of urban resistomes based on ARG relative abundance observed in Fig. 1A is consistent with the human antibiotic consumption in those countries in the period 2005–2015 (Fig. 1 and table S3). These two groups of high (HAC) and low (LAC) antibiotic consumption countries are further compared. Because of the levels of antibiotic use in Ireland (Fig. 1), it was included in the HAC group.

INSIGHTS INTO THE URBAN RESISTOME

Most of the ARGs detected in the influents corresponded to genes with widespread environmental distribution encoding resistance against first-generation antibiotics. All influent samples contained genes conferring resistance to aminoglycosides (aadA and strB), β-lactams (blaGES, blaOXA, and blaVEB), macrolide–lincosamide–streptogramin B (MLSb) (ereA, ermF, and matA/mel), sulfonamides (sul1), tetracyclines (tetM and tetQ), and multidrug resistance (gacEdelta1 and qacH). Moreover, the signatures of various genetic elements involved in gene transfer and recombination (intI1, tnpA, Tp614, ISAba3, ISPps, and ISSm2) were present in all influent samples (table S2). Regarding ARGs of high concern in clinical settings (blaNDM-1, blaKPC, blaVIM, blaIMP, mcr-1, mecA, or vanA), blaIMP and vanA were sporadically detected in the influent of different countries. The ~blaVIM~ gene was detected in Portugal, Spain, Cyprus, and Germany influents, and ~blaKPC~ in Portugal and Spain influents and sporadically in effluent samples. The ARG distribution that separated the HAC from the LAC countries was explained by a significantly ($P < 0.01$, Mann-Whitney U test) higher relative abundance of most resistance classes in HAC countries (Portugal, Spain, Cyprus, and Ireland) than in the LAC countries ($P < 0.01$, Mann-Whitney U test). The opposite was observed for tetracycline and MLSb resistance.

![Graph](image_url)

**Fig. 2.** Relative gene abundance observed in influent samples from HAC and LAC countries. Relative abundance of (A) resistance genes and (B) mobile genetic elements. The data refer to the sum of relative abundance of amplification (ratio ARG or MGE copy number: 16S rRNA gene copy) for a given pair of primers, organized in classes of “resistance” or “transfer and recombination.” In the legend, for each gene class, the country group, HAC or LAC, with significantly higher relative abundance ($P < 0.01$, Mann-Whitney U test) is indicated. Samples are organized according to the sampling campaign (C2, spring 2016; C3, autumn 2016), divided by HAC and LAC. Resistance categories: AMG (aminoglycosides), MDR (multidrug resistance), SUL (sulfonamides), BL (β-lactams), MLSb, TET (tetracycline), QUI (quinolones), AMP (amphenicols), VAN (vancomycin), and others. Note: Ireland data are missing because of restrictions on influent wastewater sample collection.
Genes (Fig. 2A). The difference in the relative abundance of determinants related to gene transfer and recombination was also noticeable. Specifically, the relative abundance of insertion sequences was higher in the LAC than in the HAC countries ($P < 0.01$, Mann-Whitney U test) (Fig. 2B). The relative abundance of genes encoding resistance to amphenicols, β-lactams, sulfonamides, and integrase enzymes presented significantly higher daily variations over the 3 days of the same sampling campaign in the HAC than in the LAC countries ($P < 0.01$, Mann-Whitney U test). This effect might have been due to the comparatively smaller size of the UWTPs of the HAC group in comparison with those of the LAC group (≤200 versus ≥300 population-equivalent and <45 versus >100 m$^3$/day) (table S4). In smaller UWTPs, peaks of exogenous ARGs may have a more pronounced effect. Given the contrasts observed between both groups of countries, we were interested in investigating whether wastewater treatment attenuates those contrasts, resulting in similar ARG abundances in all the UWTP effluents.

