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► To cite this version:

Roberto B.M. Marano, Telma Fernandes, Célia Manaia, Olga C Nunes, Donald Morrison, et al.. A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. *Environment International*, 2020, 144, pp.106035. 10.1016/j.envint.2020.106035 . hal-02931405

HAL Id: hal-02931405

<https://hal.univ-lorraine.fr/hal-02931405v1>

Submitted on 8 Sep 2020

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

Roberto BM Marano^{1,2,†}, Telma Fernandes^{3,†}, Célia M Manaia³, Olga Nunes⁴, Donald Morrison²⁴, Thomas U Berendonk⁵, Norbert Kreuzinger⁶, Tanel Telson⁷, Gianluca Corno⁸, Despo Fattakassinos⁹, Christophe Merlin¹⁰, Edward Topp^{11,12}, Edouard Jurkevitch¹, Leonie Henn²⁴, Andrew Scott¹¹, Stefanie Heß^{5,13}, Katarzyna Slipko⁶, Mailis Laht^{7,14}, Veljo Kisand⁷, Andrea Di Cesare⁸, Popi Karaolia⁹, Stella G Michael⁹, Alice L Petre¹⁵, Roberto Rosal¹⁵, Amy Pruden¹⁶, Virginia Riquelme¹⁶, Ana Agüera¹⁷, Belen Esteban¹⁷, Aneta Luczkiewicz¹⁸, Agnieszka Kalinowska¹⁸, Anne Leonard¹⁹, William H Gaze¹⁹, Anthony A Adegoke^{20,21}; Thor A Stenstrom²⁰, Alfieri Pollice²², Carlo Salerno²², Carsten U Schwermer²³, Pawel Krzeminski²³, Hélène Guilloteau¹⁰, Erica Donner²⁵, Barbara Drigo²⁵, Giovanni Libralato²⁶; Marco Guida²⁶, Helmut Bürgmann²⁷, Karin Beck²⁷, Hemda Garelick²⁸, Marta Tachão²⁹, Isabel Henriques²⁹, Isabel Martínez-Alcalá³⁰, Jose M Guillén-Navarro³⁰, Magdalena Popowska³¹, Marta Piotrowska³¹, Marcos Quintela-Baluja³², Joshua T Bunce³², Maria I Polo-López^{17,33}, Samira Nahim-Granados^{17,33}, Marie-Noëlle Pons³⁴, Milena Milakovic³⁵, Nikolina Udikovic-Kolic³⁵, Ory Jérôme^{36,37,38}, Traore Ousmane^{36,37,38}, Pilar Caballero³⁹, Antoni Oliver³⁹, Sara Rodriguez-Mozaz⁴⁰, Jose L Balcazar⁴⁰, Thomas Jäger⁴¹, Thomas Schwartz⁴¹, Ying Yang⁴², Shichun Zou⁴², Yunho Lee⁴³, Younggun Yoon⁴³, Bastian Herzog⁴⁴, Heidrun Mayrhofer⁴⁴, Om Prakash⁴⁵, Yogesh Nimonkar⁴⁵, Ester Heath⁴⁶, Anna Baraniak⁴⁷, Joana Abreu-Silva³, Manika Choudhury²⁸, Leonardo P Munoz²⁸, Stela Krizanovic³⁵, Gianluca Brunetti²⁵, Ayella Maile-Moskowitz¹⁶, Connor Brown¹⁶, Eddie Cytryn^{2*}.

[†]These authors have equal contribution.

- 22 ¹ Department of Agroecology and Plant Health, The Robert H. Smith Faculty of Agriculture, Food and
23 Environment, The Hebrew University of Jerusalem, Rehovot, Israel
- 24 ²Department of Soil Chemistry, Plant Nutrition and Microbiology, Institute of Soil Water and Environmental
25 Sciences, Volcani Center, Agricultural Research Organization, Rishon Lezion, Israel.
- 26 ***Corresponding author**, email: eddie@volcani.agri.gov.il
- 27 ³ Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado,
28 Escola Superior de Biotecnologia, Rua Arquitecto Lobão Vital, 172, 4200-374 Porto, Portugal
- 29 ⁴ LEPABE, Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia,
30 Universidade do Porto, Rua Dr. Roberto Frias, 4200-465, Porto, Portugal
- 31 ⁵ Institute of Hydrobiology, TU Dresden, Dresden, Germany
- 32 ⁶ Vienna University of Technology, Institute for Water Quality and Resources Management, Vienna, Austria
- 33 ⁷ Institute of Technology, University of Tartu, Estonia
- 34 ⁸ CNR-IRSA Molecular Ecology Group, Largo Tonolli 50, 28922 Verbania, Italy
- 35 ⁹ Civil and Environmental Engineering Department and Nireas International Water Research Center, University of
36 Cyprus, P.O. Box 20537, CY-1678, Nicosia, Cyprus
- 37 ¹⁰ UMR 7564 University of Lorraine-CNRS (LCPME), France
- 38 ¹¹ Agriculture and Agri-Food Canada, London Research and Development Centre (ON), Canada
- 39 ¹² Department of Biology, University of Western Ontario, London (ON), Canada
- 40 ¹³ Institute of Microbiology, TU Dresden, Dresden, Germany
- 41 ¹⁴ Estonian Environmental Research Centre, Estonia

- 42 ¹⁵ Department of Chemical Engineering, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain
- 43 ¹⁶ Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, USA
- 44 ¹⁷ Solar Energy Research Centre (CIESOL), Joint Centre University of Almería-CIEMAT, 04120 Almería, Spain
- 45 ¹⁸ Faculty of Civil and Environmental Engineering, Gdansk University of Technology, G. Narutowicza 11/12 street,
46 80-233 Gdańsk, Poland
- 47 ¹⁹ University of Exeter Medical School , European Centre for Environment and Human Health, Environment and
48 Sustainability Institute, University of Exeter, Penryn campus, TR10 9FE, UK
- 49 ²⁰ Institute for Water and Wastewater Technology, Durban University of Technology, Durban South Africa.
- 50 ²¹ Department of Microbiology, University of Uyo, Uyo, Nigeria
- 51 ²² CNR-IRSA Viale F. De Blasio 5, 70132 Bari, Italy
- 52 ²³ Norwegian Institute for Water Research, Gaustadalléen 21, N-0349, Oslo, Norway
- 53 ²⁴ School Applied Sciences, Edinburgh Napier University, EH11 4BN, UK
- 54 ²⁵ Future Industries Institute, University of South Australia, Adelaide, SA, 5001, Australia
- 55 ²⁶ Department of Biology, University of Naples Federico II, via Cinthia 21, 80126 Naples, Italy
- 56 ²⁷ Eawag, Swiss Federal Institute of Aquatic Science and Technology, 6047 Kastanienbaum, Switzerland
- 57 ²⁸ Department of Natural Sciences, Middlesex University, London, NW4 4BT, UK
- 58 ²⁹ CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193,
59 Portugal
- 60 ³⁰ Department of Civil Engineering, Av. de los Jerónimos, 135, 30107 Guadalupe, Murcia, Spain

