1   **Systematic review of wastewater surveillance of antimicrobial resistance in**

2   **human populations**

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Abstract

We systematically reviewed studies using wastewater for AMR surveillance in human populations, to determine: (i) the strength of the evidence for a wastewater-human AMR association, and (ii) methodological approaches which optimised identifying such an association, and which could be recommended as standard. We used Lin's concordance correlation coefficient (CCC) to quantify agreement between AMR prevalence in wastewater and human compartments, and logistic regression to identify study features (e.g. sampling methods) associated with high-agreement (defined as wastewater-human AMR prevalences within ±10%).

Of 8,867 records and 232 full-text methods reviewed, 29 studies were included. AMR prevalence data was extractable from 20 studies conducting phenotypic-only (n=11), genotypic-only (n=1) or combined (n=8) AMR detection. Overall wastewater-human AMR concordance was reasonably high for both phenotypic (CCC=0.81 [95% CI 0.74-0.87]) and genotypic comparisons (CCC=0.88 (95% CI 0.85-0.91)) despite diverse species-phenotypes/genotypes and study design. Logistic regression was limited by inconsistent reporting of study features, and limited sample size; no significant relationships between study features and high wastewater-human AMR agreement were identified. Based on descriptive synthesis, composite/flow-proportional sampling of wastewater influent, longitudinal sampling >12 months, and time/location-matched comparisons generally had higher-agreement.

Further research and clear and consistent reporting of study methods is required to confirm optimal practice.

Introduction

Antimicrobial resistance (AMR) is a significant threat to global health (O'Neill, 2016) and poses major challenges in treating infectious diseases. AMR is a multi-faceted problem compounded by diverse drivers facilitating emergence and spread. AMR surveillance is therefore critical to understanding trends, monitoring interventions and developing empiric treatment guidelines, as prioritised in the World Health Organisation's global AMR action plan (WHO, 2019). Large networks dedicated to sharing continental/global AMR data have been established to meet this need, including the European Antimicrobial Resistance Surveillance Network (EARS-Net) and the Global Antimicrobial Resistance Surveillance System (GLASS). However, current surveillance methods are limited by the reliance on individual-level sampling, which is often affected by selection bias towards healthcare-associated settings (WHO, 2018). For example, both EARS-Net and GLASS focus on AMR in clinical specimens from hospitalised patients; this however does not reliably capture AMR prevalence in commensal organisms, thought to silently constitute most of the true AMR burden (Fahrenfeld and Bisceglia, 2016; Hay et al., 2018; Hendriksen et al., 2019b). Additionally, reliance on routine clinical microbiology results often restricts data collection to a limited subset of culturable species, and focuses predominantly on susceptibility phenotypes with limited genotyping. This lack of genotyping hampers the surveillance of high-risk AMR-associated clones and the horizontal transfer of AMR determinants (Tacconelli et al., 2018).

Wastewater-based AMR surveillance has the potential to avoid biases in current surveillance methods by using wastewater to simultaneously sample both healthcare- and community-associated populations (Newton et al., 2015). The approach has already been successful in

64   illicit drug monitoring (González-Mariño, Baz-Lomba et al. 2020) and pathogen (particularly

65   enterovirus) surveillance (Asghar et al., 2014; Fernández et al., 2012), with most recent strides

66   in the surveillance of SARS-CoV-2 (Ahmed et al., 2020), but its application to AMR surveillance

67   has only relatively recently gained significant traction (Fahrenfeld and Bisceglia, 2016). Recent

68   wastewater AMR studies have investigated seasonal/geographic AMR distributions (Su et al.,

69   2017), quantified global abundances of AMR genes (Hendriksen et al., 2019b) and identified

70   associations between wastewater and clinical AMR surveillance data (Karkman et al., 2020;

71   Pärnänen et al., 2019). However, heterogeneous study designs and methods likely contribute

72   systematically to differences in outcomes/interpretations. The relative impact of study

73   features such as grab sampling (i.e. taking single samples at a single timepoint) potentially

74   collecting homogenous solids/unrepresentative samples (Reinthaler et al., 2013), interaction

75   between snapshot/longitudinal sampling design and spatiotemporal variabilities, or sampling

76   in the presence of unrepresentative, contaminating AMR-associated point sources are not

77   well understood (**Table 1**). Difficulties in standardising AMR testing across traditional

78   surveillance networks (Tacconelli et al., 2018) could be, for example, circumvented by using

79   metagenomic sequencing to universally probe wastewater resistomes (Aarestrup and

80   Woolhouse., 2020; Wright, 2007).

81

82   Despite increasing research in this area, there has been no attempt to review the available

83   data, synthesise the evidence, and assess any remaining knowledge gaps. We therefore

84   systematically reviewed studies using wastewater for AMR surveillance in human

85   populations, seeking to identify practices that could optimise the association between

86   wastewater-human AMR for surveillance purposes. We specifically focussed on study design

87   to identify the relative potential impact of specific features on study outcomes, and to

88   highlight any limitations and recommendations for future research.

89

90   **Results**

91   *Literature screen*

92   Of 8,867 de-duplicated studies identified using our search strategy, full-text methods for

93   232 relevant studies were reviewed, and based on pre-specified inclusion criteria (see

94   Methods), 29 studies were included in the review (**Fig.1 and Fig.S1**). Inter-rater reliability

95   was assessed on a subset of 701 studies and gave a Cohen kappa score of 0.76, indicating

96   consistent screening across reviewers (Banerjee et al., 1999; Landis and Koch, 1977), and

97   supporting screening of remaining study records by a single reviewer. Screening conflicts

98   were observed for only 27/701 (4%) studies and were resolved by discussion.

99

100   *Risk of bias*

101   Three of twenty-nine studies were judged at high-risk of bias, 15 with an unclear-risk and 11

102   at low-risk (**Supplementary datasets 1 and 2**). Details and rationale behind this

103   categorisation are in **Supplementary dataset 1**.

