

1 **Antimicrobial resistance in dairy slurry tanks: a critical**

2 **point for measurement and control**

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43

44 Abstract

45 Waste from dairy production is one of the world's largest sources of contamination from
46 antimicrobial resistant bacteria (ARB) and genes (ARGs). However, studies to date do
47 not provide necessary evidence to inform antimicrobial resistance (AMR)
48 countermeasures. We undertook a detailed, interdisciplinary, longitudinal analysis of
49 dairy slurry waste. The slurry contained a population of ARB and ARGs, with resistances
50 to current, historical and never-used on-farm antibiotics; resistances were associated
51 with Gram-negative and Gram-positive bacteria and mobile elements (*IS*Ecp1, *Tn*916,
52 *Tn*21-family transposons). Modelling and experimental work suggested that these
53 populations are in dynamic equilibrium, with microbial death balanced by fresh input.
54 Consequently, storing slurry without further waste input for at least 60 days was
55 predicted to reduce ARB spread onto land, with >99% reduction in cephalosporin
56 resistant *Escherichia coli*. The model also indicated that for farms with low antibiotic use,
57 further reductions are unlikely to reduce AMR further. We conclude that the slurry tank is
58 a critical point for prevalence and control of AMR, and that measures to limit the spread
59 of AMR from dairy waste should combine responsible antibiotic use, including low total
60 quantity, avoidance of human critical antibiotics, and choosing antibiotics with shorter
61 half-lives, coupled with appropriate slurry storage.

62

63 Introduction

64 Antibiotics provided to food-producing animals account for 73% of global antibiotic sales
65 (1), prompting concerns about the selection of antibiotic resistance bacteria (ARB) and
66 genes (ARGs), and their migration from livestock and their environment to humans. ARB
67 and ARGs associated with livestock can enter humans through consumption of animal
68 products, e.g. contaminated meat (2, 3) and dairy (4, 5), or more indirectly, e.g.
69 through land-application of animal waste, which may subsequently infiltrate crops (6, 7)
70 and connected water resources (8, 9).

71 Cattle production comprises 50% of global Livestock Standard Units (10), so has
72 considerable environmental impacts that need to be mitigated (11). There are
73 approximately 265 million dairy cows globally, producing high volumes of waste manure,
74 estimated at 3 billion tonnes per year (www.faostat.org). In the UK, the site of this
75 study, dairy farms are estimated to account for 80% (67 million tonnes) of total annual
76 livestock manure production (12), with more cattle waste material applied to soil in
77 England and Wales than swine and poultry combined (13).

78 Antibiotics are routinely administered to dairy cattle for treatment, and, in some cases,
79 prevention of common illnesses, including mastitis and respiratory disease (14-16).
80 Lameness, the most costly disease to UK dairy farms (17), is often prevented with
81 application of antimicrobial metals (copper, zinc) or other chemicals (formalin,
82 glutaraldehyde) in the form of footbaths (18), known to co-select for antibiotic resistance
83 (19, 20). Dairy waste can therefore contain selective and co-selective pressures in the
84 form of mixtures of antibiotics and assorted antimicrobials, as well as ARB, including
85 Extended Spectrum Cephalosporin-Resistant (ESC-R) *E. coli* (21, 22), and genetic
86 resistance determinants (23, 24). Thus, dairy waste may represent one of the world's
87 most substantial routes for AMR to enter the environment, including onto fields and
88 grasslands used for food production and into water ways.

89
90 To limit the risks of AMR, many countries have introduced responsible use policies,
91 including reducing overall agricultural use of antibiotics (25), or of human critical
92 antibiotics, including 3rd/4th generation cephalosporins (26). However, antibiotics and
93 other antimicrobials remain necessary for safeguarding animal health and welfare. Thus,
94 other countermeasures are also needed to reduce the transmission or prevalence of ARB
95 and ARGs from dairy waste into the environment. For example, current UK guidelines
96 suggest that storage of solid manure and slurry without fresh input for three months can
97 ameliorate AMR risk (27), but no evidence is provided. Slurry storage is essential in the
98 UK and other countries where dairy cows are housed indoors for large parts of the year,

99 and where slurry cannot be spread onto land that is frozen or deemed nitrate vulnerable.

100 Two European studies have assessed storage effects on dairy manure, finding that

101 certain ARGs increased during storage (28, 29); however, this 'stored' effluent regularly

102 received fresh input. Contrastingly, a survey of several US dairy farms evaluating a

103 different set of ARGs did not detect clear storage effects on ARG abundancesHurst,

104 Oliver (30).

105 Other dairy waste studies took a 'snapshot in time' (31-34), which does not allow for

106 assessment of temporal stability of the resistome and the influence of storage. Factors

107 such as temperature also influence the prevalence of enteric pathogens, indicator

108 organisms and resistance phenotypes during manure storage (35-39). Meanwhile,

109 studies assessing how cattle faecal resistomes respond to contrasting antibiotic

110 management practices generally place emphasis on individual cattle (40-42), with

111 different microbiomes, rather than the collective faecal output of the herd. Liquid-solid

112 separation of manure may also influence the persistence of AMR (43).Therefore, there is

113 a need for detailed longitudinal studies of AMR in dairy slurry and potential mitigations.

114

115 This study assessed three key questions about AMR in slurry and its relationship to

116 antibiotic use and slurry storage: (1) does the slurry tank select for or against AMR; (2)

117 how does the resistance content of the slurry tank relate to altered patterns of farm

118 antibiotic use; and (3) can slurry storage help reduce AMR in slurry before application to

119 land? Our interdisciplinary approach combined phenotypic, genomic, and metagenomic

120 microbiological analyses with chemical analyses, antibiotic use records and predictive

121 mathematical models, to provide a temporal evaluation of slurry tank content over six

122 months. This was supplemented by concurrent mini-slurry tank experiments which

123 facilitated the controlled study of isolated slurry. We designed the mathematical model

124 to enable us to study the impact of farm practices that would be impractical or unethical

125 to perform through purely empirical approaches. These included major changes to farm

126 slurry handling, antibiotic reduction to a level that would threaten animal welfare, or the

127 reintroduction of use of human critical 3rd or 4th generation cephalosporins. Thus, this
128 study enables the identification of approaches to reduce the spread of AMR into the
129 environment from an important source of such contamination.

130

131 Methods

132 *Sample site*

133 We surveyed a mid-sized, high performance commercial dairy farm in England, housing
134 ~200 milking Holstein Friesian cattle at the time of study. Practice at this farm is typical
135 of management methods at high-performance dairy farms, although all farms vary.
136 Milking cattle are housed indoors on concrete, and all excreta are regularly removed
137 from cattle yards by automatic scrapers into a drainage system terminating at the
138 3000m³ slurry tank. The drainage system also receives used cleaning materials and
139 wash water, used footbath containing zinc and copper, waste milk from cows treated
140 with antibiotics, and rainwater runoff. An automated screw press (Bauer S655 slurry
141 separator with sieve size 0.75 mm; Bauer GmbH, Voitsberg, Austria) performs liquid-
142 solid separation prior to the slurry tank. Liquids enter the slurry tank semi-continuously,
143 while solids are removed to a muck heap. Calves, dry cows, and heifers are housed
144 separately from the milking cows. Faeces and urine from calves drain into the common
145 drainage system, whilst dirty straw from calf housing is taken directly to the muck heap.
146 Excess slurry can be pumped to an 8000m³ lagoon for long term storage. Slurry from
147 either the slurry tank or lagoon is used to fertilise grassland and arable fields.

