Fate and persistence of a pathogenic NDM-1-positive *Escherichia coli* strain in anaerobic and aerobic sludge microcosms

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ABSTRACT

The presence of emerging biological pollutants in treated wastewater effluents has gained attention due to increased interest in water reuse. To evaluate the effectiveness of the removal of such contaminants by the conventional wastewater treatment process, the fate and decay kinetics of NDM-1-positive *Escherichia coli* strain PI7 and its plasmid-encoded antibiotic resistance genes (ARGs) were assessed in microcosms of anaerobic and aerobic sludge. Results showed that *E. coli* PI7 decayed at a significantly slower rate under anaerobic conditions. Approximate half-lives were 32.4 ± 1.4 h and 5.9 ± 0.9 h in the anaerobic and aerobic microcosms, respectively. In the aerobic microcosms, after 72 h of operation, *E. coli* PI7 remained detectable but no further decay was observed. Instead, 1 in every 10000 *E. coli* cells was identified to be recalcitrant to decay and persist indefinitely in the sludge. ARGs associated with the *E. coli* PI7 were detected to have transferred to other native microorganisms in the sludge, or are released to the liquid fraction upon host decay. Extracellular DNA quickly degraded in the liquid fraction of the aerobic sludge. In contrast, no DNA decay was detected in the anaerobic sludge water matrix throughout the 24 h sampling period. This study suggests an increased likelihood of environmental dispersion of ARGs associated with anaerobically treated wastewater effluents and highlights the potential importance of persister cells in the dissemination of *E. coli* in the environment during reuse events of treated wastewater.

45 **IMPORTANCE**

46 This study examines the decay kinetics of a pathogenic and antibiotic resistant strain of
47 *Escherichia coli* in microcosms simulating biological treatment units of aerobic and
48 anaerobic sludge. The results of this study points at a significantly prolonged persistence
49 of the *E. coli* and the associated antibiotic resistance gene in the anaerobic sludge.
50 However, horizontal transfer of the plasmid encoding the antibiotic resistance gene were
51 detected in the aerobic sludge by cultivation method. Detection of a subpopulation of
52 persister *E. coli* were also detected in the aerobic sludge. The findings of this study
53 suggest potential areas of concern arising from pathogenic and antibiotic-resistant *E. coli*
54 during both anaerobic and aerobic sludge treatment processes.

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INTRODUCTION

In most wastewater treatment plants (WWTPs), aerobic activated sludge processes are conventionally used as the main biological unit to achieve removal of organic materials from wastewater but such systems incur a large energy cost (1). Anaerobic digesters are increasingly being considered for use as an alternative process for wastewater treatment due to their various advantages (2). Energy can be recovered from the wastewater in the form of methane produced from anaerobic fermentation of the organic carbon, and the amount of sludge produced from anaerobic processes is lower than that from aerobic processes (1).

However, municipal wastewater often contains sub-therapeutic levels of antibiotic residues, and the continuous exposure to antibiotics can select for antibiotic-resistant bacteria (ARB) within both aerobic and anaerobic sludge processes. In recent years, WWTPs had been shown to be potential hotspot for ARB and antibiotic resistance genes (ARGs) propagation (3). Despite having undergone treatment, the treated municipal wastewater can still contain a significant amount of ARB and ARGs. To exemplify, an earlier study has shown that the treated effluents that ultimately would be intended for reuse or discharged into receiving water bodies, carried about 10^6-10^{11} heterotrophic colony forming units (CFU) per cubic meter (4, 5), from which 16-28% correspond to ARB (5). In another study, 10^7-10^9 copies of diverse tetracycline resistance genes had been found in each cubic meter of chlorinated effluents (4). To further compound this problem, genes that confer resistance to carbapenems (i.e., bla_{NDM-1}), which are antibiotics used as a last line of defense against multidrug resistant infections (6, 7), were

also detected at alarming levels in final effluents, approaching 10^9 copies per cubic meter of treated wastewater (8).

This problem is of particular concern in water-scarce countries with pressing needs to reuse the treated wastewater. The incidence of bacterial pathogens carrying ARGs that confer resistance to antibiotics of last resort (e.g. bla_{NDM-1}-positive pathogenic *Escherichia coli*) require particular attention, as the reuse of such treated wastewater effluents might pose a potential risk to the public health if disseminated into the environment during reuse events (4). As a first step to assess the risk associated to wastewater reuse, it is necessary to understand the differential fate and persistence of ARBs and ARGs in the main biological treatment unit of both, anaerobic and aerobic wastewater treatment systems. Few studies comparing the differential aerobic/anaerobic ARG removal have been performed and showed conflicting information. Molecular studies by Diel et al. (9) and Burch et al. (10) found that ARGs tend to be removed more efficiently under anaerobic conditions. However, other studies suggest that combined anaerobic-aerobic (11), or on the contrary, completely aerobic conditions are more efficient at removing ARGs and ARBs from wastewater (12).

Although informative, these earlier studies rely purely on the molecular-based detection and do not examine the factors potentially shaping the decay or persistence of the ARG and the ARB host in the sludge. The formation of specialized persister cells is a strategy adopted by different types of bacteria such as *E. coli* to endure harsh environmental conditions (13). These cells are dormant variations of vegetative cells that occur at low frequencies within the bacterial population, and are commonly known for its capacity to withstand supra-lethal concentrations of antibiotics (13-15). The contribution

105 of persisters to the establishment of chronic infections in several pathogens is well
106 documented (16-18). However, little is known about its contribution to the ARB survival
107 in aerobic and anaerobic sludge. Neither did the earlier studies examine the factors
108 shaping the decay or persistence of the ARGs associated with the antibiotic-resistant host
109 in both anaerobic and aerobic sludge. These factors include the stability and persistence
110 of ARGs as extracellular DNA, and the potential for horizontal gene transfer (HGT)
111 events when the ARG is encoded on a conjugative plasmid.