**INSIGHTS INTO THE FINAL EFFLUENT RESISTOME**

The comparative analyses of the final effluents aimed at assessing whether, irrespective of the input, the wastewater treatment could reduce the levels of ARGs to a similar baseline level across the different UWTPs examined. Similar to the influent resistomes, contrasts were observed between the HAC and LAC effluent samples. For most resistance classes (aminoglycoside, sulfonamide, β-lactam, MLSB, tetracycline, quinolones, amphenicols, and multidrug resistance), the HAC final effluents presented significantly higher relative abundance values than the LAC final effluents ($P < 0.01$, Mann-Whitney U test) (Fig. 3A). This was also observed for genes encoding integrases and transposases (Fig. 3B). The relative abundance of tetracycline and MLSB resistance genes and insertion sequences that was higher in the LAC than in the HAC influents (Fig. 2) became lower in the final effluents. The relative abundance of tetracycline and MLSB resistance genes changed after treatment and was significantly higher in the HAC than in the LAC countries ($P < 0.01$, Mann-Whitney U test) (Fig. 3). These shifts were in agreement with the differences observed in the prevalence decrease of different ARG classes in the LAC and HAC countries (Fig. 4A). The classes with sharper decreases of prevalence values in the LAC compared to the HAC countries were the ARGs encoding resistance against tetracyclines and MLSB and the insertion sequences and transposase encoding genes ($P < 0.01$, Mann-Whitney U test). Noticeably, most of these classes of genes had a higher prevalence in the influent in the LAC than in the HAC samples (Figs. 2 and 4). Also, in the

![Fig. 3. Relative gene abundance observed in effluent samples from HAC and LAC countries.](image)

The data refer to the sum of relative abundance of amplification (ratio ARG or MGE copy number: 16S rRNA gene copy) for a given pair of primers organized in classes of resistance or transfer and recombination. In the legend, for each gene class, the country group, HAC or LAC, with significantly higher relative abundance ($P < 0.01$, Mann-Whitney U test) is indicated. Samples are organized according to the sampling campaign (C1, autumn 2015; C2, spring 2016; C3, autumn 2016), divided by HAC and LAC. Resistance categories: AMG (aminoglycosides), MDR (multidrug resistance), SUL (sulfonamides), BL (β-lactams), MLSB, TET (tetracycline), QUI (quinolones), AMP (amphenicols), VAN (vancomycin), and others.
Fig. 4. Variation in average ARG prevalence between influent and effluent \(\log_{10}(\text{influent} - \text{effluent})\) in H\(_{\text{AC}}\) and L\(_{\text{AC}}\) countries. (A) Comparison of ARG log reduction values in UWTPs of the H\(_{\text{AC}}\) and L\(_{\text{AC}}\) countries, where zero indicates that treatment did not affect the cumulative ARG relative abundance, positive values indicate a reduction, and negative values indicate an increase. The classes for which H\(_{\text{AC}}\) or L\(_{\text{AC}}\) presented higher relative abundance in the influent \( (P < 0.01, \text{Mann-Whitney } U \text{ test}) \) are indicated in the table at the bottom. Asterisks indicate statistically significant differences between H\(_{\text{AC}}\) and L\(_{\text{AC}}\) log reduction values, and these cases are detailed in (B). (B) Comparison of average ARG relative abundance in influent and final effluent, for the classes with significantly different log reduction values in the H\(_{\text{AC}}\) and L\(_{\text{AC}}\) samples: (B1) MLS\(_{B}\), (B2) tetracyclines, (B3) insertion sequences, and (B4) transposase. The arrows indicate significant \( (P < 0.01; \text{Welch's } t \text{ test}) \) increases \( \-uparrow \) or decreases \( \downarrow \) after treatment (C1, autumn of 2015; C2, spring of 2016; C3, autumn of 2016). WW, wastewater.
final effluent data, the richness of the MLS$_B$ genes was significantly different ($P < 0.01$, Mann-Whitney $U$ test) in Portugal compared with Ireland and the LAC countries (data not shown). Although the relative abundance of most ARGs decreased after treatment in both the HAC and LAC countries, effluents from the HAC group had a significantly higher relative abundance of most of the ARG classes, with the exception of vancomycin resistance, integrase, and transposase encoding genes (Fig. 4). Noticeably, the exclusion of Cy-