148

149 *Microbiological sampling, strain isolation, antimicrobial susceptibility*

150 *testing and whole genome sequencing*

151 Liquid samples were collected from the slurry tank on 17 dates between May and
152 November 2017 (Table S1). *Escherichia coli* strains were isolated using Tryptone Bile X-

153 Glucuronide (TBX) or MacConkey agar or TBX/MacConkey supplemented with 16 µg ml⁻¹
154 ampicillin (AMP), or 2 µg ml⁻¹ cefotaxime (CTX); or on CHROMagar ESBL™ agar. Putative
155 *E. coli* isolates were subcultured onto TBX agar or TBX agar supplemented with 2 µg ml⁻¹
156 CTX. *E. coli* strains were confirmed using oxidase (22) and catalase tests. Antimicrobial
157 susceptibility testing (AST) using a range of antibiotic discs (Table S2) was carried out
158 on 811 *E. coli* isolates in accordance with CLSI (44) guidelines. ESC-R *E. coli* strains
159 were identified by phenotypic resistance profile as putatively *ampC* or CTX type, and
160 confirmed by PCR (22). Presence of Tn21-like mercury resistance transposons within the
161 *E. coli* isolates was initially screened for by growing isolates on LB agar containing 25 µg
162 ml⁻¹ HgCl₂. Their presence was confirmed by PCR (45). Genome assembly of selected
163 ESC-R and mercury resistant *E. coli* strains using PacBio, was carried out by the Centre
164 for Genomic Research (CGR), University of Liverpool, with methods for library preparation and
165 sequencing as previously described (46) or by Illumina short read WGS by MicrobesNG
166 (University of Birmingham, UK). Genome sequence analysis and annotation was
167 conducted using Prokka (47), CSARweb (48), Snapgene viewer (Insightful Science;
168 snapgene.com), Res Finder (49) and Plasmid Finder (50). Genome sequences are
169 deposited with NCBI under BioProject PRJNA736866.

170

171 *Metagenomics Sample collection and DNA extraction*

172

173 *Main tank Sample Collection*

174 Samples were collected from the slurry tank monthly between June and October 2017,

175 using a clean stainless steel bucket, and aliquoted into 2 large glass bottles with external
176 PE protection. Three replicate extractions were performed on 250 µl of each sample
177 using a PowerFecal Kit (Qiagen), according to manufacturer's instructions (15 extractions
178 in total). DNA was quantified using a Qubit fluorometer (Invitrogen) while quality was
179 assessed via Nanodrop 1000 (ThermoFisher). Extracted DNA was stored at 4 °C pending
180 sequencing.

181

182 *Mini-Tank Experiments*

183 Miniaturised experimental slurry tanks were set up to assess the impact of storing slurry
184 (control tanks) and to measure antibiotic stability. Twelve mini-tanks were situated on
185 the farm from 23/4/2018 to 15/6/2018 at ambient temperature (mean 24 hour
186 temperature in liquid ranged between 7° to 17°), protected from rain and direct sunlight,
187 and containing 10L grab samples of slurry from the surface of the main slurry tank. Six
188 different conditions were tested in duplicate (all amounts per litre): control; + SSD
189 (0.2mL of slurry solids homogenised by stomacher, including 67 CFU of CTX-resistant *E.*
190 *coli*); + SSD + 3µg cefquinome weekly addition; + SSD + 40µg cefalexin weekly
191 addition; + SSD + 16.8g of footbath mix (Cu + Zn); + SSD + footbath + cefquinome).
192 Mini-tanks were sampled four times (0, 2, 4 and 7 weeks after initial filling).
193 Experimental conditions were mainly used for model calibration (Supplementary Text 1).
194 *E. coli* were isolated and cultured as described above except MacConkey agar was not
195 used. DNA was extracted and processed for sequencing as above. Antibiotic
196 concentrations were measured as described previously Baena-Nogueras, Ortori (51) with
197 further methods described in Supplementary Text 3.

198

199 *Metagenomic Sequencing, Assembly and Analysis*

200 Metagenomic sequencing of DNA extracted from the main slurry tank was performed by
201 Liverpool Genomics using the Illumina HiSeq platform, and from the mini-slurry tanks by
202 Edinburgh Genomics using the Illumina NovaSeq platform (150 bp paired end libraries in
203 both cases). For the main tank, reads were trimmed with Cutadapt v1.2.1 (52) and
204 Sickle v1.2.0.0 (53), while mini-tank reads were trimmed with Fastp v0.19.07 (54).
205 Assembly was performed on trimmed reads using Megahit v1.1.3 (55). Main tank
206 technical replicates were pooled by date and assembled using the settings: k-step 10; k-
207 range 27-87. Mini-tank metagenomes were assembled individually (k-step ~20, k-range:
208 21-99). Metagenome sequences are deposited with the ENA under Study Accession
209 PRJEB38990.

210

211 Read-based searches for ARGs were performed with DeepARG v2 (56). ARGs were also
212 identified on contigs (>1.5 kb length) in order to investigate the wider genetic context of
213 the core resistome using ABRicate v1.0.1 (57), using MegaRes 2.0 for ARGs and metal
214 resistance genes (MRGs) (58) (including experimentally verified MRGs; genes requiring
215 SNP validation were excluded) and ACLAME 0.4 for MGEs (59). All data were analysed
216 with stringencies: >60% gene coverage, >80% identity(60). Lastly, the BacMet2
217 database (61) was screened against translated peptides (based on Prodigal (62) output)
218 from meta-assemblies of the main and mini-tanks (stringencies: >60% sequence
219 identity and match length >50% of peptide length).

220 Taxonomic assignment of reads was performed using Kaiju v1.6.2 (63), with default
221 settings. The reference database used was a microbial subset of the NCBI database (64),
222 including additional fungal and other microbial eukaryotic peptide sequences. Contigs of
223 interest were assigned putative identities using NCBI-nucleotide BLAST (65)
224 (Megablast(66), highly similar sequences).

225 For both ARG and taxonomic assignments, statistical comparisons were carried out using
226 the DirtyGenes likelihood ratio test (67), using randomized resampling (n=1000) from
227 the null distribution to establish p-values.

228

229 *Water Quality Analysis*

230 Water quality analysis was performed on the same samples as for microbiological
231 culturing. For each sample, 2.5L was initially sampled. Probes were used to assess the
232 pH (Hach PHC201), dissolved oxygen (Hach LDO101) and NaCl (Hach). The probe tip
233 was rinsed in Milli-Q water (Merck), dabbed dry and submerged into the bottle
234 containing slurry and left to equilibrate. The sample was then homogenized by shaking
235 vigorously before decanting into a 250mL bottle for analysis using a Hach DR3900
236 Laboratory Spectrophotometer with cuvette test kits: sulphate (LCK153); ammonium
237 (LCK303); chloride (LCK311); copper (LCK329); LATON total nitrogen (LCK338); nitrate
238 (LCK340); nitrite (LCK342); phosphate (LCK348); zinc (LCK360); COD (LCK514); and
239 TOC (LCK381). Standard procedures are available from <https://uk.hach.com>.

240

241 *Mathematical Model*

242 A mechanistic, multi-strain model of AMR in the slurry tank was constructed to simulate
243 a range of relevant farm management scenarios that would have been impractical or
244 unethical to carry out empirically. In brief, it is a coupled ordinary differential equation
245 model of bacterial populations including logistic growth, death (baseline and
246 antimicrobial induced), horizontal transfer and fitness cost of resistance, inflow and
247 outflow (68, 69). The model considered mobile resistance to penicillin, tetracycline,
248 cephalexin, cefquinome, copper, and zinc, and was simulated for a full year in order to
249 capture the recorded input of cephalexin and other antibiotics. The choice of resistances
250 reflects our interests in ESC-R *E. coli* strains, and the risk of environmental
251 contamination by mobile genes following slurry spreading. Full model description is
252 provided in Supplementary Text 1, equations in Supplementary Text 2 and parameter
253 values in Table S4. This model was deposited in BioModels (70) as MODEL1909100001.
254 The secondary storage model is derived from this model by duplicating equations for
255 each storage vessel (70) and also deposited as MODEL1909120002. A reduced model
256 was used for parameter inference from mini-tank data. Model simulations were carried
257 out in Matlab using the ode45 solver.

