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A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants

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ABSTRACT

The World Health Organization Global Action Plan recommends integrated surveillance programs as crucial strategies for monitoring antibiotic resistance. Although several national surveillance programs are in place for clinical and veterinary settings, no such schemes exist for monitoring antibiotic-resistant bacteria in the environment. In this transnational study, we developed, validated, and tested a low-cost surveillance and easy to implement approach to evaluate antibiotic resistance in wastewater treatment plants (WWTPs) by targeting cefotaxime-resistant (CTX-R) coliforms as indicators. The rationale for this approach was: i) coliform quantification methods are internationally accepted as indicators of fecal contamination in recreational waters and are therefore routinely applied in analytical labs; ii) CTX-R coliforms are clinically relevant, associated with extended-spectrum β-lactamases (ESBLs), and are rare in pristine environments. We analyzed 57 WWTPs in 22 countries across Europe, Asia, Africa, Australia, and North America. CTX-R coliforms were ubiquitous in raw sewage and their relative abundance varied significantly (< 0.1% to 38.3%), being positively correlated ($p < 0.001$) with regional atmospheric temperatures. Although most WWTPs removed large proportions of CTX-R coliforms, loads over 10^3 colony-forming units per mL were occasionally observed in final effluents. We demonstrate that CTX-R coliform monitoring is a feasible and affordable approach to assess wastewater antibiotic resistance status.

1. Introduction

Over the past few decades, the global spread of antibiotic resistance has increased to alarming levels, approaching what has been coined as the "post-antibiotic era" (Heymann et al., 2007). While this phenomenon is traditionally linked to healthcare-associated infections (Ventola, 2015), it is believed that animal husbandry, aquaculture facilities and urban wastewater treatment plants (WWTPs) also contribute to propagating antibiotic resistance by discharging antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and residual concentrations of antibiotic compounds into downstream aquatic and terrestrial environments (Berendonk et al., 2015; Tripathi and Cytryn, 2017). Standard microbial water quality assessment methods, including quantification of intestinal coliforms and enterococci, are frequently applied for monitoring microbial quality in wastewater treatment effluents (Tallon et al., 2005). While the methods based on these indicators are considered to be reliable for the detection of fecal contamination, they do not assess antibiotic resistance levels, a new threat that increasigly needs routine evaluation. When associated with mobile genetic elements (MGEs), ARGs can be horizontally transferred between bacterial cells, even across distinct lineages, thereby facilitating antibiotic resistance dissemination (Smets and Barkay, 2005). Consequently, ARGs have been described as "contaminants of emerging concern" (Pei et al., 2006; Pruden et al., 2013) and have motivated a plethora of studies targeting ARGs in WWTPs effluents and downstream environments (Marano et al., 2019; Narciso-da-Rocha et al., 2018; Rizzo et al., 2013; Wang et al., 2014). Most of these studies rely on molecular biology methods, which provide highly informative data on the ARGs present in these environments. These studies facilitated efforts to standardize analytical methods for measuring selected ARGs in WWTP effluents and receiving water bodies (Rocha et al., 2018). However, the application of molecular methods also requires specialized equipment, expensive reagents and proficient technical staff, making them less suitable for routine, widespread WWTP monitoring (Bürgmann et al., 2018; Manaia et al., 2018). Moreover, these methods do not interrelate with conventional (culture-based), globally standardized microbiological water quality indicators. Although culture-based methods often overlook a large fraction of the wastewater microbiota, they can provide fundamental data on antibiotic resistance trends of individual species and strains coupling antibiotic resistance phenotypes to individual bacterial isolates of clinical concern. A major bias of

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culture-based methods comes from omitting unknown and often strictly environmental bacteria that are profuse in wastewater samples but do not grow on standard culture media, as suggested by Bengtsson-Palme et al. (2016). The use of culturable coliforms indicators, such as *Escherichia coli*, has been globally adopted due to its low-cost and ease of implementation for assessing fecal contamination; however, previous experience has shown that antibiotic-resistant bacteria of human/animal origin thriving in wastewater are not only fecal coliforms but include a wider range of enterobacteria (Ferreira da Silva et al., 2007; Vaz-Moreira et al., 2015). Therefore, a surveillance system that could tackle these aspects was designed and tested as presented in this paper. Targeting antibiotic-resistant coliforms may be especially relevant from human health perspective given the realization that horizontal transfer of MGEs occurs much more frequently between phylogenetically closely related organisms and that this process is especially prevalent among coliforms (Popa and Dagan, 2011; Vaz-Moreira et al., 2015).

Within the framework of the European Union's COST Action ES1403: *New and emerging challenges and opportunities in wastewater reuse* (NEREUS), we conceived a simple experimental scheme for estimating the abundance of antibiotic resistance using standard methods that can easily be applied and interpreted by stakeholders like WWTP operators. The method makes use of standard membrane fecal coliform agar (mFC Agar) medium to quantify coliforms (Rompré et al., 2002), but in tandem also quantifies antibiotic-resistant coliforms by amending the mFC Agar medium with cefotaxime, a third-generation cephalosporin, at a clinically-relevant concentration (Heil and Johnson, 2016). Resistance to this antibiotic has considerable clinical significance due to the increasing abundance of resistant bacteria-harboring extended-spectrum β–lactamase (ESBL) genes, which are frequently associated with multidrug resistance and widespread in the environment (Bradford, 2001; Pitout and Laupland, 2008). This is especially true for clinically relevant "priority pathogens" from the *Enterobacteriaceae* family including *Escherichia coli*, *Klebsiella pneumoniae,* and *Enterobacter* spp., but is also pertinent to other Gram-negative "ESKAPE" pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Boucher et al., 2009).

We evaluated the applicability and the potential epidemiological indicative capacity of this method by launching a global initiative that encompassed WWTPs from five continents (Fig. 1) in two independent sampling campaigns. For most of the analyzed WWTPs, the method was applied to test raw (influent) and treated (effluent) sewage. Stakeholders provided metadata related to specific treatment plant parameters, further used to test correlations between specific factors and antibiotic resistance levels. The aims of this study were (1) to quantify the absolute and relative abundance of cefotaxime-resistant (CTX-R) coliforms in raw sewage (influent) in different locations and to investigate potential predictors of their estimated abundances; (2) to estimate the ability of WWTPs to remove CTX-R coliforms from wastewater, and (3) to quantify the load of CTX-R coliforms discharged (effluent) from WWTPs to downstream aquatic and terrestrial environments.

2. Materials and methods

2.1. Site description and WWTP characteristics

A total of 57 WWTPs from 22 countries were sampled. Most (47 of 57) of the analyzed WWTPs were located in Europe, one was located in Africa, six were in Asia, one in Australia and two WWTPs were in North America. In 14 of the 22 participating countries, two or more WWTPs were sampled (Supplementary Table 1).

