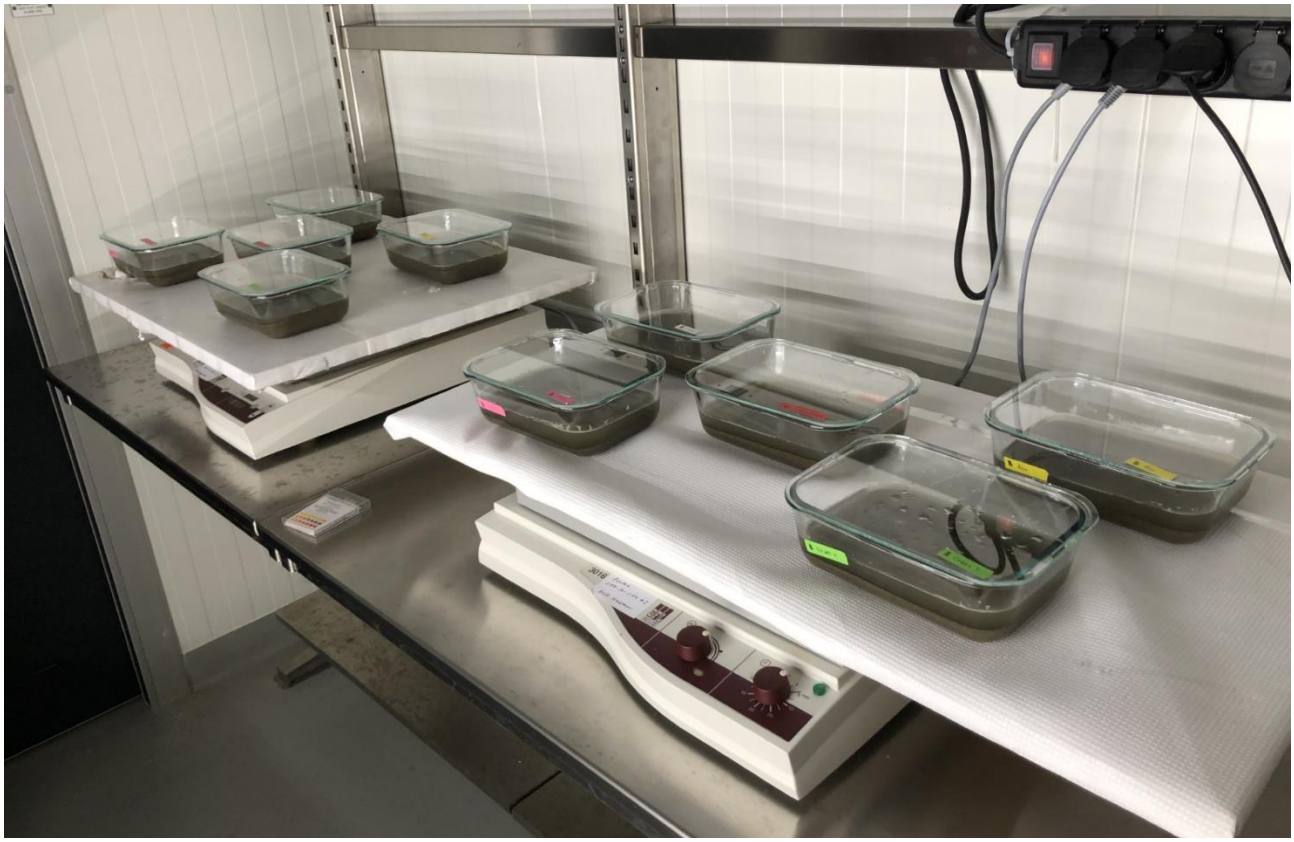
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