258

259 **Results**

260 **Resistance to antibiotics with historic, current and no documented farm use**

261 The majority of antibiotics administered to milking cows during the sampling period were
262 aminocoumarins, aminoglycosides and beta-lactams delivered in combination, and beta-
263 lactams and tetracyclines administered individually (Table S3). The last recorded use of
264 sulphonamides (sulfadoxine) was in June 2016; of first generation cephalosporins
265 (cephalexin) was in April 2017 (shortly before the start of the sampling period); of third
266 generation cephalosporins (ceftiofur) was in January 2016; and of fourth generation
267 cephalosporins (cefquinome) was in August 2015. Residual antibiotics or ARB associated
268 with historical use could potentially be present in sludge at the bottom of the tank that

269 cannot be piped for spreading. Smaller quantities of antibiotics are also given to
270 youngstock; their waste does not enter the slurry system.

271 The dominant resistance phenotypes of cultured *E. coli* isolates from the slurry tank
272 (Figure 1a) were ampicillin (34.6%), cefpodoxime (39.3%), cefotaxime (29.6%) and
273 streptomycin (26.5%); other common phenotypes included tetracycline (13.6%),
274 chloramphenicol (10.7%) and nalidixic acid (9.6%). Multidrug resistant *E. coli* strains
275 (≥ 3 different antibiotic classes, Magiorakos, Srinivasan (71)) represented 37% of the
276 cultured isolates (Figure 1b), detected in strains isolated on both antibiotic-
277 supplemented and non-supplemented media. Of these isolates, 12 cefotaxime resistant
278 *E. coli* strains were sequenced to characterize the resistance genes and mobile elements
279 carrying them. Three carried *ISEcp1* CTX-M-15, additionally carrying *qnrS* and *tetM*
280 within the *ISEcp1* element. The other sequenced ESC-R strains were chromosomal *ampC*
281 mutants.

282 In main slurry tank metagenomes, eight resistance classes account for 98% of the ARGs
283 identified in reads (Figure 1c): multidrug resistance genes (36.7%); tetracycline
284 resistance genes (21.6%); macrolide-lincosamide-streptogramin (MLS) resistance genes
285 (21.4%); aminoglycosides (7.3%); beta lactams (4.5%); peptides (4.0%); bacitracins
286 (1.6%) and glycopeptides (1.2%). MRGs were also identified (*mer*: mercury; *cop*, *cus*,
287 *pco/sil*: copper, copper/silver; *cad*, *czc*: cadmium, cadmium/zinc/cobalt; *ars*,
288 arsenic/antimony; *pbr* lead resistance). In equivalent metagenome read assemblies, MLS
289 and tetracycline ARGs were most frequently detected (70 and 46 contigs, respectively).
290 Few MRGs were detected in main tank metagenome assemblies, limited to TCR copper
291 resistance genes (5 contigs).

292

293 Overall, the identification of aminoglycoside, beta-lactam (excepting 3rd/4th generation
294 cephalosporins) and tetracycline resistance genes and phenotypes reflect current or
295 recent farm antibiotic use, while the presence of zinc and copper resistance genes reflect
296 transition metal use. The presence of sulphonamide and cephalosporin resistance genes
297 and phenotypes may be due to historical use, or reflect widespread environmental

298 occurrence (72). The prevalence of MLS resistance genes is unlikely to be associated
299 with antibiotic use, as there is no recorded MLS use for milking cows.

300

301 **Slurry tank properties and AMR remained stable due to frequent inputs**
302 Water quality measures were largely stable (Figure 2a), with some fluctuations in July
303 and August likely to be associated with mixing of slurry in the tank prior to spreading on
304 fields. The relative contribution of the dominant drug-resistance categories listed above
305 remained unchanged throughout the sampling period (Figure 1c; $p=0.172$, DirtyGenes
306 test). Likewise, taxonomic analyses of read data showed the time-stable dominance of
307 six bacterial phyla with at least 1% prevalence (Figure 2b; $p=0.254$, DirtyGenes test):
308 Bacteroidetes (13.8%), Firmicutes (13.7%), Proteobacteria (4.7%), Spirochaetes
309 (2.9%), Euryarchaeota (1.9%) and Tenericutes (1.4%). These phyla only account for
310 38% of the microbial community: there is considerable diversity in the tank with 178
311 phyla identified (Table S4).

312 The overall numbers of *E. coli* identified through culture-based enumeration also
313 remained stable (Figure 2c), with concentrations of 4.23 ± 0.40 (Log_{10} CFU mL^{-1}) on TBX
314 plates and 4.29 ± 0.46 (Log_{10} CFU mL^{-1}) on MacConkey media. *E. coli* strains resistant to
315 ampicillin (TBX/Amp 16 $\mu\text{g mL}^{-1}$) were stable at concentrations of 3.99 ± 0.43 (Log_{10} CFU
316 mL^{-1}), i.e. $\sim 58\%$ of cultured *E. coli* strains. *E. coli* that could be cultured on cefotaxime
317 selective plates (TBX/CTX 2 $\mu\text{g L}^{-1}$) were detected on only five of 17 sampling dates, with
318 counts below 10 colonies per plate on all but one day (22nd August). Thus, cefotaxime
319 resistant *E. coli* were present at low levels, but could not be reliably quantified. The full
320 AST profiles of the 811 isolates also show consistency over time, with some random
321 variation, both on antibiotic-free and antibiotic-supplemented media (Figure 3).

322

323 **Model predictions are consistent with microbial data**

324 In the mathematical model, predicted resistance to penicillins fluctuated between 0.4%
325 and 6.4% and cephalosporins between 0.5% and 7.9% (Figure 4a), i.e. both present but
326 low, despite frequent inflow of antibiotics into the tank (Figure 4b). Resistance to

327 tetracycline increased from low initial levels to fluctuate around ~25% of the *E. coli*
328 population (Figure 4a), before slowly declining over the longer term, reflecting the
329 decline in tetracycline use later in the year. These predicted levels of tetracycline and
330 cephalosporin resistances are concordant with the empirical phenotype above. Penicillin
331 resistance in the model is lower than observed empirically, probably because resistance
332 in the model is plasmid-borne, while many strains have chromosomal mutations of *ampC*
333 or chromosomally located resistance genes that could be mobilised (e.g. *ISEcp1CTX-M*-
334 15 elements). The model predicts that zinc resistance is highly prevalent, rising to
335 fluctuate around 80%, with co-occurrence of tetracycline and zinc resistance, typically
336 fluctuating between 10 and 15%, consistent with predictions that the metal
337 concentrations in the tank are co-selective (69).

338 **Associations of ARGs with other ARGs, integrons and Gram-positive taxa**

339 Several metagenome contigs contained two or more ARGs, MRGs or associations with
340 MGE markers in both the main tank (37 contigs) and mini-tank metagenome assemblies
341 (101 contigs) (Figures S1 and S2). These include ARGs belonging to the same resistance
342 gene group, e.g. *aph3* and *aph6* (both aminoglycoside resistance genes; Figure S3a)
343 which were co-localised on five main-tank and eight mini-tank contigs; as well as genes
344 associated with entirely different antibiotic resistance classes, e.g. *ant6* and *tet44*
345 (aminoglycoside and tetracycline resistance, respectively; Figure S3b) were co-localised
346 on two main-tank and eight mini-tank contigs. In other mini-tank contigs, *aph3-aph6*
347 were additionally co-resident with either a sulphonamide (*sul2*, 1 contig) or tetracycline
348 (*tetY*, 1 contig) resistance gene. *tetM* was embedded within the widely documented
349 Tn916 transposon (18 *tetM* contigs in total, nine of which were linked with Tn916
350 elements). The two largest Tn916-like contigs (18.3-18.9 kb) appear to be carried within
351 Gram-positive bacteria, possibly *Streptococcus* spp. or *Enterococcus* spp. (NCBI-BLAST,
352 ~99.96% identity, ~91% query coverage; Figure S3c). Furthermore, 21.4% (n= 6/28)
353 of main and mini-tank contigs containing *cfxA* (class-A beta-lactamase) were co-localised
354 with mobile elements.

355 Further identification of mobile resistance cassettes was through a screen of all *E. coli*
356 strains for phenotypic mercury resistance as a marker for Tn21 carriage. Sequence
357 analysis of mercury resistant *E. coli* strains showed that three carried Tn21 variants
358 carrying the integron intI2 conferring co-occurrence of combinations of penicillin,
359 sulphonamide, aminoglycoside and quaternary ammonium compound resistances.