- 61 ³¹ Institute of Microbiology, Department of Applied Microbiology, Faculty of Biology, University of Warsaw,
62 Poland
- 63 ³² School of Engineering, Newcastle University, Newcastle Upon Tyne, UK
- 64 ³³ Plataforma Solar de Almería – CIEMAT, P.O. Box 22, 04200, Tabernas, Almería, Spain
- 65 ³⁴ Université de Lorraine, CNRS, LRGP, F-54000 Nancy, France
- 66 ³⁵ Rudjer Boskovic Institute, Bijenicka 54, Zagreb, Croatia
- 67 ³⁶ Laboratoire "Microorganisme : Génome et Environnement", Université Clermont Auvergne, BP 10448, F-63000
68 Clermont-Ferrand, France
- 69 ³⁷ CNRS, UMR 6023, LMGE, F-63170 Campus Universitaire des Cézeaux, Clermont-Ferrand, France
- 70 ³⁸ Service d'hygiène hospitalière, CHU Clermont-Ferrand, Clermont-Ferrand, France
- 71 ³⁹ Laboratori EMATSA, Ctra Valls Km 3, 43130 Tarragona, Spain
- 72 ⁴⁰ Catalan Institute for Water Research (ICRA), 17003 Girona, Spain
- 73 ⁴¹ Institute of Functional Interfaces (IFG), Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen,
74 Germany
- 75 ⁴² School of Marine Sciences, Sun Yat-sen University, Guangzhou, China
- 76 ⁴³ School of Earth Sciences and Environmental Engineering, Gwangju Institute of Science and Technology (GIST),
77 Gwangju, 61005, Republic of Korea
- 78 ⁴⁴ Chair of Urban Water Systems Engineering, Technical University of Munich (TUM), Germany
- 79 ⁴⁵ National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune-411007, India

80 ⁴⁶ Jozef Stefan Institute, Jamova 39 1000 Ljubljana, Slovenia

81 ⁴⁷National Medicines Institute, Department of Molecular Microbiology, Chelmska 30/34, 00-725 Warsaw, Poland

82 ⁴⁸University of Coimbra, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

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84

85 **Research Highlights**

- 86
- Cefotaxime-resistant (CTX-R) coliforms in wastewater were surveyed
- 87
- The survey targeted influents and effluents in 57 WWTPs across five continents
- 88
- The relative abundance of CTX-R coliforms varied significantly between WWTPs
- 89
- Some WWTPs discharged high and potentially hazardous levels of CTX-R coliforms

90 **Abstract**

91 The World Health Organization Global Action Plan recommends integrated surveillance programs
92 as crucial strategies for monitoring antibiotic resistance. Although several national surveillance
93 programs are in place for clinical and veterinary settings, no such schemes exist for monitoring
94 antibiotic-resistant bacteria in the environment. In this transnational study, we developed,
95 validated, and tested a low-cost surveillance and easy to implement approach to evaluate antibiotic
96 resistance in wastewater treatment plants (WWTPs) by targeting cefotaxime-resistant (CTX-R)
97 coliforms as indicators. The rationale for this approach was: i) coliform quantification methods are
98 internationally accepted as indicators of fecal contamination in recreational waters and are
99 therefore routinely applied in analytical labs; ii) CTX-R coliforms are clinically relevant,
100 associated with extended-spectrum β -lactamases (ESBLs), and are rare in pristine environments.
101 We analyzed 57 WWTPs in 22 countries across Europe, Asia, Africa, Australia, and North
102 America. CTX-R coliforms were ubiquitous in raw sewage and their relative abundance varied
103 significantly (<0.1% to 38.3%), being positively correlated ($p < 0.001$) with regional atmospheric
104 temperatures. Although most WWTPs removed large proportions of CTX-R coliforms, loads over
105 10^3 colony-forming units per mL were occasionally observed in final effluents. We demonstrate
106 that CTX-R coliform monitoring is a feasible and affordable approach to assess wastewater
107 antibiotic resistance status.

108 **Keywords:** Antibiotic resistance, coliforms, ESBLs, wastewater treatment, water reuse

109

110 **1 Introduction**

111 Over the past few decades, the global spread of antibiotic resistance has increased to alarming
112 levels, approaching what has been coined as the "post-antibiotic era" (Heymann et al., 2007).
113 While this phenomenon is traditionally linked to healthcare-associated infections (Ventola, 2015),
114 it is believed that animal husbandry, aquaculture facilities and urban wastewater treatment plants
115 (WWTPs) also contribute to propagating antibiotic resistance by discharging antibiotic-resistant
116 bacteria (ARB), antibiotic resistance genes (ARGs), and residual concentrations of antibiotic
117 compounds into downstream aquatic and terrestrial environments (Berendonk et al., 2015; Tripathi
118 and Cytryn, 2017). Standard microbial water quality assessment methods, including quantification
119 of intestinal coliforms and enterococci, are frequently applied for monitoring microbial quality in
120 wastewater treatment effluents (Tallon et al., 2005). While the methods based on these indicators
121 are considered to be reliable for the detection of fecal contamination, they do not assess antibiotic
122 resistance levels, a new threat that increasingly needs routine evaluation. When associated with
123 mobile genetic elements (MGEs), ARGs can be horizontally transferred between bacterial cells,
124 even across distinct lineages, thereby facilitating antibiotic resistance dissemination (Smets and
125 Barkay, 2005). Consequently, ARGs have been described as "contaminants of emerging concern"
126 (Pei et al., 2006; Pruden et al., 2013) and have motivated a plethora of studies targeting ARGs in
127 WWTPs effluents and downstream environments (Marano et al., 2019; Narciso-da-Rocha et al.,
128 2018; Rizzo et al., 2013; Wang et al., 2014). Most of these studies rely on molecular biology
129 methods, which provide highly informative data on the ARGs present in these environments. These
130 studies facilitated efforts to standardize analytical methods for measuring selected ARGs in
131 WWTP effluents and receiving water bodies (Rocha et al., 2018). However, the application of
132 molecular methods also requires specialized equipment, expensive reagents and proficient