EFFECTS OF ANTIBIOTICS AND MULTIDRUG RESISTANT *E. COLI* ON BACTERIAL SEDIMENT COMMUNITIES



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ABSTRACT

The spread of antibiotic resistance is a global health concern, with growing evidence that environmental reservoirs such as aquatic sediments play a role in antibiotic resistance persistence and dissemination. This study investigates the effects of a mixture of antibiotics and the establishment of a multidrug-resistant strain of *Escherichia coli* at levels commonly discharged by wastewater treatments plants on freshwater sediment bacterial communities. In addition, the study evaluates how the potential *E. coli* establishment was affected by the occurrence of antibiotics. To assess the effect and interaction of these two variables, a 10-day static test was performed in microcosm conditions using sediment from Lake Vänern, Sweden. Prior to the full experiment, a pilot study was performed to assess the invasion capacity of *E. coli* on sediment and how to prepare the inoculation of *E.coli* in the sediment. Results from the pilot study demonstrated that *E.coli* was able to survive and establish in the sediment for up to 28 days. In the full experiment an increase of the *blaCTX-M* gene in the sediment invaded by *E.coli* was observed after 1, 3, and 7 days after inoculation, indicating potential resistance spread to natural bacterial communities. The occurrence of antibiotic contamination positively affected the spread of antibiotic resistance genes (ARGs) on natural communities. The functions of the community were resilient to both the exposed antibiotic mixture and the invasion of the *E.coli*, attributed to functional redundancy in the bacteria communities and no-ecological effects of acquiring ARGs. The bacterial communities appear to have a higher carbon metabolism and a higher nitrification over time, attributed to an increasement on their efficiencies in using carbon and ammonium.

These findings underscore the potential of invading bacteria carrying ARGs, such as *E.coli*, to influence the resistome of bacterial communities, and how this is modulated by the occurrence of antibiotic contamination. This study highlights the importance of incorporating environmental dimensions into antibiotic resistance research and supports a One Health approach to managing antimicrobial resistance.

ABSTRAKT

Spridningen av antibiotikaresistens är ett globalt problem, och växande bevis pekar på att miljön spelar en tidigare underskattad roll i spridning av antibiotikaresistens. Det är möjligt att akvatiska miljöer såsom sediment agerar som reservoar för båda bakterier och resistensgener. Denna studie syftar till att undersöka hur en blandning av antibiotika samt multiresistenta Escherichia *coli* i koncentrationer som vanligen finnes i reningsverk påverkar bakteriesamhällen i sötvattenssediment. För att undersöka effekten av dessa två variabler så genomfördes ett 10-dagars mikrokosmexperiment med sediment och vatten hämtat från Vänern, Sverige. Innan huvudexperimentet genomfördes en pilotstudie för att utveckla en metod för att inokulera sedimentet med *E. coli* samt testa invasionskapaciteten av *E. coli.* Resultaten från pilotstudien visar att *E. coli* kan etablera och överleva i sedimentet i minst 28 dagar. I huvudstudien påvisades en ökning av *blaCTX-M* genen efter 1, 3, och 7 dagar i sedimentet som invaderats av *E. coli.* Detta indikerar potentiell spridning av resistensgener till det ursprungliga bakteriesamhället i sedimentet. Förekomst av antibiotika ledde till en signifikant ökning av resistensgener i sedimentet. Funktionerna hos bakteriesamhället i sedimentet påverkades inte noterbart vi exponering av antibiotika eller *E. coli*. Detta förklaras genom funktionell redundans hos bakterierna, samt en icke signifikant ekologisk effekt av upptagande av resistensgener. Det uppmättes en ökning av kolomsättning samt nitrifikation över tid. Detta kan vara ett resultat av ökad effektivitet i metabolismen av kol samt ammonium.

Dessa resultat understryker potentialen hos invaderande bakterier som bär på resistensgener, såsom *E. coli*, att påverka resistensen hos mikrobiella samhällen i sediment, och hur detta moduleras av förekomsten av antibiotika. Denna studie belyser vikten av att inkludera miljön i antibiotikaresistensforskningen och stöder ett ”One Health”-perspektiv för att hantera antimikrobiell resistens.

NON-TECHNICAL SUMMARY

This thesis explores how antibiotic-resistant bacteria, like *E. coli*, can affect the natural bacterial communities living in lake sediments. The sediments are crucial environments where bacteria play roles in breaking down organic matter and recycling nutrients like carbon and nitrogen and is important for a healthy and sustainable ecosystem and environment. However, they are increasingly exposed to pollution including antibiotics and bacteria coming from sources such as wastewater treatment plants.