112 In this study, *Escherichia coli* PI7 was used as a model bacterium to examine the
113 existing knowledge gaps associated with the fate and persistence of ARB and ARG in
114 anaerobic and aerobic sludge. This bacterium was previously isolated from wastewater
115 and carries an extensive repertoire of ARG, including a copy of $bla_{NMD-1,}$ in a plasmid of
116 the IncF family and earlier identified as pKOX_NDM1 (19). In the recent decade, bla_{NDM-1}
117 has undergone a pandemic spread among clinically relevant bacteria (20, 21). It has also
118 been detected at alarming levels in different environmental compartments, including
119 water (22, 23), soil (24) and wastewater (8, 25). Given the importance of wastewater in
120 the environmental mobilization of ARBs and ARGs, we aim to evaluate the fate and
121 persistence of our model bacterium *E. coli* PI7, and its associated IncF plasmid in
122 anaerobic and aerobic sludge microcosms under varying trace antibiotic concentrations.
123 Using molecular tools, this study evaluates the plasmid stability, host persistence and
124 HGT events in anaerobic and aerobic conditions. Chromosomal and plasmidic decay
125 rates were measured by quantitative PCR coupled with propidium monoazide to
126 discriminate between dead cells and extracellular DNA from cells with intact cell

membranes. In addition, the potential stability of extracellular DNA in the liquid fraction of anaerobic and aerobic sludge was also evaluated.

RESULTS

Differential decay of *bla*$_{NDM-1}$ in anaerobic and aerobic sludge microcosms. Under anaerobic conditions, *bla*$_{NDM-1}$ decayed following a first-order decay kinetic model (R^2 > 0.935, Figure 1a). After 360 h of operation, *bla*$_{NDM-1}$ copy numbers decreased by 2.5-log from 10^8 to 10^6 copies/g biomass. This decrease in the *bla*$_{NDM-1}$ copy numbers corresponded to first-order decay rates of -0.021 ± 0.002 h^{-1} ($t_{1/2}$ = 32.9 ± 2.8 h) and -0.021 ± 0.001 h^{-1} ($t_{1/2}$ = 32.3 ± 1.7 h) for the non-treated (NTB) and PMA-treated biomass (PTB) fractions, respectively (Figure 1a, Table 1).

Under aerobic conditions, *bla*$_{NDM-1}$ decay in the biomass fraction showed a biphasic behavior (Figure 1b). An initial 4-log removal in copy numbers from 10^8 to 10^4 copies /g biomass during the first 72 h of reactor operation was observed. Phase-I fits a first-order decay kinetic model (R^2 > 0.83) with a decay rate of -0.1049 ± 0.019 h^{-1} ($t_{1/2}$ = 6.6 ± 1.2 h) and -0.1196 ± 0.014 h^{-1} ($t_{1/2}$ = 5.8 ± 0.7 h; Figure 1b, Table 1) for the NTB and PTB fractions, respectively. Similar to the anaerobic conditions, differences in the *bla*$_{NDM-1}$ decay rates of the two fractions were not statistically significant (p = 0.92). After 72 h of aerobic exposure, the decay was then followed by a plateau in Phase-II, with NTB and PTB decay rates of -0.007 ± 0.005 h^{-1} (R^2 = 0.917) and -0.005 ± 0.012 h^{-1} (R^2 = 0.917), respectively. No statistically supported differences were observed between NTB and PTB decay rates, suggesting that most of the detected *bla*$_{NDM-1}$ is harbored within cells with

151 intact cell membranes. PTB decay rates (associated with cells with intact membranes)
152 were statistically undistinguishable from a decay of zero (p = 0.26). Instead, bla_{NDM-1}
153 abundance stabilized at 10^5 copies/g biomass.
154
155 **Decay of bla_{NDM-1} correlates with the *E. coli* PI7 chromosomal decay.** Due to the
156 plasmidic origin of bla_{NDM-1}, the decay kinetics of this ARG might be explained by i)
157 plasmid loss or ii) cellular decay of the bacterial host. To determine which of these two
158 factors mainly explained the bla_{NDM-1} decay observed in the microcosms, we compared
159 the PTB decay rates of bla_{NDM-1} (informative of plasmid decay rates) with the PTB decay
160 rates of a chromosome-encoded gene (*uidA*). In the anaerobic microcosms, both target
161 genes decayed at the same rate (p = 0.32) (Figure 1c). Similarly, under aerobic
162 conditions, Phase-I and plateau bla_{NDM-1} and *uidA* decay rates were statistically
163 indistinguishable among all tested trace antibiotic concentrations ($0.19 > p > 0.50$)
164 (Figure 1d). *uidA* was initially spiked in the microcosm experiments at a frequency of 10^9
165 copies / g biomass, and stabilized at 10^5 copies/ g biomass during the plateau phase.
166 These results suggest the presence of a recalcitrant subpopulation of *E. coli*, occurring at
167 a frequency of 10^{-4} (plateau uidA copies / initial uidA spiking). The occurring frequency
168 of recalcitrant *uidA* copies coincides with the frequency of persister cells of *E. coli* PI7 in
169 pure culture (Figure 2).
170
171 **Trace antibiotic concentrations do not influence the persistence of *E. coli* PI7 or its**
172 **associated ARGs.** The results from the previous section suggest that bla_{NDM-1} decay is
173 mainly explained by the cellular decay of the *E. coli* PI7 host. As residual antibiotics are

174 commonly found in wastewater (26), there is a latent possibility that such trace antibiotic
175 concentrations might provide a selective advantage to *E. coli* PI7 in the sludge, and
176 consequently prolong the persistence of bla_{NDM-1}. To evaluate this possibility, the decay
177 of bla_{NDM-1} and *uidA* was assessed in the anaerobic microcosms at 0 and 100 µg/L of
178 meropenem, and at 0, 1, 10 and 100 µg/L of meropenem in the aerobic microcosms. None
179 of the selected meropenem concentrations influenced bla_{NDM-1} or *uidA* decay kinetics in
180 either anaerobic or aerobic conditions (Figure S1 and S2). NTB and PTB decay rates
181 (Table 1) at the different antibiotic concentrations were also not statistically different
182 from their respective controls ($p > 0.15$) (i.e., reactors without antibiotic).
183
184 **Activated sludge as reservoir for ARGs upon *E. coli* PI7 decay.** Due to the plasmidic
185 origin of bla_{NDM-1}, the sludge biomass might act as sink for this gene through plasmid
186 conjugation. Accordingly, HGT was assessed by culture-based methods. After 72 h of
187 operation, transconjugants were recovered from the aerobic microcosm at three sampling
188 points (Figure 1d), and at an average frequency of 10^3-10^4 CFUs/g of biomass (SI-1).
189 Further Sanger-based sequencing revealed that the transconjugants are bla_{NDM-1}-positive
190 isolates of *Enterobacteriaceae* belonging to *Shigella* and *Citrobacter* genera. No HGT
191 events were detected by culture-dependent methods in the anaerobic microcosms.
192
193 **Differential decay of colloidal DNA between anaerobic and aerobic liquid fractions.**
194 Upon *E. coli* PI7 cellular decay, bla_{NDM-1} was detected in the supernatant fraction of the
195 microcosm experiments by end-point PCR (data not shown). To evaluate the potential
196 persistence of extracellular DNA in liquid fractions of anaerobic and aerobic sludge