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and geographic regions, were scanned for suitable predictors of the total ARG abundance in UWTP effluents (Table 1). The suggested indicator primers either amplified genes encoding resistance to first-generation antibiotics or associated to mobile genetic elements, with high prevalence in wastewater (Table 1). Among these were $aadA$, $qacEdelta1$, $ermF$, and $intI1$. Noticeably, some of these elements are associated with class 1 integrons, recognized as markers of anthropogenic impact (12). On the basis of these data, a mini-array could be designed, aimed at implementing operational screening that covers major target drug classes (aminoglycosides, β-lactam, MLS$_B$, sulfonamide, and tetracycline) or functional groups (insertion sequence and


table S2). In addition, the ARGs related with resistance to aminoglyco-

sides ($aadA$ and $strB$), β-lactams ($blaOXA$), MLS$_B$ ($ermF$), sulfon-

amides ($sul2$), tetracycline ($tetW$), multidrug resistance ($qacH$), and mobile genetic elements, such as transposase ($tnpA$), integrase ($intI1$), and insertion sequences (ISAba3 and ISPps), persisted after treatment in >90% of the analyzed samples (table S2). Resistance genes considered of highest concern in clinical settings analyzed in this study were not detected in the final effluents examined. Sporadic exceptions were, e.g., $mecA$ detected in final effluent samples from Cyprus or $blaIMP$ and $vanA$ detected in both influent and final effluent samples in all countries (except $blaIMP$ in Cyprus).

| Gene class (no. of assays) | Genes dominating the class | Most representative genes |
|---------------------------|---------------------------|--------------------------|
| Gene Assay ID % of samples | Gene Assay ID Rho |
| Aminoglycoside (24) | $aadA$ AY167 62.9 | $cmlA$ AY127 0.96 |
| Amphenicol (14) | $cmxA$ AY129 27.1 | $ermF$ AY23 0.81 |
| β-Lactam (61) | $blaOXA$ AY44 34.5 | $tetQ$ AY185 0.82 |
| MDR (40) | $qacEdelta1$ AY159 92.1 | $tetX$ AY196 0.73 |
| MLS$_B$ (30) | $ermF$ AY23 68.6 | $ISPps$ AY369 0.98 |
| Quinolone (3) | $qnrSrtF11$ AY6 100.0 | $intI1$ AY336 0.96 |
| Sulfonamide (9) | $sul1$ AY363 73.0 | $tnpA$ AY202 0.86 |
| Tetracycline (34) | $tetQ$ AY185 59.6 | $tetX$ AY196 0.73 |
| Insertion sequence (9) | $ISPps$ AY369 86.5 |
| Integrase (7) | $intI1$ AY336 80.9 |
| Transposase (10) | $tnpA$ AY202 79.3 |

**Table 1. Candidate indicator assays for major gene classes in UWTP effluents.** Assays that yielded the highest amplification in the largest number of samples are reported on the left. Assays with the most representative outcome in terms of correlation with the per-class mean are reported on the right (rho: Spearman’s rank correlation coefficient). Primer pairs corresponding to the assay are listed in table S2. 

**INFERENCERE ABOUT POSSIBLE ARGS INDICATORS IN WASTEWATER**

Surveillance can be performed with two aims, both requiring different approaches and targeting distinct ARGs. One refers to the evaluation of the overall ARGs burden, a measure of the degree of contaminant ARGs occurring in a given environment. This surveillance relies on the measurement of the total resistome and its quantitative variations and pattern fluctuations. The other, which specifically focuses on biological hazards, relies on the targeted analyses of ARGs of special clinical concern or emerging in the environment. These are normally at lower abundance than most environmental ARGs, and their detection may require the use of approaches that increase sensitivity to ensure detection at very low levels (11). Despite the importance of wastewater surveillance to assess geographic trends or, at long-term, to measure the impacts of control measures, the monitoring of a high number of ARGs may be limiting due to the cost and training required for analysis on a routine basis. The use of the traditional fecal bacteria enumeration to assess the microbiological water quality may be of limited value for resistance monitoring. We could not find significant correlations between the antibiotic-resistant counts of culturable bacteria (table S4), performed with these same samples, and the ARG quantification. High-throughput methods have the potential to generate information from which indicators can be inferred. The data gathered in this study, which cover a large number of ARGs and geographic regions, were scanned for suitable predictors of the total ARG abundance in UWTP effluents (Table 1). The suggested indicator primers either amplified genes encoding resistance to first-generation antibiotics or associated to mobile genetic elements, with high prevalence in wastewater (Table 1). Among these were $aadA$, $qacEdelta1$, $ermF$, and $intI1$. Noticeably, some of these elements are associated with class 1 integrons, recognized as markers of anthropogenic impact (12). On the basis of these data, a mini-array could be designed, aimed at implementing operational screening that covers major target drug classes (aminoglycosides, β-lactam, MLS$_B$, sulfonamides, and tetracycline) or functional groups (insertion sequence and