104

105   *Summary of general characteristics*

106   Studies explicitly using wastewater for human population-level AMR surveillance made up

107   12/29 included studies. The remaining 17 studies included relevant comparisons between

108   wastewater and human AMR, but were not directly set up as wastewater-based AMR

109   surveillance studies (e.g. studies focussed on one health and transmission). Amongst the

110   twenty-nine included studies, 72 unique countries were sampled, although most (48/72) were

111   represented as part of a single global study (Hendriksen et al., 2019b) (**Fig.2**). World Bank

112   regions covered by the studies were as follows: East Asia and Pacific (n=3 studies), Europe

113   and Central Asia (n=16), Latin America and the Caribbean (n=4), Middle East and North Africa

114   (n=8), North America (n=5), South Asia (n=2), Sub-Saharan Africa (n=4). World Bank income

115   classifications showed a sampling skew towards high-income countries (high income [n=21

116   studies]; middle income [n=10] and low income [n=3]) (**Fig.S2**). Three studies covered multiple

117   regions and income classifications. Publication dates ranged from 2007-2020, with most

118   published in the last three years (n=20). Sampling years ranged from 2004-2019, with the

119   most common being 2011-2016 (n=17), and only three studies (Haghi et al., 2019; Pot et al.,

120   2020; Urase et al., 2020) sampling in the last three years (for full study descriptions, see

121   **Supplementary datasets 2 and 3**).

122

123   *AMR evaluations in included studies*

124   Evaluations of AMR were undertaken using genotypic-only methods (n=6), phenotypic-only

125   methods (n=8), or a mixed approach combining both (n=15). Genotypic-only studies

126   employed metagenomics (n=4) and qPCR (n=2). Phenotypic-only studies employed disk-

127   diffusion (n=3), microbroth dilution (n=3), or both (n=2). Mixed approach studies combined

128   disk-diffusion/microbroth dilution with qPCR (n=1), PCR (n=6) or single isolate whole genome

129   sequencing (WGS) (n=8). For data synthesis and analysis across studies, relevant phenotypic

130   data was extracted from 19 studies and genotypic data from nine studies (20 studies in total).

131   For nine studies there were no relevant data that could be extracted for inclusion in a

132   combined summary; these were either where sample counts were not reported or only raw

133   sequencing data was available which was beyond the scope of our analysis.

134

135   *Phenotypic wastewater-human AMR comparisons*

136   Phenotypic data from 19 studies covered twelve WHO Critically Important Antimicrobials

137   (CIAs) for which overall resistance prevalence in wastewater and human isolates was

138   reasonably high (CCC=0.81 [95% CI 0.74-0.87]) (**Fig.3A**). The median number of comparisons

139   (i.e. AMR prevalence for a specific species-drug across both wastewater and human

140   compartments) per study was 4 (IQR: 2-7). For any comparison, the median number of

141   isolates analysed in humans was 130 (IQR: 50-470), and in wastewater 98 (IQR: 58-356). AMR

142   prevalences were in high agreement (defined as wastewater AMR prevalence within ±10% of

143   human AMR prevalence) for 59/99 (60%) comparisons (**Supplementary dataset 4**). Hutinel et

144   al., 2019 contributed the most comparisons in high agreement (13/16 comparisons within-

145   study) while remaining studies contributed 1/1-6/7 each. Contribution of comparisons not in

146   high agreement was similar across studies (1-4 each).

147

148   The most common species and antibiotic class investigated was *E. coli* and aminoglycosides

149   respectively. When considering discordance across the top two represented species, 29/59

150   (49%) comparisons of AMR prevalence in wastewater and human populations for *E. coli*

151   showed <5% discordance and 37/59 (63%) of comparisons ≤10% discordance, whereas 6/17

152   (35%) of comparisons of AMR prevalence across compartments in *Enterococcus* spp. showed

153   <5% discordance and 8/17 (47%) ≤10% discordance (**Fig.3A top right panel**). Regarding

154   differences between antibiotic classes, comparisons investigating cephalosporin resistance

155   showed ≤10% discordance for 14/20 (70%) of comparisons, while for beta-lactam resistance

156   this was only true for 3/10 (30%) (**Fig.3A bottom right panel**).

157

158   By individual antibiotics of interest, AMR prevalence estimates (i.e. point estimate ±95%

159   confidence intervals) in human and wastewater isolates overlapped for: (i) aminoglycosides

160   (21/26 comparisons; **Fig.S3**); (ii) amoxicillin/ampicillin (6/10 comparisons; **Fig.S4**); (iii) beta-

161   lactam/beta-lactamase inhibitor combinations (6/11 comparisons; **Fig.S5**); (iv)

162   cephalosporins (13/20 comparisons; **Fig.S6**); (v) ertapenem (1/1 comparison; **Fig.S7**); (vi)

163   fluoroquinolones/quinolones (6/16 comparisons; **Fig.S8**); (vii) vancomycin (2/3 comparisons;

164   **Fig.S9**); and (viii) erythromycin (4/5 comparisons; **Fig.S10**). Overlap was also seen specifically

165   for extended-spectrum beta-lactamase (ESBL)-producing isolates (2/3 comparisons) and

166   methicillin-resistant *Staphylococcus aureus* (MRSA) (1/1 comparison; both **Fig.S11**). Non-

167   overlapping estimates were mostly associated with five studies (Zarfel et al., 2010; Huijbers

168   et al., 2020; Reinthaler et al., 2013; Saifi et al., 2009; Zaheer et al., 2020).