For each WWTP, grab samples were taken in two campaigns. The first campaign consisted of three sampling dates, once per month, between December 2016 and February 2017; for the second campaign, two sampling dates were chosen between May and October 2017 (Supplementary Table 1). Participating groups were provided with detailed protocols for sampling, sample processing and cultivation and bacterial enumeration and completed a questionnaire on metadata

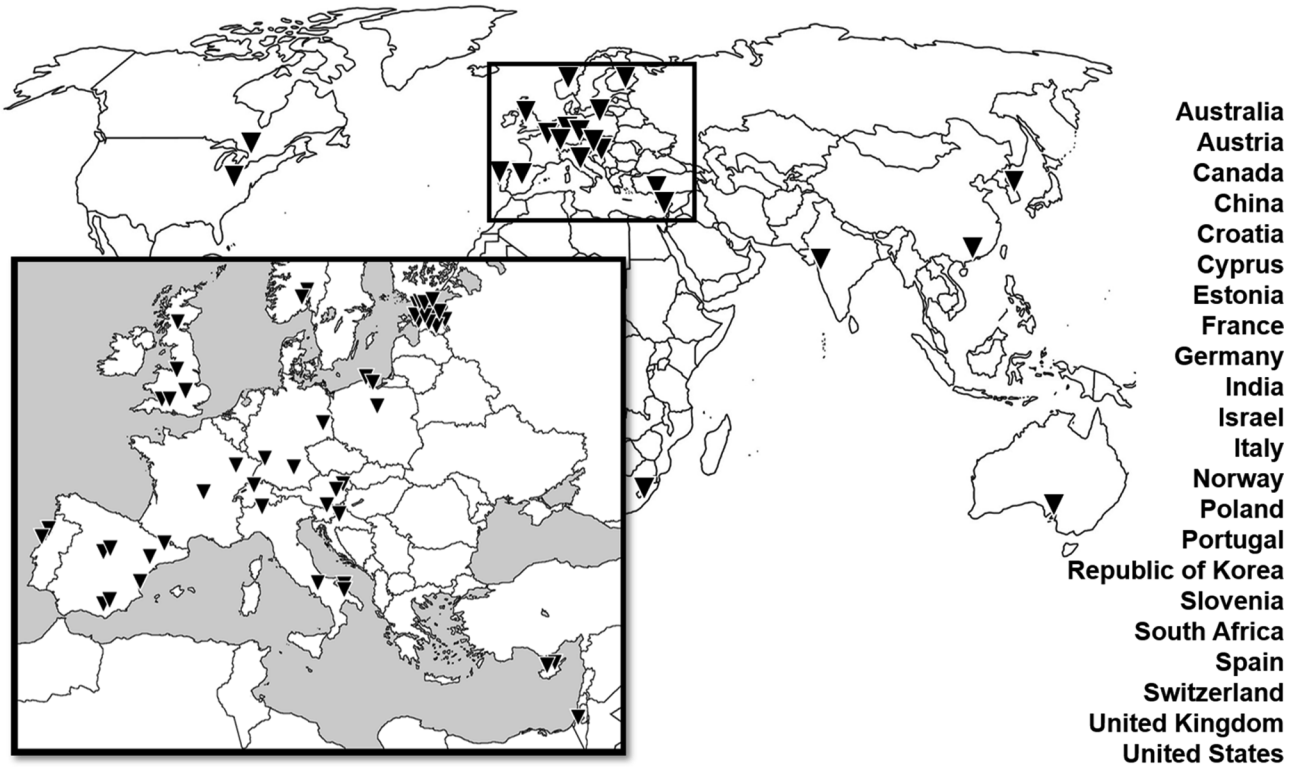

Fig. 1. A geographical overview of the global survey on cefotaxime-resistant coliforms. Black triangles show geographic locations of the participating countries where WWTP samples were collected. Square insert shows targeted European WWTPs.

related to the country and the WWTPs. All experimental data were screened, quality checked, and assembled as described below before performing statistical analyses.

2.2. Metadata collection

The participating groups filled out a questionnaire with data related to WWTP catchment population size and influent characteristics, the WWTPs' technical setup and operational conditions, physicochemical characteristics of the final effluent, meteorological conditions of WWTP sites, antibiotic consumption in each country and the WWTPs' GPS coordinates. Meteorological conditions were retrieved from official national forecast online web databases, using average monthly temperatures of sampling days. Antibiotic consumption, available for European countries only, was retrieved from the antimicrobial use database of the European Centre for Disease Prevention and Control (www.ecdc.europa.eu) using the filters: "2016", "β-lactam antibacterials and penicillins", and "primary care sectors". Antibiotic consumption is indicated as 'defined daily doses (DDD) per 1000 inhabitants per day'.

2.3. Sampling

Grab samples were collected during weekdays and processed on the day of sampling as described below. Raw sewage (influent) after primary sedimentation and effluents after secondary biological treatment and/or after disinfection were sampled. From a total of 57 WWTPs evaluated in this study 54 applied conventional activated sludge (CAS) with secondary sedimentation, two WWTPs used membrane bioreactor (MBR), and one used trickling filter (TF) treatment technologies (Supplementary Table 1). Distinct disinfection processes were applied in 22 WWTPs, using chlorination (n = 7), UV radiation (n = 11), chlorination and UV radiation (n = 1), membrane filtration (n = 2) or ozonation (n = 1). Three WWTP treatments applied the disinfection process seasonally, two used UV radiation in the summer and one chlorination in the winter (Supplementary Table 1).

