Evidence of elevated antibiotic resistance plasmid retention and gene transfer frequency in anaerobic wastewater ecosystems

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ABSTRACT

Wastewater is a common pathway for the spread of antibiotic resistance (AR) genes and bacteria into the environment. Biological treatment can mitigate this path, but horizontal gene transfer (HGT) between bacteria also occurs in such processes, although the influence of bioreactor habitat and ecology on HGT frequency is not well understood. Here, we quantified how oxidation-reduction (redox) conditions impact the fate of a Green fluorescent protein (Gfp)-tagged AR plasmid (pRP4-gfp) within an E. coli host (EcoFJ1) in the liquid and biofilms of multi-phase bioreactors. Replicate bioreactors treating domestic wastewater were operated under stable aerobic (+195 ± 25 mV), anoxic (-15 ± 50 mV), and anaerobic (-195 ± 15 mV) conditions, and flow cytometry and selective plating were used to quantify donor strain, EcoFJ1(pRP4-gfp), and putative transconjugants over time. Plasmid pRP4-gfp-bearing cells disappeared rapidly in aerobic ecosystems (~2.0 log reduction after 72 h), especially in the liquid phase. Conversely, EcoFJ1(pRP4-gfp) and putative transconjugants persisted longest in anaerobic biofilms (~1.0 log reduction, after 72 h). Apparent plasmid transfer frequencies also were higher under anaerobic conditions. Further, protozoan abundances were over 20 times higher in aerobic reactors relative to anaerobic reactors, and protozoa numbers significantly inversely correlated with pRP4-gfp signals across all reactors (p < 0.05). Overall, HGT frequency was consequentially impacted by bioreactor habitat conditions and trophic effects, especially relative oxygen level and evident predation. These observations provide guidance for new and alternate bioreactor designs aimed at minimizing AR HGT during biological wastewater treatment.
Introduction

Bacteria persist and thrive in widely varying habitats due to their genetic plasticity. One phenomenon that influences bacterial plasticity is the acquisition and loss of extraneous genes, such as those conferring antibiotic resistance (AR) via horizontal gene transfer (HGT) 2-4. In natural, clinical, and engineered systems, HGT can occur by conjugation and transduction, mediated by transmission vectors, such as plasmids and viruses, radiating AR genes across microbial communities 5-7. Broad host range plasmids, such as members of the IncP-1 incompatibility group, display particularly promiscuous inter-species mobility among biota, which is important to microbial adaptation and evolution in many environmental contexts 8-13.

One location where the HGT of AR genes is believed to be important is within biological wastewater treatment systems, which contain higher density and a myriad of microbes in activated sludge, biofilm, and other treatment processes 14-16. Numerous mobile genetic elements (MGEs), such as plasmids, transposons and integrons, also exist in such systems 17-21. For these reasons, biological treatment units have been suggested as locations where HGT occurs, including between pathogenic and commensal bacteria 22, 23. In fact, many studies have shown elevated abundances of AR genes and bacteria in bioreactors 24, 25, even in well-operated systems 26-29, but how these all relate to where and how frequently HGT actually occurs is less known. This includes the extent of transconjugant/transductant AR bacteria released to the environment in WWTP effluents.

To address this question, we developed a reporter assay to allow us to measure in situ HGT frequencies and putative transconjugant formation in different wastewater ecosystems. In parallel, we also characterised microbial food-web conditions with a view to understanding how protozoan activity might influence HGT. We speculate that habitat and ecological
factors are both important for AR HGT\textsuperscript{10,27}, but few studies have considered their influence in tandem.

Microbial communities and core resistomes vary across wastewater networks, which can be explained by differences in habitat and ecological conditions among compartments\textsuperscript{27,28}. For example, Eberl, et al.\textsuperscript{29} observed a significant decline of Gfp-labelled \textit{Pseudomonas putida} seeded into aerobic activated sludge, which was linked to protozoan predation. Mallory, et al.\textsuperscript{30} also showed that abundances of two antibiotic resistant strains, \textit{Salmonella typhimurium} and \textit{Klebsiella pneumonia}, declined dramatically in the presence of eukaryotic predators in sewage mixed liquor. However, no declines in abundance were observed in the presence of eukaryotic inhibitors. Such insights into habitat and food-webs have only been superficially extended to HGT even though they may be key to AR transmission in WWTPs.

An example of how habitat influences AR gene removal is seen in redox-sequenced wastewater treatment reactors\textsuperscript{31,32}. Sequential redox systems have been shown to reduce total bacteria, AR genes, and MGEs by passing wastewater through aerobic then oxygen-free compartments\textsuperscript{31}. It has been speculated this occurs because each sequential redox compartment selects against different sub-communities (e.g., obligate aerobes versus anaerobes), which in turn, removes a wider array of AR bacteria from the system. However, it is not known how each redox condition impacts HGT, which is key to understanding how different redox units might be used or combined to reduce AR releases from WWTPs.

Conflicting observations have been made about how different redox conditions influence on HGT, but methods have differed widely\textsuperscript{33,34}. Therefore, fair comparisons are hard to make. Here, the relative fate of a conjugative AR plasmid (pRP4) was quantified within different redox wastewater ecosystems. This plasmid was tagged with a Green-fluorescent-protein reporter (pRP4-\textit{gfp}) and transferred into a nalidixic acid-resistant environmental \textit{E. coli} strain.
(EcoFJ1). The recombinant donor, EcoFJ1(pRP4-gfp), then was seeded into identical aerobic, anoxic and anaerobic bioreactors, and pRP4-gfp, its bacterial host, and putative transconjugants were quantified in biofilms and the liquid phase, using a combination of standard microbiology methods and flow cytometry. Protozoan abundances also were quantified to assess possible trophic effects.

Materials and methods

Experimental design and bioreactors set up

Baseline work was performed to determine suitable experimental conditions for assessing HGT, including creating stable redox conditions, and reproducible seeding and sampling protocols (see Supporting Information, SI). Three sets of experiments were performed (i.e., Preliminary, Stage 1 and Stage 2), each designed to quantify EcoFJ1(pRP4-gfp) and putative transconjugants under different redox conditions and seeding regimes. The Preliminary experiment was a discrete experiment, whereas the Stage 1 and 2 experiments were performed in series.

Six bench-scale sequencing batch biofilm reactors (SBBRs) were used for all experiments, operated in duplicate under aerobic, anoxic and anaerobic conditions at 25 ± 2 °C (Figure S1). To create discrete liquid and biofilms phases within each reactor (ecological niches in WWTP ecosystems), polyurethane sponge cubes were “floated” in the reactors (Figure S2). Biofilms readily developed on the sponge cubes and individual cubes were harvested as part of the sampling protocol, as needed. All reactors were acclimatised for ~120 days prior to the experiments and, as a result, very stable redox and chemical conditions developed over time. The oxidation-reduction potentials (ORPs), measured daily, ranged from aerobic (+195 ± 25 mV; mean ± standard deviation) through anoxic (-15 ± 50 mV) to anaerobic (-195 ± 15 mV). A summary of the water chemistry data is provided in Table S1 (SI).
**E. coli donor strain construction**

A previously constructed gfp-tagged version of plasmid RP4\(^\text{35}\), here referred to as pRP4-gfp, was used in this study. In this construct the gfp gene is constitutively expressed, creating a visible reporter system for tracking the presence of pRP4 in host and recipient cells. The carriage of pRP4-gfp in donor and recipient cells can be distinguished using three resistance phenotype markers in the plasmid and molecular Polymerase Chain Reaction (PCR) analysis.