fractions, a separate experiment was carried out. Naked plasmidic DNA was spiked in a dialysis cassette and its decay was tracked by qPCR and electroporation assays in *E. coli* TOP10. As sequence-dependent DNA flexibility determines the cleave rate mediated by DNase I (27), the decay rates of the cloning vector do not necessarily represent the decay rates of bla_{NDM-1} plasmid. Specifically, given the larger size of the bla_{NDM-1} plasmid (ca. 110 kbp), it may be more inclined to degrade faster than the clone vector of smaller size. Although the substitution of the clone vector may overestimate the persistence of the actual plasmid decay, this experiment is informative about the persistence potential of DNA/ARGs in anaerobic and aerobic liquid fractions. No decay was detected by qPCR or electroporation assays in bla_{NDM-1} spiked into the anaerobic liquid fraction (p = 0.5) (Figure 3a and 3b). On the contrary, in the aerobic liquid fraction, bla_{NDM-1} decay was detected by both qPCR and electroporation assays at a rate of -0.036 ± 0.005 h^{-1} ($t_{1/2}$ = 19.4 ± 2.8 h) and -0.278 ± 0.03 h^{-1} ($t_{1/2}$ = 2.5 ± 0.25 h), respectively. Decay rates estimated by electroporation assays in the aerobic liquid fraction were significantly higher than those quantified by qPCR ($p << 0.05$).

DISCUSSION

The removal of ARB, particularly those that are resistant to new class of antibiotics, from wastewater is required to protect public health during reuse events (4). Both aerobic- and anaerobic-based wastewater treatment systems are utilized for the treatment of the municipal wastewater. However, a key parameter to consider when implementing these systems would be the decay rates of ARB and ARGs in the associated sludge and in the treated wastewater effluents.

220 Using a *bla*$_{NDM-1}$-positive *E. coli* PI7 isolated from wastewater as a model bacterium,
221 it was observed that the decay of this bacterium was one order of magnitude faster in the
222 aerobic sludge compared to the anaerobic sludge microcosms. The longer persistence of
223 *E. coli* PI7 observed in the anaerobic condition coincides with earlier microcosm studies
224 comparing the differential anaerobic/aerobic survival of *E. coli* in diluted fermenter
225 sludge (28), manure, manure slurry (29, 30), drinking water (31) and activated sludge
226 (32). These studies provide evidence that oxygen is a key factor driving decay of *E. coli*
227 in the secondary habitat, and partly explain the longer persistence of *E. coli* PI7 in the
228 anaerobic microcosms. Endogenous reactive oxygen species (ROS) are formed as
229 byproducts during aerobic metabolisms (33). ROS are highly oxidative molecules that
230 can react with cellular components and lead to cytotoxic (33-35) and mutagenic defects
231 in the cells (36). Several detoxification mechanisms have evolved in aerobic and
232 facultative organisms in order to cope with the deleterious effects of ROS (33, 37). Even
233 though *E. coli* possess several of those detoxification mechanisms (33), it is possible that
234 the cells experiencing oxidative stress undergo a decrease in fitness, and hence decayed
235 faster in the aerobic microcosms.

236 However, the presence of oxygen is most likely not the only factor driving the decay
237 of *E. coli* PI7 in the microcosms since decay is also observed in the anaerobic
238 microcosms. In addition to ROS, the microbial community has been reported to play an
239 important role in the decay of *E. coli* within the environment (38). The longer persistence
240 of *E. coli* PI7 could also be partially explained by the fact that microbial communities in
241 the gut and the anaerobic sludge tend to be more similar to each other, as exemplified
242 from the predominance of *Firmicutes* and *Bacteroidetes* in both ecosystems (39), than

between the gut microbiota and aerobic sludge (40-42). Due to these similarities in the microbial communities *E. coli* PI7 might have acclimated better and decayed slower in the anaerobic sludge than aerobic sludge.

It was further observed that the decay kinetics of *E. coli* PI7 followed a biphasic decay behavior in the aerobic microcosms. This biphasic decay behavior has been documented extensively for *E. coli* and other fecal indicator bacteria (43, 44). After the initial spiking event, bla_{NDM-1} and *uidA* were detected in the aerobic reactors at an average frequency of 10^9 copies/ g biomass. After 72 h, no further decay was observed, and both gene copy numbers plateau at an approximate frequency of 10^5 copies/g biomass (Figure 1b). As only one copy of bla_{NDM-1} or *uidA* is present per *E. coli* PI7 cell, these results indicate that 1 in 10000 cells may be recalcitrant to decay (SI-2). Previous studies on pure cultures of *E. coli* suggest this pattern resulted from heterogeneity within the bacterial population (45). We further hypothesized that the bacterial subpopulation responsible for the plateau phase phenotype corresponds to persister cells, a phenotypical variant of vegetative cells that exhibit improved tolerance to antimicrobials and other stressful environmental conditions (13). Indeed, the same *E. coli* PI7 was observed to upregulate transcriptional responses of genes related to persister cell formation upon solar irradiation (46), suggesting that *E. coli* PI7 adopts this strategy to facilitate its persistence during stressful conditions. Persisters typically occur at a fixed frequency between 10^{-4} to 10^{-6} depending on the *E. coli* strain (14). Consequently, in all aerobic microcosms (n = 12), the *E. coli* PI7 populations consistently stabilized at a frequency of 10^5 cells/ g biomass after a 4-log cell density decline (Figure 1). A further evaluation of the frequency of persister cells formation in *E. coli* PI7 revealed that for every 10000 vegetative cells, 1

266 persister cell is formed (frequency of 10^{-4}, Figure 2), coinciding with the persisting cell
267 numbers obtained in the decay experiments. Further evidence supporting the presence of
268 persister cells in the aerobic sludge is the fact that even though *uidA* was consistently
269 detected in PTB fractions at a frequency of 10^5 copies/ g sludge until the end of the decay
270 experiments, *E. coli* PI7 was no longer recovered from the sludge by culture-based
271 methods after 96 h of microcosm establishment (data not shown). These data suggest that
272 *E. coli* PI7 possibly transitioned to a viable but non-culturable (VBNC) state that is
273 consistent with the persister cell hypothesis.