2.4. Enumeration of bacteria

Membrane fecal coliform Agar (mFC, Difco® with 0.01% (w/v) Rosolic Acid (Difco®)) was prepared according to manufacturer instructions. For the quantification of CTX-R coliforms, mFC Agar was supplemented with cefotaxime sodium salt (Sigma®) at a final concentration of 4 μg mL^{-1}. This concentration was based on the minimal inhibitory concentration (MIC) breakpoint levels for *Enterobacteriaceae,* documented by CLSI (Clinical and Laboratory Standards Institute, Wayne, PA, 2010), which is more stringent than the EUCAST MIC breakpoints of > 2 μg mL^{-1} (European Committee of Antibiotic Susceptibility Testing http://www.eucast.org/clinical_breakpoints/) and would also be more selective against false positives and intermediately resistant colonies. Culture media were prepared no more than three days before sampling and dispensed in 60 mm diameter Petri plates. When the liquid culture medium reached 55 °C, the approporate volume of the fresh concentrated filter-sterilized antibiotic stock solution was added before pouring plates. Samples were serially diluted and 1 mL from 2 to 4 consecutive 10-fold serial dilutions were filtered through 47 mm diameter 0.45 μm pore size nitrocellulose sterile membrane filters on mFC Agar plates (in triplicate) with and without cefotaxime. For less turbid water samples (*i.e.*, effluents) up to 100 mL were filtered. Cultures were incubated for 24 h at 37 °C. Incubation time was not prolonged beyond 24 h to avoid biases associated with the inactivation of cefotaxime in the culture medium by the CTX-R bacteria that produce extracellular cefotaxime degrading β-lactamases. Moreover, even though the mFC Difco® protocol recommends incubation at 44 °C, we specifically chose 37 °C (i) to reduce curing of plasmids harboring ARGs, which has been shown to occur at elevated temperatures (Trevors, 1986), and (ii) to avoid possible functional thermal instability previously reported for CTX-M enzymes and other β-lactamases at higher temperatures (He et al., 2016; Vanhove et al., 1995). To validate our choice of incubation temperature we compared the identity of the bacterial groups recovered under both the conditions and criterion used (*i.e.*, incubation at 37 °C vs. 44 °C), (see Section 2.7). Under these conditions, the blue colonies enumerated on mFC Agar with or without cefotaxime were considered as presumptive coliforms. The limit of detection (LOD) of the method was calculated as the minimal number of colonies that could be enumerated in the highest volume analyzed. Extrapolation of the generated results suggests that the LOD was approximately 0.3 colony forming units (CFUs) mL^{-1} (with 1 being the minimum number of observable colonies on triplicate mFC plates for 1 mL of filtered water); however, it should be noted that in a few cases filtered effluent volume was increased to 10 or 100 mL when water samples were particularly clear. To improve reproducibility, it was recommended that each step in the procedure (media preparation, dilutions, filtration and colony enumeration) be conducted by the same person processing the samples of a given WWTP. Participating groups were provided with a standard spreadsheet to collect the results of bacterial enumeration and were asked to report the date and location of sampling.

2.5. Quality control criteria

For CFU enumeration, the following criteria were applied by all participating groups: (i) optimally, only filtering membranes with 10–80 CFUs were included in analyses; (ii) when this range of CFUs was not available, the highest dilution with a countable number of CFU was used; (iii) when a selected dilution had replicates with inconsistent CFU counts (defined as a standard deviation (SD) > 20% of the mean), a different dilution range was chosen according to the above inclusion criteria; and (iv) whenever technical or methodological issues from WWTPs or during sample preparation were reported, data were discarded regardless of the above inclusion criteria.

After the initial data trimming and quality control, data from 228 influent samples, 199 secondary effluents (MBR or CAS final effluent or prior to disinfection) and 79 effluents after a disinfection step were collected (Supplementary Table 1).

The relative abundance of CTX-R coliforms was calculated by dividing the number of CFUs mL^{-1} on mFC Agar supplemented with cefotaxime by the number of CFUs mL^{-1} on mFC agar without antibiotic for each of the three replicates. The WWTP removal efficacy for coliforms and CTX-R coliforms was calculated as either percent or log-unit removal (respectively) using the following equations:

$$\frac{CFUsinfluent - CFUsoutflow}{CFUsinfluent} \times 100;\ \log\left(\frac{CFUsinfluent}{CFUsoutflow}\right)$$

2.6. Statistical analyses

To evaluate whether coliform abundance in influents and effluents would be a predictor for CTX-R coliform levels we used F-tests to compare variances. Predictor factors for resistance in influents were also investigated in linear regression analyses comparing coliforms and CTX-R coliforms, to local geographical areas of WWTPs and to the described β-lactam antibiotic consumption indicator (2.2). Non-parametric Mann Whitney U-tests were used to compare coliforms and CTX-R coliforms CFU mL^{-1} in influents at selected regional atmospheric temperature ranks. Removal efficacy of coliforms and CTX-R coliforms from influents after CAS process (*i.e.* secondary effluents) from WWTPs located in regions with different temperatures were compared with non-parametric Mann Whitney *U* test. Statistical analyses were conducted using GraphPad Prism® version 6.00 for Windows (GraphPad Software, La Jolla, CA, United States) and were interpreted using a significance level set at $p \leq 0.05$.

2.7. Methodological validation through bacterial characterization

In parallel with the surveillance study, the diversity of bacteria targeted by the procedure used was assessed and the taxonomy of selected bacteria was determined in a parallel series of tests, with the following objectives in mind: (i) assess the taxonomic composition of wastewater derived bacteria forming blue colonies on mFC Agar at 37 °C and compare with that of blue colonies recovered at 44 °C (the recommended incubation temperature for isolating fecal coliforms on mFC Agar); (ii) assess if, as hypothesized, bacteria with acquired antibiotic resistance are more prevalent when incubated at 37 °C than at 44 °C.

These bacterial characterization experiments involved eight partners of different geographic locations (USA, Australia, Israel, Portugal, UK, Germany, Croatia, Poland). Samples from secondary effluents (only) were processed as described above and incubated at 37 °C and 44 °C. From plates incubated at 37 °C or 44° (on agar with and without cefotaxime) a total of 30 blue CFUs were selected, isolated, and taxonomically characterized using either 16S rRNA gene sequence anaylsis or MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectronomy; detailed protocols for both are described in the Supplementary Material). To elucidate the potential impact of incubation temperature on the relative abundance of CTX-R isolates, the recovery capacity of CTX-R *E. coli* isolates was evaluated at the two compared incubation temperatures; only *E. coli* were investigated due to their clinical relevance and the fact that they were the most abundant species identified, suitable for statistical investigation. The amount of total and CTX-R *E. coli* CFU per mL in raw samples was estimated, for each assembly of 30 isolates, from each group, from the four tested conditions using the formula below:

$$\left(\frac{\text{N identified E. coli}}{\text{N total identified}}\right) \times (\text{N blue CFUs on filter} \times \text{Df})$$

where the first factor of the multiplication is the proportion of validated *E. coli* out of the 30 isolates from each condition, and the second factor is the total number of blue CFUs per mL in the raw sample (blue CFUs on filter multiplied by the dilution factor, Df). Thereafter, the ratios between the estimated *E. coli* CFU per mL retrieved at 37 °C and 44 °C were calculated for mFC Agar and mFC Agar + CTX (respectively).