*Pärnänen et al., Sci. Adv. 2019; 5 : eaau9124     27 March 2019* 6 of 10
transposase). Notably, the total abundance of resistance genes could be predicted using a subset of primers. For example, the data from assay AY159 (qsEdelta1) alone explain almost 90% of the variance in total ARG abundance. Using a linear model with three inputs, e.g., AY167 (aadA), AY23 (ermF), and AY337 (intI1), the predictive power could be moderately increased (adjusted $R^2 = 0.95$). This information provides a promising direction for the future wastewater surveillance with a substantial workload and cost reduction as well as ease of data analyses when compared with nontargeted and high-throughput approaches.

**DISCUSSION**

A major expectation of this first integrated surveillance of European wastewaters was to assess whether the relative abundance of urban wastewater resistome would follow the same trend observed in the prevalence of antibiotic resistance in clinical isolates, also coincident with antibiotic use profiles herein designated by HAC and LAC. The distribution of ARGs in the urban resistome showed the separation of the north and south countries studied, which is consistent with what has been shown for clinical isolates (5). The results suggest that studied UWTPs, based on activated sludge secondary treatment processes, with different configurations and, in a few cases, with a tertiary treatment or simply a disinfection stage, were able to reduce the relative abundance of all examined ARG classes, except genes encoding resistance to vancomycin (Fig. 4). However, the ARG burden after wastewater treatment was significantly higher in the south (HAC) than in the north (LAC) countries (Fig. 3). As a consequence, it is hypothesized that the final effluents of the HAC countries may have a higher impact on the receiving environment than those of the LAC countries. A result that deserves future research is the basis why the ARG prevalence decrease is higher in some cases in the LAC countries. This observation may be related to the dynamics of the ARG bacterial hosts during treatment (13, 14), influenced by operational conditions (e.g., temperature) and microbiota composition. In addition, or alternatively, it may be a consequence of the differences in the wastewater treatment reactors which are larger in the LAC countries than in the HAC countries (Table S4). Although the diversity of ARGs in activated sludge samples, assessed based on metagenomes, seems to be fairly conserved in different wastewater treatment plants (15, 16), investigation of the enriched microbiota and operational conditions on ARG reduction seems warranted. The potential influence of the presence of selective pressures, particularly antibiotic residues, during treatment has been suggested in different studies as capable of influencing the fate of antibiotic resistance (17–20). The analyses of more than 50 antibiotic residues in the same effluent samples used in this study did not show any correlation with relative abundance of ARGs [(Rodriguez-Mozaz et al., in preparation). Similarly, no statistically significant correlations could be established between the relative abundance of ARGs in the final effluents and country-level information on antibiotic consumption in the primary care sector [data from ESAC-Net; (21)]. The influence of the treatment process or specific operational conditions on ARG relative abundance was beyond the scope of this surveillance. However, the variations in ARG prevalence observed in the HAC and LAC countries revealed by this study are highly relevant and novel findings that deserve future research investigation.

The evolution and spread of antibiotic resistance is a complex process, resulting from the interplay of different and often confounding variables. The HAC and LAC countries differed in the rate of antibiotic use in humans, but also in other, probably non-negligible factors, such as temperature, precipitation, or antibiotic use in pets and livestock. Although the antibiotics used in livestock production are not discharged into the municipal sewage system, these residues may contribute to an overall increase of antibiotic or antibiotic resistance load in that region. The HAC countries, excluding Ireland, also had higher animal antibiotic use and higher average minimal temperatures (biomass, 187.2 to 425.8 mg/kg; >11°C) than the LAC countries (biomass, 37.7 to 179.7 mg/kg; <5°C) (Fig. S2). Curiously, Ireland, with lower temperature and animal antibiotic use, had final effluents with lower relative ARG abundance ($P < 0.01$, Mann-Whitney U test) than the other HAC countries, except Spain. Germany, on the contrary, had higher antibiotic animal use than the other LAC countries; however, its relative abundance of ARGs was not significantly different from other LAC countries ($P < 0.01$, Mann-Whitney U test).

