1   **The dilution effect limits plasmid horizontal transmission in multispecies bacterial communities**

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27   **Abstract**

28   By transferring ecologically important traits between species, plasmids drive genomic divergence and

29   evolutionary innovation in their bacterial hosts. Bacterial communities are often diverse and contain

30   multiple coexisting plasmids, but the dynamics of plasmids in multispecies communities are poorly

31   understood. Here, we show, using experimental multispecies communities containing two plasmids,

32   that bacterial diversity limits the horizontal transmission of plasmids due to 'the dilution effect'; an

33   epidemiological phenomenon whereby living alongside less proficient host species reduces the

34   expected infection risk for a focal host species. In addition, plasmid horizontal transmission was also

35   affected by plasmid diversity, such that the rate of plasmid conjugation was reduced from coinfected

36   host cells carrying both plasmids. In diverse microbial communities, plasmid spread may be limited by

37   the dilution effect and plasmid-plasmid interactions reducing the rate of horizontal transmission.

38

39   **Introduction**

40   Mobile genetic elements (MGE) are an important source of potentially beneficial accessory traits for

41   host bacteria, equipping these bacterial cells with new ready-to-use functions and thereby allowing them

42   to expand their ecological niche [1-3]. Plasmids are common in bacterial communities, infecting diverse

43   bacterial taxa [4], and often multiple plasmids co-exist in natural microbial communities [5-6]. The

44   long-term persistence of plasmids in bacterial communities will depend both on the proficiency of host

45   species to stably maintain plasmids in their populations by vertical transmission [7], and the rate of

46   horizontal transmission of plasmids within and between species by conjugation [8].

47   Previous studies have shown that plasmids are not equally maintained across different host

48   species [9-10], while plasmid transmission dynamics are affected by bacterial community structure [8].

49   Thus, in communities where plasmids rely on horizontal transmission for their maintenance [8,11],

50   plasmid dynamics could be affected by the diversity of the community, especially if the different host

51   species differ in their proficiency and transmission rates. Studies focused on parasite transmission in

52   host communities have shown that the transmission of multi-host parasites can be limited by species

53   richness, which is termed the 'dilution effect' [12-13]: A focal host species has a reduced risk of parasite

54 infection when in a diverse community than would be expected from its intraspecific transmission rate,

55 if transmission from other species in the community is less efficient [14]. We hypothesise that the

56 dilution effect may also apply to plasmids in communities where hosts differ in their ability to maintain

57 and transmit plasmids.

58   To gain a better understanding of plasmid dynamics in complex multi-plasmid / multi-host

59 communities we constructed simple bacterial communities in effectively sterile potting soil (soil

60 microcosms) under controlled laboratory conditions and tracked plasmid dynamics over-time.

61 Specifically, communities contained two distinct conjugative plasmids, pQBR57 and pQBR103, that

62 are known to vary in their rate of conjugation within populations of the focal species, *P. fluorescens*

63 SBW25 [15]. *P. fluorescens* SBW25 populations were embedded within a community of five

64 *Pseudomonas* species, and these were compared to controls where *P. fluorescens* SBW25 was

65 propagated in monoculture. We report that presence of the *Pseudomonas* community reduced the rate

66 of plasmid co-infection in *P. fluorescens* SBW25 in line with there being a dilution effect limiting the

67 rate of horizontal transmission in more diverse communities.

68

69 **Materials and methods**

70 **Bacterial strains and plasmids**

71 *P. fluorescens* SBW25 [16] was the plasmid-donor in this study, carrying either the plasmid pQBR57

72 or pQBR103. *P. fluorescens* SBW25 was labelled by directed insertion of gentamicin resistance (Gm^R)

73 as previously described [17]. The plasmids used in this study, pQBR103 and pQBR57 are large

74 conjugative plasmids (425 kb and 307 kb respectively) that confer mercury resistance via a *mer* operon

75 encoded on a Tn5042 transposon [5, 15, 18]. Both plasmids were independently conjugated into

76 gentamicin resistant (Gm^R) *P. fluorescens* SBW25 from streptomycin resistant (Sm^R) plasmid-bearing

77 *P. fluorescens* SBW25. pQBR57 was also conjugated from *P. fluorescens* SBW25 Sm^R into *P.*

78 *fluorescens* SBW25(pQBR103) Gm^R in order to obtain *P. fluorescens* SBW25 (pQBR103:pQBR57).