274 Persister cells had been studied in clinical settings as this dormant state allows
275 antibiotic-susceptible bacterial populations to survive antimicrobial treatments (14). To
276 the best of our knowledge, there is only one report of environmental incidence of
277 dormant but infective state of the fish pathogen *Pasteurella piscicida* in the environment
278 (47). However, the importance of these specialized cells on the survival and
279 dissemination of *E. coli* in the environment, particularly in the wastewater treatment
280 system, has been overlooked (48). The occurrence of persisters of pathogenic strains in
281 the sludge (such as *E. coli* PI7) has implications on the management and disposal of the
282 sludge originating from WWTPs and their respective treated effluents. As this
283 subpopulation of bacteria exhibits high tolerance to antimicrobials, this raises the
284 question on whether our current disinfection practices are effective at fully inactivating
285 persisters of pathogenic bacteria that remained in the treated wastewater. This can raise
286 potential concerns when treated wastewater is intended for reuse, as improper
287 inactivation might represent a direct risk to the public health.

An earlier study in reactors treating wastewater have suggested that as a general trend, ARGs are removed more efficiently in anaerobic conditions (9). However, in the study by Diehl and LaPara (9), the anaerobic reactors were operated at a longer solid retention time (SRT) compared to the aerobic reactors. This difference in the operational parameters of both systems makes it difficult to determine whether the improved ARG removal corresponded to factors related to the anaerobic/aerobic condition or to differences in the SRTs of both types of systems. Nonetheless, it is important to highlight that Diehl and LaPara (9) observed that some particular ARGs showed prolonged persistence under the anaerobic condition, suggesting that the differential ARG decay is dependent on each particular ARG. In agreement with this observation, Burch and collaborators (10) concluded that the removal rates of ARGs vary substantially depending on the specific ARG. In a more detailed study, Rysz and collaborators (49) indicated that the effect of oxygen availability in the maintenance of tetracycline resistance genes is also dependent on the host cell, or more specifically, in the particular plasmid-host pair. Anaerobic conditions lead to complete loss of a plasmid carrying the tetracycline resistance gene (RP1 plasmid) in *Pseudomonas aeuroginosa*, while *E. coli* retained its tetracycline resistance plasmid (pSC101) for over 500 generations. Both pSC101 and the bla_{NDM-1}-plasmid harbored by *E. coli* PI7 (pKOX_NDM-1) carry a toxin-antitoxin system that might improve their retention in the *E. coli* host. Moreover, both plasmids belong to the IncF family, which show high stability in *Enterobacteriaceae* (50-53). These two factors shared by pSC101 and pKOX_NDM-1 might have accounted for their retention under energy-deprived anaerobic conditions. As the particular interaction

of pKOX_NDM-1 and *E. coli* PI7 is highly stable, cellular decay (*E. coli* PI7 decay) is the main factor driving the decay kinetics of bla_{NDM-1} in our microcosm experiments.

In a complex microbial community where competition for resources takes place (43), we expected that the addition of meropenem would have resulted in increased fitness of *E. coli* PI7 in the anaerobic and aerobic sludge. On the contrary, such antibiotic concentrations did not provide an evident selective advantage to *E. coli* PI7 that resulted in prolonged persistence. It is important to highlight that bla_{NDM-1} is fully functional in *E. coli* PI7 (MIC of 64 µg/mL (25)), suggesting that the possible adaptive advantage imposed by the meropenem addition might be negligible compared to the other negative pressures experienced by *E. coli* in the sludge environment.

Although we did not detect any effects of trace antibiotic concentration on the survival of *E. coli* PI7 or bla_{NDM-1} persistence, other studies had suggested that exposure to sub-lethal antibiotic and disinfectant concentrations stimulate the plasmid conjugation rates in activated sludge (54) and in water (55). Indeed, transconjugants were recovered from the aerobic microcosms. As IncF plasmids are unique to *Enterobacteriaceae* (56), all transconjugants isolated fall within this taxonomical unit, which includes many genera associated with waterborne pathogens such as *Salmonella*, *Yersinia, Klebsiella*, *Shigella, Citrobacter* etc. (57, 58). In contrast, no transconjugants were recovered from the anaerobic microcosms, most likely because such HGT events took place with fastidious bacteria highly prevalent in anaerobic environments (59), and that these bacteria cannot be easily cultivated. Similarly, after 120 h of aerobic microcosm establishment, transconjugants were no longer recovered from the aerobic sludge (Figure 1d). Lower plasmid stability of the new host-plasmid interaction is a plausible explanation for the

loss of transconjugants recovery. However, IncF plasmids are highly stable in *Enterobacteriaceae* (50-53), and such stability is likely improved by the toxin-antitoxin system (25). Due to the phylogenetic proximity of *Shigella* and *Citrobacter* to *E. coli* (60), it is speculated that this plasmid might also be stable in these hosts. Hence, an alternative explanation for the lack of transconjugant recovery is the loss of culturability of such bacteria since the progression to a viable but non-culturable (VBNC) state is a survival strategy widely common in diverse groups of bacteria (61).