Although the north-to-south clinical antibiotic resistance gradient reported by the EARS-Net was also present in the European wastewaters analyzed in this study, we were interested in assessing the possible correlation between the clinical settings and the final effluents of the UWTPs studied. The relative abundance of several ARGs was significantly correlated with the prevalence of phenotypic resistance in clinical isolates of E. coli, K. pneumoniae, P. aeruginosa, or S. aureus (Fig. 5). This result is interesting and supported by the recognized ubiquity of these bacterial groups. Ubiquity and environmental fitness may be key concepts when discussing antibiotic resistance in the environment. Among the bacterial groups surveyed by EARS-Net, those that show an evident north-to-south gradient of resistance prevalence are ubiquitous bacteria that can thrive in the human body as well as in wastewater. These bacteria have optimal growth temperatures above 30°C (E. coli, 37°C; K. pneumoniae, 30° to 35°C; S. aureus, 25°C to 37°C; P. aeruginosa, 10°C to 45°C).

**Fig. 5. Association between the relative abundance of ARGs in UWTP effluents (aggregated by drug classes) and country-level data on phenotypic resistance of clinical isolates [data from EARS-Net].** (31) Numbers represent Spearman’s rank correlations; color codes indicate statistical significance. No statistically significant correlations were observed for Acinetobacter spp., Enterococcus faecium, or S. pneumoniae.
higher culturable bacterial counts than the LAC countries (table S4). Also evident in this study. However, we have concluded that the use of antibiotic resistance persistence and proliferation in the environment. Curiously, the three UWTP Germany samples that clustered with HAC influent samples (DE2 in Fig. 1) were from the treatment plant located in a region with the highest average temperature, which also presented higher cultivable bacterial counts than the LAC countries (table S4).

This is the first European integrated antibiotic resistance surveillance in wastewaters. Although this survey represents a limited number of UWTPs at the European scale, the fact that they were selected on the basis of the single criterion of location along the north-to-south gradient is supportive of our conclusions. The results not only reinforce the strong relationship between clinical and environmental antibiotic resistance but also signal the importance of taking into consideration in designing possible strategies to control antibiotic resistance. The importance of surveillance to improve antibiotic resistance control is also evident in this study. However, we have concluded that the use of traditional fecal indicators may have a limited capacity to provide reliable comparisons of antibiotic resistance status in wastewaters. Although culture-dependent methods continue to have their place in water quality monitoring, they may lack the necessary sensitivity for comparative purposes. Therefore, another interesting outcome of this comprehensive study is the possibility of designing simplified and low-cost surveillance protocols. These protocols, supported by high-throughput data, can be feasibly used on a routine basis for wastewater antibiotic resistance monitoring worldwide.

MATERIALS AND METHODS

Sampling and sample processing

The study included seven European countries, where UWTP samples were collected over three sampling campaigns in early autumn 2015 and 2016 and early spring 2016. Volumes between 1 and 2 liters were collected, in sterile flasks, and stored at 4°C until analysis for a maximum of 12 hours. In each sampling campaign, were collected three 24-hour composite samples, one per day, over three consecutive days (Tuesday, Wednesday, and Thursday) (table S1). Geographic, demographic, and technical characterization provided by the UWTPs is available in table S4. All participants followed a common protocol for sampling, sample processing, and DNA extraction.

DNA extraction and processing

All wastewater samples (n = 168) were filtered in triplicate through sterile polycarbonate membranes (0.22-μm porosity; Whatman, UK) and stored at −80°C until DNA extraction. Volumes of 25 to 50 ml of influent wastewater samples or of 150 to 400 ml of final effluent wastewater samples were filtered. Total DNA was extracted in triplicate, at once for the same sampling campaign, using the PowerWater DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) according to the manufacturer’s instructions, and quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). For each sampling date, a DNA pool from the triplicates was prepared, resulting in one DNA extract per day and UWTP and three extracts per sampling campaign. DNA extract pools were shipped to the Michigan State University and run using the qPCR array, as previously described (24). Each partner kept an aliquot of the DNA pools for additional analyses as needed.

qPCR array

The qPCR array contained 384 primer sets as previously described (10), including some additional primer sets: 16S rRNA gene (AY2), blaCTX-M (AY326 and AS360), blaox-A (AY361), blasheV (AY272), blatem (AY3), blav89 (AY260), mecA (AY284), qnrS1F1 (AY3), tetM (AY357), vanA (AY4; AY368), mer-1 (AY80), and int11 (AY45) (table S2). The concentration and quality of the DNA samples were verified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). Only the samples fulfilling the quality criteria were processed. The qPCR array uses a microfluidic SmartChip multisample Nanodispenser (WaferGen at the time of the study, now Takara) to load primer sets and samples from 384-well plates into a SmartChip (Takara) with 5 184-reaction wells. The same 384-well plate of primers was used for all experiments, and three technical replicates were run for each sample. Samples were diluted to have the same mass of DNA per reaction. Following amplification on the SmartChip Real-Time PCR cycler, Cq values were calculated using default parameters provided with the SmartChip analysis software.