An indigenous wastewater-borne *E. coli* strain was isolated from bioreactor samples, using HiCrome coliform agar (Sigma Aldrich, UK), from an existing laboratory SBBR treating domestic wastewater. Prior to genetic modification, antibiotic susceptibility testing showed that the isolate was sensitive to 21 clinical antibiotics, including three resistance phenotype markers encoded in pRP4-gfp (i.e., ampicillin (Amp), kanamycin (Km) and tetracycline (Tc)). To facilitate subsequent work and allow this strain to be detected against “background” *E. coli* strains in the bioreactors and the wastewater source, a chromosomal nalidixic acid (Nal) resistant variant was isolated. To do this, the *E. coli* was plated onto Luria-Bertani (LB) agar plates containing 25 µg/mL nalidixic acid and it was incubated for 48 hours at 30°C. A spontaneous Nal-resistant mutant was isolated (named EcoFJ1) and used for subsequent experiments. Plasmid pRP4-gfp was transferred to EcoFJ1 via conjugative plate mating (see SI). The final recombinant donor organism, named EcoFJ1(pRP4-gfp), had a chromosomal resistance marker to nalidixic acid, plasmid encoded resistance to Amp, Km, and Tc, and constitutive Gfp fluorescence (see Figure S3).

**Bioreactor seeding procedures**

Prior to seeding experiments, samples from the bioreactors and wastewater source were screened for the presence of pRP4-like plasmids and Nal-resistant phenotypes using nutrient agar media containing selective antibiotics (see Enumeration section). In parallel, samples were screened for naturally Gfp-fluorescing strains using flow cytometry (FCM) and
fluorescent microscopy techniques. Results were negative in all samples, indicating the absence of background pRP4-like plasmids, donor-like phenotypes, Gfp-fluorescing cells, or pRP4-bearing Nal-resistant bacteria present in the bioreactors or the wastewater source.

Inoculation of EcoFJ1(pRP4-gfp) into the reactors was performed under pseudo-steady state operating conditions (after acclimation). EcoFJ1(pRP4-gfp) was added to achieve a final concentration of 1 x 10^6 cells/mL, an abundance predetermined to provide a donor to recipient ratio of 1:100 (i.e., 1% donor within the total cell population in the reactors). This ratio was chosen to reflect typical levels of *E. coli* in local wastewater. EcoFJ1(pRP4-gfp) was added to each reactor during the daily feeding routine, immediately after the mixing step.

Two seeding regimes were used: 1) a single seeding event used in the Preliminary and Stage 1 experiments, and 2) a semi-continuous seeding regime used in Stage 2. For Preliminary and Stage 1 experiments, the reactors were seeded once and then sampled over a 72-hour period, which testing had shown to be adequate for comparing differences between reactors. The Preliminary and Stage 1 experiments were initiated by seeding at time zero, whereas the Stage 2 experiment was extended directly from the Stage 1 experiment. In Stage 2, reactors were re-seeded with EcoFJ1(pRP4-gfp) during the routine SSBR hydraulic exchange cycle every 24-hour (targeting ~1 x 10^6 cells/mL) for three days. After three re-seeding cycles, the reactors were operated for four additional days without re-seeding and sampled a final time after 168 hours. Sample regimes for Stage 1 and 2 are shown in Table 1. Sampling in the Preliminary experiment was the same as Stage 1, except sampling was less frequent in the first 12 hours.
Table 1 Sampling time points designated in Stage 1 and Stage 2 experiments

<table>
<thead>
<tr>
<th>Redox</th>
<th>Aerobic</th>
<th>Anoxic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample matrix</td>
<td>Sponge biofilm</td>
<td>Sponge biofilm</td>
<td>Sponge biofilm</td>
</tr>
<tr>
<td>Liquid phase</td>
<td>0, 3, 6, 9, 12, 24, 48, 72</td>
<td>0, 3, 6, 9, 12, 24, 48, 72</td>
<td>0, 3, 6, 9, 12, 24, 48, 72</td>
</tr>
</tbody>
</table>

**Stage 1**
- Sampling time (h)
  - Aerobic: 0, 3, 6, 9, 12, 24, 48, 72
  - Anoxic: 0, 3, 6, 9, 12, 24, 48, 72
  - Anaerobic: 0, 3, 6, 9, 12, 24, 48, 72

**Stage 2**
- Sampling time (h)
  - S1_0, S1_24
  - S2_24, S2_48
  - S3_48, S3_72
  - S3_168

Notes:
- Stage 2 commenced after the completion of Stage 1 sampling;
- Re-seeding and sampling at time = 0 h;
- Sampling at time = 24 h;
- Re-seeding and sampling at time = 48 h;
- Re-seeding and sampling at time = 72 h;
- Sampling at time = 168 h.

**Sampling and sample processing**

Samples were collected aseptically in quadruplicate per sampling event from each reactor.

All samples were collected during the mixing mode, and included both sponges (i.e., biofilm) and liquid phase samples. After sampling, sponge cubes and liquid samples were divided into two aliquots: one for FCM and the other for microbial culturing. Liquid samples for FCM were immediately fixed using sterile formaldehyde (Sigma Aldrich, UK) to a final concentration of 1% w/v formaldehyde in sample, which preserved Gfp activity. Sponge cubes were collected using a sterile galvanised wire sampling coil. After removal, cells were recovered from the sponge cubes by aseptically squeezing and washing using sterile phosphate buffer saline (PBS). Cells were then fixed with 1% formaldehyde. All fixed samples were stored in dark at 4 °C for less than 14 days prior to FCM analysis.

**Flow cytometry quantification**

FCM was used to quantify total bacterial cells and to determine the proportion of cells exhibiting Gfp signal. Pre-treatment of cells for FCM analysis was adapted from previous
studies\textsuperscript{36,37} and further optimised for this study (see FCM workflow in SI; Figure S4).

Homogenised pre-treated cell suspensions were initially counterstained with the DNA stain, 4',6-diamidino-2-phenylindole (DAPI), to quantify the total cell number within each sample. DAPI was chosen as it has high affinity to dsDNA and has an emission spectrum that overlaps minimally with Gfp signal, allowing simultaneous quantification of both signals. All samples were analysed at low flowrates (25 µL/min) using technical triplicates. FCS Express 6 software was used to evaluate the FCM data.