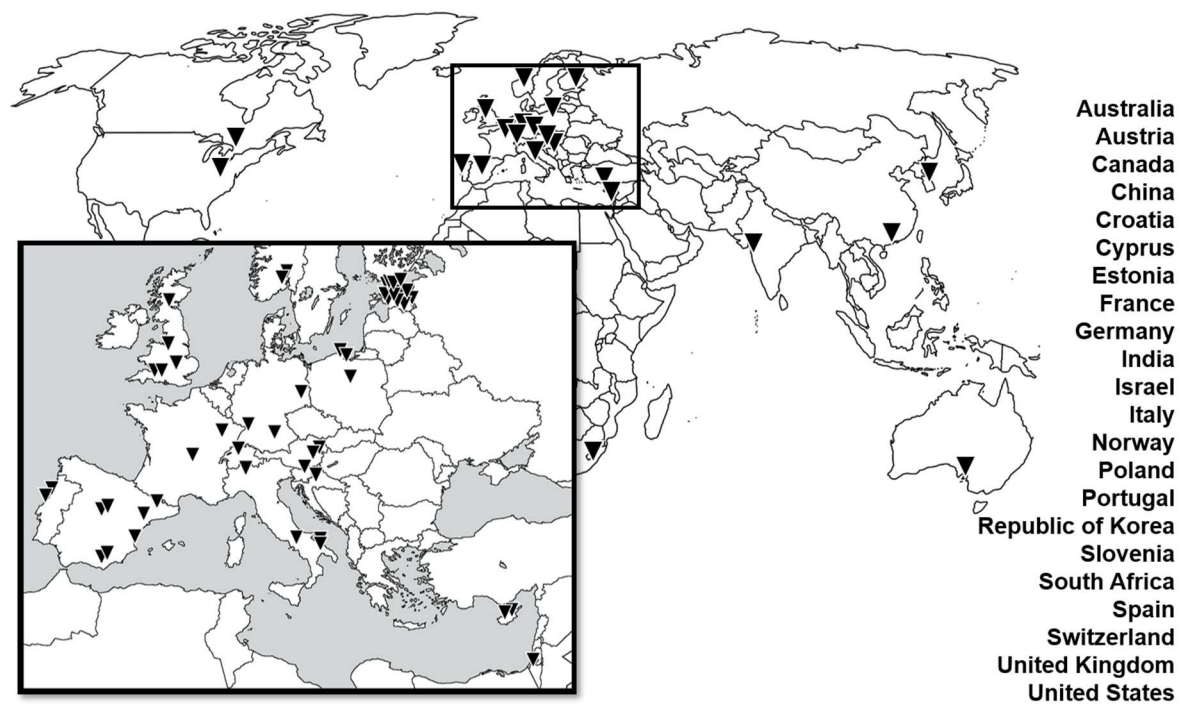
133 technical staff, making them less suitable for routine, widespread WWTP monitoring (Bürgmann
134 et al., 2018; Manaia et al., 2018). Moreover, these methods do not interrelate with conventional
135 (culture-based), globally standardized microbiological water quality indicators. Although culture-
136 based methods often overlook a large fraction of the wastewater microbiota, they can provide
137 fundamental data on antibiotic resistance trends of individual species and strains coupling
138 antibiotic resistance phenotypes to individual bacterial isolates of clinical concern. A major bias
139 of culture-based methods comes from omitting unknown and often strictly environmental bacteria
140 that are profuse in wastewater samples but do not grow on standard culture media, as suggested
141 by Bengtsson-Palme et al. (2016). The use of culturable coliforms indicators, such as *Escherichia*
142 *coli*, has been globally adopted due to its low-cost and ease of implementation for assessing fecal
143 contamination; however, previous experience has shown that antibiotic-resistant bacteria of
144 human/animal origin thriving in wastewater are not only fecal coliforms but include a wider range
145 of enterobacteria (Ferreira da Silva et al., 2007; Vaz-Moreira et al., 2015). Therefore, a surveillance
146 system that could tackle these aspects was designed and tested as presented in this paper. Targeting
147 antibiotic-resistant coliforms may be especially relevant from human health perspective given the
148 realization that horizontal transfer of MGEs occurs much more frequently between
149 phylogenetically closely related organisms and that this process is especially prevalent among
150 coliforms (Popa and Dagan, 2011; Vaz-Moreira et al., 2015).

151 Within the framework of the European Union’s COST Action ES1403: *New and emerging*
152 *challenges and opportunities in wastewater reuse* (NEREUS), we conceived a simple
153 experimental scheme for estimating the abundance of antibiotic resistance using standard methods
154 that can easily be applied and interpreted by stakeholders like WWTP operators. The method
155 makes use of standard membrane fecal coliform agar (mFC Agar) medium to quantify coliforms

156 (Rompré et al., 2002), but in tandem also quantifies antibiotic-resistant coliforms by amending the
157 mFC Agar medium with cefotaxime, a third-generation cephalosporin, at a clinically-relevant
158 concentration (Heil and Johnson, 2016). Resistance to this antibiotic has considerable clinical
159 significance due to the increasing abundance of resistant bacteria-harboring extended-spectrum
160 β -lactamase (ESBL) genes, which are frequently associated with multidrug resistance and
161 widespread in the environment (Bradford, 2001; Pitout and Laupland, 2008). This is especially
162 true for clinically relevant "priority pathogens" from the *Enterobacteriaceae* family including
163 *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp., but is also pertinent to other
164 Gram-negative "ESKAPE" pathogens such as *Acinetobacter baumannii* and *Pseudomonas*
165 *aeruginosa* (Boucher et al., 2009).

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

177



178

179 **Figure 1. A geographical overview of the global survey on cefotaxime-resistant coliforms.**

180 Black triangles show geographic locations of the participating countries where WWTP samples
181 were collected. Square insert shows targeted European WWTPs.

182

183 **2 Materials and methods**

184 *2.1 Site description and WWTP characteristics*

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

189 For each WWTP, grab samples were taken in two campaigns. The first campaign consisted
190 of three sampling dates, once per month, between December 2016 and February 2017; for the
191 second campaign, two sampling dates were chosen between May and October 2017
192 (Supplementary Table 1). Participating groups were provided with detailed protocols for sampling,
193 sample processing and cultivation and bacterial enumeration and completed a questionnaire on
194 metadata related to the country and the WWTPs. All experimental data were screened, quality
195 checked, and assembled as described below before performing statistical analyses.

196

197 *2.2 Metadata collection*

198 The participating groups filled out a questionnaire with data related to WWTP catchment
199 population size and influent characteristics, the WWTPs' technical setup and operational
200 conditions, physicochemical characteristics of the final effluent, meteorological conditions of
201 WWTP sites, antibiotic consumption in each country and the WWTPs' GPS coordinates.
202 Meteorological conditions were retrieved from official national forecast online web databases,
203 using average monthly temperatures of sampling days. Antibiotic consumption, available for
204 European countries only, was retrieved from the antimicrobial use database of the European Centre

205 for Disease Prevention and Control (www.ecdc.europa.eu) using the filters: "2016", "β-lactam
206 antibacterials and penicillins", and "primary care sectors". Antibiotic consumption is indicated as
207 'defined daily doses (DDD) per 1000 inhabitants per day'.