To understand what happens when resistant bacteria enter these natural communities, this study set up controlled microcosm experiments using lake sediment and water. The experiments tested how adding multidrug-resistant E. coli, with or without a mixture of antibiotics, affected the local bacterial communities over time. Changes in the abundance of bacteria, the spread of antibiotic resistance genes, and overall metabolic activity (such as carbon metabolism and nitrification), were detected.

The results showed that introducing resistant *E. coli*, especially together with antibiotics, led to an increase in resistance gene levels within the sediment. This suggests that invading bacteria can potentially transfer resistance genes to local bacteria or persist in the environment themselves. While the overall bacterial abundance and community functions did not collapse, there were detectable shifts that highlight how even brief exposure to contaminants can leave an impact.

Importantly, the findings underscore the need to consider the environmental dimensions of antibiotic resistance. Not only is it a clinical problem affecting human and animal health — it also has ecological impacts that can change the makeup and function of microbial ecosystems. This supports the idea of using a One Health approach, which recognizes the interconnectedness of human, animal, and environmental health when addressing antimicrobial resistance on a broader scale.

Contents

1 Introduction

Antibiotic resistance is a growing global health threat that extends into the environment. Aquatic sediments can act as reservoirs for both antibiotics and antibiotic-resistant bacteria, yet their role in resistance dissemination remains underexplored. This thesis investigates how environmentally relevant concentrations of antibiotics, and the invasion of multidrug-resistant *Escherichia coli* affect bacterial communities and their functions in freshwater sediments, while contributing to a broader understanding of resistance dynamics within the One Health framework.

1.1 Antibiotics

Antibiotics are naturally occurring or synthetically produced chemicals that have bactericidal or bacteriostatic properties, meaning that the chemicals either kill or inhibit the growth of bacteria (Walsh & Wright, 2005). The first discovery of a naturally occurring antibiotic compound was made by Alexander Fleming in 1928 (Bennett & Chung, 2001), leading to the "golden age" of antibiotic discovery through the 1940s-1960s, when discovery and development of antibiotics was at its peak (Hutchings et al., 2019). Since then, antibiotics have been an essential tool to treat bacterial infections, saving millions of lives annually (C. J. L. Murray et al., 2022; Naddaf, 2024).

Currently, there are hundreds of antibiotic compounds in use. Antibiotics can be classified into groups depending on their chemical structure and/or mode of action. Some of the main classes are shown in Table 1. One important feature of antibiotics is that they target sites found in bacterial cells that are absent in eukaryotic cells, minimizing the risk of effects in humans or other animals during treatment (Ribeiro da Cunha et al., 2019). Most common targets are either nucleic acid or protein synthesis, cell walls or cell membranes (Gualerzi et al., 2013).

Table 1. Major classes of antibiotics, as well as an example compound within each class, and the molecular targets of each class. Table was constructed using sources from (Grenni et al., 2018; Hutchings et al., 2019).

Class	Antibiotic compound	Target
Aminoglycosides	Kanamycin	Inhibits protein synthesis, 30S ribosomal subunit
β-lactams	Cefotaxime	Inhibits cells wall synthesis
Diaminopyrimidine	Trimethoprim	Inhibits dihydrofolate reductase, precursor in DNA synthesis
Macrolides	Erythromycin	Inhibits protein synthesis, 50S ribosomal subunit
Quinolones	Ciprofloxacin	Inhibits DNA gyrase and topoisomerase IV, inhibits DNA synthesis

Rifamycin	Rifampicin	Inhibits RNA polymerase, thus inhibiting RNA synthesis
Sulphonamides	Mafenide	Inhibits folic acid synthesis
Tetracyclines	Tetracycline	Inhibits protein synthesis, 30S ribosomal subunit

Medicinal and agricultural use of antibiotics has been increasing over the past century, and as a consequence, antibiotic residues are found ubiquitously in the environment (Kraemer et al., 2019). The main societal concern with the current use, overuse, and misuse of antibiotics is the rise in antibiotic resistant bacteria (ARB), which poses a major threat to human health by limiting our ability to fight bacterial infections. In 2019, 1.27 million deaths were directly attributed to antibiotic resistance, and this number is expected to increase (Murray et al., 2022). Estimates show that by 2050 close to 2 million people could die annually as a direct result of antibiotic resistance, which means that around 40 million people are predicted to die between 2024-2050 because of resistant bacteria (Naddaf, 2024).