**Enumeration of *E. coli* donor strain and putative transconjugants**

In parallel to FCM, the prevalence of pRP4-\textit{gfp} in cells was determined using plate culturing on selective media. All samples were serially diluted ($10^0$ to $10^5$) in sterile PBS, and then 10 µL of diluted samples were spotted on LB nutrient agar and with either: (i) the three antibiotics encoded by the resistance marker genes in pRP4-\textit{gfp} (100 µg/mL Amp, 12.5 µg/mL Km and 50 µg/mL Tc), or (ii) the same three antibiotics plus nalidixic acid (25 µg/mL) to counter-select for recipient cells that had acquired pRP4-\textit{gfp} from EcoFJ1(pRP4-\textit{gfp}). Agar plates were incubated at 30ºC for 24 hours before colony counting. Colony counting was performed to estimate the proportion of original EcoFJ1 donor cells carrying the pRP4-\textit{gfp} plasmid versus putative transconjugant cells that had received pRP4-\textit{gfp} by gene transfer. Transfer frequencies were estimated from the number of pRP4-\textit{gfp} encoding colonies (i.e., bacteria resistant to Amp, Km and Tc) and the number of EcoFJ1(pRP4-\textit{gfp}) colonies (i.e., *E. coli* resistant to Amp, Km, Tc and Nal). The transfer frequency is reported as the transconjugant:recipient cell ratio (T/R), according to equations from previous work (see SI)\textsuperscript{15}. A “transconjugant” here was defined as a cell that successfully and stably acquired the plasmid (based on sequential sub-culturing), whereas “recipient” is a cell that does not have the plasmid.
Identification and confirmation analysis of transconjugants

Colonies collected after 24 hours in Stage 1 were used to identify specific transconjugants. A total of 191 isolates were assessed (~60 to 65 per redox reactor) and 24 stable transconjugants were isolated from different samples following a decision tree (see Figure S5). Genomic DNA from these isolates was extracted using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, UK) according to the manufacturer’s protocols. To confirm the presence of pRP4-gfp in these isolates, a diagnostic PCR was performed for the gfpmut3b gene which is located on the plasmid, using forward primer Pgfpu (5'CACTGGAGTTGTCCCAATTCTTG-3') and reverse primer Pgfpd (5'CAGATTGTGGACAGGTAATGG-3')38. After confirming the presence of pRP4-gfp in the transconjugant pool, the isolate DNA was subjected to 16S rRNA gene PCR amplification and Sanger sequencing39. Cleaned sequences were searched against the National Centre for Biotechnology Information (NCBI) 16S rRNA gene database for archaea and bacteria to identify possible host species. Nucleotide sequences were aligned, and phylogenetic analysis was conducted with MEGA software using the neighbour-joining (NJ) method, to construct a distance-based tree to compare donor and transconjugant identities.

Quantifying protozoa

Biofilm and liquid phase samples from the Stage 1 reactors were sub-sampled to quantify protozoa levels over time using Neubauer haemocytometry (ThermoFisher Scientific, UK). The purpose was to estimate levels of possible predators over time under each redox condition to compare with EcoFJ1(pRP4-gfp) abundances. Samples were collected at 0, 12, 24, 48, and 72 hours, in triplicate. Protozoa were counted in four 9 mm² squares per sample and concentrations were calculated according to manufacturer’s guidelines. Images of selected samples were captured under fluorescent light to assess whether any protozoa displayed Gfp fluorescence, indicative of possible ingestion of bacteria containing pRP4-gfp.
Statistical and data analysis

All data were analysed using R statistical software 3.5.0\(^{40}\). One-way analysis of variance (ANOVA) sample tests followed by multiple pairwise-comparisons using post-hoc Tukey test were performed to compare differences in Gfp levels among contrasting redox conditions and time points. Kruskal-Wallis and Games-Howell post-hoc tests were used as non-parametric alternatives for the ANOVA and Tukey tests when data distributions were not normal. A two-way ANOVA test was performed to evaluate the effect of time and redox conditions on relative pRP4-gfp abundances. Unless otherwise noted, differences between data groups with p values < 0.05 were defined as significant.

Results and Discussion

Initial assessment of EcoFJ1(pRP4-gfp) gene transfer assay

Preliminary experiments were performed to validate the assay and estimate possible abundances of EcoFJ1(pRP4-gfp) and putative transconjugants over time under different redox conditions. In this experiment, FCM was used to distinguish pRP4-gfp hosts from the native and non-transconjugant populations because they display constitutive Gfp fluorescence. Based on FCM data, EcoFJ1(pRP4-gfp) comprised 1.1 ± 0.9% of reactor populations immediately after seeding. However, Gfp signals significantly increased in all reactors for seven hours after seeding, both in the liquid phase and biofilms, before dropping below initial levels after 24 and 48 hours, respectively (Figure 1). Proportional increases in the Gfp signal detected in the first seven hours were much greater in biofilm samples, suggesting some partitioning of cells into the biofilms, comparatively rapid acclimation, and possible new growth.

Although trends were similar among redox conditions, Gfp signals were higher and were retained longer in the anaerobic reactors, compared with the anoxic and aerobic units (up to
The highest relative Gfp signals were found in anaerobic reactors, especially in biofilms, with almost 28% of the bacterial signal in biofilms associated with the Gfp signal. In contrast, only ~3.0% of the Gfp signal was associated with the liquid phase. Further, elevated Gfp signal prevailed beyond 24 hours in the anaerobic and anoxic units, but not in the aerobic reactors.

**Figure 1.** FCM data for the Preliminary experiments showing percentage of pRP4-gfp encoding cells relative to total cell population. Biofilms and liquid phase samples were taken across the different reactors over 72 hours. Error bars indicate standard error (n=4).

**Abundance and fate of pRP4-gfp over time**

Based on data from the Preliminary experiment, follow-up experiments were performed in two Stages. Stage 1 was like Preliminary work, but had more frequent sampling and additional analyses, whereas Stage 2 used a different seeding regime. Stage 1 data confirmed that changes in pRP4-gfp (measured Gfp) signals over time were very different between the aerobic and the two oxygen-free reactors (Figure 2A). Absolute pRP4-gfp levels dropped dramatically within hours after seeding in the aerobic systems (one-log lower in liquid phase after 24 hours). In contrast, although pRP4-gfp levels initially dropped in biofilms in the anoxic and anaerobic systems, absolute levels were equal or higher than initial concentrations after 24 hours in both the liquid phase and biofilms.
Figure 2. Spatial and temporal pattern of the pRP4-gfp levels under different redox conditions within the biofilm and liquid phases with (A) single seeding (Stage 1) and (B) semi-continuous seeding (Stage 2). Error bars indicate standard error (n=4).