208 *2.3 Sampling*

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

219 *2.4 Enumeration of bacteria*

220 Membrane fecal coliform Agar (mFC, Difco® with 0.01% (w/v) Rosolic Acid (Difco®))
221 was prepared according to manufacturer instructions. For the quantification of CTX-R coliforms,
222 mFC Agar was supplemented with cefotaxime sodium salt (Sigma®) at a final concentration of 4
223 µg mL⁻¹. This concentration was based on the minimal inhibitory concentration (MIC) breakpoint
224 levels for *Enterobacteriaceae*, documented by CLSI (Clinical and Laboratory Standards Institute,
225 Wayne, PA, 2010), which is more stringent than the EUCAST MIC breakpoints of >2 µg mL⁻¹
226 (European Committee of Antibiotic Susceptibility Testing

227 http://www.eucast.org/clinical_breakpoints/) and would also be more selective against false
228 positives and intermediately resistant colonies. Culture media were prepared no more than three
229 days before sampling and dispensed in 60 mm diameter Petri plates. When the liquid culture
230 medium reached 55 °C, the appropriate volume of the fresh concentrated filter-sterilized antibiotic
231 stock solution was added before pouring plates. Samples were serially diluted and 1 mL from 2-4
232 consecutive 10-fold serial dilutions were filtered through 47 mm diameter 0.45 µm pore size
233 nitrocellulose sterile membrane filters on mFC Agar plates (in triplicate) with and without
234 cefotaxime. For less turbid water samples (*i.e.*, effluents) up to 100 mL were filtered. Cultures
235 were incubated for 24 hours at 37 °C. Incubation time was not prolonged beyond 24 hours to avoid
236 biases associated with the inactivation of cefotaxime in the culture medium by the CTX-R bacteria
237 that produce extracellular cefotaxime degrading β-lactamases. Moreover, even though the mFC
238 Difco® protocol recommends incubation at 44 °C, we specifically chose 37 °C (i) to reduce curing
239 of plasmids harboring ARGs, which has been shown to occur at elevated temperatures (Trevors,
240 1986), and (ii) to avoid possible functional thermal instability previously reported for CTX-M
241 enzymes and other β-lactamases at higher temperatures (He et al., 2016; Vanhove et al., 1995). To
242 validate our choice of incubation temperature we compared the identity of the bacterial groups
243 recovered under both the conditions and criterion used (*i.e.*, incubation at 37 °C vs. 44 °C), (see
244 section 2.7). Under these conditions, the blue colonies enumerated on mFC Agar with or without
245 cefotaxime were considered as presumptive coliforms. The limit of detection (LOD) of the method
246 was calculated as the minimal number of colonies that could be enumerated in the highest volume
247 analyzed. Extrapolation of the generated results suggests that the LOD was approximately 0.3
248 colony forming units (CFUs) mL⁻¹ (with 1 being the minimum number of observable colonies on
249 triplicate mFC plates for 1 mL of filtered water); however, it should be noted that in a few cases

250 filtered effluent volume was increased to 10 or 100 mL when water samples were particularly
251 clear. To improve reproducibility, it was recommended that each step in the procedure (media
252 preparation, dilutions, filtration and colony enumeration) be conducted by the same person
253 processing the samples of a given WWTP. Participating groups were provided with a standard
254 spreadsheet to collect the results of bacterial enumeration and were asked to report the date and
255 location of sampling.

256

257 *2.5 Quality control criteria*

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

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

268 The relative abundance of CTX-R coliforms was calculated by dividing the number of
269 CFUs mL⁻¹ on mFC Agar supplemented with cefotaxime by the number of CFUs mL⁻¹ on mFC
270 agar without antibiotic for each of the three replicates. The WWTP removal efficacy for coliforms

271 and CTX-R coliforms was calculated as either percent or log-unit removal (respectively) using the
272 following equations:

273
$$\frac{CFUs\ influent - CFUs\ outflow}{CFUs\ influent} \times 100 ; \quad \log\left(\frac{CFUs\ influent}{CFUs\ outflow}\right).$$

274

275 *2.6 Statistical analyses*

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

287

288 *2.7 Methodological validation through bacterial characterization*

289 In parallel with the surveillance study, the diversity of bacteria targeted by the procedure
290 used was assessed and the taxonomy of selected bacteria was determined in a parallel series of
291 tests, with the following objectives in mind: (i) assess the taxonomic composition of wastewater
292 derived bacteria forming blue colonies on mFC Agar at 37 °C and compare with that of blue
293 colonies recovered at 44 °C (the recommended incubation temperature for isolating fecal coliforms

294 on mFC Agar); (ii) assess if, as hypothesized, bacteria with acquired antibiotic resistance are more
295 prevalent when incubated at 37 °C than at 44 °C.

296 These bacterial characterization experiments involved eight partners of different
297 geographic locations (USA, Australia, Israel, Portugal, UK, Germany, Croatia, Poland). Samples
298 from secondary effluents (only) were processed as described above and incubated at 37 °C and
299 44 °C. From plates incubated at 37 °C or 44 ° (on agar with and without cefotaxime) a total of 30
300 blue CFUs were selected, isolated, and taxonomically characterized using either 16S rRNA gene
301 sequence analysis or MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight
302 mass spectrometry; detailed protocols for both are described in the Supplementary Material). To
303 elucidate the potential impact of incubation temperature on the relative abundance of CTX-R
304 isolates, the recovery capacity of CTX-R *E. coli* isolates was evaluated at the two compared
305 incubation temperatures; only *E. coli* were investigated due to their clinical relevance and the fact
306 that they were the most abundant species identified, suitable for statistical investigation. The
307 amount of total and CTX-R *E. coli* CFU per mL in raw samples was estimated, for each assembly
308 of 30 isolates, from each group, from the four tested conditions using the formula below:

$$309 \left(\frac{\text{N identified } E. coli}{\text{N total identified}} \right) \times (\text{N blue CFUs on filter} \times \text{Df})$$

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

315

316 **3 Results and discussion**

317 Numerous studies have explored antibiotic resistance in WWTP influents and effluents using
318 both traditional cultivation techniques (Hoelle et al., 2019) and culture-independent molecular
319 analyses that target ARGs (Petrovich et al., 2018). Data generated from these studies is very
320 important, but because they are generally constrained to individual facilities or local regions, they
321 lack perspective on the global dimensions of antibiotic resistance in WWTPs. In contrast, two
322 recent international collaborative initiatives have facilitated comprehensive perceptions of
323 antibiotic resistance in WWTPs. By applying shotgun metagenomic analysis of untreated sewage
324 in 79 sites from 60 countries around the world, Hendriksen et al. (2019) found systematic
325 geographical differences in abundance and diversity of ARGs, with little correlation to antibiotic
326 use or bacterial taxonomy. While specific factors were difficult to identify, they concluded that
327 socioeconomic factors such as poor sanitation were primary drivers of ARG propagation. A recent
328 pan-European study conducted by Pärnänen et al. (2019) that targeted over 250 ARGs and
329 associated MGEs in influents and effluents of 12 WWTPs from seven countries using highly-
330 parallel quantitative PCR found a north-south distribution of ARGs, consistent with trends
331 observed for clinical isolates (ECDC, 2018). While both of these studies significantly contribute
332 to our understanding of antibiotic resistance in WWTPs, they are based on methodologies that are
333 extremely costly and require (i) advanced expertise in manipulation and data analyses, (ii)
334 DNA/RNA extraction kits, (iii) expensive equipment (e.g. real-time PCR, sequencing facilities)
335 and (iv) Physical Containment facilities (*i.e.* PC2, PC3), all normally not available in WWTP labs.
336 We therefore focused on implementing a simple culture-based approach that can be routinely
337 applied or analyzed on a global scale for elucidating the abundance of clinically-relevant
338 antibiotic-resistant bacteria in WWTPs. Such a method would only require a simple modification

339 of already existing microbial analytical procedures at WWTPs compared to the costly alternative
340 methods described above (i.e qPCR and metagenomics). A comparative cost analysis to better
341 elucidate on such expenses is given in Supplementary Table 5. Of note, the described method
342 should not be intended as an alternative to the use of mFC Agar® plates incubated at 44 °C, as the
343 populations targeted by the two conditions (i.e. 44 °C and 37 °C) are slightly different as elaborated
344 below).