3. Results and discussion

Numerous studies have explored antibiotic resistance in WWTP influents and effluents using both traditional cultivation techniques (Hoelle et al., 2019) and culture-independent molecular analyses that target ARGs (Petrovich et al., 2018). Data generated from these studies is very important, but because they are generally constrained to individual facilities or local regions, they lack perspective on the global dimensions of antibiotic resistance in WWTPs. In contrast, two recent international collaborative initiatives have facilitated comprehensive perceptions of antibiotic resistance in WWTPs. By applying shotgun metagenomic analysis of untreated sewage in 79 sites from 60 countries around the world, Hendriksen et al. (2019) found systematic geographical differences in abundance and diversity of ARGs, with little correlation to antibiotic use or bacterial taxonomy. While specific factors were difficult to identify, they concluded that socioeconomic factors such as poor sanitation were primary drivers of ARG propagation. A recent pan-European study conducted by Pärnänen et al. (2019) that targeted over 250 ARGs and associated MGEs in influents and effluents of 12 WWTPs from seven countries using highly-parallel quantitative PCR found a north–south distribution of ARGs, consistent with trends observed for clinical isolates (ECDC, 2018). While both of these studies significantly contribute to our understanding of antibiotic resistance in WWTPs, they are based on methodologies that are extremely costly and require (i) advanced expertise in manipulation and data analyses, (ii) DNA/RNA extraction kits, (iii) expensive equipment (e.g. real-time PCR, sequencing facilities) and (iv) Physical Containment facilities (*i.e.* PC2, PC3), all normally not available in WWTP labs. We therefore focused on implementing a simple culture-based approach that can be routinely applied or analyzed on a global scale for elucidating the abundance of clinically-relevant antibiotic-resistant bacteria in WWTPs. Such a method would only require a simple modification of already existing microbial analytical procedures at WWTPs compared to the costly alternative methods described above (i.e qPCR and metagenomics). A comparative cost analysis to better elucidate on such expenses is given in Supplementary Table 5. Of note, the described method should not be intended as an alternative to the use of mFC Agar® plates incubated at 44 °C, as the populations targeted by the two conditions (*i.e.* 44 °C and 37 °C) are slightly different as elaborated below).

Comparison of the two incubation temperatures described in section 2.7 revealed that mFC Agar cultures (with/without cefotaxime) incubated at 44 °C selected for members of the *Enterobacteriaceae* family, with the majority of these being *E. coli* (> 71% of the identified), as expected. *E. coli* was also the most abundant species detected from the mFC screened cultures incubated at 37 °C, although at a lower frequency (> 31% of the identified). Other genera of the family *Enterobacteriaceae* were detected on plates incubated at 37 °C with or without cefotaxime, and these included *Citrobacter, Enterobacter,* and *Klebsiella* spp., all genera that include opportunistic human pathogens

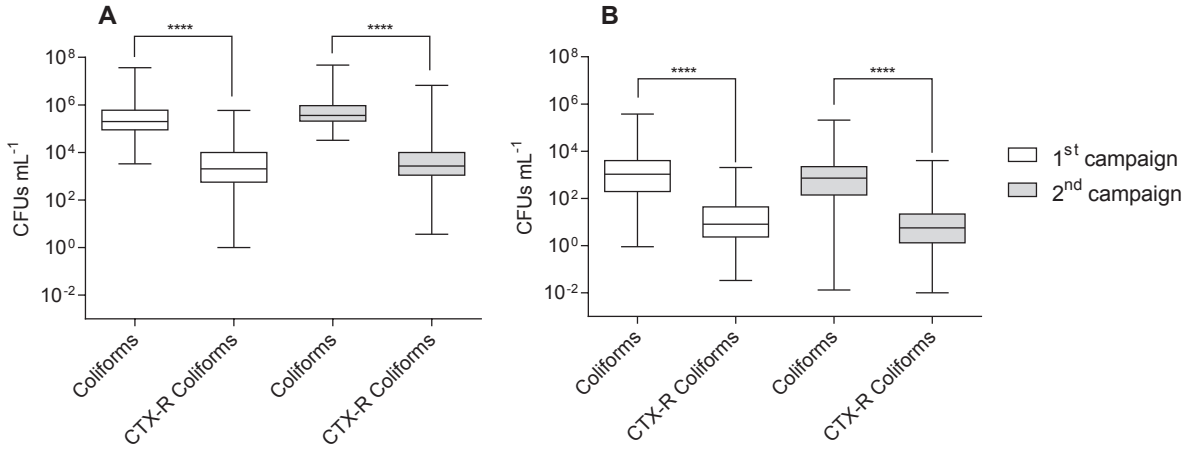

Fig. 2. Distribution of coliforms and CTX-R coliforms in 57 sampled WWTPs. A: influents, based on 228 samples (146 from the 1st campaign, and 82 from the 2nd). **B**: effluents, on 244 samples (150 from the first campaign and 94 from the second). Asterisks refer to a p-value < 0.0001 on an F–test to compare the variance between samples. Box plots show means and quartiles, whiskers indicate the minimum and maximum values.

(Supplementary Fig. 1). Non–*Enterobacteriaceae* were also detected in cultures incubated at 37 °C, mainly *Aeromonas* spp. (*A. caviae* and *A. hydrophila*), representing < 23% of all identified isolates, while other genera and species represented < 3% (Supplementary Fig. 2). Noticeably, although the amount of *E. coli* among the blue colonies counted on mFC Agar incubated at 37 °C was lower than those counted on mFC Agar incubated at 44 °C, both estimates were within the same order of magnitude when considered as CFU per mL (average ratio of 6 comparisons between the two incubation temperature = 1.1 ± 0.4; Supplementary Fig. 3). On the contrary, incubation of mFC Agar plates supplemented with cefotaxime at 37 °C facilitated higher ($p < 0.05$) recovery of CTX–R *E. coli* than the same medium incubated at 44 °C (by a factor 2.8 ± 1.5 across 6 comparisons), supporting the hypothesis that acquired antibiotic resistance is more stable at 37 °C than at 44 °C. These findings suggest that despite the slightly elevated number of false positives (non coliforms and aeromonads) generated, the significantly higher level of CTX–R *E. coli* detected at 37 °C supports its application in targeting the resistant *E. coli* fraction of wastewater. At least for the tested fraction of the coliform community, these findings support the hypothesis that CTX–R coliforms might be selected against or lose resistance determinants such as plasmids at higher temperatures, biasing the evaluation of their proportion in the original sample (Trevors, 1986).

3.1. Abundance of CTX-R coliforms in raw sewage

It can be assumed that CTX-R coliforms in influents primarily originate from fecal matter in the sewage entering the individual WWTP. Fig. 2A and Supplementary Table 2 summarize the abundance of CTX-R coliforms in influents observed in the two global survey campaigns. For both the sampling campaigns, between WWTPs, the variance of CTX-R coliforms was significantly higher than that of total coliforms (Fig. 2A). This variance was also reflected in the relative abundance of CTX–R coliforms (as percentage) in the various WWTPs, which ranged between < 0.1% and 38.3%, with a global mean of 2.7%. These observations highlight both, the ubiquity of CTX-R coliforms in untreated wastewater sewage and the highly unequal geographical distribution of this resistance phenotype.