The contribution of HGT and the factors affecting the dissemination of plasmids in activated sludge have been well-documented in laboratory conditions. These factors include plasmid–host-range (62), sludge retention times (63), stressful environmental conditions (54) and host/donor phylogenetic affiliations (64). Significant enrichment of the ratios of multiple ARGs compared to 16S rRNA genes throughout the wastewater treatment process confirms the potential for mobility and proliferation of ARGs within the activated sludge microbial communities in full-scale treatment systems (65). bla_{NDM-1} HGT events involving activated sludge microorganisms and soil bacteria had also been reported (8). In agreement with these studies, our results emphasizes the role of activated sludge as environmental reservoir of plasmid-encoded ARGs, as well as their potential role in mediating the dissemination of ARGs into the environment and to other *Enterobacteriaceae*.

Subsequently, upon cellular decay of *E. coli* PI7 and the previously identified transconjugants, bla_{NDM-1} can be released to the non-settleable or colloidal fraction of the sludge. The pDNA decay experiments showed that although the decay at the gene level is considerably slower (measured by qPCR), the fragmentation process of naked DNA in

356 the liquid aerobic fraction leads to a rapid decay in the replicative structure of the plasmid
357 (measured by electroporation assays). Circular plasmids replicate by rolling-circle, strand
358 displacement or theta replication. In these three replication mechanisms, the circular
359 structure of at least one DNA strand must be maintained in order to complete the
360 replication cycle (66). A single double-strand break would linearize the plasmid, resulting
361 in the disruption of the replication process by any of these mechanisms. Ultimately, the
362 lack of replicative functions would compromise the ability of the plasmid to be
363 disseminated and maintained in the transformed bacterial host.

364 DNA degradation in the environment is a complex multifactorial process
365 involving chemical and biological aspects (67). DNase-mediated degradation is the main
366 biological process driving the decay of extracellular DNA in the environment (68, 69),
367 while low temperatures, high salinity, high levels of organic matter and anoxic
368 environments are some of the physicochemical factors that contribute to the preservation
369 of environmental DNA (67, 70). Determining the factors influencing the persistence of
370 DNA in sludge liquid fraction is out of the scope of this study. However, it was identified
371 that DNA persisted for a longer time in the liquid fraction of the anaerobic sludge
372 compared to that of the aerobic sludge, ultimately increasing the probability of
373 subsequent bla_{NDM-1} uptake and fixation by environmental bacteria. In a previous study, it
374 was found that membranes of 100 kDa and smaller could achieve significant removal of
375 ARGs, attaining up to 4.5-log removal of colloidal DNA (71). Hence, coupling
376 membrane separation with the activated sludge process, can serve to mitigate microbial
377 risk associated with the presence of persisters and extracellular DNA in the treated
378 effluent.

379 In summary, the results from this study highlights the higher potential of
380 dissemination of *E. coli* PI7 and ARGs associated to prolonged host and extracellular
381 DNA persistence in anaerobic sludge. In the aerobic sludge, this study demonstrates
382 transconjugation of plasmids encoding ARG to compatible bacteria within the sludge,
383 highlighting the likelihood of potential horizontal gene transfer events. Furthermore, this
384 study emphasizes the potential importance of persister cells in the survival and
385 dissemination of enteric pathogens into then natural environment and suggest a certain
386 extent of indirect and direct risk on the public health imposed by the presence of persister
387 cells of pathogenic strains remaining in the sludge and effluents of WWTP.
388
389 **MATERIALS AND METHODS**
390 **Microcosm preparation.** Two sets of microcosm experiments, representing anaerobic
391 and aerobic biological reactors, were established in 1 L sterile Pyrex bottles. Anaerobic
392 and aerobic microcosms were seeded with 800 mL (mix liquor suspended solids, MLSS
393 of 4-5 g/L) of sludge from a lab-scale anaerobic bioreactor (39) and an aerobic full-scale
394 WWTP, respectively. The sludge in the anaerobic bioreactor is comprised of camel feces
395 and anaerobic sludge from an industrial WWTP in Riyadh, and the anaerobic bioreactor
396 had been in operation for more than 3 years (39, 72, 73). The full-scale WWTP was
397 located at King Abdullah University of Science and Technology in Thuwal, Saudi
398 Arabia, and had a capacity of 1600 m^3/day. Hydraulic retention time (HRT) and sludge
399 retention time (SRT) in the activated sludge tank were 2.5 h and 40 d, respectively. The
400 sludge tanks were operated at an average temperature of 33 °C, 1-2 mg/L of dissolved
401 oxygen and pH 7-8. Sludge for each replicate run (n = 3) was recovered at three different

time points scattered along a one-year period for both anaerobic and aerobic seed sludge. Prior to the *E. coli* PI7 spiking event, the seed sludge was acclimated for 10 d and screened for the presence of bla_{NDM-1} or *uidA*. No bla_{NDM-1} or *uidA* copies were detected in anaerobic and aerobic sludge by both end-point PCR and cultivation methods before *E. coli* PI7 spiking.

Decay experiments. The *E. coli* PI7 inoculum to be spiked into the microcosms was grown in LB broth at 37 °C for 8 h to an OD_{600} of 0.7. Once this cell density was reached, 100 mL of culture were spiked into each microcosm, resulting in a final volume of 900 mL per microcosm. The addition of this large quantity of *E. coli* PI7 into each individual microcosm, albeit not representative of actual conditions in WWTP, was similar to approach undertaken by earlier studies (74, 75). The high cell density spiked into the microcosm was required to ensure final cell density upon decay remains within the limits of detection by qPCR, and to also allow detection of subpopulations (e.g. persister cells) that generally occur in low cell densities. Decay experiments were performed at different trace antibiotic concentrations. In the anaerobic microcosms, decay was evaluated at 0 and 100 µg/L of meropenem (n = 2 per replicate run) (Sigma-Aldrich, St Louis, MO, USA). In the aerobic microcosm, concentrations of 0, 1, 10 and 100 µg/L of meropenem were tested (n = 4 per replicate run). Anaerobic microcosms were not tested with 1 and 10 µg/L meropenem because of the low sludge production from anaerobic bioreactor, and this restricted the number of microcosms that can be set up. Concentrations were chosen to represent the concentration range of organic micropollutants commonly reported in municipal wastewater (76, 77). Three independent replicate runs for each aerobic and