1.2 Antibiotic resistance

Antibiotic resistant bacteria can arise by two main pathways, mutations in individual organisms or through horizontal gene transfer (HGT), which are mechanisms by which bacteria can take up foreign DNA (Larsson & Flach, 2022). Horizontal gene transfer can occur through multiple different pathways, however the three main ones are transduction, transformation, and conjugation (Berglund, 2015). Of these, conjugation is the main pathway involved in the dissemination of antibiotic resistance genes (ARGs).

Conjugation is most likely to be successful between bacteria that are phylogenetically close to each other (Philippot et al., 2010; Smillie et al., 2011), but there is evidence showing that conjugation can occur even between bacteria and eukaryotes (Bates et al., 1998; S. Li et al., 2023). Conjugation is only possible when the donor cell and recipient cell are in contact. For gram-negative bacteria this contact occurs using a pilus (Figure 1), while in gram-positive bacteria this is done using adhesins (Kohler et al., 2019; Liu et al., 2020). Once contact has been established the ARGs can be transferred between cells. The process of conjugation depends on environmental factors such as nutrient levels and temperature, but also on the bacterial species, and the genes involved (Piscon et al., 2023; Shafieifini et al., 2022).

The transfer of ARGs are facilitated by mobile genetic elements (MGE) (Partridge et al., 2018). MGEs are genetic material that have intracellular or intercellular mobility, such as integrons, transposons, and plasmids. ARGs are frequently found on conjugative plasmids. In recent years it has been shown that many plasmids contain multiple ARGs, conferring resistance towards multiple antibiotics (Lund et al., 2025). Acquisition of such MGEs create what is often referred to as “superbugs”, bacteria that are multidrug resistant. Their rise is causing major concerns in clinical settings (Alpert, 2017).

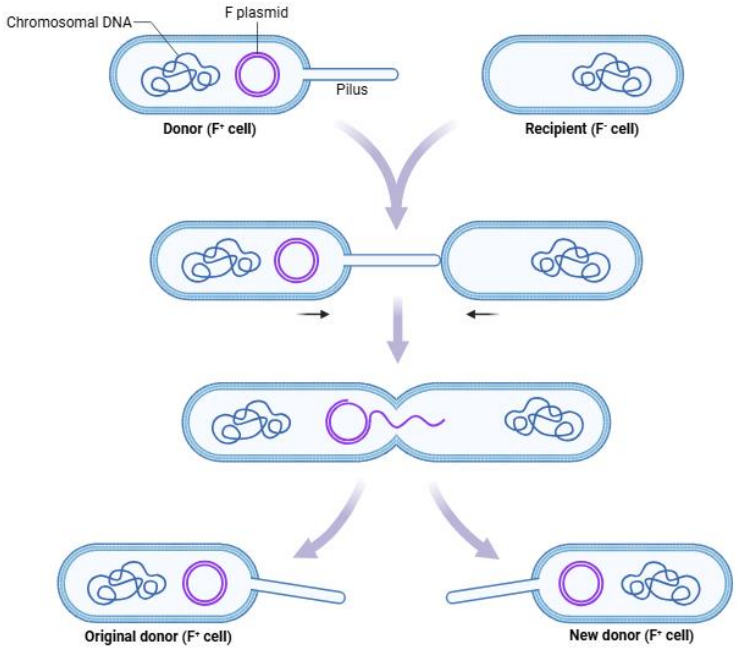

Figure 1. Schematic showing conjugation between to gram-negative cells using a pilus that is extended from the donor cell. Image taken from (BioRender 2021).

Conjugative plasmids contain the necessary genes for its own conjugation, such as the type of pilus, and vary between plasmids (Partridge et al., 2018). Some plasmids have been categorized based on their incompatilibty (Inc), as their genes for DNA replication and cell partitioning is shared, and as a result of this they cannot stably exist in the same cell (Yu et al., 2024). The Inc groups IncA/C, IncF, IncH, IncI, IncL, and IncN together carry the largest variety of ARGs (Rozwandowicz et al., 2018). Of these, IncH, IncL, and IncN have a broad host-range, enabling them to replicate in a large number of bacterial species.