79 Each plasmid-donor was mixed in 1:1 ratio with the plasmid-recipient strain, incubated for 48 h and

80 spread on King's B (KB) agar plates containing 10 µg ml^{-1} gentamicin and 20 µM of mercury(II) chloride

81 to select for transconjugant colonies [19]. As previously described, the conjugation assays were

82 conducted in 6 ml KB growth medium in 30 ml universal vials ('microcosms') at 28°C in shaking

83 conditions (180 rpm). Background communities consisted of five different *Pseudomonas* species; *P.*

84 *stutzeri* JM300 (DSM 10701) [20], *P. putida* KT2440 [21], *P. protegens* Pf-5 [22], *P. fluorescens* Pf0-

85 1 [23], *P. aeruginosa* PAO1 [24].

86

87 **Selection experiment**

88 Twelve colonies of the plasmid-bearing *P. fluorescens* SBW25(pQBR103) and *P. fluorescens*

89 SBW25(pQBR57) were grown overnight in KB microcosms at 28°C with shaking 180 rpm. Six

90 colonies of each of the plasmid-free *Pseudomonas* species (*P. stutzeri* JM300 (DSM 10701), *P. putida*

91 KT2440, *P. protegens* Pf-5, *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1) were also grown overnight in

92 KB microcosms using the same culture conditions. Six replicate populations containing equal

93 proportions of *P. fluorescens* SBW25(pQBR103) and *P. fluorescens* SBW25(pQBR57) were

94 propagated either with or without the background community of five *Pseudomonas* species. Populations

95 were grown in potting soil microcosms supplemented with mercury (16 µg g^{-1} Hg(II)). Each community

96 had a starting ratio of 1:1 between *P. fluorescens* SBW25(pQBR103) and *P. fluorescens*

97 SBW25(pQBR57) (~each 1x10^6 cfu g^{-1}) such that the starting frequencies of pQBR103 and pQBR57

98 were approximately 50%. The background community of *Pseudomonas* species contained each species

99 in equal proportion (~each 4x10^5 cfu g^{-1}). To prepare the soil inoculum, the mix of each community

100 (final volume: 100 µl) was centrifuged for 1 min at 10,000 rpm and resuspended in 1 ml M9 salt solution

101 [25]. Next, the soil microcosms (10 g twice-autoclaved John Innes No. 2 compost soil) were inoculated

102 with 100 µl of the mix, briefly vortexed to disperse the inoculum in the soil, and incubated at 28°C at

103 80% humidity [8]. Every 4 days, 10 ml of M9 buffer and 20 glass beads were added to each soil

104 microcosm and mixed by vortexing for 1 min, and 100 µl of soil wash was transferred to a fresh soil

105 microcosm as previously described by Hall *et al.* [8]. The communities were propagated for 6 transfers

106 (24 days, estimated to be approx. 42 bacterial generations).

107   At each transfer, total population counts were estimated by plating onto non-selective KB agar

108   plates. Bacterial counts for the plasmid-bearing *P. fluorescens* SBW25 strains were estimated by plating

109   onto selective media: 10 μg ml^{-1} gentamicin KB agar plates. Each of these plates were then replica

110   plated onto mercury KB agar plates (100 μM mercury(II) chloride) in order to assess the frequency of

111   mercury resistance within *P. fluorescens* SBW25 and at the whole-community level. Twenty-four

112   colonies of *P. fluorescens* SBW25 were sampled every 2 transfers from the mercury containing plates

113   and tested for the presence of the plasmids and mercury transposon by PCR screening. Twenty-four

114   colonies of the total community were randomly sampled from the mercury containing plates at two

115   time-points (transfers 4 and 6) and also tested for the presence of the plasmids and mercury transposon.