345 Comparison of the two incubation temperatures described in section 2.7 revealed that mFC
346 Agar cultures (with/without cefotaxime) incubated at 44 °C selected for members of the
347 *Enterobacteriaceae* family, with the majority of these being *E. coli* (>71% of the identified), as
348 expected. *E. coli* was also the most abundant species detected from the mFC screened cultures
349 incubated at 37 °C, although at a lower frequency (>31% of the identified). Other genera of the
350 family *Enterobacteriaceae* were detected on plates incubated at 37 °C with or without cefotaxime,
351 and these included *Citrobacter*, *Enterobacter*, and *Klebsiella spp.*, all genera that include
352 opportunistic human pathogens (Supplementary Figure 1). Non-*Enterobacteriaceae* were also
353 detected in cultures incubated at 37 °C, mainly *Aeromonas spp.* (*A. caviae* and *A. hydrophila*),
354 representing less than 23% of all identified isolates, while other genera and species represented
355 less than 3% (Supplementary Figure 2). Noticeably, although the amount of *E. coli* among the blue
356 colonies counted on mFC Agar incubated at 37 °C was lower than those counted on mFC Agar
357 incubated at 44 °C, both estimates were within the same order of magnitude when considered as
358 CFU per mL (average ratio of 6 comparisons between the two incubation temperature = 1.1 ± 0.4 ;
359 Supplementary Figure 3). On the contrary, incubation of mFC Agar plates supplemented with
360 cefotaxime at 37 °C facilitated higher ($p < 0.05$) recovery of CTX-R *E. coli* than the same medium
361 incubated at 44 °C (by a factor 2.8 ± 1.5 across 6 comparisons), supporting the hypothesis that

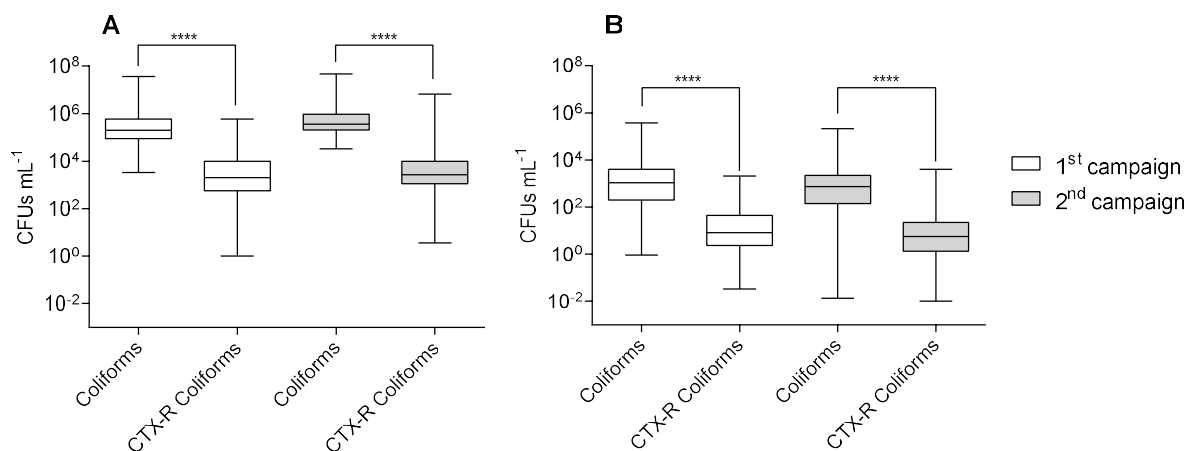
362 acquired antibiotic resistance is more stable at 37 °C than at 44 °C. These findings suggest that
363 despite the slightly elevated number of false positives (non coliforms and aeromonads) generated,
364 the significantly higher level of CTX-R *E. coli* detected at 37 °C supports its application in
365 targeting the resistant *E. coli* fraction of wastewater. At least for the tested fraction of the coliform
366 community, these findings support the hypothesis that CTX-R coliforms might be selected against
367 or lose resistance determinants such as plasmids at higher temperatures, biasing the evaluation of
368 their proportion in the original sample (Trevors, 1986).

369

370 *3.1 Abundance of CTX-R coliforms in raw sewage*

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

380

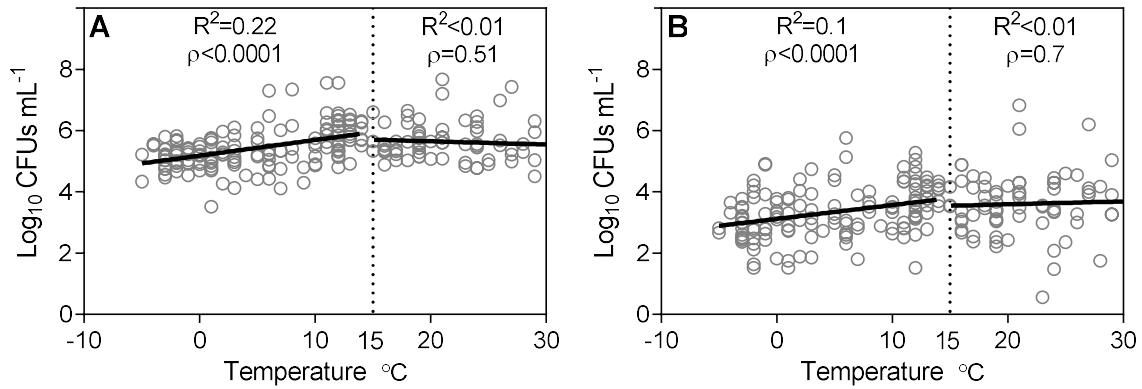


381

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

386 Strong variation in CTX-R coliforms levels between the different WWTP influents may
387 stem from a myriad of factors such as antibiotic use, sanitary conditions, and various abiotic
388 factors. Recent studies have suggested significant correlations between climate (aerial
389 temperature) and antibiotic resistance. MacFadden et al. (2018) performed a meta-analysis of
390 antibiotic-resistant *E. coli*, *K. pneumoniae* and *Staphylococcus aureus* data from different
391 geographical regions of the US (1.6 million strains, from 223 facilities across 41 states between
392 2013–2015). They found that regional temperature was the most significant factor associated with
393 antibiotic resistance, where a 10 °C increase in minimum temperature coincided with
394 approximately 2-6% increase in resistance in the three targeted pathogens. Temperature is very
395 often underestimated in wastewater microbial ecology, in contrast to freshwater microbial
396 ecology, where it is considered one of the most significant parameters in determining the bacterial