169

170   *Genotypic wastewater-human AMR comparisons*

171   For extracted genotypic data (single isolate WGS and PCR), prevalence of AMR genes in

172   wastewater isolates was highly correlated with prevalence in human isolates (CCC=0.88 (95%

173   CI 0.85-0.91)) (**Fig.3B**), with high agreement (wastewater AMR prevalence within ±10% of

174   human AMR prevalence) in 169/228 (74%) comparisons. The median number of comparisons

175   (i.e. AMR prevalence for a specific species-AMR gene combination across both wastewater

176   and human compartments) per study was 12 (IQR: 8-44). For any comparison, the median

177   number of isolates analysed in humans was 94 (IQR: 25-437), and in wastewater 91 (IQR: 30-

178   388). Most high agreement comparisons were observed in Raven et al., 2019 (71/169),

179   followed by Adator et al., 2020b (33/169) and Zaheer et al., 2020 (19/169). Studies

180   contributing the most comparisons not in high agreement were Pot et al., 2020 (17/59) and

181   Adator et al., 2020b (12/59), while remaining studies contributed 1-8 each.

182

183   The most common species and AMR gene family investigated was *E. coli* and CTX-M

184   respectively. When considering discordance across species, 95/145 (66%) comparisons of

185   AMR prevalence in wastewater and human populations for *E. coli* showed <5% discordance

186   and 120/145 (83%) of comparisons ≤10% discordance, while comparisons in *Enterococcus*

187   spp. showed 17/44 (39%) <5% discordance and 33/44 (74%) ≤10% discordance. *Enterobacter*

188   *cloacae and Campylobacter* spp. comparisons shared more even distributions of discordance

189   levels (**Fig.3B top right panel**). For AMR gene family, comparisons of most gene families across

190   compartments were in high agreement (i.e. ≤10% discordance) such as for CTX-M 20/28

191   (71%), *dfr* 18/20 (90%) and *aad* 14/16 (88%) (**Fig.3B bottom right panel**).

192

193   For a subset of the eight most common genes conferring resistance to WHO CIA antibiotics

194   represented across studies, 95% CI around prevalence estimates in both compartments

195   overlapped as follows: (i) *aac* (12/15 comparisons; **Fig.S12** [displayed for individual allelic

196   variants]); (ii) *arr* (1/2 comparisons; **Fig.S13**); (iii) CTX-M 22/26 comparisons; **Fig.S14**); (iv) *erm*

197   (4/6 comparisons; **Fig.S15**); (v) *fos* (2/2 comparisons; **Fig.S16**); (vi) *oxa* (8/8 comparisons;

198   **Fig.S17**); (vii) *van* (3/3 comparisons; **Fig.S18**) and (viii) *qnr* (3/4 comparisons; **Fig.S19**).

199

200   *Study features potentially associated with higher wastewater-human AMR agreement.*

201   If more than 70% of wastewater-human AMR prevalence comparisons conducted were in

202   high agreement, studies were classed as high agreement overall; this was the case for 6/19

203   studies (32%) with phenotypic data and 4/9 (44%) studies with genotypic data. Eight studies

204   had both phenotypic and genotypic data available but only 1/8 (13%) showed overall high

205   agreement for both approaches (Adator et al., 2020b); remaining 7/8 either showed high

206   agreement for only one approach or no high agreement at all. Total comparisons across

207   approaches were used to classify studies for logistic regression. The strongest association was

208   with the type of human sample analysed (p=0.23, **Table S20**); the limited number of eligible

209   studies, and the substantial heterogeneity of combinations of approaches deployed across

210   studies, meant power to detect independent associations was low.

211

212   We therefore synthesised study features descriptively, also assigning studies to moderate

213   (30-70%) and low agreement categories (<30%); keeping phenotypic- and genotypic-

214   approaches separate. Sampling of influent, either alone or in conjunction with effluent,

215   appeared most consistently associated with moderate-/high-agreement in estimates of AMR

216   prevalence between wastewater and human compartments (**Fig.4-Table S22**). Most studies

217   with extractable data conducted longitudinal sampling (24/28; 1 conducted both); this

218   approach occurred in all three agreement categories (**Fig.4A**). The three studies undertaking

219   snapshot (i.e. single-timepoint) sampling all had high-agreement (n=2) and moderate-

220   agreement (n=1). For longitudinal studies, the timeframe of sampling was potentially

221   relevant: Of the seven low-agreement longitudinal studies, 6/7 sampled for ≤12 months and

222   only 1/7 for >12 months. Conversely, 14/17 medium/high-agreement longitudinal studies

223   sampled for >12 months, and only 3/17 for <6 months. (**Fig.4B**). Moderate-/high-agreement

224   studies deployed several wastewater sampling methods, with composite, grab and flow-

225   proportional sampling showing no clear relationship with agreement (**Fig.4C**). Of note,

226   sampling point or method was not reported by six studies.   Most studies performed

227   comparisons on wastewater at least in part derived from the human population sampled (i.e.

228   direct comparisons, 19/28), while five conducted indirect comparisons, one conducted both

229   and four were unclear/unreported. Most moderate-/high-agreement studies conducted

230   direct comparisons (**Fig.4D**). Other study features showed even more variation across

231   agreement categories but a smaller number of WwTWs sampled was potentially more

232   associated to moderate-/high-agreement studies (**Fig.S21 – Table S22**).

233

234   *Studies without extractable data*

235   The nine studies without extractable data that could be synthesised are summarised below

236   in terms of their overall ability to detect wastewater-human AMR associations based on

237   reported conclusions. Full descriptive summaries including study details and specific findings

238   are in **supplementary dataset 5**.

239

240   *Targeted qPCR-based studies*

241   Two studies performed direct AMR gene detection using qPCR of either 229 (Pärnänen et al.,

242   2019) or eight AMR genes (Colomer-Lluch et al., 2014); both reported a relationship between

243   wastewater AMR and national AMR data.

244

245   *Metagenomic-based studies*

246   Four studies employed metagenomics to identify potential wastewater-human AMR

247   associations. Two of these studies appeared to demonstrate an association while the other

248   two were inconclusive.

249

250   *Mixed-approach and phenotypic-only studies*

251   Two studies used mixed approaches combining phenotypic AST with qPCR (2-targets) (Meir-

252   Gruber et al., 2016) and single isolate WGS (Gouliouris et al., 2019); one study used a

253   phenotypic approach only (YoungKeun et al., 2015). All three studies appeared to show a

254   wastewater-human AMR association.

255

256   **Discussion**

257   From our review and synthesis of the available data, we found characterisation of AMR in

258   wastewater shows promise in reflecting AMR in human populations, irrespective of diverse

259   target species, target resistances and study locations, although associations may be stronger

260   for some species and AMR mechanisms than others. The strength of this relationship varied

261   across studies and was likely influenced by study design, setting, spatiotemporal sampling

262   strategies, and testing approach (i.e. sample preparation, DNA extraction, target

263   species/resistances and genotypic/phenotypic); the heterogeneity of methodological

264   approaches and lack of clear reporting of key study features made any quantitative

265   synthesis very difficult.