360 **Waste management for AMR reduction**

361 We investigated the use of slurry storage to ameliorate resistance through a combination
362 of empirical and modelling work. In the mini-tanks, we found that storage of slurry
363 without inflow rapidly decreased the total concentration of cultured *E. coli* cells (Figure
364 S6a), as well as *Escherichia*, *Pseudomonas* and *Klebsiella* spp. sequences identified by
365 metagenomics (Figure 5). Reads assigned to gut-associated anaerobes belonging to
366 Bacteroidetes including *Bacteroides* spp., *Alistipes* spp. and *Prevotella* spp. declined in
367 steps. In contrast, the relative abundance of *Acinetobacter* spp. gradually increased until
368 week four, before declining again by the end of the experiment (Figure 5).

369 The prevalence of beta-lactam resistance genes declined considerably in <2 weeks
370 (Figure 6a). The overall relative abundance of tetracycline resistance genes declined
371 marginally over 7-weeks of storage (Figure 6b); however, different patterns were
372 observed with different gene groups: *tetY* (Figure 6c) and *tet40* (Figure 6d) declined
373 sharply within two weeks, while others, e.g. *tetM* (Figure 6e) were maintained in stored
374 slurry. According to BLAST analysis against the NCBI database, mini-tank contigs
375 containing *tetY* (2 contigs) were likely associated with Gamma-Proteobacteria, while
376 *tet40* (6 contigs) was consistently linked to Firmicutes. Similarly, *tetM* was typically
377 associated with Firmicutes (7 of 16 contigs; >89% sequence coverage, >99% sequence
378 identity), more specifically Bacilli. The proportion of MLS ARGs remained comparatively
379 stable throughout (Figure 6f), consistent with their presence not connected with patterns
380 of MLS use on the farm.

381 We implemented a two-stage in series storage mathematical model to consider whether
382 the storage of slurry in the main tank, without fresh inputs, would reduce AMR in slurry
383 prior to land application. The model predicted that after only four days of storage, 50%

384 of the amoxicillin- and cefalexin-resistant *E. coli* are removed, and after 60 days of
385 storage, only 0.29% of cefalexin-resistant and 0.00001% of amoxicillin resistant *E. coli*
386 remained (Figure 7a). However, the model predicts that tetracycline resistant bacteria
387 will increase over this time by 25% due to ongoing selective pressure and low fitness
388 cost. Importantly, multidrug resistant *E. coli* become undetectable.

389

390 **Simulations of altered antibiotic use support criteria for responsible use**

391 Simulations of on-farm antibiotic use (~9.7 mg/Population Correction Unit (PCU) in
392 2017) result in low levels of penicillin and cephalosporin resistance, consistent with the
393 empirical data. We simulated further reductions in antibiotics entering the tank to either
394 50% or 10% of current use. Neither reduction had a material impact on either resistance
395 (Figure 7b) but there is a small reduction in tetracycline resistance (33% reduction in
396 resistance at 10% usage) because of the reduced selective pressure for tetracycline
397 resistance.

398 Very few cephalosporin resistant *E. coli* were detected in the farm samples (detailed
399 above). Thus, we also simulated a return to use of the critically important 4th generation
400 cephalosporin (cefquinome) in place of cefalexin (1st generation), assuming that
401 cefquinome resistance also confers resistance to cefalexin. After accounting for the lower
402 recommended dosage of cefquinome relative to cefalexin, we found cefquinome use
403 increased resistance to both cefquinome and cefalexin of only 0.65% and 0.35%
404 increase respectively (Figure 7c). To represent high antibiotic use following an outbreak
405 of disease, we simulated 50 mg/PCU of cefquinome used in place of cefalexin. Such a
406 scenario was predicted to select an increase of cefquinome resistance of only 3.55%.

407

408 **Discussion**

409

410 **The slurry tank is a critical measurement and control point for AMR**

411 The bacterial community and ARGs in the slurry tank appear to be maintained in a state
412 of dynamic equilibrium, with a balance between input of fresh microorganisms from the

413 cattle, and decline, as observed in the mini slurry-tank experiments. This equilibrium is
414 also evident in the observed stability of the virome of the same tank over the same
415 sampling period (73). The slurry tank maintains an array of ARGs, many of which have
416 been found in other animal wastes. These include MLS genes such as *mefA* (24, 29, 74)
417 and the *cfxA* group of beta-lactamase genes (24, 74, 75). The association of *cfxA* with
418 Gram-positive organisms suggests that AMR phenotyping should routinely include a
419 Gram-positive sentinel; *Enterococcus* spp. may be suitable because of their use in water
420 quality analysis (76) and the inclusion of *E. faecium* in the ESKAPE pathogens list (77).
421 Tetracycline resistance genes such as *tetW* and *tetM* have also been frequently found in
422 cattle and swine waste (29, 78, 79). Although present in low quantities relative to other
423 ARGs, *tetM* has the potential for selection and possible mobilisation (e.g., *ISEcp1* or
424 *Tn916*-like elements). Consequently, the tank appears to be a critical sampling location,
425 representative of the AMR status of the farm as a whole, reflecting current and previous
426 antibiotic use. The presence of resistance genes to antibiotics with no recorded use (e.g.
427 quinolone resistance, MLS genes) are likely to reflect broader environmental, and
428 possibly human, input into the farm microbiome.

429 At a superficial level, the slurry tank appears to meet many criteria presumed to define a
430 'hotspot' for AMR, which cite a high abundance of bacterial populations and the routine
431 presence of antimicrobial residue (80). However, the concept of an AMR 'hotspot', where
432 bacterial and antimicrobial abundance are assumed to lead to increases in AMR
433 prevalence, alongside the related concept of 'reservoir', assumed to represent the
434 nascent AMR genes circulating in the environment poised to be mobilised through
435 antimicrobial exposure, are open to critique (81). Our findings suggest that the tank,
436 rather than generating resistance, can ameliorate resistance, depending on the waste
437 management practice, and that slurry be stored for at least two months without fresh
438 slurry inputs to the system/tank. Thus, the tank is neither a hotspot nor a reservoir, but,
439 if managed appropriately, can be a critical control point for reducing the transmission of
440 ARGs and ARB from livestock into the wider environment.

441

442
443 **Agricultural AMR policy should combine responsible antibiotic use with**
444 **effective waste management**
445 Policy and industry guidance to reduce AMR focus on reduced or responsible agricultural
446 antimicrobial use (25, 82, 83), including the cessation of use of human critical
447 antibiotics. Our findings provide evidence in support of responsible use. Simulations of
448 reductions below the already low level of 9 mg/PCU did not predict reductions in
449 penicillin and cephalosporin resistance below current levels. However, reduced
450 tetracycline use led to reduced tetracycline resistance, associated with the environmental
451 stability of this antibiotic, suggesting that prudent antibiotic use could also include
452 antibiotic choice encouraging use for those with shorter half-lives where medically
453 appropriate. While our findings suggested that use of 3rd and 4th generation
454 cephalosporins did not lead to substantial increases associated resistances, once such
455 resistances are established, relevant genes, e.g. CTX-M, can be selected for by 1st
456 generation use. Although UK policy initiatives have greatly reduced the use of 3rd/4th
457 generation cephalosporins on UK dairy farms, globally their use remains prevalent, e.g.
458 Ceftiofur (3rd generation cephalosporin) is routinely used in the US to treat metritis (84,
459 (85) and mastitis (86). Eliminating the use of these antibiotics in agricultural production
460 should still be an important goal of national and global policies to mitigate the
461 environmental dissemination of AMR (87).
462 A policy focus on antibiotic use is limited because of the need to use antibiotics to treat
463 sick livestock. We also showed that waste management practice provides an additional
464 mechanism to control AMR, by reducing the prevalence of resistance genes and key
465 microbial phyla in slurry prior to soil amendment. Specifically, secondary storage of
466 slurry for a period of 60 days, without fresh inflow, would significantly reduce the levels
467 of ARB within the tank, representing an opportunity for rational farm design and practice
468 to minimize AMR outcomes. This result is also concordant with other practices for
469 mitigating AMR on farms, including the use of anaerobic digestion (79, 88),
470 vermicomposting and solid-liquid separation (43).