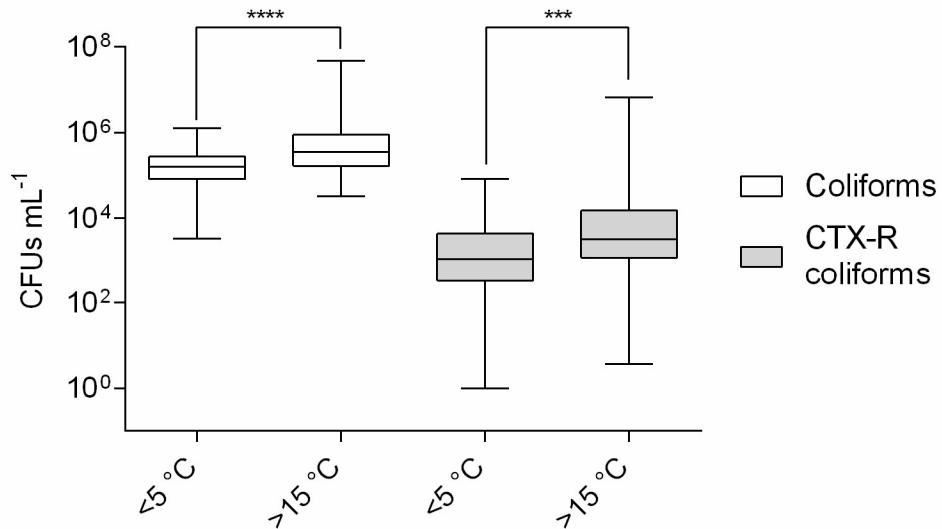
397 community composition of rivers and lakes (Likens, 2010). To investigate the potential correlation
398 between local temperature (monthly temperature, at the collection site, of the day of sampling
399 based on meteorological data) and the abundance of coliforms and CTX-R coliforms in raw
400 sewage, 228 influent samples comparing monthly mean temperatures with an absolute abundance
401 of coliforms and CTX-R coliforms were analyzed. The rationale for addressing ambient
402 temperature stemmed from three factors: (i) ambient temperature can significantly influence
403 sewage temperature within activated sludge where aeration is applied (due to differences in air
404 temperature); (ii) temperature can have a major impact in combined sewer systems that receive
405 rain/melted snow; (iii) temperature is often a surrogate parameter for geographical latitude as
406 north-south gradients, which are frequently reported in clinical ABR monitoring studies
407 (MacFadden et al., 2018; Pärnänen et al., 2019). We observed a slightly positive correlation
408 between \log_{10} converted CFU values and temperature, which was more prominent below 15 °C
409 for both coliforms ($R^2=0.22$) and CTX-R coliforms ($R^2=0.1$) (Figure 3, Supplementary Figure 9).
410 Upon the observed trend inversion of the dataset above and below this indicated temperature, we
411 compared the CFU abundance in samples from places with monthly regional temperatures below
412 5 °C and above 15 °C (as monthly averages), represented by two groups with a similar number of
413 samples (77 samples <5 °C and 72 samples >15 °C) for both coliforms and CTX-R coliforms at
414 both temperature ranges (Figure 4). A version of Figure 4 of the complete datapoint split into three
415 ranks is presented in Supplementary Figure 10.



416

417 **Figure 3. Linear regression of log₁₀ converted coliforms (A) and CTX-R coliforms (B) in WWTP influents as a**
418 **function of monthly average temperature in the sampled areas.** Datasets are split into temperatures ranks below
419 and above 15 °C (highlighted by dotted lines); R² and *p*-values from linear regression are reported for each tested
420 interval. A version of these data analyses without splitting of temperature intervals is given in Supplementary Figure
421 9.

422



423

424 **Figure 4. Comparison of coliforms and CTX-R coliforms abundance in WWTPs' influent sewage in selected**
425 **temperature ranks.** Asterisks refer to a Mann-Whitney U-test with given *p*-values <0.0001 (****) and *p*<0.001 (***).

426

Sample size coliforms : <5 °C n=77, >15 °C n=72; sample size CTX-R coliforms <5 °C n=76, >15 °C n=72.

427

428

Box plots show percentiles together with medians; whiskers show the minimum and maximum values. A version of these data

429

analyses with complete dataset intervals is given in Supplementary Figure 10.

430

Both, total and CTX-R coliforms (CFUs mL⁻¹) were generally more abundant in WWTPs

431

influent from areas with higher temperatures (Figure 4) and this observation was not dependent

432

on specific locations or sampling campaign, as data from the WWTPs were evenly distributed

433

across the two temperature ranks. However, no clear statistical correlation with temperature was

434

observed when looking at the relative abundance of CTX-R coliforms (Supplementary Figure 4)

435

suggesting that other confounding variables might have influenced the observed relative

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abundance in sewage. Despite the fact that correlation does not necessarily entail causation, it was

437

speculated that a higher abundance of coliforms in WWTPs operating in geographical areas with

438

temperatures above the 15 °C threshold may be explained by a multitude of climatic and potential

439

indirect socioeconomic factors that were not taken into consideration in this study. The difficulty

440

in disentangling temperature from other factors such as antibiotic use, population density, and

441

hygiene, complicates the identification of specific factors linked to antibiotic resistance, as

442

indicated in the two recent molecular-based global studies described above (Hendriksen et al.,

443

2019; Pärnänen et al., 2019). It is possible that water temperature might be more informative in

444

deciphering such trends and we recommend future studies to couple CFU-related data analyses

445

with water temperatures together with air temperatures.

446

Average defined daily doses (DDD) of β -lactams per 1000 inhabitants in European

447

countries were compared to CFU counts in influents using linear regression analysis, but the tests

448

failed to differentiate correlations between DDD values and coliforms, or between DDD values

449 and CTX-R coliforms, therefore this parameter was considered to be not informative in this study
450 (data not shown). However, it should be noted that DDD values used here reflect general antibiotic
451 usage trends of entire countries and not necessarily the local areas that feed a given WWTP.
452 Previous studies by Collignon et al. (2018) likewise found that annual average β -lactam antibiotics
453 usage in individual communities may not be a reliable predictor of resistance levels of fecal
454 coliforms in wastewater. This may be due to the low resolution of national antibiotic consumption
455 data and may be resolved if coupled with accurate local surveys on antibiotic prescriptions in the
456 area served by a given WWTP, as shown by Caucci et al. (2016). Together with β -lactam
457 antibiotics usage, the contribution of hospital effluents to CTX-R coliforms relative abundance
458 observed in WWTPs' sewage was evaluated comparing (i) the ratio between the number of beds
459 equivalent and population served, to (ii) the observed CTX-R coliforms relative abundance in the
460 influents. From the 17 WWTPs analyzed where data were available (Supplementary Table 1), no
461 correlation was observed (slope not significantly different from 0; Supplementary Figure 5). The
462 most plausible explanation for these observations is that antibiotic resistance dynamics are dictated
463 by complex ecological interactions that may not be explained solely based on the use of antibiotics
464 in clinical settings or even on the occurrence of antibiotic residues in wastewater (Novo et al.,
465 2013; Varela et al., 2014). Other factors related to the individual wastewater network at
466 catchment points might further influence such diversity (e.g. volume of flow, combined or
467 separated sewer systems, etc.) (Choi et al., 2018).