Cultivable bacteria

Wastewater samples were characterized for the fecal coliform and enterococci counts, two groups of bacteria frequently used as microbiological indicators of water quality. The bacterial enumeration was performed on membrane coliform agar (mFC) or on m-Enterococcus agar and on these culture media supplemented with one of the following antibiotics: amoxicillin (32 mg/liter), tetracycline (TET; 16 mg/liter), or ciprofloxacin (1 mg/liter). A volume of 1 ml of wastewater or of the adequate serial dilution was filtered through cellulose nitrate membranes (0.22-μm porosity; Sartorius Stedim Biotech, Göttingen, Germany), placed onto the adequate culture medium and incubated at 30°C for 24 hours for fecal coliforms or 48 hours for enterococci. Counts were done in triplicate, and the results were used as part of wastewater characterization provided in table S4.

Data analysis

The quality criteria used to select the qPCR array data to be used in the analyses were (i) samples with at least two replicates, (ii) samples with more than 5 ng of DNA, and (iii) quantifications with a Cq ≤ 27. A Cq value of 27 corresponded to a limit of quantification lower than 100 gene copies (25). The qPCR array semiquantitative analyses did not support estimations based on gene abundance per volume of water because calibration curves were not obtained for each gene. However, for a subset of 10 genes, calibration curves that supported the assessment of the relationship between relative (16S rRNA based) and absolute quantification (fig. S3) were obtained. Some samples (Portugal) used for the array were also tested by real-time qPCR for the quantification of selected genes (fig. S4). Those analyses allowed us to conclude that the overall trends are similar between the quantitative qPCR and the semiquantitative qPCR array.

The 384 primer sets target 259 different genetic determinants. These comprised 229 ARGs organized in classes according to the class of antibiotics to which they confer resistance (AMG, aminoglycosides; SUL, sulfonamides; BL, β-lactams; MLSB; TET, tetracycline; QUI,
quino-ones; AMP, amphenicols; VAN, vancomycin; and others), as MDR (multidrug resistance) when conferring resistance to more than one class of antibiotics; 16 genetic transfer and recombination elements (integrase, transposase, and insertion sequence); 9 plasmid-associated genetic determinants; and 5 housekeeping genes. The plasmid-associated assays were excluded from the analyses because of the poor coverage (table S2). All the analyses were performed considering the remaining 375 primer pairs independently. The results of amplions’ relative abundance were calculated using the $C_i$ values of the reference gene and the genes of interest, applying the following formula: \[ \text{log}_{10} \left( \frac{C_i}{C_{\text{target gene}}} \right) \] (26).

The statistical analyses were performed using the R environment v3.4.0. To compare the average values between two groups, Mann-Whitney $U$ tests (27) were performed. Correlations between the relative abundance of each gene and the sum of the relative abundance of all genes from the same group (Table 1) were computed using Spearman’s rank-based approach (28). Statistical modeling of the total abundance of ARGs involved a power transformation of the gene’s relative abundances and the complete removal of zero-inflated assays (no measurable amplification in >25% of the samples).

All reported $P$ values were adjusted for multiple testing according to the respective hypotheses (29). To calculate the log reduction of the abundance of resistance and genetic transfer and recombination classes, the formula $\log_{10}(\text{influent relative abundance}) − \log_{10}(\text{effluent relative abundance})$ was applied, and Mann-Whitney $U$ test was used to find significant differences between $H_{AC}$ and $L_{AC}$. PCoA was calculated using the Bray-Curtis dissimilarities calculated using the package vegan v2.4 (30) and the cmdscale command from stats package. The latitude was defined by the latitude of the city of the UWTP. The data for human consumption were collected from the antimicrobial consumption database (available at https://ecdc.europa.eu/en/antimicrobial-consumption/database/country-overview), considering the consumption of antimicrobials for systemic use (ATC group J01) in the community (primary care sector) and the hospital sector expressed as defined daily dose (DDD) per 1000 inhabitants and per day using the mean from years 2005–2015.