425 anaerobic set-up were performed, comprising a total of 6 and 12 anaerobic and aerobic
426 microcosm respectively. Microcosms were operated as sequencing batch reactors at a
427 constant temperature of 37 °C. The aerobic microcosms were aerated with atmospheric
428 air at 250 mL/min, while the anaerobic microcosms were established in air-tight bottles
429 and mechanically stirred at 250 rpm. To prevent oxygen intrusion, anaerobic microcosms
430 were established and sampled in a vinyl anaerobic chamber (COY Lab Products, MI,
431 USA). After each feeding/sampling event, anaerobic microcosms were purged with
432 99.9% nitrogen for 15 min. MLSS and pH were measured every 24 h for all microcosms,
433 and pH was maintained at 7.2 ± 0.4 using HEPES buffer at a final concentration of 25
434 mM. Daily, 100 mL of liquid was removed from the aerobic and anaerobic microcosms,
435 and replaced with synthetic wastewater (39) that had the corresponding concentration of
436 antibiotic, achieving a food-to-microorganism ratio (F/M) of 0.2. Biomass was separated
437 from the non-settleable liquid fraction by centrifugation at 9800 g for 20-30 min in a
438 sterile ultracentrifuge bottle, and re-introduced into the respective microcosm.
439
440 **Sample processing.** At each sampling event, 14 mL of sludge was collected from each
441 microcosm and used for i) total biomass DNA isolation (2 mL), ii) sludge exposure to
442 propidium monoazide, PMA (0.5 mL) and iii) detection of horizontal gene transfer
443 (HGT) events by culture-based methods (0.5 mL). Sludge exposure to PMA was
444 performed as previously described (78). Given that PMA can be limited in its accuracy to
445 differentiate between cells with compromised and intact cell membranes, more details on
446 PMA exposure protocol and validation are given in SI-3 and Table S1.
447

448 Sludge samples for total DNA isolation and PMA-treated samples were centrifuged at
449 10000 g for 5 min and the supernatant fraction was discarded. The remaining biomass
450 pellet was immediately frozen at -80 °C, and subsequently lyophilized using Alpha 1-2
451 LDplus freeze dryer (Martin Christ GmbH, Germany). After completion of the drying
452 cycle, dry biomass weights were recorded and samples were ready for DNA extraction.
453
454 **Horizontal gene transfer (HGT) detection by culture-based methods.** To detect
455 potential conjugation events, the remaining 0.5 mL of the initial 14 mL sludge sample
456 were serially diluted and plated on MUG-EC (Sigma-Aldrich, St Louis MO) with 1.5%
457 w/v agar and 8 µg/L meropenem. MUG-EC allows the rapid screening of *E. coli* as MUG
458 cleavage by the glucuronidase enzyme leads to the formation of colonies that exhibit blue
459 florescence under UV (79). Non-fluorescent colonies were selected as potential
460 transconjugants, and subsequently confirmed by colony PCR using bla_{NDM-1} specific
461 primers as shown in Table 2). Strain identity was determined by 16S rRNA gene
462 sequencing using the 11F/1492R primer pair (80). Detection limit of culture-based
463 methods performed in this study was determined to be 10^4 CFU / g sludge (SI-4).
464
465 **Colloidal DNA decay.** *E. coli* PI7 carries a 110 kbp plasmid encoding for NDM-1 (25).
466 Plasmid integrity was determined by electroporation into Invitrogen TOP10
467 electrocompetent cells (Thermo Fisher Scientific, Carlsbad, CA, USA). Due to the low
468 electroporation frequencies of this large plasmid, decay experiments were carried out
469 using the pCR®2.1 cloning vector (Thermo Fisher Scientific, Carlsbad, CA, USA) that
470 harbored a 640 bp bla_{NDM-1} gene insertion (i.e., total plasmid vector size of ~ 4.6 kbp).

471 Decay experiments of the plasmid were performed in the liquid fraction of either aerobic
472 or anaerobic sludge collected from a local WWTP and a lab-scale AnMBR, respectively.
473 The sludge liquid fraction was separated from the biomass by centrifugation at 10000 g
474 for 20 min. Supernatant was recovered and filtered with cheesecloth to further remove
475 biomass in suspension. Subsequently, 1.6 mL of plasmidic DNA (10^{10} copies/µL) were
476 dosed into a Float-A-Lyzer® G2 Dialysis device with MWCO of 100 kDa (Spectrum
477 Laboratories, Rancho Dominguez, CA), and the device was submerged in the liquid
478 fraction of either aerobic or anaerobic sludge for a period of 24 h. Anaerobic decay
479 experiments were performed under anaerobic conditions in a vinyl anaerobic chamber
480 with an atmosphere of 95% nitrogen and 5% Hydrogen (COY Lab Products, MI, USA).
481 50 µL samples were taken from the dialysis device at each sampling point, and used for i)
482 plasmidic DNA quantification with qPCR and ii) plasmid integrity quantification by
483 electroporation in TOP10 cells (SI-5).
484
485 **DNA extraction.** Lyophilized PMA-treated sludge and non PMA-treated sludge
486 fractions, were subjected to DNA extraction using the PowerSoil® DNA extraction kit
487 (MO BIO) with slight modifications to the manufacturer's protocol, as previously
488 described (4).
489
490 **Gene quantification and statistical tests.** *uidA* and bla_{NDM-1} genes are used as the
491 chromosomal and plasmidic marker, respectively. β-D-glucuronidase (*uidA*) was selected
492 as the chromosomal marker since only one copy of this gene is present per *E. coli*
493 genome (19). Differences in the decay of the chromosomal and plasmidic material of *E.*