266

267   Our estimates of concordance (**Fig.3A, 3B**) supporting a wastewater-human AMR correlation

268   are in line with estimates in individual studies (Huijbers et al., 2020; Hutinel et al., 2019;

269   Karkman et al., 2020). In particular, Huijbers et al., 2020 reported coefficients of

270   determination as 0.62-0.72 for individual antibiotics and 0.85 when data was combined for

271   four antibiotic classes – similar to our findings of 0.81 and 0.88 for class-unrestricted

272   phenotypic and genotypic data respectively. Although data was too limited to robustly

273   estimate Lin's CCC for individual species and AMR, variability in the level of discordance of

274   wastewater-human comparisons (**Fig.3A, 3B – right panels**) and overlap in 95% CI around

275   point estimates (**Fig.S3-19**) is likely attributed in part to specific species and AMR that work

276   best for wastewater-based AMR surveillance, which may vary with setting or over time, and

277   those where the approach consistently works less well for. This phenomenon was also

278   reported in several studies without extractable prevalence data where specific AMR

279   classes/genes exhibited notably higher/lower wastewater-human agreement. The

280   performance increase (non-significant) of genotypic comparisons seen in both Lin's CCC and

281   in the reduced proportion of strongly discordant comparisons (**Fig.3B right panels)** may

282   reflect the relatively species- and mechanism-agnostic nature of genotypic AMR detection

283   methods (mostly WGS in extracted data) over phenotypic methods which may be more

284   susceptible to variations from differing growth media/conditions and interpretation of

285   resistance breakpoints. Problems with accurately characterising AMR prevalence when only

286   small numbers of isolates are analysed (median of 94-130 for human and 91-98 for

287   wastewater compartments across pheno-/genotypic comparisons here) is also a concern

288   highlighted by previous researchers, particularly when few resistant isolates are available

289   (Huijbers et al., 2020).

290

291   Although not a focus in our review, genotypic profiling potentially affords some additional

292   advantages over phenotypic analyses, and is relevant to confirming that genetic

293   mechanisms underpinning phenotypes are also similar. Genomic approaches, such as

294   sequencing of isolates or whole sample metagenomics, enable a more agnostic approach to

295   be adopted than for qPCR, in that analyses do not need to be restricted to a subset of

296   predefined genes/gene variants. Genomic data and profiles can also be more readily shared

297   with the wider community to allow for cross-study comparisons and data synthesis; as

298   demonstrated by Karkman et al. Genomics approaches also allow for the evaluation of

299   genetic relatedness and quantitation of either isolates or microbial populations across

300   compartments (e.g. through phylogenetics, taxonomic/strain-level profiling and strain-

301   based comparisons using metagenomes). Genomic approaches may be higher-resolution

302   and more flexible, but at a higher resource cost; sensitivity for the detection of AMR genes

303   is also dependent on sequencing depth, and accurately associating specific AMR gene

304   markers with strains or species in short-read based metagenomes remains difficult (Gweon

305   et al., 2019).

306

307   Our review highlights the need for clear guidance on performing these studies in a more

308   standardised way, with a view to consolidating best-practice approaches in a workflow

309   whilst enabling some flexibility to account for differences in any given setting. WwTW

310   influent is likely the most population-representative wastewater sample for AMR

311   surveillance using either phenotypic or genotypic approaches. This is not unexpected, as

312   previous studies have described transformation of microbial and AMR gene composition

313   during treatment (Tong et al., 2019; Zhang et al., 2020). Transformed samples may remain

314   useful for wastewater-based AMR surveillance, potentially dependent on treatment

315   process; however differing levels of treatment have been shown to select for different

316   species/AMR determinants (Tong et al., 2019). Additionally, a temporospatial overlap of

317   wastewater sampled and the target surveillance population is likely helpful; a feature of the

318   majority of moderate-/high-agreement studies as well as sampling fewer WwTWs which may

319   also relate to the closeness of the populations compared.

320

321   Composite sampling also seems sensible as wastewater composition changes significantly

322   over short timescales (Guo et al., 2019) and individual grab samples may be "flooded" by

323   homogenous solid material (Reinthaler et al., 2013). However, grab sampling is convenient

324   and avoids significant autosampler-associated workload and capital costs, and was the most

325   common sampling method used in the studies analysed. Further research is needed into

326   characterising how effectively single timepoint grab samples versus composite/proportional

327   samples reflect temporal AMR changes.

328

329   Longitudinal sampling with timeframes over 12 months was most common in both

330   phenotypic and genotypic high agreement studies, consistent with data from two studies

331   which could not be directly synthesised (Hendriksen et al., 2019a; Pignato et al., 2010). In

332   these studies, both used two-weekly sampling intervals over 12- and 3-month timeframes

333   respectively. The former observed an association between ampicillin-resistant wastewater

334   and contemporaneous clinical isolates, whereas the latter found no relationship between

335   contemporaneous public health surveillance and wastewater metagenomic read

336   abundances.

337

338   Future studies should clearly report sewer inputs, including any unique AMR-associated

339   inputs (e.g. hospitals, agricultural sources) that may obscure the detection of a wastewater-

340   human AMR association (Fahrenfeld and Bisceglia, 2016). The importance of specific AMR-

341   associated inputs is likely linked to whether the AMR mechanisms under evaluation are

342   associated with that specific source, or already widely disseminated in the community. For

343   example, one study (Raven et al., 2019) sampled WwTWs with and without hospital input,

344   and found the most clinically-prevalent *E. coli* ESBL gene was ubiquitous in all WwTWs,

345   indicating prior widespread dissemination. Another study (Jakobsen et al., 2008) focussing

346   on *E. coli* gentamicin resistance in hospital effluent, receiving WwTW influent and domestic-

347   only wastewater, found significantly lower prevalence in domestic-only wastewater

348   compared to hospital effluent and WwTW influent which shared similar prevalence,

349   indicating that the presence of any hospital-associated wastewater in influent would