116   The PCR screening was designed to use three set of primers that targeted the *mer* operon-Tn5042

117   transposon [forward primer: 5'-TGCAAGACACCCCCTATTGGAC-3', reverse primer: 5'-

118   TTCGGCGACCAGCTTGATGAAC-3'], the pQBR103-plasmid specific origin of replication *oriV*

119   [forward primer: 5'-TGCCTAATCGTGTGTAATGTC-3', reverse primer: 5'-

120   ACTCTGGCCTGCAAGTTTC-3'] and the pQBR57-plasmid specific *uvrD* gene [forward primer: 5'-

121   CTTCGAAGCACACCTGATG-3', reverse primer: 5'-TGAAGGTATTGGCTGAAAGG-3'] [26].

122

123   **Competitive fitness assay**

124   Four individual colonies of the ancestral *P. fluorescens* SBW25(pQBR103:pQBR57) were competed

125   against the plasmid-free *P. fluorescens* SBW25 with and without the five-species community. The

126   fitness assay was performed with and without mercury in soil microcosms. Relative fitness was

127   measured by mixing differentially the plasmid-bearer (Gm^{R}) and plasmid-free (Sm^{R}) in 1:1 ratio. The

128   five-species community was added in the same ratio as at the beginning of the selection experiment.

129   The inoculum was diluted 100-fold in M9 salts before being added into soil microcosms and incubated

130   at 28°C and 80% humidity for 4 days. Samples were plated on KB agar plates supplemented with

131   selective concentration of 10 μg ml^{-1} gentamicin and 50 μg ml^{-1} streptomycin at the beginning and end

132   of the competition to estimate the density of plasmid-bearing and plasmid-free bacteria. The relative

133   fitness was calculated as the selection rate (r) [27].

134

135 **Conjugation assay**

136 Four individual colonies of each ancestral *P. fluorescens* SBW25 (pQBR103:pQBR57), *P. fluorescens*

137 SBW25 (pQBR103) and *P. fluorescens* SBW25 (pQBR57) were conjugated into the isogenic plasmid-

138 free strain. Conjugation rate of the different plasmids was measure by mixing differentially the plasmid-

139 bearer (Gm^R or Sm^R) and plasmid-free (Sm^R or Gm^R respectively) in 1:1 ratio. The mix was centrifuged

140 for 1 min at 10,000 rpm to remove spent media, resuspended in M9 salt solution, diluted 100-fold in

141 high (KB), medium (0.1x KB) and low (0.01x KB) resource media and incubated at 28°C for 48 h. KB

142 agar plates were supplemented with 10 μg ml^{-1} gentamicin or 50 μg ml^{-1} streptomycin to estimate the

143 density of plasmid-donor and plasmid-recipient bacteria at the beginning and end of the assay. KB agar

144 plates were supplemented with 10 μg ml^{-1} gentamicin and 20 μM mercury(II) chloride or 50 μg ml^{-1}

145 streptomycin and 20 μM mercury(II) chloride to estimate the density of the transconjugant bacteria at

146 the end of the assay. The conjugation rate was calculated with the method firstly described by Simonsen

147 *et al.* [19].

148

149 **Statistical analyses**

150 Statistical analyses were performed using RStudio version 3.2.3 [28]. The prevalence of each plasmid

151 status (pQBR103 only, pQBR57 only, or both) in *P. fluorescens* SBW25 was estimated as the area

152 under the curve using the function *auc* of the package 'flux' [29]. One-way ANOVA tests compared

153 plasmid prevalence in *P. fluorescens* SBW25 with versus without the community. Kruskal-Wallis test

154 was used to analyse the end-point frequency of each plasmid at a whole-community level since the data

155 was not normally distributed. Welch's t-test was used to analyse the effect of the background

156 community on the relative fitness of *P. fluorescens* SBW25 carrying both plasmids. Kruskal-Wallis test

157 was used to assess the differences between the conjugation rates of pQBR57 and pQBR57: pQBR103

158 in the different resource media as the data were not normally distributed; the conjugation rate of

159 pQBR103 plasmid was not in detectable range in medium and low resource media thus pQBR103 was

160 not included in this statistical analysis. Welch's t-test was used to compare the conjugation rate of

161   pQBR57 to pQBR57:pQBR103 and pQBR103 plasmid in high resource media where the conjugation

162   rate of each plasmid was in detectable range.