471 Two qPCR-based studies surveying Finnish swine and dairy farms reported that storage
472 of animal manure slurry coincided with significant increases in select tetracycline,
473 sulphonamide and aminoglycoside resistance genes when compared to fresh manure
474 (28, 29). However, the farms involved in these studies used storage systems which
475 received regular fresh inflow during the sampling period. Our metagenomic analyses of
476 mini-tanks indicate that in the absence of fresh input a range of ARG classes decline
477 (e.g. aminoglycoside and beta-lactam ARGs) or remain relatively stable (e.g. MLS
478 ARGs). Moreover, culture-based results confirm an overall reduction in antibiotic
479 resistant *E. coli* in slurry stored without inflow. Collectively, this provides empirical
480 evidence supporting existing UK guidelines regarding the storage of slurry without
481 further input as a means of reducing environmental exposure to AMR determinants.

482

483 **Evaluation of co-selection needs alternative approaches**

484 Aminoglycoside, tetracycline and sulphonamide resistance genes were found on the
485 same contigs. The result is consistent with sulphonamide resistance being co-selected by
486 concurrent use of multiple antimicrobials because aminoglycosides and tetracycline were
487 the two antibiotic classes used most during the sampling period. We anticipated finding
488 evidence of co-occurrence of ARGs and MRGs in assembled metagenomic data, in
489 accordance with other studies (19, 24, 89). However, apart from antibiotic resistance
490 associated with Tn21-like elements carrying integrons, we found no evidence for such
491 linkage in the slurry metagenomes or sequenced *E. coli* strains. This lack of evidence
492 might not be evidence of absence of ARG-MRG co-occurrence, as these genes may not
493 necessarily be genetically linked on a chromosome or on plasmids, and yet still be
494 subject to co-selection if they reside in the same cell. Accordingly, the use of long-read
495 or hybrid genome sequencing of strains selected for zinc or copper resistance may be
496 more appropriate for detecting the co-occurrence of ARGs and MRGs (90).

497

498 **Conclusions**

499

500 We have conducted a longitudinal, interdisciplinary study of the dynamics of AMR in a
501 dairy slurry tank. The microbiota was in a state of dynamic equilibrium, with fresh input
502 of bacteria from the animals balanced by natural decay. Antibiotic resistance was
503 maintained, reflecting current and previous veterinary practice, as well as interaction
504 with the broader environment. The slurry tank is therefore both a natural measurement
505 point for on-farm resistance, as well as a control countermeasure point for resistance
506 being released into the wider environment (land and water). The spread of antibiotic
507 resistance into the wider environment through slurry application can be mitigated by a
508 combination of responsible antibiotic use, including low total quantity, avoidance of
509 human critical antibiotics, and antibiotic choice with shorter half-lives, with slurry
510 storage. These approaches can mitigate spread of AMR into the environment from one of
511 the world's largest sources of AMR pollution.

512

513

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554 Editing, Project Administration, Acquisition of Funding

555

556 Data Availability

557
558 Genome sequences are deposited with NCBI under BioProject PRJNA736866.
559 Metagenome sequences are deposited with the ENA under Study Accession PRJEB38990.
560 Mathematical models are deposited in BioModels as MODEL1909100001 and
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849 Figure Legends

850 **Figure 1: Antimicrobial resistance phenotypes and reads in the slurry tank** (a)

851 Resistances to a panel of 16 antibiotics (Supplementary Table 2) largely do not depend

852 on the type of supplemented media used. (b) The number of resistances per isolates;

853 37% of cultured isolates resistant to three or more antibiotic classes. These resistances

854 are seen on all types of media. (c) Proportion of ARGs mapped to different antibiotic

855 resistance classes (% reads). The metagenomic resistance profile is largely stable over

856 time. There appears to be a gradual increase in the proportion of aminoglycoside and
857 beta lactam resistance genes, which could be seen as consistent with antibiotic use
858 during that period, but there is no statistical significance to the changes in proportions.
859 ARGs are also reasonably consistent with observed phenotype data.

860 **Figure 2: Stability of microbial ecosystem, *E. coli* counts and water quality**
861 **measures.** (a) water quality analysis from samples taken from the slurry tank over a
862 five month period concurrent with microbial counts. Water quality measures are
863 generally stable, with some fluctuations concordant with slurry use. (b) Six taxonomic
864 groups accounting for at least 1% each of microbial reads show stable abundance in
865 time. There is considerable diversity; these groups only account for 38% of reads, with
866 all reads mapped to 178 different microbial phyla. (c) Counts of *E. coli* concentrations
867 showing *E. coli* on TBX and MaConkey plates (all *E. coli*), TBX and AMP plates (*E. coli*
868 resistant to ampicillin) and on CTX plates (ESC-R *E. coli*). Overall *E. coli* abundance is
869 stable throughout the sampling period, as are counts of ampicillin resistant strains. CTX-
870 resistant *E. coli* are only observed on five sampling days, and on all of those occasions
871 at levels too low to be reliably quantified. The data for the other days are below the limit
872 of detection of the method used and are plotted at 0 for ease of display.

873
874 **Figure 3: Antibiotic susceptibility testing of *E. coli* isolates shows diverse but**
875 **stable range of phenotypic resistances.** In each panel, the heatmap shows the
876 proportion of strains resistant to each of 16 different antibiotics on each of the sampling
877 dates. Grey bars indicate no use of those plate types on those dates. (a) plates without
878 antibiotic supplement; (b) plates supplemented with ampicillin; (c) plates supplemented
879 with cephalosporins. In all cases, the patterns of resistances are stable in time.
880 Cephalosporin supplemented plates identify more resistant strains than other plates,
881 including to other antibiotic classes, including tetracyclines and quinolones.

882
883 **Figure 4: Model simulations of antimicrobials and antimicrobial resistance in the**
884 **slurry tank.** (a) Model prediction of resistant *E. coli* populations in the slurry tank over a

885 year's period, given (b) antibiotic usage on farm in 2017. Resistance groups are not
886 mutually exclusive. The resistances are reasonably stable once the model simulation
887 reaches its steady state, with fluctuations resulting from periodic removal of slurry for
888 use as fertilizer. (b) Mass (in mg) of oxytetracycline, cefalexin and amoxicillin given
889 during 2017 together with model simulation predicting concentrations (in mg L⁻¹) of
890 these antibiotics in the slurry tank over the same period. Observe that tetracycline is
891 present in the tank, despite intermittent use, due to its high environmental stability. This
892 explains the consistent proportion of tetracycline resistance. The two beta lactam
893 antibiotics decay more rapidly after use.

894

895 **Figure 5: Storage without further waste-addition leads to a decline in select**
896 **bacteria.** Relative abundances of *Escherichia* spp., *Pseudomonas* spp., *Klebsiella* spp.,
897 *Bacteroides* spp., *Prevotella* spp. and *Alistipes* spp. in stored slurry based on
898 metagenomic short-read data. *Escherichia* reads from metagenomics are concordant
899 with culturing data (viable *E. coli* counts in CFU/ml over time are given in Figure S6a),
900 both showing a stepwise decline. *Pseudomonas* and *Klebsiella* also show a stepwise
901 decline. *Bacteroides*, *Prevotella*, and *Alistipes* show a gradual decline. *Acinetobacter*
902 increase over the first four weeks before declining.

903

904 **Figure 6: Impact of mini tank storage on selected ARGs based on DeepARG**
905 **analysis.** Relative abundance (percentage of reads) of (a) beta lactam ARGs; (b)
906 tetracycline ARGs; (c) *tetY*; (d) *tet40*; (e) *tetM*; (f) MLS ARGs. The decline in beta
907 lactam reads is consistent with other data. Tetracycline ARGs show different patterns for
908 different genes. The persistence of MLS ARGs is consistent with their presence not
909 related to lack of MLS use on the farm.