After 24 h, pRP4-gfp levels continued to drop through 72 hrs in the aerobic reactors in both the biofilm and liquid phase samples, ultimately achieving a two-log reduction in the liquid phase (final concentrations = 4.0 to 4.4 x 10^4 cells/mL), significantly lower than initial concentrations (p < 0.001). Whereas, pRP4-gfp levels did not change significantly from initial levels in the anoxic and anaerobic units, even after 72 hrs (p > 0.05). Clearly, pRP4-gfp, either in EcoFJ1 or transconjugant cells, was retained longer and at higher levels under lower redox conditions. The presence of oxygen consistently negatively correlated with the levels of pRP4-gfp, although the effects over time were greater in the liquid phase (two-way ANOVA; p-values ≤ 0.001) than in biofilms (two-way ANOVA; 0.0098 < p-values < 0.004).
As the experiment proceeded to Stage 2, daily re-seeding and sampling was performed every 24 hours for the next three days and a final sampling was performed four days later. During the course of re-seeding (Stage 2), the levels of pRP4-gfp in the biofilms did not significantly differ among the three redox conditions (Figure 2B) and remained similar until the final sampling at 168 hours (i.e., between S3_72 to S3_168). This suggests that pRP4-gfp, either in EcoFJ1 or in transconjugants, developed a temporary pseudo-steady-state within the biofilms. This may be due to the sheltering of bacteria once in biofilms, greater persistence of the seed strain (either by growth or death avoidance), and/or via conjugational transfer of pRP4-gfp to other bacteria.

As in Stage 1, the rate of decline of pRP4-gfp in Stage 2 was greatest in liquid phase in the aerobic reactors, followed by the anoxic and anaerobic systems. Although pRP4-gfp levels increased after each re-seeding (to ~ 10^6 cells/mL), abundances dropped soon thereafter in the aerobic liquid phase, a drop that was less evident in the anoxic reactors. In the anaerobic reactors, pRP4-gfp levels increased, possibly due to new growth and/or the progressive accumulation of seeded EcoFJ1(pRP4-gfp) under anaerobic conditions.

Final samples were collected at 168 hours. At this time, pRP4-gfp levels were similar across all biofilm samples (~ 10^5 cells/mL), whereas liquid phase levels decreased as a function of the redox conditions: i.e., aerobic < anoxic < anaerobic. pRP4-gfp numbers (either in EcoFJ1 or transconjugants) were almost two orders of magnitude greater in the anaerobic reactors (1.3 x 10^6 cells/mL) compared with the aerobic systems (4.5 x 10^4 cells/mL). Once again, oxygen conditions strongly influenced the fate of the tagged plasmid in the reactors, although it was not clear whether residual pRP4-gfp signals were original host cells (EcoFJ1) versus transconjugants. In summary, based on single pulse-seeding, pRP4-gfp counts (either in EcoFJ1 or transconjugants) decreased most rapidly when oxygen was present, whereas under
semi-continuous seeding, pRP4-gfp persisted longer in biofilms and was less dependent on the redox conditions.

**Impact of redox conditions on the spatial fate of pRP4-gfp and its hosts**

Statistical comparisons showed pRP4-gfp levels were significantly lower in aerobic versus anaerobic conditions in single-seeding experiments (Tukey’s comparisons for liquid phase and biofilm samples; \( p < 0.05 \)) (Table S2). This was also seen with semi-continuous seeding in Stage 2, but only in liquid phase samples (Kruskal-Wallis; \( p \)-values < 0.05). Whereas, no significant differences were seen in the liquid phase in Stage 2 between aerobic and anoxic samples. During semi-continuous seeding, the pRP4-gfp levels were not statistically differ in the biofilms regardless of redox conditions (Kruskal-Wallis; \( p > 0.05 \)).

The relative level of survival of the pRP4-gfp host cells (either EcoFJ1 or transconjugants) can be inferred from data in Figure 2. Overall, pRP4-gfp host cells survived for a shorter time under aerobic conditions, with numbers declining in the liquid phase in Stage 1 and in the latter part of Stage 2 when re-seeding is not done. In contrast, the host strain survived much longer in biofilms, especially when re-seeding was performed. The data show relatively constant levels of the host strain in biofilms, independent of reactor redox conditions. This could be because the host strains were more sheltered within biofilms. The fate of antibiotic resistance under aerobic versus anaerobic conditions has been the subject of several studies. Some studies have suggested that anaerobic conditions are favourable for reducing AR determinants, whereas other studies show reductions are greater under wholly aerobic and aerobic-anaerobic conditions in series. Our results with EcoFJ1(pRP4-gfp) are most comparable to the findings by Mantilla-Calderon and Hong. Both studies showed seeded donor organisms decreased more rapidly under aerobic condition relative to anaerobic conditions, although observed HGT frequencies were greater.
in their studies. This discrepancy probably relates to different experimental protocols between the two studies; e.g., much higher seed concentrations were used by Mantilla-Calderon and Hong\textsuperscript{37}. Other differences include different bioreactor set-ups, the addition of an antibiotic to their reactor, and using plasmids from different incompatibility groups (i.e., IncP vs IncF).

Both studies showed habitat (redox) conditions influenced HGT frequency with external variables (e.g., donor levels and selective agents) influencing relative HGT levels.

**Transfer frequencies of pRP4-gfp, including possible HGT**

Various factors might potentially influence the fate of the EcoFJ1(pRP4-gfp) in the reactors, including the relative persistence of EcoFJ1(pRP4-gfp) prior to plasmid transfer, the donor-to-recipient ratio, conjugational competence of pRP4-gfp to new hosts, and-or the survival of transconjugants after pRP4-gfp transfer. It is not possible to clearly separate these drivers based on our data, but it is possible to estimate net relative pRP4-gfp transfer frequencies and possible host survival, either of EcoFJ1(pRP4-gfp) or transconjugants.

Mean conjugal transfer frequencies are summarised in Figure 3 for samples collected at 24 h in the Stage 2 experiment (see Table S3 for details). Transfer frequencies ranged from 0.00001 to 0.01 transconjugants per recipient (T/R), based on selective culturing. Frequencies varied widely among redox conditions, and between liquid and biofilm samples. Lower transfer frequencies were consistently seen in aerobic and anoxic samples (T/R range = 0.000132 to 0.00147), whereas ten-fold higher frequencies were observed under anaerobic conditions (e.g., T/R = 0.0078 ± 0.0023 in the liquid phase). The range of transfer frequencies were in good agreement with relative pRP4-gfp levels observed across the FCM data for the same system. Transfer frequencies were higher in biofilms as redox conditions became more reducing (i.e., aerobic < anoxic < anaerobic), although the highest frequencies were seen in the anaerobic liquid phase. Transfer frequencies seen here are consistent with those observed previously using the widely-used filter mating assay\textsuperscript{10, 45}. 

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Transconjugant abundances were also quantified in the Stage 2 experiment. The highest relative numbers of transconjugants also were found under anaerobic conditions, especially in the liquid phase (T/R range = 0.007 to 0.0085). Aerobic and anoxic samples had much lower relative transconjugant levels (T/R range = 0.000026 to 0.00061), which has been seen in previous activated sludge and effluent samples \(^{15,46}\). Despite the observed differences, ANOVA analysis indicated transfer frequencies in biofilms did not significantly differ among redox conditions (ANOVA, \(0.12 < p < 0.56\)), although significantly higher numbers of transconjugants were detected in anaerobic liquid phase samples (ANOVA; \(p < 0.05\)). This could be due to a variety of factors, but FCM data suggest longer host cell survival may be very important, as suggested by different \(p\)RP4-\(gfp\) levels over time among redox environments.