494 *coli* PI7 was therefore assessed by comparing PMA-treated (PTB) decay rates of *uidA*
495 and bla_{NDM-1} genes, respectively. bla_{NDM-1} and *uidA* copy numbers were determined by
496 absolute quantification on a Applied Biosystems 7900HT Fast Real-Time PCR system
497 (Thermo Fisher Scientific, Carlsbad, CA, USA). PCR primers and TaqMan probe
498 sequences are listed in Table 2. MLSS measurements were fairly stable in the microcosm
499 experiments during the whole length of the experiment (Figure S3), suggesting stability
500 of both aerobic and anaerobic microbial communities in the microcosms. Gene copy
501 numbers were normalized by dry weight of biomass. Decay rates expressed as Ln (N/N_o),
502 where N corresponds to the copy numbers at $t = X_i$, and N_o corresponds to the copy
503 numbers at $t = 0$. Half-life was calculated using a first order decay kinetic model. Linear
504 regressions were performed using the least squares method and the significance of the
505 slopes of the regression models ($\beta \neq 0$) were evaluated using t-test. Decay curves were
506 compared using the model $Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i1} X_{i2}$ (81). All statistical analysis
507 was done using StatPlus at 95% confidence unless otherwise stated.
508
509 **Determination of persister cell frequency in pure cultures of *E. coli* PI7.** Persister
510 cells are dormant variations of vegetative cells that can withstand harsh environmental
511 conditions including exposure to supra-lethal concentrations of antibiotics. This
512 resistance to antibiotics is not encoded in the chromosome but it is rather a consequence
513 of their dormant phenotype. The frequency of persister cells in this study was determined
514 using a modified protocol described by Keren *et al*. (15). Modifications were made on the
515 type of antibiotic used and the working concentration. As *E. coli* PI7 exhibits extremely
516 high tolerance to ampicillin, the antibiotic challenge was performed with meropenem, a

carbapenem exhibiting a similar mode of action (7). *E. coli* PI7 was challenged with a supra-lethal meropenem concentration of 640 µg/mL that corresponds to a 10-fold increase of the minimum inhibitory concentration (MIC), and a 5-fold increase of the lethal meropenem concentration reported for this strain (25). In summary, overnight cultures of *E. coli* PI7 were diluted 1:1000 in LB broth without meropenem, and incubated at 37 °C and 200 rpm to a final OD_{600} of 0.2. To provide a baseline cell count (t = 0), 10 mL of culture from each flask (n = 4) were pooled and placed at 4 °C. Subsequently, cell cultures were challenged with meropenem (640 µg/mL), and incubated at 37 °C and 200 rpm for 180 minutes. Cell counts were determined by serial dilution and plating in MUG-EC agar plates. Persister cell frequency was expressed as the ratio of the cell count at t = 180 min to the cell count at t = 0.

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805
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TABLES

808

809 **Table 1.** *bla*$_{NDM-1}$ decay rates in PMA-treated biomass fractions of anaerobic and aerobic
810 sludge microcosms under different trace meropenem concentrations. P-values correspond
811 to the comparison of each decay curve derived from meropenem-spiked microcosms (i.e.,
812 1, 10 and 100 µg/L meropenem), with the decay model obtained in the respective control
813 microcosms (0 µg/L). No significant differences were observed in their *bla*$_{NDM-1}$ decay at
814 the different meropenem concentrations tested.

Microcosm	Replicate run	Meropenem concentration (µg/L)[1]	k (h^{-1})[2]	$t_{1/2}$ (h)	*p-value*[3]
Anaerobic sludge	1	Control	-0.0202	34.3	-
		100	-0.0223	31.1	0.33
	2	Control	-0.0219	31.7	-
		100	-0.0213	32.5	0.15
	3	Control	-0.0223	31.1	-
		100	-0.0205	33.8	0.98
Aerobic sludge	1	Control	-0.1361	5.1	-
		1	-0.1460	4.7	0.77
		10	-0.1436	4.8	0.82
		100	-0.1386	5.0	0.94
	2	Control	-0.1107	6.3	-
		1	-0.1095	6.3	0.70
		10	-0.0993	7.0	0.71
		100	-0.0923	7.5	0.46
	3	Control	-0.1119	6.2	-
		1	-0.1253	5.5	0.49
		10	-0.1095	6.3	0.85
		100	-0.1133	6.1	0.96

815 1. Control corresponds to 0 µg/L of meropenem

816 2. k values for the aerobic microcosms correspond to Phase-I decay

817 3. *p-value* corresponds to the testing of H_o: $k_{Control} = k_{Xi\ \mu g/L}$.

818 **Table 2.** List of primers used in this study. *Primer pair NDM-154 and probe NDM-22 qPCR amplification efficiency = 98%.
819 **Primer pair uidA159 and probe uidA-23 qPCR amplification efficiency = 102%.

Primer	*Gene Target*	*Amplicon size(bp)*	*Sequence*	*Cycling conditions*	*Use*
*NDM154-F**	*blaNDM-1*	154	ATTAGCCGCTGCATTGAT	50°C x 2 min; 95°C x 20 s; 40 cycles of 95°C x 1s and 60°C x 20s *	qPCR
*NDM154-R**			CATGTCGAGATAGGAAGTG		
*uidA159-F***	*uidA*	159	CGAATCCTTTGCCACGCAAG	50°C x 2 min; 95°C x 20 s; 40 cycles of 95°C x 1s and 60°C x 20s *	qPCR
*uidA159-R***			TCACAGCCAAAAGCCAGACA		
NDM640-F	*blaNDM-1*	640	TAGTGCTCAGTGTCG	95°C x 3 min; 35 cycles of 95°C x 30s, 60°C x 30 s and 72°C x 1 min; final elongation 72°C x 5min	Sequencing (Transconjugant screening)
NDM640-R			CATTAGCCGCTGCA		
11F	16S	1481	GTTYGATYCTGGCTCAG	95°C x 5 min; 35 cycles of 95°C x 1 min, 45°C x 45 s and 72°C x 2 min; final elongation 72°C x 10 min	Sequencing (Transconjugant identity)
1492R			GGYTACCTTGTTACGACTT		

Probe	*Gene Target*	*Sequence*	*Cycling conditions*	*Use*
*NDM-22**	*blaNDM*	56-FAM/AGACATTCG/ZEN/GTGCGAGCTGGCGGA/3	As described for primer pair	qPCR
*uidA-23***	*uidA*	56-FAM/TCGCCCTTC/ZEN/ACTGCCACTGACCG/31A	As described for primer pair	qPCR

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826 **FIGURE LEGENDS**
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828 **Figure 1.** *bla*$_{NDM-1}$ decay kinetics in non-treated (black circles) and PMA-treated (white
829 circles) biomass fractions under **(a)** anaerobic and **(b)** aerobic conditions, at 0 µg/L of
830 meropenem (n = 3). Plasmid stability under **(c)** anaerobic and **(d)** aerobic conditions is
831 evaluated by comparing the *bla*$_{NDM-1}$ (white circles) and *uidA* (white triangles) decay
832 rates in PMA-treated biomass fractions (n = 3). Dotted lines in frame 1C indicate
833 sampling points in which transconjugants were recovered by plating techniques.
834