Strong variation in CTX-R coliforms levels between the different WWTP influents may stem from a myriad of factors such as antibiotic use, sanitary conditions, and various abiotic factors. Recent studies have suggested significant correlations between climate (aerial temperature) and antibiotic resistance. MacFadden et al. (2018) performed a *meta*-analysis of antibiotic-resistant *E. coli*, *K. pneumoniae* and *Staphylococcus aureus* data from different geographical regions of the US (1.6 million strains, from 223 facilities across 41 states between 2013 and 2015). They found that regional temperature was the most significant factor associated with antibiotic resistance, where a 10 °C increase in minimum temperature coincided with approximately 2–6% increase in resistance in the three targeted pathogens. Temperature is very often underestimated in wastewater microbial ecology, in contrast to freshwater microbial ecology, where it is considered one of the most significant parameters in determining the bacterial community composition of rivers and lakes (Likens, 2010). To investigate the potential correlation between local temperature (monthly temperature, at the collection site, of the day of sampling based on meteorological data) and the abundance of coliforms and CTX-R coliforms in raw sewage, 228 influent samples comparing monthly mean temperatures with an absolute abundance of coliforms and CTX-R coliforms were analyzed. The rationale for addressing ambient temperature stemmed from three factors: (i) ambient temperature can significantly influence sewage temperature within activated sludge where aeration is applied (due to differences in air temperature); (ii) temperature can have a major impact in combined sewer systems that receive rain/melted snow; (iii) temperature is often a surrogate parameter for geographical latitude as north–south gradients, which are frequently reported in clinical ABR monitoring studies (MacFadden et al., 2018; Pärnänen et al., 2019). We observed a slightly positive correlation between $\log_{10}$ converted CFU values and temperature, which was more prominent below 15 °C for both coliforms ($R^2 = 0.22$) and CTX-R coliforms ($R^2 = 0.1$) (Fig. 3, Supplementary Figure 9). Upon the observed trend inversion of the dataset above and below this indicated temperature, we compared the CFU abundance in samples from places with monthly regional temperatures below 5 °C and above 15 °C (as monthly averages), represented by two groups with a similar number of samples (77 samples < 5 °C and 72 samples > 15 °C) for both coliforms and CTX-R coliforms at both temperature ranges (Fig. 4). A version of Fig. 4 of the complete datapoint split into three ranks is presented in Supplementary Figure 10.

Both, total and CTX-R coliforms (CFUs mL^{-1}) were generally more abundant in WWTPs influents from areas with higher temperatures (Fig. 4) and this observation was not dependent on specific locations or sampling campaign, as data from the WWTPs were evenly distributed across the two temperature ranks. However, no clear statistical correlation with temperature was observed when looking at the relative abundance of CTX-R coliforms (Supplementary Fig. 4) suggesting that other confounding variables might have influenced the observed relative abundance in sewage. Despite the fact that correlation does not necessarily entail causation, it was speculated that a higher abundance of coliforms in WWTPs operating in geographical areas with temperatures above the 15 °C threshold may be explained by a multitude of climatic and potential indirect socioeconomic factors that were not taken into consideration in this study. The difficulty in disentangling temperature from other factors such as antibiotic use, population

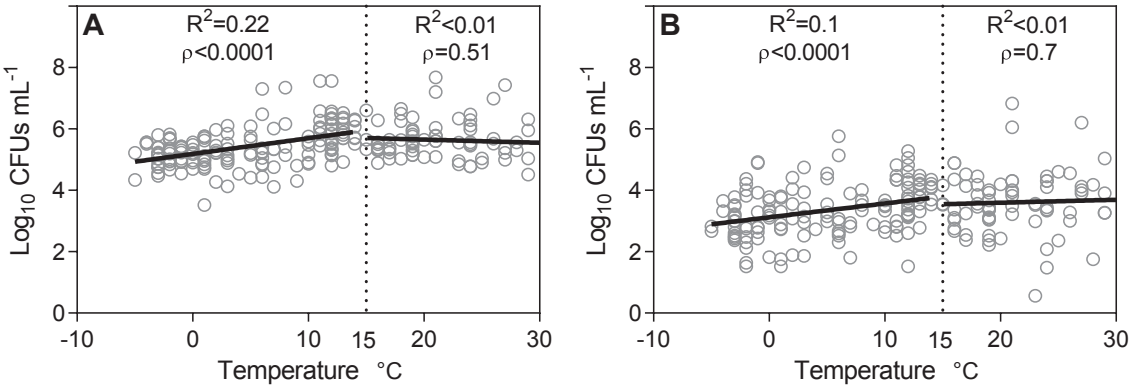

Fig. 3. Linear regression of $\log_{10}$ converted coliforms (A) and CTX-R coliforms (B) in WWTP influents as a function of monthly average temperature in the sampled areas. Datasets are split into temperatures ranks below and above 15 °C (highlighted by dotted lines); R^2 and p-values from linear regression are reported for each tested interval. A version of these data analyses without splitting of temperature intervals is given in Supplementary Figure 9.

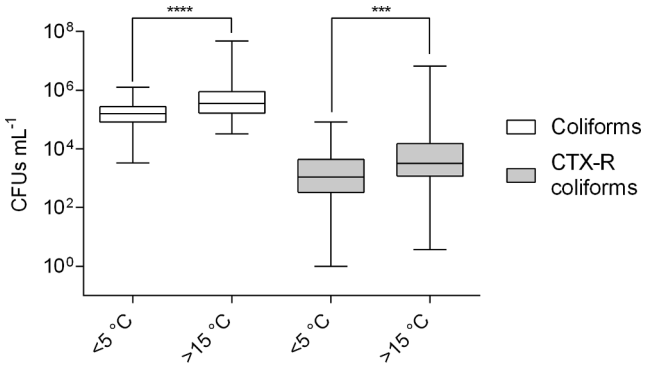

Fig. 4. Comparison of coliforms and CTX-R coliforms abundance in WWTPs' influent sewage in selected temperature ranks. Asterisks refer to a Mann-Whitney *U* test with given p-values < 0.0001 (****) and p < 0.001 (***). Sample size coliforms: < 5 °C n = 77, > 15 °C n = 72; sample size CTX-R coliforms < 5 °C n = 76, > 15 °C n = 72. Box plots show percentiles together with medians; whiskers show the minimum and maximum values. A version of these data analyses with complete dataset intervals is given in Supplementary Figure 10.

density, and hygiene, complicates the identification of specific factors linked to antibiotic resistance, as indicated in the two recent molecular-based global studies described above (Hendriksen et al., 2019; Pärnänen et al., 2019). It is possible that water temperature might be more informative in deciphering such trends and we recommend future studies to couple CFU-related data analyses with water temperatures together with air temperatures.