163

164   **Results**

165   **Plasmid co-infection limited in community**

166   While mercury resistance remained at fixation in all replicates, we observed contrasting plasmid

167   dynamics in the *P. fluorescens* SBW25 population with versus without the background *Pseudomonas*

168   community. In the presence of the background community, in the majority of replicates the *P.*

169   *fluorescens* SBW25 population was dominated by pQBR103, such that bacteria were typically either

170   singly-infected by pQBR103 or co-infected with both pQBR103 and pQBR57. By contrast, in the

171   absence of the background community we observed higher rates of co-infection with both pQBR103

172   and pQBR57, or, in a single replicate, the fixation of pQBR57. Overall, we observed that the frequency

173   of plasmid co-infection was higher in the absence of the background community (ANOVA $F_{1,10}$=5.569,

174   p=0.039; Figure 1). To test if this effect could be caused by higher fitness costs of plasmid co-infection

175   in the presence versus absence of the community, perhaps due to more intense resource competition,

176   we competed *P. fluorescens* SBW25(pQBR103:pQBR57) against plasmid-free *P. fluorescens* SBW25

177   with or without the background community. We found, however, that the presence of the background

178   community had no effect on the relative fitness of *P. fluorescens* SBW25(pQBR103:pQBR57) (Welch's

179   t-test, $t_{13.68}$=0.698, p=0.496; Figure 2).

180   pQBR57 is known to have a far higher conjugation rate than pQBR103 in potting soil [15],

181   therefore it is likely that co-infection would have often resulted from pQBR57 conjugating into cells

182   that already carried pQBR103. This process of infectious transmission through the *P. fluorescens*

183   SBW25 population could have been less efficient in the presence of the background community if,

184   rather than conjugating into *P. fluorescens* SBW25(pQBR103), pQBR57 conjugated into the other

185   *Pseudomonas* species. This is conceptually similar to the dilution effect in epidemiology whereby

186   biodiversity reduces infection risk in a focal species [13]. Consistent with this idea, we observed high

187   levels of mercury resistance in the total community, of which *P. fluorescens* SBW25 made up only

188   ~18% of the total mercury resistant fraction at the end of the experiment, confirming plasmid

189   transmission of the *mer* operon into the other taxa (Figure 3). Within the mercury resistant fraction of

190   the total community, we were able to detect the more highly conjugative plasmid pQBR57, but not

191   pQBR103, at an appreciable frequency (X^2(2, N=18)=12.176, p=0.002; Figure 4). Together these

192   suggest that, indeed, the transmission of pQBR57 into *P. fluorescens* SBW25(pQBR103) cells was

193   impeded by dilution by the community leading to reduced co-infection of *P. fluorescens* SBW25.

194   Finally, we tested whether the rate of conjugation to plasmid-free recipient cells varied

195   depending on whether the donor was singly-infected or co-infected, and whether conjugation rates were

196   affected by resource level to mimic the effects of increased resource competition in more diverse

197   communities. Conjugation rates from all backgrounds — *P. fluorescens* SBW25(pQBR103), *P.