468

469 *3.2 CTX-R coliform removal in WWTPs*

470 The removal of coliforms and CTX-R coliforms was evaluated in WWTPs where both
471 influent and effluent samples were available. Overall, from all sampling dates, a total of 220 count

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

481 The data from the seven WWTPs that included a final disinfection step were specifically
482 analyzed to evaluate the abundance and removal dynamics of CTX-R coliforms along the
483 treatment continuum (influent, secondary effluent, and disinfected effluent). Overall multiple
484 sampling dates (1st and 2nd campaign combined) from these seven investigated WWTPs
485 contributed to assemble 23 full continuum profiles. All these WWTPs applied CAS treatment
486 combined with one of the following disinfection treatments: chlorination ($n=2$), UV radiation
487 ($n=3$), chlorination and UV radiation ($n=1$) or ozonation ($n=1$). In these WWTPs, concerning the
488 raw sewage, the CAS treatment reduced the coliforms and CTX-R coliform loads by
489 approximately 2 log-units, whereas average log removal in the final disinfected effluents was
490 4.4 ± 2 for coliforms and 3.5 ± 1.4 for CTX-R coliforms. However, we observed a high data
491 dispersion of coliforms and CTX-R coliforms loads in the final effluents (Supplementary Figure
492 6). Indeed, the abundance of CTX-R coliforms in all disinfected effluents analyzed were below
493 the LOQ in 28 sampling instances (35% of total disinfected final effluent samples analyzed).

494 The scope of secondary treatment removal of coliforms and CTX-R coliforms was
495 investigated as a function of WWTP regional temperature to evaluate a possible correlation
496 between removal efficiency and temperature. Such an association was evaluated in both percentage
497 removal relative to influent levels and absolute log-units removal of CTX-R coliforms choosing
498 two aeriels temperature ranks, $<5\text{ }^{\circ}\text{C}$ and $>15\text{ }^{\circ}\text{C}$ (Supplementary Figure 7 and Supplementary
499 Figure 8). Overall, no significant differences were observed between the two groups following
500 secondary biological treatment; however, in geographical locations where temperature was below
501 $5\text{ }^{\circ}\text{C}$, we identified a greater number of WWTPs with removal efficiencies of $<95\%$ of resistant
502 coliforms (Supplementary Figure 7). Specifically, the lowest efficiencies were observed for POL2,
503 EST10, DEU1, EST2, GBR4, POL3, EST5, AUT2, and EST4, suggesting that these low removal
504 efficiencies are not nationally or geographically associated, and can be eventually sporadic failures
505 of the systems, potentiated or not by lower temperature. Similar observations from previous studies
506 had also showed that higher ambient temperatures and correlate with increased activated sludge
507 removal rates of coliforms (Miranzadeh et al., 2013). It should be noted however, that other
508 WWTPs with higher removal efficiency were present in the same regions. Furthermore,
509 temperature in secondary aeration tanks can be substantially different from air temperature,
510 therefore the climate may not be a strong predictable indicator of removal efficiency.

511

512 *3.3 CTX-R coliforms in discharged effluents*

513 WWTP effluents are either discharged into natural water bodies such as streams, rivers,
514 lakes, seas, oceans, or used for irrigation of land. In both scenarios, antibiotic-resistant bacteria
515 (and specifically human commensals or pathogens) can potentially be introduced into the urban
516 water cycle and food webs, subsequently leading to increased human exposure. Stakeholders

517 routinely monitor fecal coliforms to assess contamination in effluent-receiving recreational water
518 bodies, and treated wastewater used for irrigation. However, these indicators do not provide insight
519 into the potential contribution of effluents to antibiotic resistance dissemination, which can have
520 substantial environmental and epidemiological significance (Berendonk et al., 2015; Manaia,
521 2017). The advantage of the microbiological method described in this study is that it can be adopted
522 with only minor variation by existing national and regional surveillance programs, with minimal
523 need for additional infrastructure or professional requirements, and with minimal added costs.

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

529 The analysis of the effluent samples with high CTX-R coliforms counts did not reveal
530 noticeable geographic trends, suggesting that these values may be dependent on local WWTP
531 characteristics and performance, although more robust surveillance of a broader range of WWTPs
532 is required to validate this observation. One exception was the Pune WWTP analyzed in this study,
533 which had the highest influent and effluent resistance levels in both absolute and relative
534 abundance in both campaigns. While one WWTP from India is surely not enough to reveal
535 geographic trends, the high influent CTX-R coliforms levels observed therein may be associated
536 with multiple factors including non-regulated antibiotic consumption, contamination from local
537 antibiotic production facilities that may promote the selection of resistance genes. India is reported
538 as one of the largest consumers of antibiotics (Laxminarayan and Chaudhury, 2016) and previous
539 studies had linked fecal coliforms to high levels of ESBL levels in WWTPs in the country (Lamba

540 and Ahammad, 2017). Combined with fragile sanitary infrastructure, the above-mentioned factors
541 might significantly contribute to the observed values (Bengtsson-Palme et al., 2014). Of note,
542 comparatively higher temperatures of India during samplings could have also contributed to the
543 higher coliforms and CTX-R counts observed (in accordance with MacFadden et al., (2018))
544 possibly due to faster bacterial growth at higher temperatures (Marano and Prakash personal
545 communication). On the other hand, such simplistic inference might not be applied to other
546 countries where a high abundance of CTX-R coliforms was also measured in the treated effluent,
547 such as the WWTPs in Poland, where regulations follow EU requirements. Despite various
548 attempts of geographical sorting indeed, apart from Poland, we could not identify any clear links
549 between CTX-R coliform effluent levels and specific countries. This was evident for example for
550 Spain ($n_{WWTP}=7$), UK ($n_{WWTP}=5$), Italy ($n_{WWTP}=4$), and in particular Estonia ($n_{WWTP}=12$) where
551 different WWTPs exhibited both high and low CTX-R coliforms effluent values.