Average defined daily doses (DDD) of β-lactams per 1000 inhabitants in European countries were compared to CFU counts in influents using linear regression analysis, but the tests failed to differentiate correlations between DDD values and coliforms, or between DDD values and CTX-R coliforms, therefore this parameter was considered to be not informative in this study (data not shown). However, it should be noted that DDD values used here reflect general antibiotic usage trends of entire countries and not necessarily the local areas that feed a given WWTP. Previous studies by Collignon et al. (2018) likewise found that annual average β-lactam antibiotics usage in individual communities may not be a reliable predictor of resistance levels of fecal coliforms in wastewater. This may be due to the low resolution of national antibiotic consumption data and may be resolved if coupled with accurate local surveys on antibiotic prescriptions in the area served by a given WWTP, as shown by Caucci et al. (2016). Together with β-lactam antibiotics usage, the contribution of hospital effluents to CTX-R coliforms relative abundance observed in WWTPs' sewage was evaluated comparing (i) the ratio between the number of beds equivalent and population served, to (ii) the observed CTX-R coliforms relative abundance in the influents. From the 17 WWTPs analyzed where data were available (Supplementary Table 1), no correlation was observed (slope not significantly different from 0; Supplementary Fig. 5). The most plausible explanation for these observations is that antibiotic resistance dynamics are dictated by complex ecological interactions that may not be explained solely based on the use of antibiotics in clinical settings or even on the occurrence of antibiotic residues in wastewater (Novo et al., 2013; Varela et al., 2014). Other factors related to the individual wasterwater network at catchement points might further influence such diversity (e.g. volume of flow, combined or separated sewer systems, etc.) (Choi et al., 2018).

3.2. CTX-R coliform removal in WWTPs

The removal of coliforms and CTX-R coliforms was evaluated in WWTPs where both influent and effluent samples were available. Overall, from all sampling dates, a total of 220 count values for colifoms and 215 count values for CTX–R coliforms were compared between influents and effluents. Globally, the average abatement of both coliforms and of CTX-R coliform populations in secondary (CAS and MBR) effluents was 2.3 ± 1.2 log units. MBR treatments showed significantly higher coliform removal capacity when compared to CAS treatments ($p < 0.0001$, mean 5.8 ± 0.6 and 2.1 ± 0.8 log removal for MBR and CAS, respectively). The higher removal observed for MBRs is likely provided by the membranes, especially in the case of ultrafiltration (CYP WWTP), where small pore-size, contributes to the retention of coliforms due to size exclusion and cell-colloid interactions (Schwermer et al., 2017). However, the dataset contained only eight sampling points from MBR whereas CAS accounted for 140 sampling points.

The data from the seven WWTPs that included a final disinfection step were specifically analyzed to evaluate the abundance and removal dynamics of CTX-R coliforms along the treatment continuum (influent, secondary effluent, and disinfected effluent). Overall multiple sampling dates (1st and 2nd campaign combined) from these seven investigated WWTPs contributed to assemble 23 full continuum profiles. All these WWTPs applied CAS treatment combined with one of the following disinfection treatments: chlorination ($n = 2$), UV radiation ($n = 3$), chlorination and UV radiation ($n = 1$) or ozonation ($n = 1$). In these WWTPs, concerning the raw sewage, the CAS treatment reduced the coliforms and CTX–R coliform loads by approximately 2 log-units, whereas average log removal in the final disinfected effluents was 4.4 ± 2 for coliforms and 3.5 ± 1.4 for CTX-R coliforms. However, we observed a high data dispersion of coliforms and CTX-R coliforms loads in the final effluents (Supplementary Figure 6). Indeed, the abundance of CTX-R coliforms in all disinfected effluents analyzed were below the LOQ in 28 sampling instances (35% of total disinfected final effluent samples analyzed).

The scope of secondary treatment removal of coliforms and CTX-R coliforms was investigated as a function of WWTP regional temperature to evaluate a possible correlation between removal efficiency and temperature. Such an association was evaluated in both percentage removal relative to influent levels and absolute log-units removal of CTX-R coliforms choosing two aerials temperature ranks, < 5 °C and > 15 °C (Supplementary Figure 7 and Supplementary Figure 8). Overall, no significant differences were observed between the two groups following secondary biological treatment; however, in geographical locations where temperature was below 5 °C, we identified a greater number of WWTPs with removal efficiencies of < 95% of resistant coliforms (Supplementary Figure 7). Specifically, the lowest efficiencies were observed for POL2, EST10, DEU1, EST2, GBR4, POL3, EST5, AUT2, and EST4, suggesting that these low removal efficiencies are not nationally or geographically associated, and can be eventually sporadic failures of the systems, potentiated or not by lower temperature. Similar observations from previous studies had also showed that higher ambient temperatures and correlate with increased activated sludge removal rates of coliforms (Miranzadeh et al., 2013). It should be noted however, that other WWTPs with higher removal efficiency were present in the same regions. Furthermore, temperature in secondary aeration tanks can be substantially different from air temperature, therefore the climate may not be a strong predictable indicator of removal efficiency.

3.3. CTX-R coliforms in discharged effluents

WWTP effluents are either discharged into natural water bodies such as streams, rivers, lakes, seas, oceans, or used for irrigation of land. In both scenarios, antibiotic–resistant bacteria (and specifically human commensals or pathogens) can potentially be introduced into the urban water cycle and food webs, subsequently leading to increased human exposure. Stakeholders routinely monitor fecal coliforms to assess contamination in effluent-receiving recreational water bodies, and treated wastewater used for irrigation. However, these indicators do not provide insight into the potential contribution of effluents to antibiotic resistance dissemination, which can have substantial environmental

and epidemiological significance (Berendonk et al., 2015; Manaia, 2017). The advantage of the microbiological method described in this study is that it can be adopted with only minor variation by existing national and regional surveillance programs, with minimal need for additional infrastructure or professional requirements, and with minimal added costs.

A total of 244 individual effluent samples were analyzed from both sampling campaigns. Coliforms and CTX-R coliform levels in the analyzed WWTP effluents in the first and second campaigns are shown in Fig. 2B, and the abundance of CTX-R coliforms in individual WWTPs based on the geographic region is shown in Fig. 5. CTX-R coliform abundance in these effluents was highly variable, ranging from values below the LOQ to values exceeding 10^3 CFUs mL^{-1}.

The analysis of the effluent samples with high CTX-R coliforms counts did not reveal noticeable geographic trends, suggesting that these values may be dependent on local WWTP characteristics and performance, although more robust surveillance of a broader range of WWTPs is required to validate this observation. One exception was the Pune WWTP analyzed in this study, which had the highest influent and effluent resistance levels in both absolute and relative abundance in both campaigns. While one WWTP from India is surely not enough to reveal geographic trends, the high influent CTX-R coliforms levels observed therein may be associated with multiple factors including non-regulated antibiotic consumption, contamination from local antibiotic production facilities that may promote the selection of resistance genes. India is reported as one of the largest consumers of antibiotics (Laxminarayan and Chaudhury, 2016) and previous studies had linked fecal coliforms to high levels of ESBL levels in WWTPs in the country (Lamba and Ahammad, 2017). Combined with fragile sanitary infrastructure, the above-mentioned factors might significantly contribute to the observed values (Bengtsson-Palme et al., 2014). Of note, comparatively higher temperatures of India during samplings could have also contributed to the higher coliforms and CTX–R counts observed (in accordance with MacFadden et al., (2018)) possibly due to faster bacterial growth at higher temperatures (Marano and Prakash personal communication). On the other hand, such simplistic inference might not be applied to other countries where a high abundance of CTX-R coliforms was also measured in the treated effluent, such as the WWTPs in Poland, where regulations follow EU requirements. Despite various attempts of geographical sorting indeed, apart from Poland, we could not identify any clear links between CTX-R coliform effluent levels and specific countries. This was evident for example for Spain (n_{WWTP} = 7), UK (n_{WWTP} = 5), Italy (n_{WWTP} = 4), and in particular Estonia (n_{WWTP} = 12) where different WWTPs exhibited both high and low CTX–R coliforms effluent values.