350   confound any community-based estimates. Additional metadata such as sample storage

351   conditions or freeze/thaw cycles are also pertinent to interpretation as investigated by

352   Poulsen et al., who similarly suggested detailed reporting of these features.

353

354   Our study has several limitations. We were not resourced to conduct duplicate screening by

355   two reviewers; however, the risk of single reviewer bias was mitigated by validating the

356   screening strategy using three reviewers on a subset of records. We excluded non-English

357   publications, potentially missing some relevant studies. Studies were highly diverse in

358   reported features, design and outcomes, making a comprehensive synthesis difficult. In

359   particular, many features were poorly characterised and could not be explored in our

360   analyses. For our study feature analysis we focused *a priori* on features that optimised the

361   identification of an association between wastewater and human AMR prevalence, however

362   it may be that in some circumstances there is genuinely no such association. In many

363   settings globally, established wastewater infrastructures are not available, and an analysis

364   of, for example, WwTW influent may not be feasible.

365

366   **Conclusion**

367   In conclusion our review suggests that overall, wastewater-based surveillance of population-

368   level AMR appears relatively robust, despite high diversity in study design, methods and

369   metadata. However, based on limited available data, we would recommend that where

370   feasible, composite sampling of influent with longitudinal timeframe >12 months, and

371   contemporaneous sampling of wastewater and human samples that are directly associated

372   (i.e. the human population sampled contributes to the wastewater sampled) are used to

373   generate more robust data to better evaluate the strengths and limitations of this approach

374   for surveillance purposes. Clear reporting of study methods and features are essential, and

375   this will facilitate the development of optimal practice guidelines for this emerging

376   surveillance tool.

377

Materials and Methods

379   For this systematic review, we adopted the "Population Intervention Comparator Outcome"

380   (PICO) framework using the following domains: Wastewater, antimicrobial resistance,

381   bacteria and public health surveillance/methods. A PRISMA checklist is included in

382   (**Supplementary dataset 6),** and the complete PROSPERO protocol is available at:

383   https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019134946.

384

Literature search

386   The search string was developed through iterative preliminary searches in consultation with

387   a librarian experienced with systematic reviews. Full search strings adapted for each database

388   are presented in (**Supplementary dataset 7**). Searches were conducted on 01/02/2019 in:

389   MEDLINE, EMBASE, Global Health, CAB Abstracts, Scopus and Web of Science Core Collection.

390   Searches were updated on 09/01/2021 using identical search strings. Results were limited to

391   the English language and de-duplicated.

392

Eligibility criteria

394   Study titles/abstracts were screened with a series of questions (**see Fig.S1**) to determine if

395   the study was: (i) primary research, (ii) collected human-associated wastewater, (iii) reported

396   AMR prevalence, and (iv) performed a comparison between the wastewater dataset and

397   another human-associated dataset. Studies explicitly performing wastewater-based

398   surveillance of human AMR were included using parallel criteria. If it was unclear whether a

399   study met criteria based on title and abstract alone, the study was passed onto the next stage.

400   For studies that passed the initial screen, full-text methods were reviewed and studies

401   included if they: (i) analysed wastewater samples/data from a wastewater treatment facility,

402   and (ii) represented a human population through their non-wastewater dataset; or (iii)

403   conducted a self-defined wastewater-based AMR surveillance study as the study aim.

404

405   *Study selection and data extraction*

406   Three reviewers (KKC, LB, NSi) independently screened a random subset of 8% of total

407   retrieved study records to estimate Cohen's Kappa score (Cohen, 1960) as a measure of inter-

408   reviewer reliability beyond chance. As this score supported sufficiently consistent screening

409   across reviewers, the remaining records were screened by a single reviewer (KKC).

410

411   For included records, data were extracted by two reviewers (KKC, LB) using a pre-tested

412   data extraction form piloted on five random included records (**Supplementary dataset 8**),

413   including (non-exhaustive): study design (wastewater sampling strategy/site/methods, AMR

414   detection methods, human sample type, sample sizes) and outcomes (AMR prevalence,

415   wastewater-human comparison results). Raw resistance prevalence data (total and resistant

416   isolate counts) were extracted if available. Phenotypic data extraction was limited to

417   antibiotics on the WHO critically important antimicrobials (CIAs) list (AGISAR, 2018) to

418   maintain clinical relevance. Genotypic data was extracted in full; individual gene analysis

419   was however only undertaken for a subset of AMR genes that conferred resistance to CIAs

420   and were characterised in multiple studies. Any statistical methods or modelling approaches

421   were also recorded.

422

423   *Risk of bias assessment*

424   Risk of bias was assessed independently by two reviewers (KKC, LB) using a qualitative

425   approach based on the Cochrane risk of bias tool (Higgins et al., 2011) addressing five bias

426   domains (**Supplementary dataset 1**); the modified tool focused on systematic differences at

427   the study level as outcomes reported were highly diverse. Studies at "high-risk" of bias were

428   those with inconsistencies in sampling, AMR detection methods, measurement and

429   reporting of outcomes; "low-risk" studies broadly maintained consistency across

430   comparators. If information present was insufficient to assess risk of bias the classification

431   "unclear" was assigned. Discrepancies were resolved by discussion until consensus was met,

432   and an overall qualitative measure (high, low and unclear) was assigned to each study;

433   primarily based on the majority of domain judgements (details in **Supplementary dataset 1**).

434

435   *Data synthesis and analysis*

436   For extracted resistance prevalence data, we used Lin's concordance correlation coefficient

437   (CCC – R package DescTools) with 95% confidence intervals (CIs) to quantify the agreement

438   between the proportion of resistant wastewater isolates and the proportion of resistant

439   human isolates, with the latter representing the reference standard. As perfect concordance

440   is unrealistic, we arbitrarily defined high agreement to be a ±10% difference in resistance

441   prevalence between wastewater and human compartments. Since Lin's CCC does not reflect

442   error in resistance proportion estimates, we also plotted and compared resistance

443   prevalence and Clopper-Pearson 95% CIs by study and antibiotic or AMR gene.