198   fluorescens* SBW25(pQBR57), and *P. fluorescens* SBW25 (pQBR103:pQBR57) — were reduced in

199   diluted media (effect of resource media, X^2(2, N=22)=16.85, p<0.001; Figure 5; conjugation of

200   pQBR103 was not detectable in medium and low resource media). Consistent with previous studies,

201   conjugation rates from pQBR57-containing backgrounds were far higher than those from *P. fluorescens*

202   SBW25(pQBR103) (Welch's t-test, $t_{5.581}$= -14.973, p<0.001), but co-infected donors had a reduced

203   conjugation rate compared to *P. fluorescens* SBW25(pQBR57) donors (Welch's t-test, $t_{5.773}$= -5.751,

204   p=0.001; Figure 5). These results suggest that co-infection itself may have reduced the rate at which

205   pQBR57 spread in the *P. fluorescens* SBW25 population, and that greater resource competition in the

206   presence of the background community may have reduced the rate of infectious spread of both plasmids.

207

208   **Discussion**

209   Using simple soil bacterial communities, we show that plasmid co-infection in a focal host species was

210   reduced in the presence of a community of other bacterial species. This was not caused by differential

211   fitness effects of plasmid-carriage in monocultures versus communities, but rather appears to have been

212   determined by the effect of bacterial species richness on the epidemiology of horizontal transmission

213   of plasmids in the focal host population. Whereas, in monocultures, the highly conjugative plasmid

214   pQBR57 spread into the *P. fluorescens* SBW25(pQBR103) sub-population, in communities this spread

215   was impeded. Detection of pQBR57 at appreciable frequencies in the total community suggests that

216   this effect was due to a substantial fraction of conjugation events leading to the infection of non-SBW25

217   cells by pQBR57. Because the conjugation rate of pQBR57 may also be lower from other *Pseudomonas*

218   species (e.g., this is known to be the case for *P. putida* [8]), this interspecific conjugation is likely to

219   have had the effect of reducing the overall conjugation rate to *P. fluorescens* SBW25(pQBR103) cells

220   and thus lowering the probability of plasmid co-infection.

221   Similar to plasmids, the transmission of parasites has often been found to be lower in species-

222   rich communities where a focal species is diluted in the diverse community and therefore has a reduced

223   risk of infection [14, 30-32]. The dilution effect is supported by experimental studies and

224   epidemiological models which suggest that introducing communities of alternative hosts could help to

225   control the transmission of vector-borne diseases caused by parasites (zooprophylaxis) [33-36]. The

226   identity of the introduced host species has important implications in preventing the parasites'

227   transmission, as different host species are likely to vary in their susceptibility to hosting the parasite

228   [37]. Highly susceptible host species could amplify the disease reservoir of a parasite instead of

229   suppressing it, therefore in order to prevent the dissemination of a parasite, the enrichment of these host

230   species should be restricted in the community [37]. Similar dynamics could apply to plasmids, where

231   host species are known to vary widely in their proficiency to host and transmit plasmids [8].

232   Parasite epidemiological models also suggest that the species richness of the parasite

233   community can affect the transmission of a focal parasite [38-39]. Both parasite diversity and co-

234   infection have been found to reduce the transmission rate of parasites in a community [38]. Similarly,

235   here we found that the conjugation rate from the donor *P. fluorescens* SBW25(pQBR103:pQBR57) was

236   lower compared with the *P. fluorescens* SBW25(pQBR57) donor. This suggests that plasmid co-

237   infection itself could limit the transmission rate of highly conjugative plasmids, like pQBR57. We

238   speculate that plasmid co-infection affected the plasmid transmission as a result of plasmid-plasmid

239   interactions in the host cell [40]. Co-existing plasmids could trigger a stronger cellular response in the

240   host cell, while the increase in genetic sequence and encoded genes is likely to amplify the physiological

241   and metabolic cost to the host cell, moreover co-infecting plasmids are likely to compete for limited

242   cellular resources (i.e host's replication factors; [41]). Indeed, we predict that intracellular competition

243   is likely to be more intense between related plasmids, since these will have the greatest overlap in their

244   resource requirements e.g. similar suites of tRNAs.

245   In nature, bacteria inhabit species-rich communities wherein they co-exist with multiple diverse

246   plasmids [42-43]. The experiments reported here highlight that plasmid dynamics can be affected by

247   both bacterial and plasmid diversity. Plasmids are currently of clinical concern as they often carry and

248   disseminate antimicrobial resistance genes (ARGs) [44]. ARGs are found in bacterial communities

249   colonizing diverse environments where microbial communities can act as resistance reservoirs [45-46].