3.4. Implications and potential standards for discharged CTX-R coliforms in receiving environments

This study provides data that can facilitate the development of urgently required criteria for the permitted levels of CTX-R coliforms in effluents and various water reuse scenarios. Existing fecal coliform standards for wastewater recognize the potential threat that wastewater effluent poses to human health, although criteria for 'safe' levels of fecal indicators vary greatly between regions and use. For example, in California and Israel, the fecal coliform limits are 2.2 and 10 CFU per 100 mL, respectively (EPA, 2012). In Greece and Cyprus, these are 100 CFU per 100 mL for fecal coliforms, whereas the World Health Organization (WHO) standards for water re-use for irrigation are 1,000 CFU per 100 mL for fecal coliforms (Blumenthal et al., 2000). Similar criteria exist for discharge into water bodies intended for recreational use. For example, the US Environmental Protection Agency recommends a limit of 200 CFU per 100 mL of fecal coliforms in rivers and streams used for swimming (U.S. EPA, 1976). While most of the effluent samples contained low levels of CTX–R coliforms, it is troubling that 37% (89 out of 243 individual sampling points) exceeded the WHO

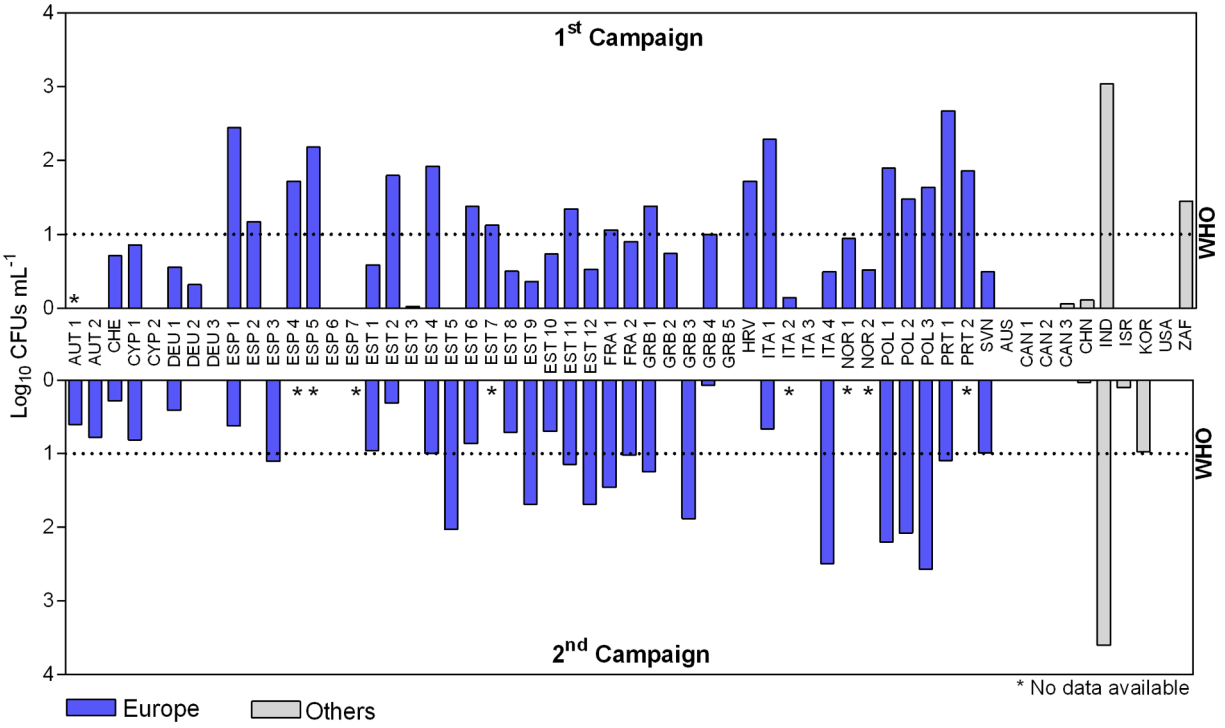

Fig. 5. Average CTX-R coliforms mL^{-1} in WWTPs' effluents (country-code + WWTP's assigned number from the same country). Fifty-seven WWTPs sampled in the first campaign (top) and forty-eight in the second campaign (bottom). The dotted line shows the WHO limits for water reuse regulation referred to Escherichia coli (CFUs mL^{-1}) log converted. Australia (AUS); Austria (AUT); Canada (CAN); China (CHN); Croatia (HRV); Cyprus (CYP); Estonia (EST); France (FRA); Germany (DEU); India (IND; Pune); Israel (ISR); Italy (ITA); Republic of Korea (KOR); Norway (NOR); Poland (POL); Portugal (PRT); Slovenia (SVN); South Africa (ZAF); Spain (ESP); Switzerland (CHE); United Kingdom (GRB); United States (USA). Asterisks indicated missing participation in the sampling campaign.

cutoff formulated for fecal coliforms (resistant and not; Fig. 5). Of note, as discussed above, whilst the hereby proposed method detects also coliforms other than *E. coli* (conventionally the most used fecal coliform indicator), *E. coli* still represent up to 50% of the pool of coliforms detected on mFC Agar plates incubated at 37 °C. If one considers that an average large WWTP (e.g. 50,000 person equivalent) can release more than 10^7 L per day of effluent, we estimate that under suboptimal treatment conditions (CTX–R coliforms $>$ 10 CFU mL^{-1} referring to the above mentioned WHO indicated threshold for fecal coliforms), up to 10^{11} CTX-R coliforms may be released daily into effluent receiving rivers. Additionally, if we consider that under Mediterranean climate conditions seasonal irrigation can exceed 1 m^3 of water per square meter of soil, this would mean that under the same assumptions each square meter of soil could potentially receive 10^8 CTX-R coliforms per season. While many countries have developed methods for optimizing the irrigation of edible crops, long-term exposure to antibiotic resistance determinants still might increase the risks of systematic transmission of these determinants throughout natural environments. Different types of downstream environments might present different permissiveness in terms of antibiotic-resistant bacteria dissemination and persistence, and release of antibiotic-resistant bacteria into surface water bodies can have a different and broader impact than the release in soil (Eckert et al., 2018; Leonard et al., 2018; Munck et al., 2015). Future studies should seek to ascertain whether antibiotic-resistant bacteria in these different environments are transmitted to humans, as well as to describe the impacts on human health resulting from such exposures. Additionally, they should characterize the genetic and phenotypic diversity of CTX-R coliforms and evaluate their capacity to persist in downstream environments (Karkman et al., 2018). Finally, effluents intended for re-use or discharged to bathing waters should be further investigated to assess the presence of pathogens, such as resistant *K. pneumoniae*, and enterohemorrhagic *E. coli* (EHEC) strains (Nguyen and Sperandio, 2012).