To study the association between relative ARG abundance in UWTP effluents and phenotypic resistance in the primary care sector [data from EARS-Net, (37); Fig. 5], the information from the two sources was matched in terms of target antibiotics. Specifically, phenotypic resistance against penicillins, aminopenicillins, third-generation cephalosporins, and methicillin was classified as (and compared to) genotypic $\beta$-lactam resistance. Phenotypic macrolide resistance was compared with the relative abundance of ARGs conferring resistance to MLS.$\beta$.

**Supplementary Materials**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/3/eaau9124/DC1

Fig. S1. Average richness values (number of positive assays) for the different influent and effluent wastewater samples from high ($H_{AC}$) and low ($L_{AC}$) antibiotic consumption countries, for resistance genes and mobile genetic elements.

Fig. S2. Food-producing animals’ antibiotics consumption (expressed in biomass (mg/kg), values for 2013) and average maximal and minimal annual temperature and precipitation (yellow, average $T_{\text{max}} > 6^\circ$C; blue, average $T_{\text{max}} < 5^\circ$C).

Fig. S3. Average abundance (copies/ml; upper bars; left-hand legend) and prevalence values (gene copies/16S rRNA gene copies; lower bars; right-hand legend) for the different influent (Inf) and effluent (Eff) wastewater samples from high ($H_{AC}$) and low ($L_{AC}$) antibiotic consumption countries, determined on the basis of qPCR array for the genes: 16S rRNA, intI1, blaKPC, sul1, tetM, ermF, aadA, tnpA, and gag/EdE1.

Fig. S4. Average abundance (copies/ml; upper bars; left-hand legend) and prevalence values (gene copies/16S rRNA copies; lower bars; right-hand legend) calculated by traditional real-time qPCR and qPCR array for Portuguese influent and effluent wastewater samples.

Table S1. Influent and effluent wastewater samples used in the study.

Table S2. qPCR primer sets and the percentage of samples that gave positive results for the genes: 16S rRNA copies; lower bars; right-hand legend) calculated by traditional real-time qPCR and qPCR array for Portuguese influent and effluent wastewater samples.

Table S3. Consumption of antibacterials for systemic use (ATC group J01) in the community (primary care sector) in different European countries from 2005 to 2015 (see also Fig. 1), defined as daily dose per 1000 inhabitants and per day.

Table S4. Characterization of the UWTPs examined in this study, in terms of dimension, geographic conditions, treatment, and microbiological indicators.

Data S1. qPCR array data.

Data S2. List of samples.

Data S3. List of assays.

Reference (32)

**REFERENCES AND NOTES**

1. WHO, Global antimicrobial resistance surveillance system (GLASS) report: Early implementation 2016–2017 (Geneva, 2018); www.who.int/glass/resources/publications/early-implementation-report/en/.

2. T. U. Berendonk, C. M. Manaia, C. Merlin, D. Fatta-Kassinos, E. Cytryn, F. Walsh, H. Bürgmann, H. Serum, M. Norström, M.-N. Pons, N. Kreuzinger, P. Huovinen, S. Stefani, T. Schwartz, V. Kisand, F. Baquero, J. L. Martinez, Tackling antibiotic resistance: The environmental framework. Nat. Rev. Microbiol. 13, 310–317 (2015).

3. D. G. J. Larsson, A. Andreomort, J. Bengtsson-Palme, K. K. Brandt, A. M. de Roda Husman, P. Fagerstedt, J. Fick, C.-F. Flach, W. H. Gaze, M. Kuroda, K. Kvist, R. Laxminarayan, C. M. Manaia, K. M. Nielsen, J. Plant, M.-C. Ploy, C. Segovia, P. Simonet, K. Smalla, J. Snape, E. Topp, A. J. van Hengel, D. W. Verner-Jeffreys, M. P. J. Viria, E. M. Wellington, A.S. Wernersson, Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138 (2018).

4. WHO (World Health Organization), Global action plan on antimicrobial resistance (2015), p. 28; www.who.int/iris/handle/10665/193736.