250   Expansion of the resistance reservoirs via HGT between bacterial communities is currently an

251   increasing concern [47]. Understanding the transmission dynamics of ARG-encoding plasmids at the

252   community-level is therefore imperative in order to constrain the emergence of resistance in natural

253   microbial communities. This work suggests that plasmid dissemination along with the resistance genes

254   they encode in a focal taxon (e.g. a pathogen) could be limited in more species-rich communities, where

255   plasmid transmission is constrained by the dilution effect.

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268

269   **Conflicts of interest**

270   None declared.

271

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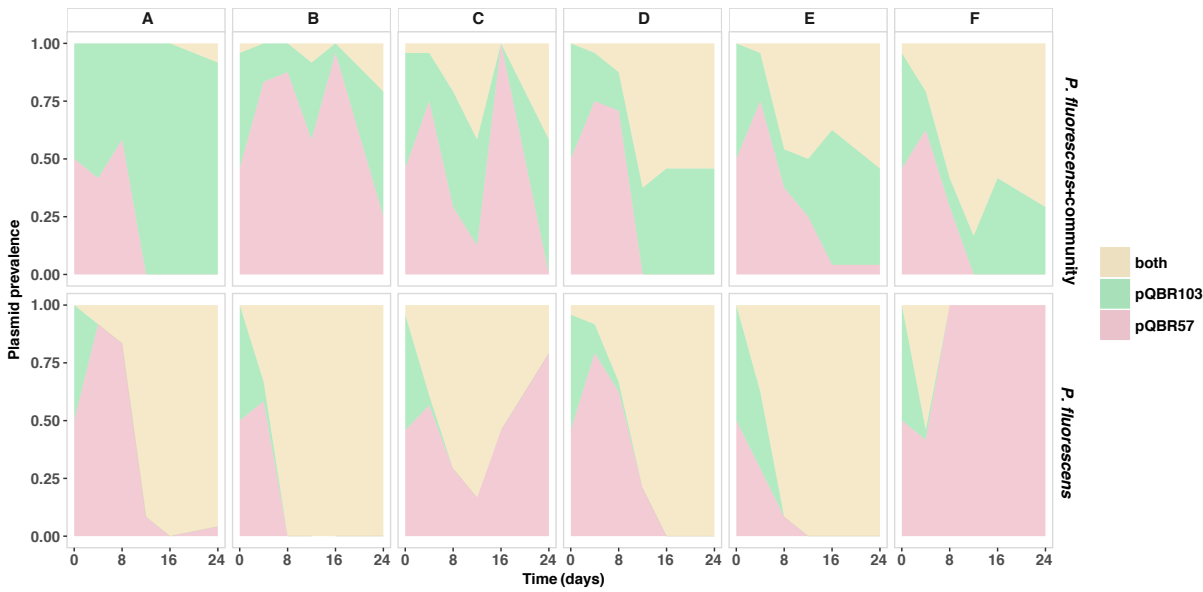
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402

403   **Figure legends**

404   **Figure 1.** Plasmid ratio and plasmid profile in *P. fluorescens* SBW25. *P. fluorescens* + community

405   panels show the plasmid prevalence in *P. fluorescens* when plasmid-bearing *P. fluorescens* species were

406   co-cultured with the five-species community; *P. fluorescens* panels show the plasmid prevalence in *P.*

407   *fluorescens* when *P. fluorescens* was cultured as single-species. Co-existence of both, pQBR57 and

408   pQBR103 plasmids (yellow); pQBR103 plasmid (green); pQBR57 plasmid (red).

409

410   **Figure 2.** Relative fitness of *P. fluorescens* (pQBR103:pQBR57) in absence and presence of the five-

411   species community. 0 µg g^{-1} Hg(II) (pink), 16 µg g^{-1} Hg(II) (blue). Circles represent the individual data

412   points of four clonal replicates. Error bars represent the SEM of four clonal replicates.