835 **Figure 2.** *E. coli* PI7 cell count before (t = 0 min) and after meropenem challenge (t =
836 180 min) at 640 µg/mL (n = 4). The persister cell frequency was expressed as the ratio of
837 the cell count at t = 180 min and the cell count at t = 0 min, which corresponded to a
838 frequency of $3.14 \times 10^{-4} \pm 1.5 \times 10^{-4}$ (n = 4).
839

840 **Figure 3.** Decay of extracellular colloidal DNA in anaerobic (white diamonds) and
841 aerobic (black diamonds) sludge liquid fraction measured by **(a)** qPCR and **(b)**
842 electroporation assays.
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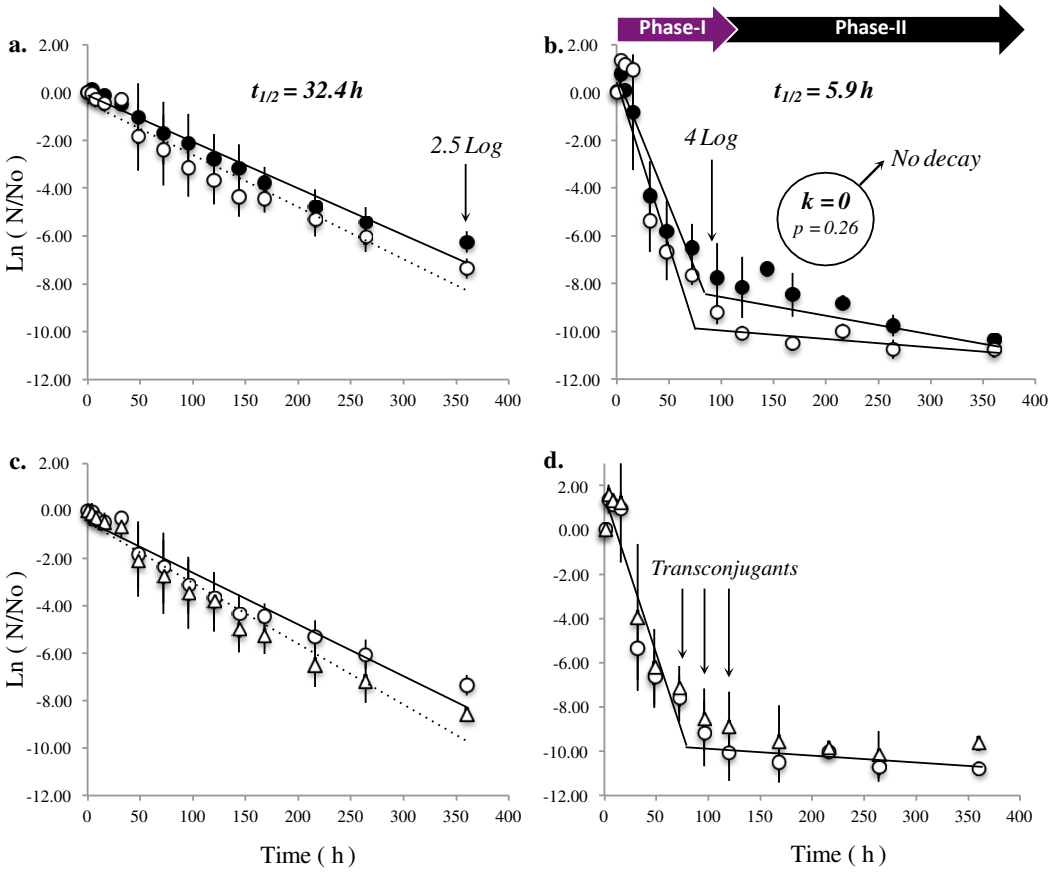

Figure 1

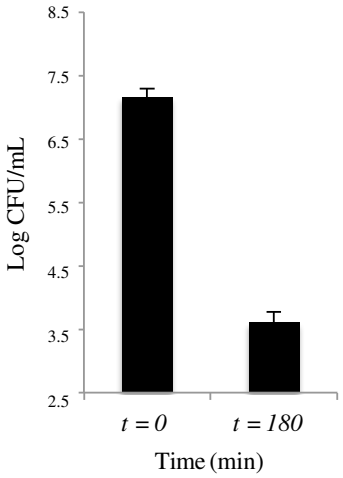

Figure 2

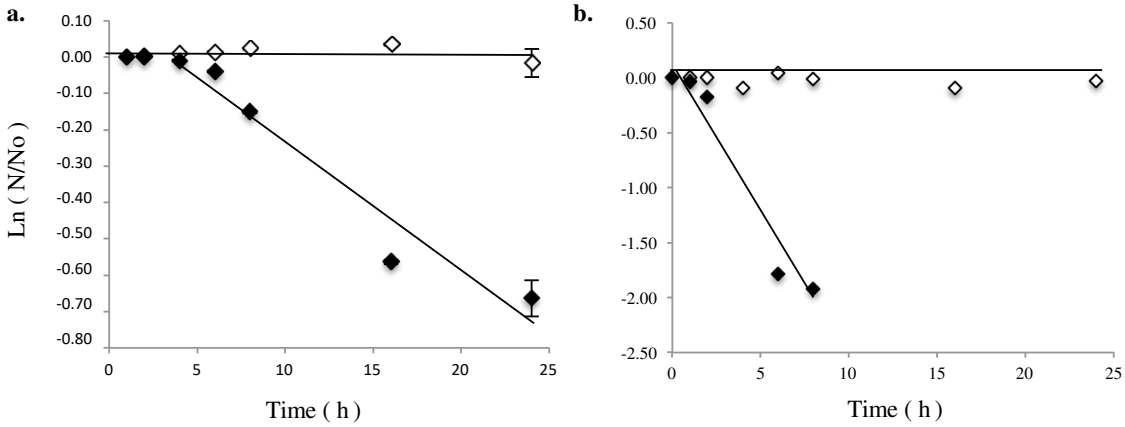

Figure 3