Lower HGT frequencies in the biofilms might be explained by various factors, such as the extent of the extracellular polymeric matrix, which might provide a barrier that restricts access by external donors to potential recipients. Other possible explanations are shearing forces present at the biofilm-liquid interface, which may impede or interrupt mating pair formation \(^{12}\), and the impact of cell \(in situ\) phenomena of cell-to-cell contact and signalling \(^{47}\), nutrients and temperature \(^{48}\). Greater access to nutrients in the liquid phase may explain why higher transfer frequencies were observed in the liquid phase. However, these need to be proven.
Beyond quantifying transfer frequencies, DNA was extracted from 24 of the stable transconjugant isolates (after repeated subculture on antibiotic selective media) for 16S rRNA sequencing to determine their identity and phylogeny. All isolates were closely related strains to *E. coli* (Table S4 and Figure S6). Microbiological media (nutrient agar containing the selective antibiotics) was used to identify transconjugants, the data suggests a higher frequency of stable conjugation between related strains. It is noteworthy that 22 of the 24 stable transconjugants came from anaerobic reactors (16 from biofilm samples and six from the liquid phase) with only two coming the aerobic and anoxic units, both from biofilms. This further implies conjugation is more prevalent under anaerobic systems.

**Relationship among redox condition, protozoa count, and transfer frequencies**

Microscopic analysis of samples from the Preliminary experiment indicated that protozoa levels varied widely among the different redox reactors, suggesting differences in protozoan grazing might influence the persistence of pRP4-*gfp* in the systems (see Figure S7). To test this hypothesis, protozoa abundances were quantified in samples from the Stage 1 experiment. Significantly higher abundances of protozoa were present in both biofilm and the
liquid phase samples from the aerobic reactors compared with anoxic and anaerobic units (Figure 4A; Wilcoxon test; \( p < 0.05 \)). Further, protozoa numbers increased for 24 hours after seeding the aerobic reactors, suggesting protozoan growth after the addition of EcoFJ1(pRP4-gfp). Protozoa abundances declined by 72 h. This increase and decline were seen in both aerobic biofilm and liquid phase samples. In contrast, protozoa numbers were initially lower in the anoxic and anaerobic reactors, and no significant changes in protozoa levels were seen in these reactors.

Protozoa increases closely matched higher rates of pRP4-gfp host disappearance in the aerobic systems, suggesting protozoan predation may be important in reducing pRP4-gfp numbers (Figure 4B) and, presumptively, the time available for HGT in the aerobic reactors. In fact, protozoa abundances were significantly inversely correlated with pRP4-gfp host levels in the liquid phase (Pearson’s correlation = -0.71, \( p < 0.05 \)) and in biofilms (Pearson’s correlation = -0.64, \( p < 0.05 \)) across the samples (Figure 4B).

Although not quantitative, physical evidence of predation also is shown in Figure 5. Twelve randomly chosen aerobic samples were screened using fluorescent microscopy and gfp-fluorescing protozoa were evident in all images. This suggests possible ingestion of pRP4-gfp host strains and retention of Gfp signal in the “gut” of the predators. Evidently, after seeding the aerobic units with EcoFJ1(pRP4-gfp), protozoan activity increased as a response. This was not seen in the anoxic and anaerobic reactors because protozoa that live under oxygen-free conditions are not typically bacterial predators, suggesting that pRP4-gfp host strains (mainly EcoFJ1) survived longer because predation was low (Figure 2).
Figure 4. Protozoa quantification in liquid phase and biofilm samples showing (A) absolute abundance of protozoa and (B) Correlations between pRP4-gfp levels and protozoa count. Shaded areas show 95% confidence levels. Error bars represent standard errors (n=2).
Figure 5. Microscopy analysis showing (A & C) Phase contrast, and (B & D) epifluorescence images of food vacuoles expressing Gfp fluorescence suggesting pRP4 host cells potentially engulfed by predatory protozoa.

Practical implications to HGT in wastewater ecosystems

Previous work designed to estimated conjugative plasmid exchange used simple mass-action models from liquid broth data\textsuperscript{49}, including when studying horizontal gene flow within environmental samples (using mating assays on solid surfaces)\textsuperscript{8,50}. However, such assays neglected the wider influence of habitat and ecological factors, which probably influence plasmid transfer rates and frequencies in real world systems. Here we confirm that such factors may be key to observed plasmid transfer frequencies and hence HGT. Irrespective to spatial location, pRP4-gfp host strains survived longer under oxygen-free conditions, especially under anaerobic conditions. This is possibly explained by longer donor survival times due to reduced predation when oxygen is not present (Figures 4 and 5).
Protozoa abundances significantly increased after EcoFJ1(pRP4-gfp) seeding and protozoa level inversely correlated with pRP4-gfp levels across samples. Further, when re-seeding was not performed in the later part of Stage 2, pRP4-gfp host cells abundances only declined in the aerobic reactors (not anoxic or anaerobic), especially in the liquid phase. Finally, pRP4-gfp survived longer in biofilms and abundances were always higher during semi-continuous seeding. Extended survival in biofilms is consistent with sheltering from possible predators, whereas semi-continuous seeding provides replacement of new host strains every 24 hours. Predation does not appear to be the only pressure acting against pRP4-gfp survival under aerobic conditions. Apparent HGT transfer frequencies were also much lower under aerobic conditions. It is not clear whether this is a function of the presence of oxygen and how it might influence cell stress responses (and hypothetically transfer frequency) or simply due to reduced time available because of shorter host survival time for possible gene exchange. Our data suggest it is probably a combination both. pRP4-gfp hosts generally survived longer in the biofilms, even under aerobic conditions when protozoa levels increased. Biofilm bacteria produce extracellular polymeric substances and develop metabolic networks within their structure, limiting the exposure of the resident bacteria to external physical, chemical and biological stresses. However, transfer frequency was also much higher under anaerobic conditions, which combined with greater shielding, suggests why HGT and transconjugant formation was greater in the anaerobic reactors. pRP4-gfp host cells survive longer under these conditions, creating an increased opportunity for mating-pair formation. It is noteworthy that 22 of the 24 stable transconjugants came from anaerobic units and 16 were from biofilm samples. Although the data here are for a single host-plasmid combination, the work has important practical implications. Our model system shows HGT in wastewater ecosystems is strongly influenced by the microecological habitat in which the cells reside. Here, greater predation
provides one possible explanation for reduced plasmid transfer under aerobic conditions. This is plausible because ciliated protozoa have already been shown to be important for the removal of faecal coliforms from sewage in biological treatment processes\textsuperscript{42}. Protozoa are present in many natural habitats and proliferate in engineered ecosystems, such as wastewater treatment systems. Given most protozoans are phagocytic heterotrophs that oxidise their prey in order to obtain organic nutrients\textsuperscript{53}, they need oxygen to survive. Predation therefore provides a plausible explanation for why pRP4-\textit{gfp} declines most significantly under aerobic conditions, and less so under anaerobic conditions where protozoa are primarily parasitic symbionts rather than bacterial predators.