4. Conclusion

From the comprehensive analysis conducted here, differential patterns in the distribution of CTX-R coliforms are clear, although the factors influencing the observed differences are yet to be fully elucidated. This approach could be even more informative if such monitoring approaches are adopted to conduct large-scale national, regional and international surveillance projects that target many WWTPs across a given country or region, and if individual WWTPs are robustly monitored as part of routine monitoring campaigns. The global observed discrepancy between the variance of CTX-R coliforms and the total coliforms in the influents worldwide provides evidence that drivers of such variety might subsist. In order to better understand the factors that dictate this scope in WWTPs, future studies should perform more exhaustive analysis of specific WWTPs and link them to a range of *meta*-parameters related to the WWTP's surrounding area and the served population over time, in order to elucidate the factors influencing the distribution of CTX-R coliforms, such as β-lactam usage only in the population served by the targeted WWTPs (including hospital and livestock untreated/pre-treated wastewater effluents when relevant) to contextualize the outcomes to the 'big picture' of antibiotic resistance. Collectively, these approaches would facilitate: (i) detection of external factors and selective pressures that potentially contribute to antibiotic resistance levels in WWTPs, (ii) identification of local and global antibiotic resistance trends in WWTPs, (iii) understanding of the effects of quantified emission values of CTX-R coliforms in effluent-receiving environments, and (iv) help identify possible unexpected high peaks increasing the risk of clinical outbreaks when effluent wastewater is discharged into surface water or reused in agriculture.

Cefotaxime-amended chromogenic selective media are increasingly applied in surveillance studies (Snow et al., 2011) and can potentially serve as an alternative to the cefotaxime-amended mFC medium used here; however we recomend the used cefotaxime concentration to be 4 μg mL^{-1}. Given the growing epidemiological relevance of carbapenemase-producing *Enterobacteriaceae*, future surveys targeting WWTPs should concomitantly apply mFC medium amended with imipenem or meropenem, or use commercial available chromogenic media (García-Fernández et al., 2017). Finally, a subset of the isolates obtained should be subject to screening for cefotaxime-resistance genes (*i.e.* those encoding CTX-M enzymes), using PCR and/or whole genome sequencing and comparative analyses of ARGs and MGEs to detect possible geographic patterns.

Credit authorship contribution statement

Roberto B.M. Marano: Investigation, Data curation, Project administration. **Telma Fernandes:** Investigation, Data curation, Formal analysis, Visualization. **Célia M. Manaia:** Conceptualization, Methodology, Supervision, Validation, Resources. **Olga Nunes:** Conceptualization, Methodology, Supervision, Validation, Resources. **Donald Morrison:** Supervision, Validation, Resources. **Thomas U. Berendonk:** Supervision, Resources, Validation. **Norbert Kreuzinger:** Supervision, Resources. **Tanel Telson:** Resources. **Gianluca Corno:** Supervision, Resources. **Despo Fatta-Kassinos:** Resources. **Christophe Merlin:** Supervision, Resources. **Edward Topp:** Supervision, Resources. **Edouard Jurkevitch:** Supervision, Validation. **Leonie Henn:** Resources, Validation. **Andrew Scott:** Resources. **Stefanie Heß:** Resources. **Katarzyna Slipko:** Resources. **Mailis Laht:** Resources. **Veljo Kisand:** Resources. **Andrea Di Cesare:** Supervision, Resources. **Popi Karaolia:** Resources. **Stella G. Michael:** Resources. **Alice L. Petre:** Resources. **Roberto Rosal:** Resources. **Amy Pruden:** Supervision, Validation, Resources. **Virginia Riquelme:** Resources. **Ana Agüera:** Resources. **Belen Esteban:** Resources. **Aneta Luczkiewicz:** Validation. **Agnieszka Kalinowska:** Validation. **Anne Leonard:** Resources. **William H. Gaze:** Resources. **Anthony A Adegoke:** Resources. **Thor A Stenstrom:** Resources. **Alfieri Pollice:** Resources. **Carlo Salerno:** Resources. **Carsten U. Schwermer:** Resources. **Pawel Krzeminski:** Resources. **Hélène Guilloteau:** Resources. **Erica Donner:** Supervision, Validation, Resources. **Barbara Drigo:** Supervision, Validation, Resources. **Giovanni Libralato:** Resources. **Marco Guida:** Resources. **Helmut Bürgmann:** Resources. **Karin Beck:** Resources. **Hemda Garelick:** Validation. **Marta Tacão:** Resources. **Isabel Henriques:** Resources. **Isabel Martínez-Alcalá:** Resources. **Jose M. Guillén-Navarro:** Resources. **Magdalena Popowska:** Resources. **Marta Piotrowska:** Resources. **Marcos Quintela-Baluja:** Resources. **Joshua T. Bunce:** Resources. **Maria I. Polo-López:** Resources. **Samira Nahim–Granados:** Resources. **Marie-Noëlle Pons:** Resources. **Milena Milakovic:** Resources, Validation. **Nikolina Udikovic-Kolic:** Resources, Validation. **Jérôme Ory:** Resources. **Traore Ousmane:** Resources. **Pilar Caballero:** Resources. **Antoni Oliver:** Resources. **Sara Rodriguez-Mozaz:** Resources. **Jose L. Balcazar:** Resources. **Thomas Jäger:** Validation. **Thomas Schwartz:** Resources. **Ying Yang:** Resources. **Shichun Zou:** Resources. **Yunho Lee:** Resources. **Younggun Yoon:** Resources. **Bastian Herzog:** Resources. **Heidrun Mayrhofer:** Resources. **Om Prakash:** Resources, Validation. **Yogesh Nimonkar:** Resources. **Ester Heath:** Resources. **Anna Baraniak:** Validation. **Joana Abreu-Silva:** Validation. **Manika Choudhury:** Validation. **Leonardo P. Munoz:** Validation. **Stela Krizanovic:** Resources, Validation. **Gianluca Brunetti:** Validation. **Ayella Maile-Moskowitz:** Validation. **Connor Brown:** Validation. **Eddie Cytryn:** Conceptualization, Methodology, Supervision, Validation, Resources.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106035.

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