910

911 Figure 7: **Model simulations of altered farm practise or antibiotic use** (a) Storing
912 slurry without fresh inflow for 60 days is predicted to reduce resistance. Cephalexin
913 resistance is reduced by more than 99.99% while amoxicillin resistance is reduced by
914 more than 99%. (b) Model predictions of current antibiotic usage (9.7 mg/PCU)
915 compared to a 50% reduction (4.85mg/PCU) and 90% reduction (0.97mg/PCU) show
916 negligible impact on slurry tank resistance levels. (c) Model predictions of the change in
917 resistant *E. coli* in the tank when using a 4th generation cephalosporin instead of a 1st
918 generation cephalosporin on low, medium and high antibiotic usage farms showing
919 increased resistance to all relevant antibiotics.

920

921

Figure 1

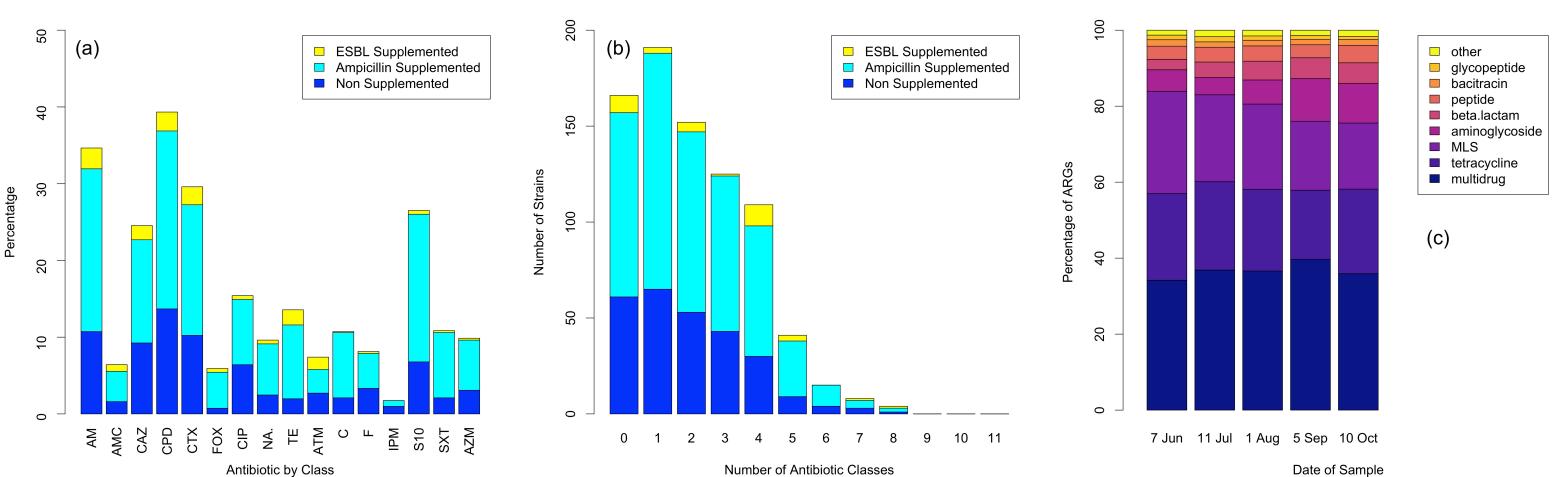


Figure 2

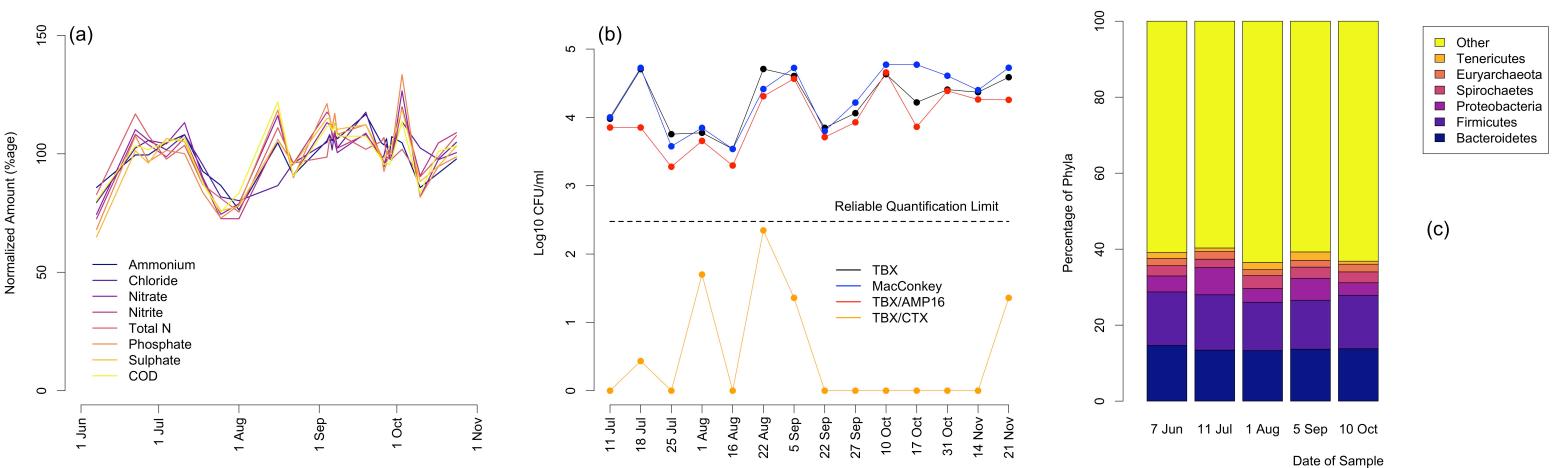


Figure 3

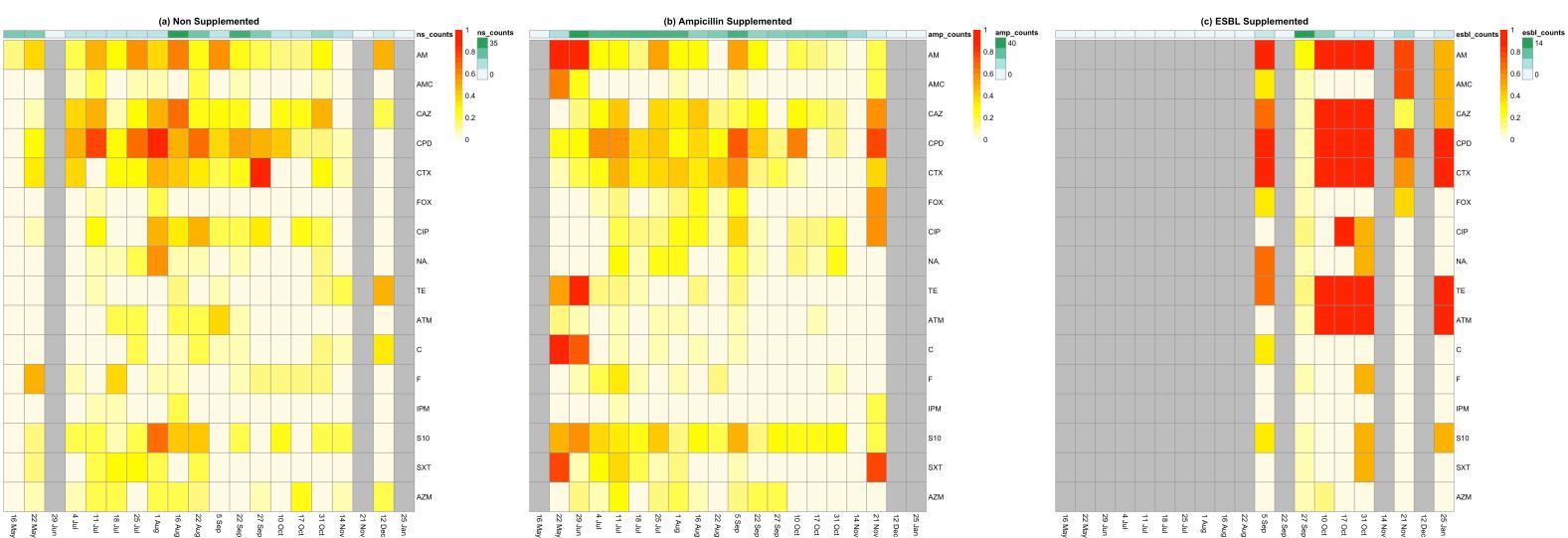
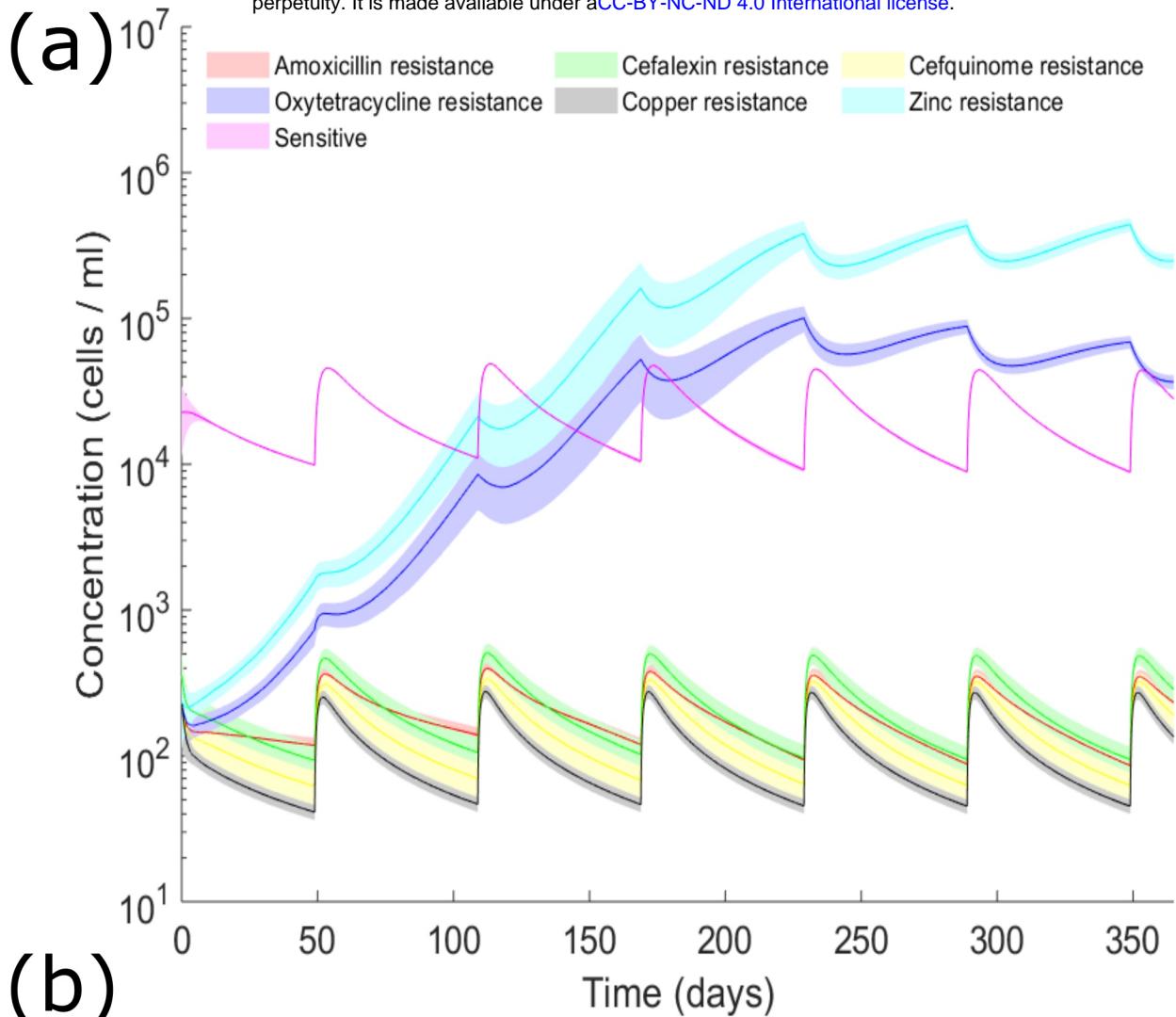


Figure 4



(b)