Work herein suggests controls on the rate of HGT in wastewater ecosystems are more complex than previously thought, being impacted by habitat, ecology, and genetics. However, to validate these finding in a broader context, other hosts and recipients need to be assessed under different wastewater habitat conditions. If ecological factors do influence host fate and prospective HGT, optimal wastewater treatment processes to reduce AR genes and bacteria need to be considered. In fact, this is already being done, employing aerobic and anoxic compartments in series to reduce AR genes from wastewater \textsuperscript{32,33} and improved solids separation \textsuperscript{28} to reduce AR genes from wastewater streams. Although we do not fully understand the factors of wastewater processes that affect the persistence and transmission of AR genes and plasmids, data here suggest the microecology of the system must also be considered in future studies addressing this key question.

**Acknowledgement**

The authors acknowledge funding support from the Engineering and Physical Science Research Council (EPSRC) and AstraZeneca Global Sustainability. Sincere thanks to Professor Barth F. Smets from Danish Technical University for providing the \textit{gfp}-labelled RP4 plasmid and Dr. Susanne Pohl for assisting with the microbial genetics.
References


Supporting information

Evidence of elevated antibiotic resistance plasmid retention and gene transfer frequency in anaerobic wastewater ecosystems

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Total number of pages: S1-S17; four tables; seven figures

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**Figure S4.** Step-by-step workflow for sample preparation prior to flow cytometry analysis using the Attune NxT flow cytometer (ThermoFisher Scientific).

**Figure S5.** Decision tree for the screening and isolation of presumptive transconjugants from sub-samples using selective media.

**Figure S6.** Phylogenetic tree based on neighbour-joining (NJ) method showing relationships between the *EcoFJ2* seed strain and transconjugants isolated from difference samples.

**Figure S7.** Microscopic analysis of samples abstracted from (A) liquid phase; and (B) sponge biofilm from the aerobic SBBR before any seeding. Samples specimens were viewed at 400x magnification and example presumptive predators are indicated by red arrows.
**Methods development and system operations**

**Bioreactors set up.** Sequencing batch biofilm bioreactors (SBBRs), designed to simulate three different redox environments, were set up in a 3 x 2 matrix to characterise host and plasmid fate in biofilms and the reactor liquid phase. Six bioreactors, comprising identical 1-L glass vessels were equipped with submersible magnetic stirrers at 200rpm (2mag AG, Germany) were operated in parallel at a constant temperature of 25 ± 2 ºC regulated in a water bath. The bioreactors were used to treat domestic wastewater in sequencing batch mode under aerobic, anoxic and anaerobic conditions (duplicate per redox condition; Figure S1).

![Figure S1. Bioreactor assembly. Schematic overview of the series of SBBR bioreactors containing sponge cubes for biofilm attachment. Gas bags were employed to equalise the pressure in the airtight system during feeding and decanting liquid exchange in anoxic and anaerobic SBBR.](image)

All reactors were amended with identical numbers of sponge cubes to facilitate biofilm formation on elements that could be easily removed for sampling (see Figure S2). Specifically, two hundred cubes of 1 cm x 1 cm x 1 cm polyurethane sponge, comprising total sponge volume equivalent to 0.2 L, were added into each of biofilm bioreactor, to act as supporting media for biofilm development and growth.
Figure S2. (A) Clean polyurethane sponge cut into 1cm x 1cm x 1cm cubes for use as immobilisers to support biofilm growth in bioreactors; (B) Sponge cubes containing biofilms taken from a SBBR during pseudo-steady state.

Specific reactor conditions were maintained as follows:

1. “Aerobic” conditions were maintained by pumping ambient air through air stone diffusers using a peristaltic pump (Watson Marlow, Cornwall UK). The pumping rate was adjusted at 120 mL/min to supply aeration that achieved a DO concentration of 4 ± 2 mg/L.

2. “Anoxic” conditions were ensured to be airtight by applying a layer of silicone grease sealant to prevent the intrusion of air and adding sodium nitrate stock solution to a final concentration at 25 mg/L NO₃-N, which has been shown previously to be adequate to sustain denitrification¹.

3. “Anaerobic” reactors were sealed similar to anoxic units, but no additional sodium nitrate was added and they were regularly sparged with N₂ gas.

The four oxygen free reactors were provided with gas collection bags to determine whether CH₄ gas was being produced. Achieving methanogenesis was not a goal of the reactors, but gas was still measured to fully characterise conditions in each system. Primary settled sewage for use as bioreactor feed was collected weekly from Tudhoe Mill sewage works, Northeast of England, throughout the experiment and stored at 4 ºC prior to use.
**Bioreactor start up and operation.** The daily feeding procedure was as follows: (i) stop mixing, ii) settle (one-hour), (iii) decant, (iv) refill, and (v) re-commence mixing. However, each redox condition was sustained differently. The aerobic SBBRs were created by pumping ambient air into the units through stone diffusers using a peristaltic pump, which achieved a DO concentration of 4 ± 2 mg/L. Anoxic conditions were created by the daily addition of sodium nitrate to a final concentration of 25 mg/L NO₃-N, which sustained stable denitrification. Oxygen “leakage” in the anoxic reactors was minimised by applying a layer of silicone grease around the reactors lids to prevent air intrusion. The anaerobic reactor units were maintained similar to the anoxic units, except sodium nitrate was not added and N₂ was sparged daily into the reactors immediately after feeding.

Reactors for preliminary, Stage 1 and Stage 2 experiments had working volumes of 0.9 L, with designed hydraulic (HRT) and sludge retention times (SRT) of 3 days and 10 days, respectively. They were stirred at 200 rpm for homogenous mixing during the treatment cycle. Mixed liquor suspended solids (MLSS) concentrations were maintained between 2750 ± 205 mg/L across systems, as recommended by Metcalf & Eddy for SBBRs (with 10-15 day biomass retention times). Daily feeding and decanting routines were performed manually using the following sequence: (i) stop mixing, ii) settle, (iii) decant, (iv) refill, and (v) re-commence mixing. The settling time was one hour to allow the biosolids to settle before decanting the settled liquid from each bioreactor by siphoning.

**Inoculum and start up.** Common start-up approaches were used across all experiments. Activated sludge (AS), anaerobic sludge, and primary settled wastewater were collected from two local waste treatment facilities in the Northeast of England, UK, for use as the inoculum. AS and primary settled wastewater were collected from the nitrifying aeration tank and the holding chamber downstream of a primary clarifier in Tudhoe Mill sewage works
Donor strains construction. The pRP4-gfp original donor strain, *P. putida* KT2442, and the nalidixic acid resistant *E. coli* recipient (*E. coli* NalR), were grown overnight in LB medium at 30°C and 37 °C, respectively, with shaking at 165 rpm. Overnight cultures were harvested, and the optical density of each bacterial suspension was determined and adjusted with sterile saline solution (1 x PBS) to approximately 5 x 10^7 CFU/ml (optical density at 600 nm [OD600] = 0.5). Conjugal mating was initiated by mixing equal volumes of the *P. putida* donor (D) and the *E. coli* NalR recipient (R) suspensions. A sample (30-µL) of the mixed suspension was transferred to a LB agar plate and incubated at 30°C for 48 h. Following incubation, the cell mass was harvested and resuspending in sterile PBS. Samples of the resulting bacterial suspensions were then streaked onto Hicrome chromogenic agar, supplemented with a combination of the three antibiotics to which the plasmid encodes
resistance. Presumptive *E. coli* transconjugants (blue colonies on the chromogenic medium) were isolated and further purified. The colonies were viewed under UV-light using a transilluminator and epifluorescence microscope (Figure S3) to confirm the presence of the *gfp* gene.