5. ECDC (European Centre for Disease Prevention and Control), Surveillance of antimicrobial resistance in Europe 2016. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), Stockholm (2017).

6. C. M. Manaia, Assessing the risk of antibiotic transmission from the environment to humans: Non-direct proportionality between abundance and risk. Trends Microbiol. 25, 173–181 (2017).

7. L. Rizzo, C. Manaia, C. Merlin, T. Schwartz, C. Dagot, M. C. Ploy, I. Michael, D. Fatta-Kassinos, Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360 (2013).

8. V. F. Lanza, F. Baquero, J. L. Martinez, R. Ramos-Ruiz, B. González-Zorn, A. Andreomort, A. Sánchez-Valenzuela, S. D. Ehrlich, S. Kennedy, E. Ruppé, W. van Schaik, R. J. Willems, F. de la Cruz, T. M. Coque, In-depth resistome analysis by targeted metagenomics. Microbiome 6, 11 (2018).

9. A. Karkman, T. A. Johnson, C. Lyra, R. D. Stedfeld, M. Tamminen, J. M. Tiedje, M. Viria, High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 92, fiw014 (2016).

10. F. Wang, R. D. Stedfeld, O.-S. Kim, B. Chai, L. Yang, T. M. Stedfeld, S. G. Hong, D. Kim, H. S. Lim, S. A. Hashsham, J. M. Tiedje, W. J. Sul, Influence of soil characteristics and proximity to Antarctic research stations on abundance of antibiotic resistance genes in soils. Environ. Sci. Technol. 50, 12621–12629 (2016).

11. C. M. Manaia, J. Rocha, N. Scaccia, R. Marano, E. Radu, F. Bianullo, F. Cerqueira, G. Fortunato, I. C. Iakovides, I. Zammit, I. Kampouris, I. Vaz-Moreira, O. C. Nunes, Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324 (2018).

12. M. R. Gillings, Class 1 integrons as invasive species. Curr. Opin. Microbiol. 38, 10–15 (2017).

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

14. Y. Yang, B. Li, S. Zou, H. H. P. Fang, T. Zhang, Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res. 62, 97–106 (2014).

15. C. Munk, M. Albertsen, A. Telke, M. Ellabaan, P. H. Nielsen, M. O. A. Sommer, Limited dissemination of the wastewater treatment plant core resistome. Nat. Commun. 6, 8452 (2015).

16. X.-X. Zhang, T. Zhang, Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. Environ. Sci. Technol. 45, 2598–2604 (2011).
17. J. Li, W. Cheng, L. Xu, P. J. Strong, H. Chen, Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Res.* **22**, 4587–4596 (2015).

18. P. Gao, M. Munir, I. Xagoraraki, Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Water Res.* **69**, 234–242 (2015).

20. J. Bengtsson-Palme, R. Hammarén, C. Pal, M. Östman, B. Björlenius, C.-F. Flach, J. Fick, E. Kristiansson, M. Tysklind, D. G. J. Larsson, Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci. Total Environ.* **572**, 697–712 (2018).

22. G. Garrity, D. J. Brenner, N. R. Krieg, J. T. Staley, *Berger's Manual of Systematic Bacteriology- Vol 2: The Proteobacteria, Part C-The Alpha-, Beta-, Delta- and Epsilonproteobacteria* (Springer-Verlag, 2005).

25. R. D. Stedtfeld, S. W. Baushke, D. M. Tourlousse, S. M. Miller, T. M. Stedtfeld, E. Gulari, J. M. Tiedje, S. A. Hashsham, Isothermal assay targeting class 1 integrase gene for environmental surveillance of antibiotic resistance markers. *J. Environ. Manag.* **198**, 213–220 (2017).

26. K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCT) Method. *Methods* **25**, 402–408 (2001).

27. H. B. Mann, D. R. Whitney, On a test of whether one of two random variables is stochastically larger than the other. *Ann. Math. Stat.* **18**, 50–60 (1947).

28. D. J. Best, D. E. Roberts, Algorithm AS 89: The upper tail probabilities of spearman’s rho. *Appl. Stat.* **24**, 377–379 (1975).

29. Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300 (1995).

30. J. Oksanen, J. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, vegan: Community Ecology Package, vegan R Packag. version 2.4-4 (2017); https://cran.r-project.org/package=vegan.