444

445   As we aimed to identify study approaches that could maximise the association between

446   wastewater-human AMR for surveillance purposes, we investigated study features

447   associated with "high agreement", defined as >70% of the comparisons of species-

448   phenotype or species-genotype AMR prevalence estimates in wastewater and human

449   compartments within-study being within ±10% of each other. As study feature reporting

450   was highly inconsistent, we only considered features that were broadly reported across

451   studies. We then used logistic regression to identify if any study features were associated

452   with this high agreement classification in STATA/IC v.16.1 (StataCorp, College Station, USA).

453

454   In addition, given the heterogeneity of study features, their inconsistent reporting across

455   studies, and the small number of studies limiting power to detect associations, we also

456   descriptively synthesised features potentially associated with wastewater-human AMR

457   agreement, using a modified version of the harvest plot, specifically designed to help

458   assimilate findings where a quantitative synthesis is not feasible (Ogilvie et al., 2008). For

459   this, in addition to the high agreement category specified above, we also assigned studies to

460   moderate (30-70%) and low agreement categories (<30%); keeping phenotypic- and

461   genotypic-approaches separate.

462   **Tables and figures**

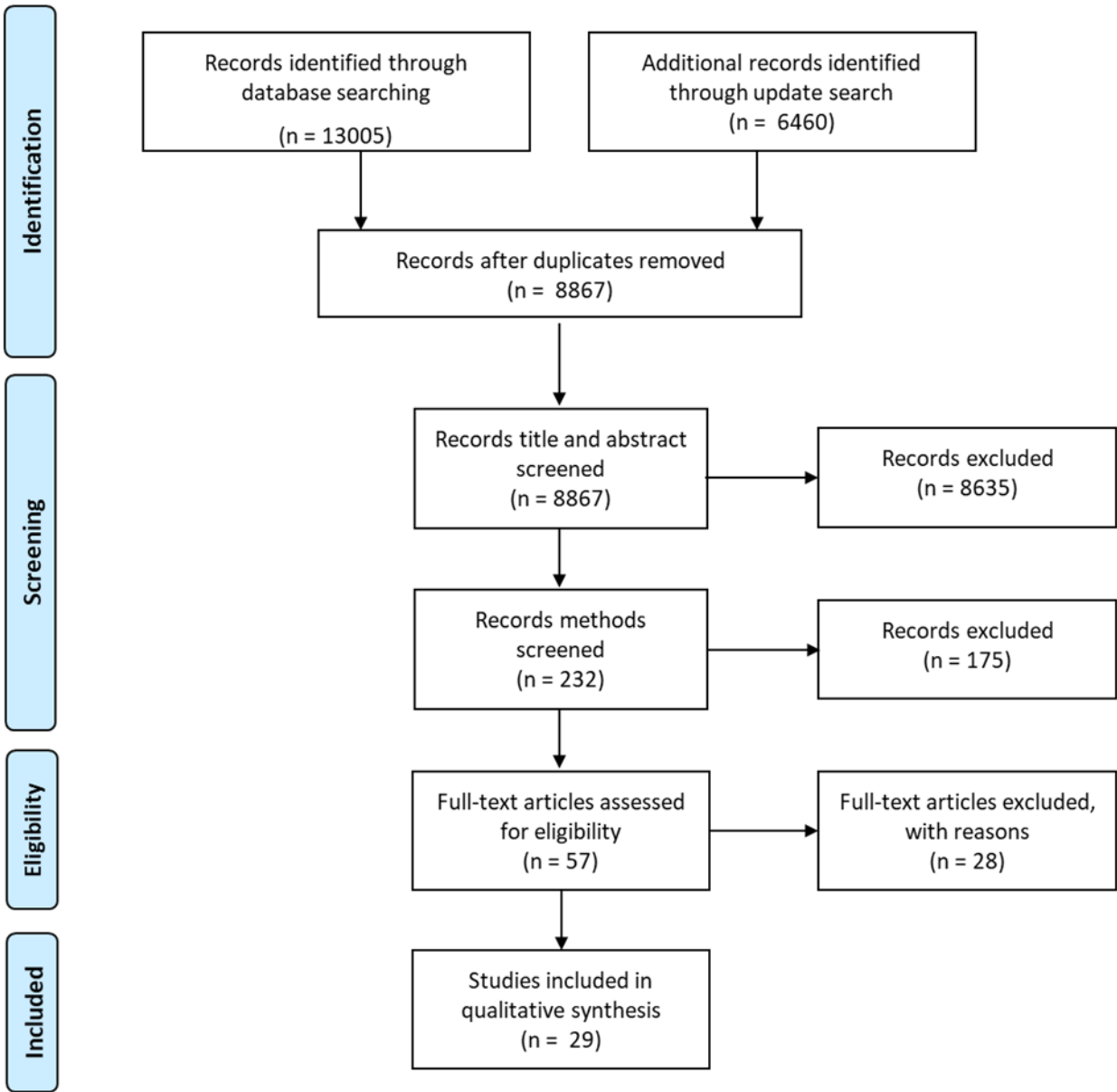
463   **Table 1: Potential sources of variation in wastewater-based AMR surveillance studies**