413

414   **Figure 3.** Densities of the total community and of the *P. fluorescens* SBW25 population over time.

415   Solid lines show mean density of the total community (blue) and of the *P. fluorescens* SBW25

416   population (pink). Dotted lines show mean density of mercury resistant cells in the total community

417   (blue) and the *P. fluorescens* SBW25 population (pink). Grey shaded areas show standard errors (n =
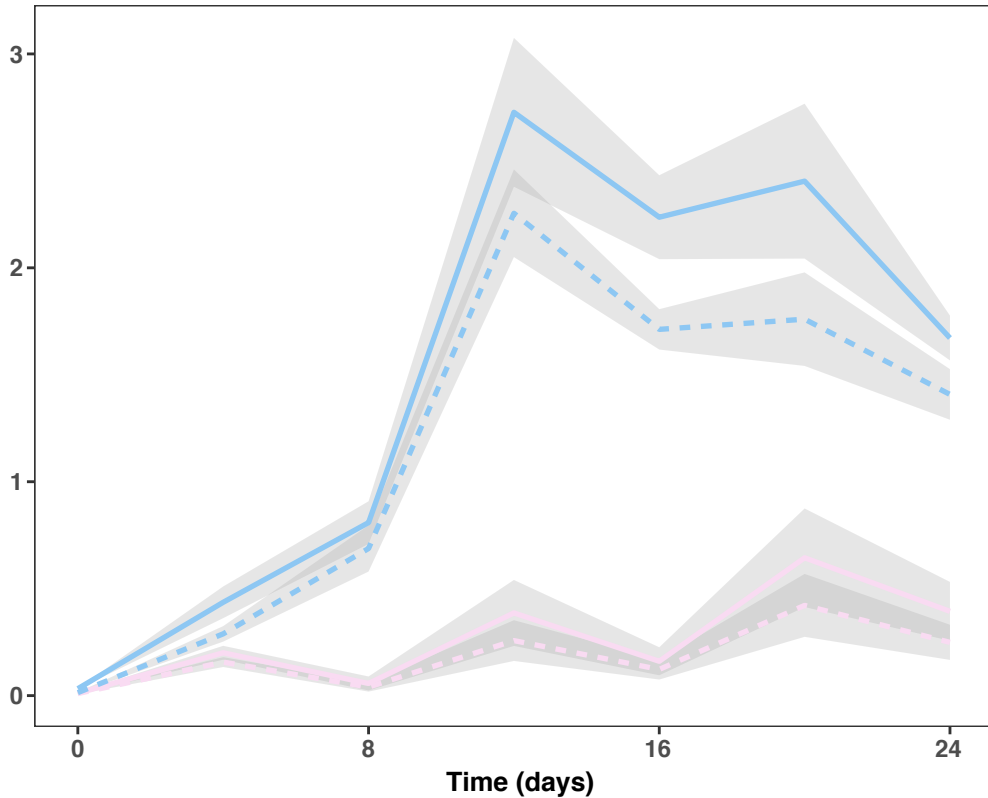
418   6).

419

420   **Figure 4.** a. Plasmid genotype frequencies in the total community at the end of the experiment. Each

421   box shows the upper and lower quartile, the interquartile range (IDR, length of box) and the median

422   (solid line across the box) of each plasmid genotype frequency in the replicate populations (A-F, n=6).

423   Circles show the outliers of the data. b. Counts of plasmid genotypes in each replicate population (A-

424   F) from twenty-four colonies sampled from the mercury resistant fraction of the total community at the

425   end of the experiment. Co-existence of both, pQBR57 and pQBR103 plasmids (yellow); pQBR103

426   plasmid (green); pQBR57 plasmid (red).

427

428   **Figure 5.** Conjugation rate of *P. fluorescens* (pQBR103:pQBR57), *P. fluorescens* (pQBR103) and *P.*

429   *fluorescens* (pQBR57) in high, medium and low resource media. Error bars represent the SEM of four

430   clonal replicates.