**Figure S3.** Enumeration and screening of *E. coli* donor derivative strain, EcoFJ1(pRP4-*gfp*), encoding Nal resistance and the pRP4-*gfp* plasmid. (A) UV illuminated colonies of the EcoFJ1(pRP4-*gfp*) strain showing Gfp fluorescent colonies (left) and non-fluorescent colonies of the original environmental *E. coli* strain, EcoFJ1 (right), as a negative control. Plates were irradiated at a wavelength of 366 nm from a benchtop UV-transilluminator. (B) Phase contrast and (C) epifluorescence micrographs of EcoFJ1(pRP4-*gfp*) showing green fluorescent colonies on a Nikon Eclipse Ti fluorescence microscope.
Samples and data processing

Flow cytometry quantification workflow. Pre-treatment methods for the preparation of cells for analysis by flow cytometry were adapted from past studies and further optimised for samples collected in this study. Pre-treatment is essential to ensure optimal passage of individual cells (rather than clumps) within the fluidic flow cell of the cytometer. A combination of mild sonication and treatment with surfactant were applied to optimise the disaggregation of the bacterial cells from the sludge and biofilm specimens, which tends to exist as biological flocs\(^3\). The complete routine for FCM sample preparation is shown in Figure S4, which involves sample dilution, surfactant dispersal, ultrasound sonication, and filtration.

Sample dilution. Samples that are too dense tend to mask the GFP signal while excess dilution leads to a failure to detect rare populations. The cells present in the formaldehyde-preserved samples were too concentrated for FCM analysis and were therefore diluted in 1:1000 using 0.2\(\mu\)m filtered DI water to avoid congestion of the fluidic flow cell during data acquisition.

Surfactant dispersion. Diluted samples were subjected to treatment with the surfactant Tween 80 (5\%) in a solution of sodium pyrophosphate (10 mM) to disperse and disaggregate biofilm agglomerates. Samples were mixed with magnetic spin vanes (for stirring in V-vials) on a magnetic stirring plate at 200 rpm for 15 minutes in the dark.

Sonication and filtration. After chemical treatment, samples underwent mild sonication in an ultrasound sonicating bath to further dislodge floc-bound bacterial cells. Sonication was performed for four minutes with one-minute intervals. Subsequent samples were filtered through a 20-\(\mu\)m sterile cell strainer (ThermoFisher Scientific, UK) to separate out the large particles that are often present in wastewater samples.
Sample counterstain. The pre-treated, uniformly suspended samples (formaldehyde-fixed) were firstly permeabilised to allow DAPI to gain access to the interior of the cells by treatment with Triton X-100 (Sigma Aldrich, UK) at 0.1% for 15 minutes at room temperature. DAPI (ThermoFisher Scientific) was added to the permeabilised bacterial suspension to a final concentration of 3 µM and incubated at room temperature for 30 min. DAPI counter-stained samples were immediately analysed by the Attune NxT flow cytometer equipped with acoustic assisted hydrodynamic focusing system (ThermoFisher Scientific). Fluorescence was detected using the blue and violet laser with excitation/emission wavelength of 488/530 nm and 405/610 nm for GFP and DAPI, respectively.
Figure S4. Step-by-step workflow for sample preparation prior to flow cytometry analysis using the Attune NxT flow cytometer (ThermoFisher Scientific).
Transfer frequency estimation. The number of indigenous potential recipients (Nr) was determined using FCM prior to the seeding experiment. The EcoFJ1(pRP4-gfp) donor seed strain (Nd) was enumerated using nutrient agar medium amended with the three pRP4-mediated antibiotic markers: ampicillin (Amp), kanamycin (Km) and tetracycline (Tc), plus the chromosomally-mediated nalidixic acid (Nal). The number of bacteria carrying pRP4-gfp (Np) was determined by culturing on nutrient agar plates containing Amp, Km, and Tc. The transfer frequency (f) at 24 h after inoculation was estimated as the difference between to total number of cells in the population encoding pRP4-gfp (i.e., resistant to Amp, Km, Tc and Nal) using the formula in Equation S1:

Equation S1. The equation used to determine the transfer frequency from original seed organism to potential recipient cells present in the bioreactors\(^4\).

\[
f = (Np - Nd)/Nr
\]

Where:

\(Np\) = The number of bacteria carrying plasmid pRP4-gfp;

\(Nd\) = The number of original donor seed organism; and

\(Nr\) = The number of indigenous potential recipients

Isolation of presumptive transconjugants. Samples were enumerated on nutrient agar (LB agar) supplemented with X-gluc (ThermoFisher Scientific) and the three antibiotics for the pRP4-based plasmid. Cleavage of X-gluc by the \(E.\ coli\) glucuronidase produce an intense blue precipitate of chloro-bromoindigo, and the resulting blue colonies distinguish colonies of \(E.\ coli\) from other species. Appropriate dilutions for plating were based on the spot plating results and 100 µL aliquots were plated evenly on prepared agar plates and incubated at 30 °C for 24 h (Figure S5).
Figure S5. Decision tree for the screening and isolation of presumptive transconjugants from sub-samples using selective media.

16S rRNA sequencing for identification of transconjugants. PCR assays using the Q5 High-Fidelity DNA polymerase (New England Biolabs, UK) were conducted in a 100-μl volume reaction system containing 1 μl diluted DNA extract as the template, and 2.5 μl of each primer (10 μM), 10 μl of the 5x Q5 reaction buffer, 1 μl of 10 mM dNTPs, 0.5 μl Q5 DNA polymerase (0.02 U/μL) and 82.5 μl nuclease free water. Prior to sequencing, the PCR products were purified using the QIAquick PCR purification kit (QIAGEN, UK) and sent for Sanger sequencing of both the forward and reverse reactions at GATC Biotech, UK. The same primers used for PCR were also employed to sequence both strands of the PCR.
products. Quality of nucleotide sequences were viewed and cleaned using the FinchTV chromatogram viewer program.
Supplementary data

Table S1. Summary of wastewater characteristics and reactor performance as a function of redox conditions.