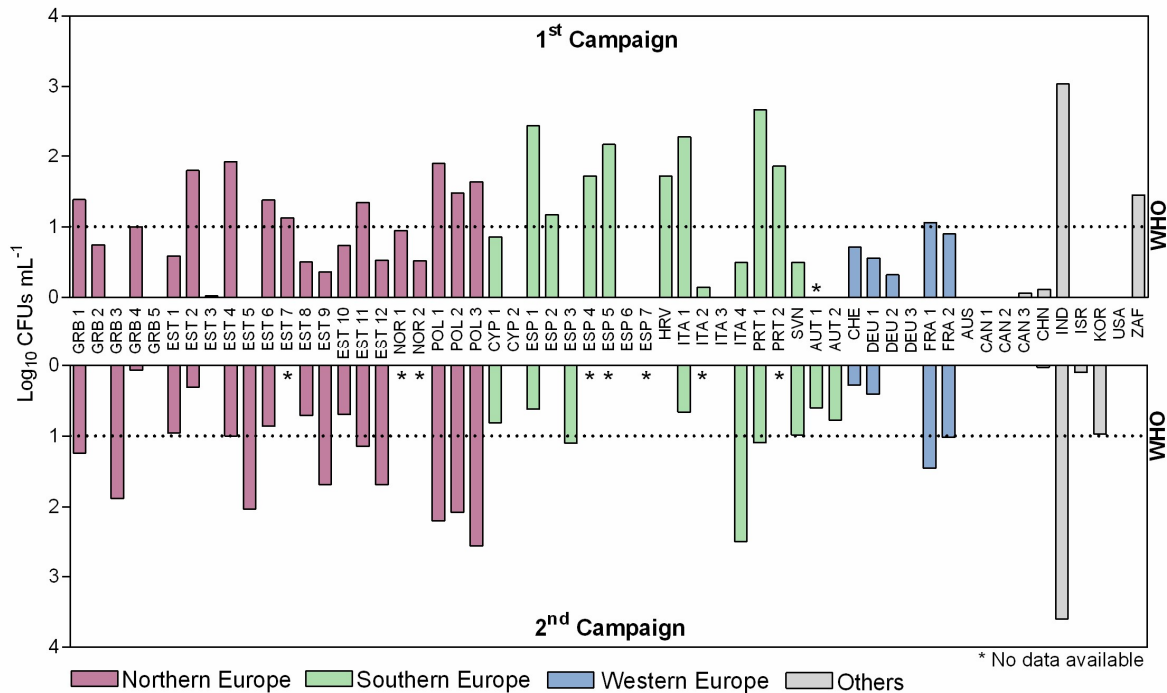
552

553 *3.4 Implications and potential standards for discharged CTX-R coliforms in receiving* 554 *environments*

555 This study provides data that can facilitate the development of urgently required criteria
556 for the permitted levels of CTX-R coliforms in effluents and various water reuse scenarios.
557 Existing fecal coliform standards for wastewater recognize the potential threat that wastewater
558 effluent poses to human health, although criteria for ‘safe’ levels of fecal indicators vary greatly
559 between regions and use. For example, in California and Israel, the fecal coliform limits are 2.2
560 and 10 CFU per 100 mL, respectively (EPA, 2012). In Greece and Cyprus, these are 100 CFU per
561 100 mL for fecal coliforms, whereas the World Health Organization (WHO) standards for water
562 re-use for irrigation are 1,000 CFU per 100 mL for fecal coliforms (Blumenthal et al., 2000).

563 Similar criteria exist for discharge into water bodies intended for recreational use. For example,
564 the US Environmental Protection Agency recommends a limit of 200 CFU per 100 mL of fecal
565 coliforms in rivers and streams used for swimming (U.S. EPA, 1976). While most of the effluent
566 samples contained low levels of CTX-R coliforms, it is troubling that 37% (89 out of 243
567 individual sampling points) exceeded the WHO cutoff formulated for fecal coliforms (resistant
568 and not; Figure 5). Of note, as discussed above, whilst the hereby proposed method detects also
569 coliforms other than *E. coli* (conventionally the most used fecal coliform indicator), *E. coli* still
570 represent up to 50% of the pool of coliforms detected on mFC Agar plates incubated at 37 °C. If
571 one considers that an average large WWTP (e.g. 50,000 person equivalent) can release more than
572 10⁷ L per day of effluent, we estimate that under suboptimal treatment conditions (CTX-R
573 coliforms >10 CFU mL⁻¹ referring to the above mentioned WHO indicated threshold for fecal
574 coliforms), up to 10¹¹ CTX-R coliforms may be released daily into effluent receiving rivers.
575 Additionally, if we consider that under Mediterranean climate conditions seasonal irrigation can
576 exceed 1 m³ of water per square meter of soil, this would mean that under the same assumptions
577 each square meter of soil could potentially receive 10⁸ CTX-R coliforms per season. While many
578 countries have developed methods for optimizing the irrigation of edible crops, long-term exposure
579 to antibiotic resistance determinants still might increase the risks of systematic transmission of
580 these determinants throughout natural environments. Different types of downstream environments
581 might present different permissiveness in terms of antibiotic-resistant bacteria dissemination and
582 persistence, and release of antibiotic-resistant bacteria into surface water bodies can have a
583 different and broader impact than the release in soil (Eckert et al., 2018; Leonard et al., 2018;
584 Munck et al., 2015). Future studies should seek to ascertain whether antibiotic-resistant bacteria
585 in these different environments are transmitted to humans, as well as to describe the impacts on

586 human health resulting from such exposures. Additionally, they should characterize the genetic
 587 and phenotypic diversity of CTX-R coliforms and evaluate their capacity to persist in downstream
 588 environments (Karkman et al., 2018). Finally, effluents intended for re-use or discharged to
 589 bathing waters should be further investigated to assess the presence of pathogens, such as resistant
 590 *K. pneumoniae*, and enterohemorrhagic *E. coli* (EHEC) strains (Nguyen and Sperandio, 2012).



591
 592 **Figure 5. Average CTX-R coliforms mL⁻¹ in WWTPs' effluents (country-code+WWTP's assigned number from**
 593 **the same country).** Fifty-seven WWTPs sampled in the first campaign (top) and forty-eight in the second campaign
 594 (bottom). The dotted line shows the WHO limits for water reuse regulation referred to *Escherichia coli* (CFUs mL⁻¹)
 595 log converted. Australia (AUS); Austria (AUT); Canada (CAN); China (CHN); Croatia (HRV); Cyprus (CYP);
 596 Estonia (EST); France (FRA); Germany (DEU); India (IND; Pune); Israel (ISR); Italy (ITA); Republic of Korea
 597 (KOR); Norway (NOR); Poland (POL); Portugal (PRT); Slovenia (SVN); South Africa (ZAF); Spain (ESP);
 598 Switzerland (CHE); United Kingdom (GRB); United States (USA). Asterisks indicated missing participation in the
 599 sampling campaign.

600 **4 Conclusion**

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

621 Cefotaxime-amended chromogenic selective media are increasingly applied in surveillance
622 studies (Snow et al., 2011) and can potentially serve as an alternative to the cefotaxime-amended

623 mFC medium used here; however we recommend the used cefotaxime concentration to be
624 $4 \mu\text{g mL}^{-1}$. Given the growing epidemiological relevance of carbapenemase-producing
625 *Enterobacteriaceae*, future surveys targeting WWTPs should concomitantly apply mFC medium
626 amended with imipenem or meropenem, or use commercial available chromogenic media (García-
627 Fernández et al., 2017). Finally, a subset of the isolates obtained should be subject to screening for
628 cefotaxime-resistance genes (*i.e.* those encoding CTX-M enzymes), using PCR and/or whole
629 genome sequencing and comparative analyses of ARGs and MGEs to detect possible geographic
630 patterns.

631 **Acknowledgments**

632 The authors would like to acknowledge the COST Action ES1403 NEREUS “New and emerging
633 challenges and opportunities in wastewater reuse”, supported by COST (European Cooperation in
634 Science and Technology, www.cost.eu), for enabling the collaboration among the authors of the
635 paper.

636 Part of this work was financed by the Water JPI through the national funding agencies
637 supporting the consortium WaterJPI/0001/2013 STARE – “Stopping Antibiotic Resistance
638 Evolution”.

639 The authors would also like to thank all the wastewater treatment plants’ staff for their
640 support of the sampling campaigns.

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