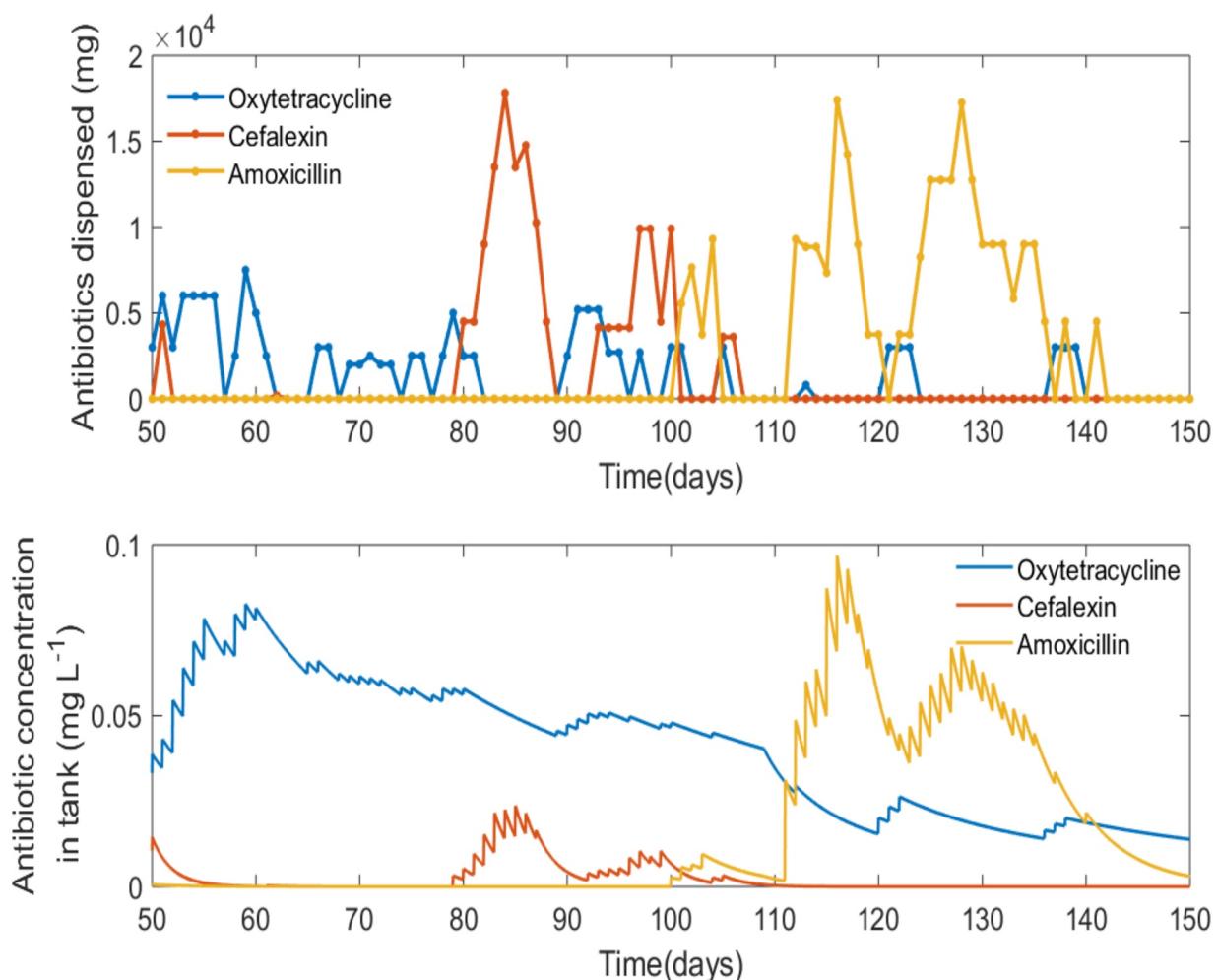


Figure 5

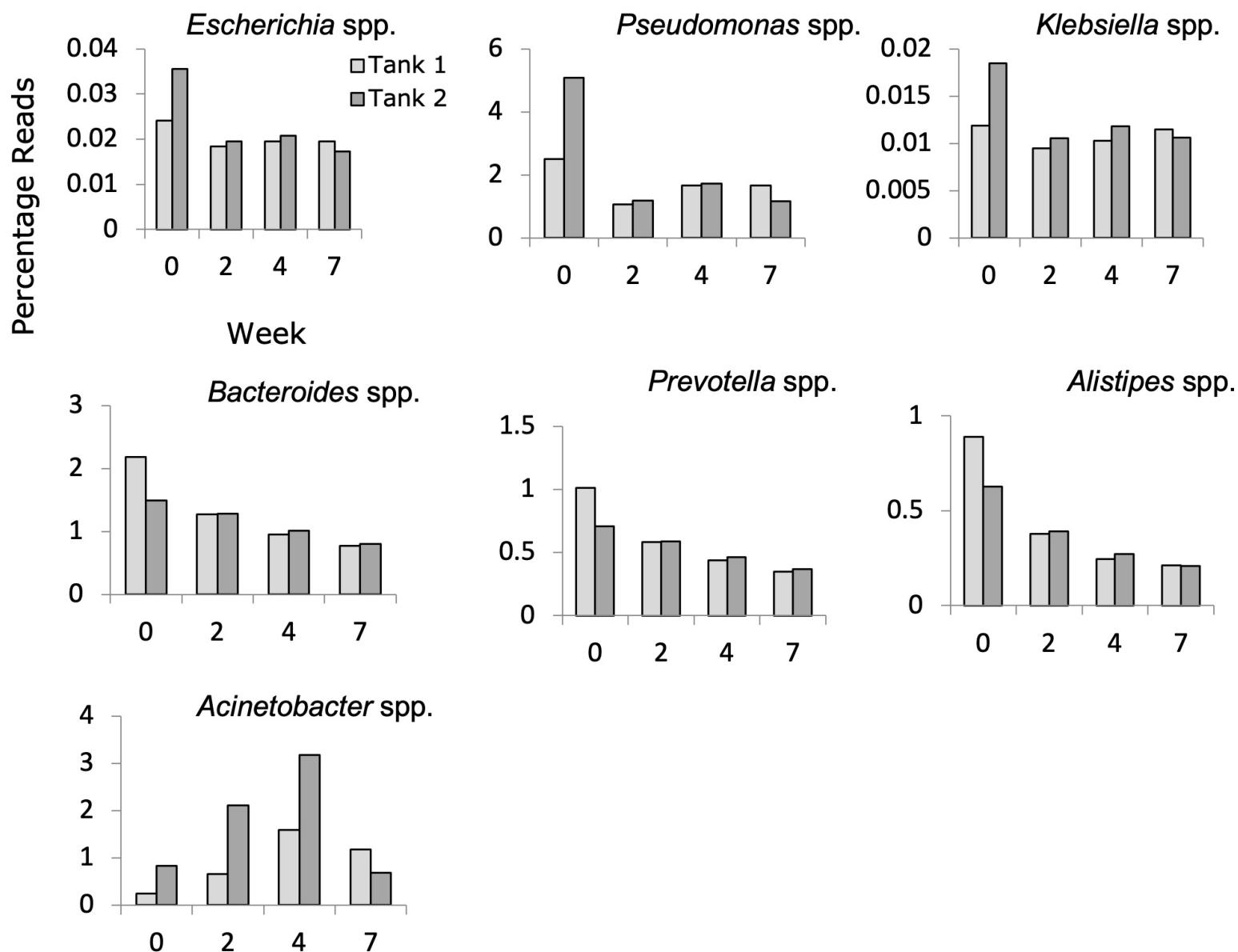


Figure 6

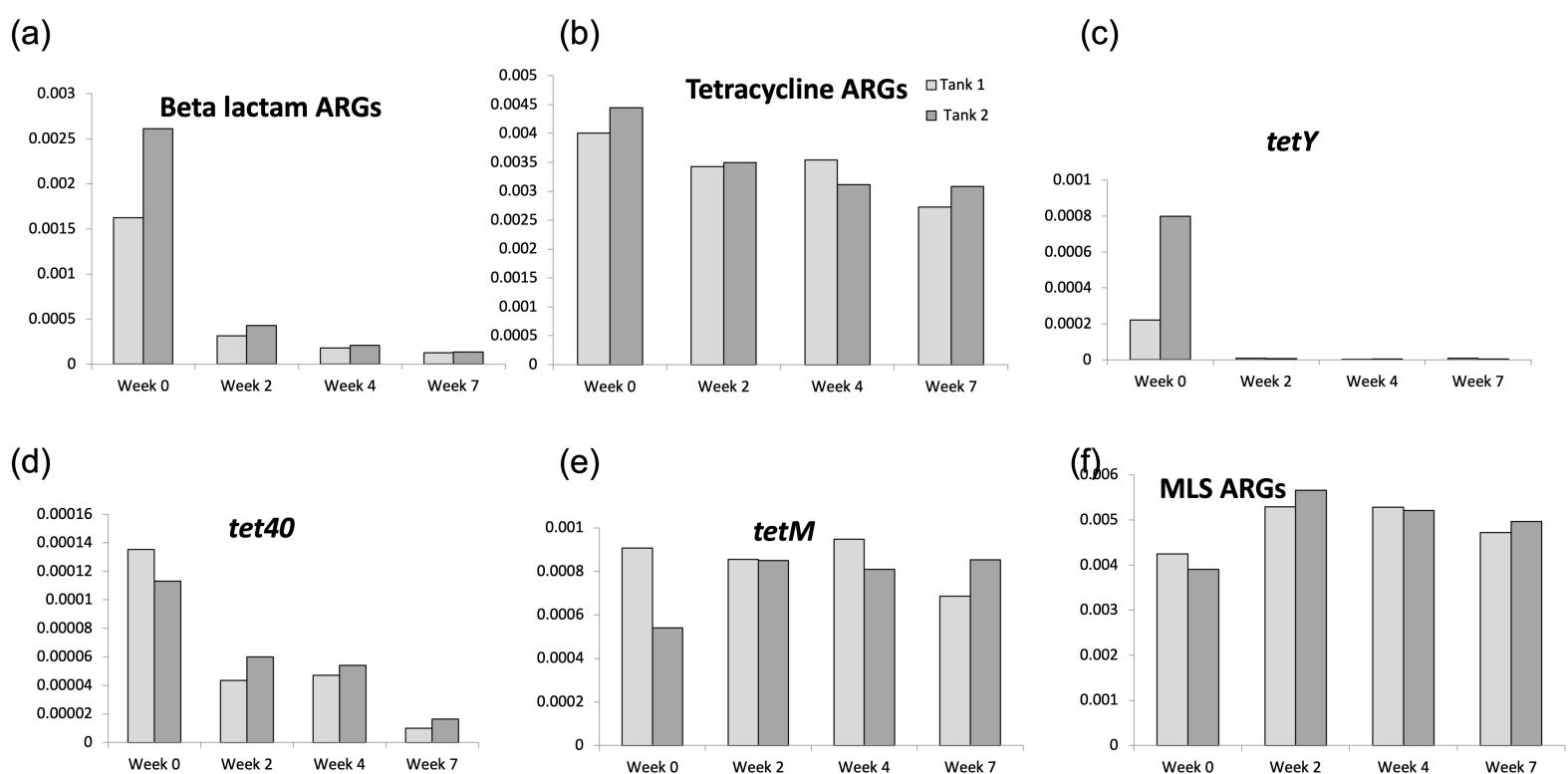


Figure 7