<table>
<thead>
<tr>
<th></th>
<th>Influent</th>
<th>Aerobic</th>
<th>Anoxic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total COD</strong></td>
<td>315 ± 81.1</td>
<td>30.5 ± 13.7</td>
<td>71.2 ± 35.0</td>
<td>167 ± 60.0</td>
</tr>
<tr>
<td><strong>Soluble COD</strong></td>
<td>109 ± 54.1</td>
<td>19.2 ± 7.4</td>
<td>34.9 ± 12.1</td>
<td>48.9 ± 16.9</td>
</tr>
<tr>
<td><strong>Ammonia</strong></td>
<td>34.9 ± 8.4</td>
<td>0.2 ± 7.5</td>
<td>32.0 ± 7.1</td>
<td>41.4 ± 8.9</td>
</tr>
<tr>
<td><strong>Nitrite</strong></td>
<td>1.0 ± 0.5</td>
<td>0.4 ± 0.7</td>
<td>2.3 ± 3.1</td>
<td>0.5 ± 0.9</td>
</tr>
<tr>
<td><strong>Nitrate</strong></td>
<td>0.8 ± 0.6</td>
<td>114 ± 59</td>
<td>27.5 ± 38.1</td>
<td>1.7 ± 5.9</td>
</tr>
<tr>
<td><strong>Phosphate</strong></td>
<td>11.9 ± 12.8</td>
<td>9.2 ± 5.8</td>
<td>7.9 ± 4.7</td>
<td>20.1 ± 17.5</td>
</tr>
</tbody>
</table>

Table S2. P-values, showing significant differences of pRP4-gfp levels for comparisons between liquid and biofilm samples at different redox conditions in sequencing batch reactors during Stage 1 and Stage 2 experiments.

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>Samples</th>
<th>Games-Howell/Tukey post-hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 1 Seeding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic - Anaerobic</td>
<td>Liquid phase</td>
<td>0.03*</td>
</tr>
<tr>
<td>Aerobic - Anoxic</td>
<td>Liquid phase</td>
<td>0.06</td>
</tr>
<tr>
<td>Anaerobic - Anoxic</td>
<td>Liquid phase</td>
<td>0.94</td>
</tr>
<tr>
<td>Aerobic - Anaerobic</td>
<td>Biofilm</td>
<td>0.02*</td>
</tr>
<tr>
<td>Aerobic - Anoxic</td>
<td>Biofilm</td>
<td>0.56</td>
</tr>
<tr>
<td>Anaerobic - Anoxic</td>
<td>Biofilm</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Stage 2 Seeding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic - Anaerobic</td>
<td>Liquid phase</td>
<td>&lt; 0.01**</td>
</tr>
<tr>
<td>Aerobic - Anoxic</td>
<td>Liquid phase</td>
<td>0.29</td>
</tr>
<tr>
<td>Anaerobic - Anoxic</td>
<td>Liquid phase</td>
<td>&lt; 0.01**</td>
</tr>
<tr>
<td>Aerobic - Anaerobic</td>
<td>Biofilm</td>
<td>0.18</td>
</tr>
<tr>
<td>Aerobic - Anoxic</td>
<td>Biofilm</td>
<td>0.81</td>
</tr>
<tr>
<td>Anaerobic - Anoxic</td>
<td>Biofilm</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Note: Asterisks represent p-values; * denotes p ≤ 0.05; ** denotes p ≤ 0.01.
**Table S3.** Transfer frequencies during Stage 2 seeding experiment, estimated as per potential recipient cells across bioreactors via selective culturing.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Sample matrix</th>
<th>Time</th>
<th>Transfer</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td>Biofilm</td>
<td>S1_24</td>
<td>2.4E-04</td>
<td>4.0E-05</td>
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<tr>
<td></td>
<td>Liquid phase</td>
<td>S1_24</td>
<td>7.6E-04</td>
<td>5.0E-05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2_48</td>
<td>7.0E-04</td>
<td>0.0E+00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S3_72</td>
<td>1.0E-04</td>
<td>0.0E+00</td>
</tr>
<tr>
<td><strong>Anoxic</strong></td>
<td>Biofilm</td>
<td>S1_24</td>
<td>1.8E-03</td>
<td>5.0E-05</td>
</tr>
<tr>
<td></td>
<td>Liquid phase</td>
<td>S1_24</td>
<td>1.4E-03</td>
<td>2.0E-04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2_48</td>
<td>3.8E-04</td>
<td>0.0E+00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S3_72</td>
<td>1.2E-03</td>
<td>2.9E-04</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td>Biofilm</td>
<td>S1_24</td>
<td>4.1E-03</td>
<td>2.6E-03</td>
</tr>
<tr>
<td></td>
<td>Liquid phase</td>
<td>S1_24</td>
<td>8.6E-04</td>
<td>6.4E-04</td>
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<tr>
<td></td>
<td></td>
<td>S2_48</td>
<td>8.6E-04</td>
<td>5.0E-04</td>
</tr>
<tr>
<td></td>
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<td>S3_72</td>
<td>7.8E-03</td>
<td>2.3E-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2_48</td>
<td>8.5E-03</td>
<td>5.0E-04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S3_72</td>
<td>7.1E-03</td>
<td>2.1E-03</td>
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</tbody>
</table>
Table S4. Significant species detected based on sample DNA sequencing and database sequences in the NCBI nucleotide database.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isolate</th>
<th>Identified species (NCBI)</th>
<th>Significant match (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic biofilm</td>
<td>FL1</td>
<td><em>Escherichia coli</em></td>
<td>99.8</td>
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<tr>
<td>Anoxic biofilm</td>
<td>FL2</td>
<td><em>Escherichia coli</em></td>
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</tr>
<tr>
<td>Anaerobic biofilm</td>
<td>FL3</td>
<td><em>Escherichia coli</em></td>
<td>99.9</td>
</tr>
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<td>Anaerobic liquid phase</td>
<td>FL4</td>
<td><em>Escherichia coli</em></td>
<td>99.9</td>
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<td>Anaerobic liquid phase</td>
<td>FL5</td>
<td><em>Escherichia coli</em></td>
<td>99.9</td>
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<td>Anaerobic liquid phase</td>
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<td>Anaerobic liquid phase</td>
<td>FL7</td>
<td><em>Escherichia coli</em></td>
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<td>Anaerobic biofilm</td>
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<td><em>Escherichia coli</em></td>
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<td>Anaerobic liquid phase</td>
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<td><em>Escherichia coli</em></td>
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<td><em>Escherichia coli</em></td>
<td>99.9</td>
</tr>
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<td><em>Escherichia coli</em></td>
<td>100.0</td>
</tr>
<tr>
<td>Anaerobic biofilm</td>
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<td><em>Escherichia coli</em></td>
<td>99.9</td>
</tr>
<tr>
<td>Anaerobic biofilm</td>
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<td><em>Escherichia coli</em></td>
<td>99.9</td>
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Figure S6. Phylogenetic tree based on neighbour-joining (NJ) method showing relationships between the EcoFJ2 seed strain and transconjugants isolated from difference samples.
Figure S7. Microscopic analysis of samples abstracted from (A) liquid phase; and (B) sponge biofilm from the aerobic SBBR before any seeding. Samples specimens were viewed at 400x magnification and example presumptive predators are indicated by red arrows.
References