Aspects for consideration	Examples	Key variabilities	References
Wastewater sampling point	• WwTW influent/during treatment/effluent • Hospital effluent • Domestic sewers/manholes • Informal sewer systems (LMICs)	• Treatment processes can transform microbial and AMR composition • Focussed sampling may only represent specific sub-populations* • Informal sewer systems (often with low flow) may be susceptible to homogeneity	(Fahrenfeld and Bisceglia, 2016; Jakobsen et al., 2008; Larson et al., 2020; Tong et al., 2019; Zhang et al., 2020)
Wastewater sampling method	• Grab (single sample) • Composites • Proportional sampling (flow/time/volume)	• Single grab samples can be flooded by homogenous solids • Wastewater composition can vary significantly over short time periods • Composite and proportional samples will capture average composition but may be unable to discriminate peak values during sampling period*	(Guo et al., 2019; Michael-kordatou et al., 2020; Reinthaler et al., 2013)
WwTW sewershed inputs	• Hospitals • Other healthcare facilities (e.g. carehomes) • Agriculture • Industry	• Effluent from AMR-associated sources may obscure detection of true population-level trends (e.g. elevated levels of unique AMR, co-selection of plasmids, non-human associated AMR)*	(Fahrenfeld and Bisceglia, 2016; Jakobsen et al., 2008; Larson et al., 2020)
WwTW properties and sampling conditions	• Size of WwTW sewershed • Treatment methods • Infrastructure/design • Weather (seasons, rainfall, temperature) • Flow rate	• Long conveyance times from population to sampling point may impact composition due to transformation in unique environment of the sewer system (anaerobic, temperature, biofilms) • When sampling treated wastewater, differing levels of treatment can selectively transform AMR and microbial composition • Presence of pre-treatment infrastructure (e.g. pumping stations, balancing tanks) may also play a role in transforming wastewater • Heavy rainfall may dilute wastewater in combined sewer systems via rainwater runoff and by infiltration of groundwater (dislodged biofilms, infiltration of freshwater microorganisms) • Combined sewer overflows will impact composition of post-treatment samples collected during events • Flow rate is associated to hydraulic retention time and the level of WwTW treatment - impacting treated samples	(Fahrenfeld and Bisceglia; Pehrsson et al., 2016; Shanks et al., 2013; Tong et al., 2019)

Aspects for consideration	Examples	Key variabilities	References
Wastewater processing methods	• Filtration • Storage conditions • Addition of sodium thiosulfate (chlorine neutraliser) • Freeze-thaw cycles • DNA extraction methods	• Different wastewater processing methods may selectively affect recovery yields of specific species* • Multiple freeze-thaw cycles shown to select for Firmicutes, Actinobacteria, and eukaryotic microorganisms • Use of different metagenomic DNA extraction kits and procedures has been shown to modulate inferred microbial composition*	(Ahmed et al., 2020; Knudsen et al., 2016; Michael-kordatou et al., 2020; Poulsen et al., 2021)
AMR detection method	• Phenotypic culture-based (selective media, disk-diffusion, microbroth dilution) • Genotypic culture-based (WGS, PCR, qPCR) • Direct-from-sample genotypic (qPCR, metagenomics)	• Culture-based methods may be subject to variations from phenotyping method and breakpoint selection • Methods based on culturing isolates may only capture a fraction of the diversity present even with detailed sampling • Targeted genomic approaches are restricted to a subset of predefined genes/gene variants • Bioinformatic deconvolution can be subject to variation based on tools/databases/references utilised – especially for short-reads and plasmids	(Davies, 2019; Davies et al., 2020; Lal Gupta et al., 2020; Shaw et al., 2021)

464 *Also recently studied as sources of variation in SARS-CoV-2 wastewater-based epidemiology and

465 likely relevant for AMR surveillance

Figure 1: PRISMA flowchart of search strategy and study inclusions/exclusions

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473   **Figure 2: Geographic distribution of wastewater sampling and test approach of included studies**

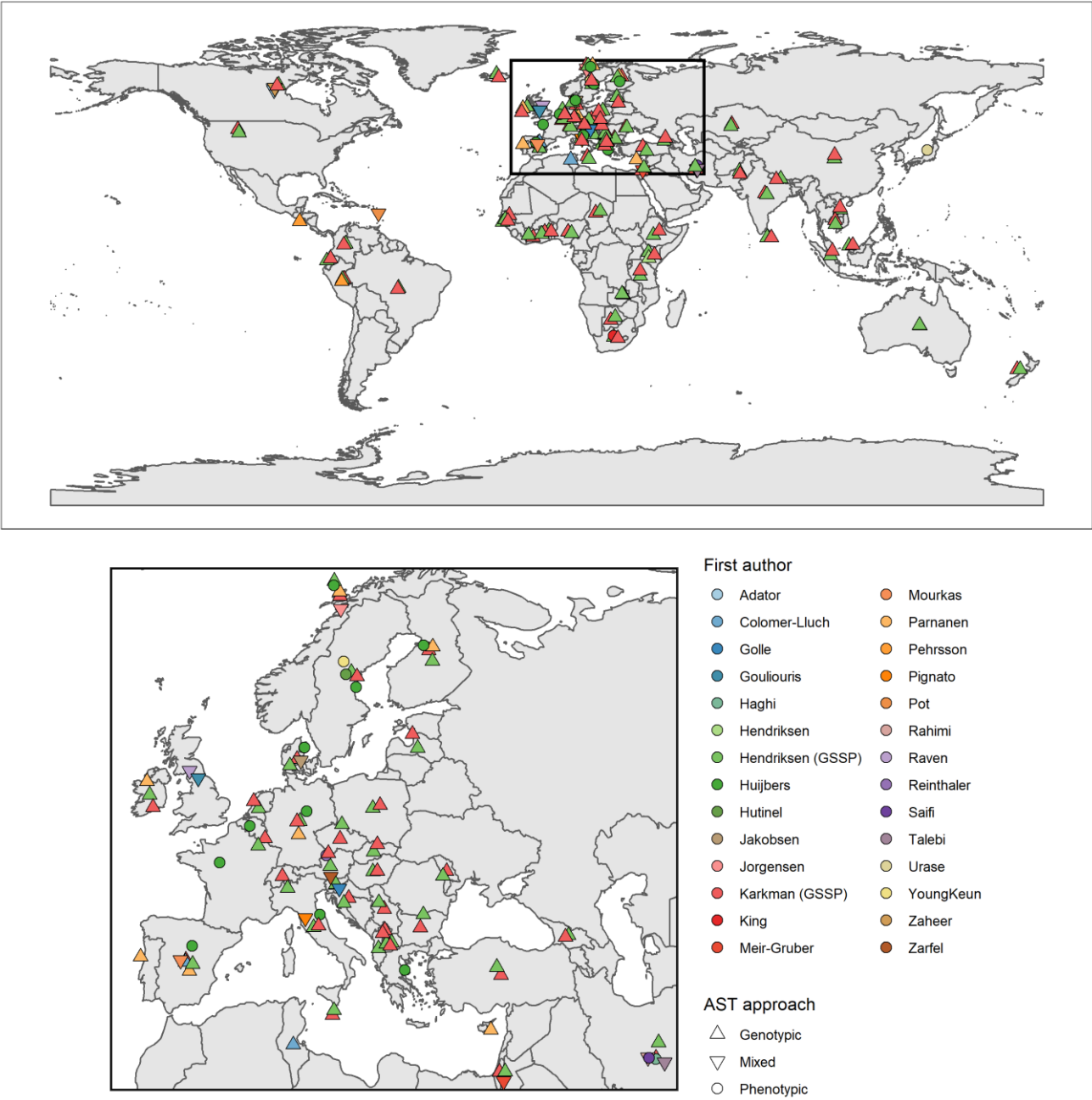
474   Centroids of countries sampled by included studies are plotted with colours and shapes according to

475   citation and test approach respectively. Non-Hendriksen (GSSP-global sewage surveillance project)

476   studies are plotted with jitter around the centroid for the map focussing on Europe. AST=antimicrobial

477   susceptibility testing

478   **Figure 3: AMR in wastewater isolates and human isolates for phenotypic (A) and genotypic (B)**

479   **comparisons.**

480   Left: Concordance plot of AMR prevalence in wastewater and human isolates stratified by AMR

481   detection approach (i.e. phenotypic versus genotypic approaches). Each point represents a single

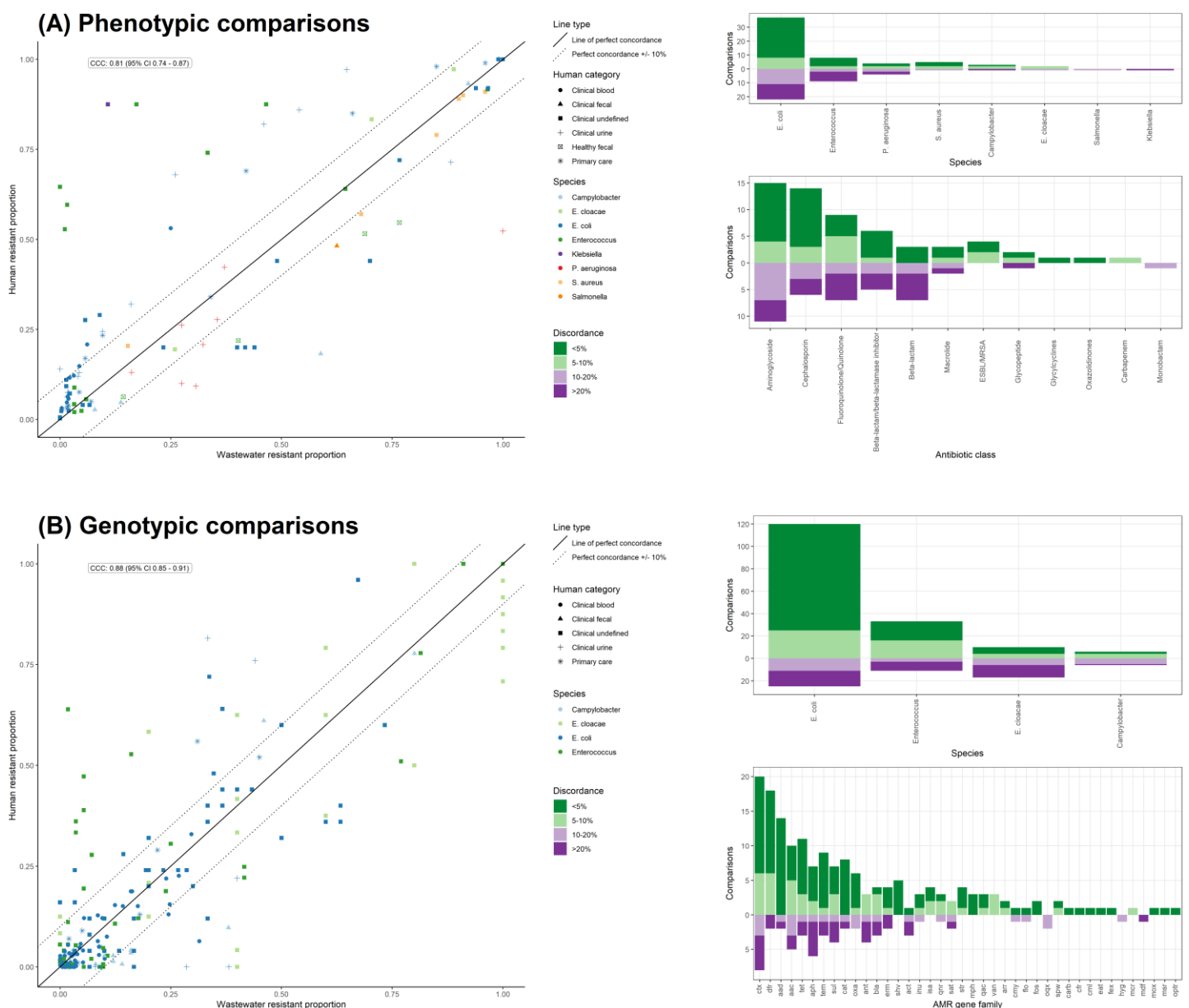
482   wastewater-human comparison conducted and is coloured by bacterial species tested and human

483   sample type used. Lin's concordance coefficient (CCC) is labelled with 95% confidence intervals.

484   Unbroken line of $y=x$ is plotted as perfect concordance between wastewater and human resistance.

485   Dashed lines of $y=x+0.1$ and $y=x-0.1$ represent high agreement, i.e. ±10% from perfect concordance

486   respectively. Right: Grouping of comparisons from concordance plot by level of discordance into

487   categories for species and antibiotic class or AMR gene family.

488   **Figure 4: Study design features split by study-level agreement and wastewater sampling point.**

489   Modified harvest plot of most widely reported study features (A, B, C, D) in relation to wastewater

490   sampling point (horizontal facet) and overall study-level agreement (vertical facet). Study features are

491   coloured and bar height relates to number of studies with the feature.

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494

495   **Contributors**

496   A complete list of author contributions is given in the appendix (p17).

497   **Competing interests**

498   The authors report no conflicts of interest.

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