1.3 Bacterial communities in aquatic sediment

In aquatic environments such as the sediment in rivers and lakes, bacteria make up the largest part of the biomass (Nealson, 1997). In addition, they play key roles in the degradation of organic matter and biogeochemical cycles of nutrients like C, N, P, and S (Figure 2). Such roles are accomplished by the huge bacterial diversity found in the sediment. In general, bacterial metabolic activity occurs mostly in the first few centimetres of the aquatic sediment as a result of more oxygen and organic matter being available (Lewandowski et al., 2019). Outside of being reservoirs for organic matter and microbes, high levels of pollutants are also commonly found in freshwater sediments (Heim & Schwarzbauer, 2013; Salomons et al., 1987)

The dominating phylum of bacteria in freshwater sediments are usually Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, with Proteobacteria being the most common (Ge et al., 2021; Lim et al., 2011; Nam et al., 2008; Song et al., 2012; Wan et al., 2014; Yi et al., 2021). Bacterial community composition is affected by biogeochemical factors and thus varies throughout time and space (Ge et al., 2021; Torsvik et al., 1996; Yi et al., 2021).

However, not only environmental factors but also anthropogenic ones affect the composition of bacterial communities. For instance, human pathogenic bacteria, such as Bacilli and Enterobacterales, are more prone to be found in urban areas (Ge et al., 2021).

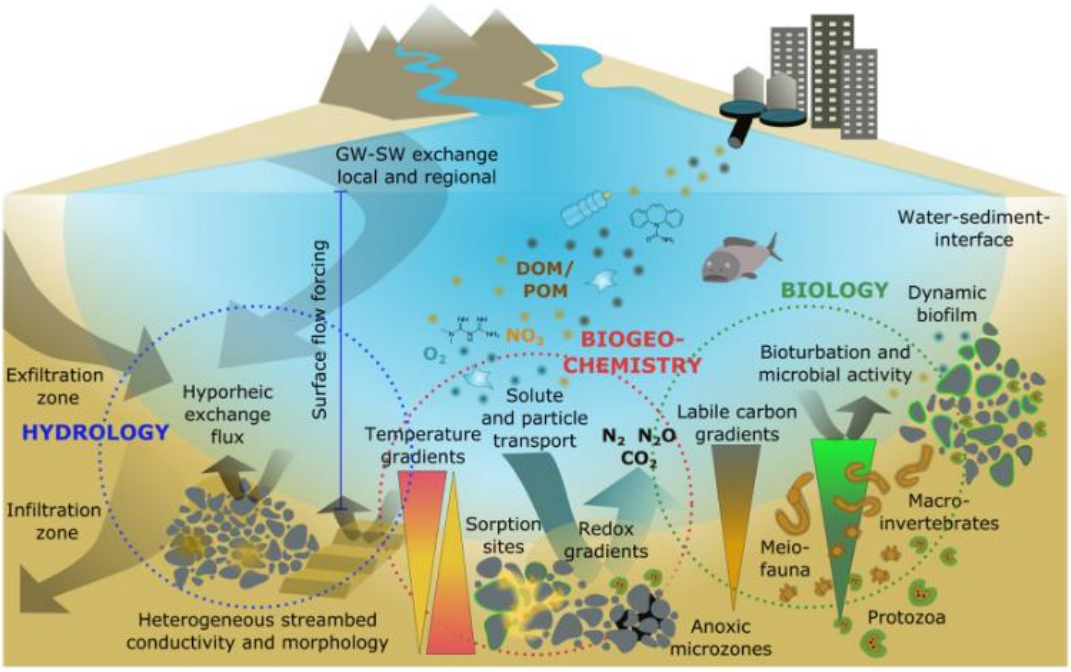

Figure 2. Model of the main processes which the sediments is involved in within freshwater systems. Model from (Lewandowski et al., 2019).

1.4 Antibiotics and antibiotic resistance in the environment

In addition to the threat of antibiotic resistance to human health, there is a growing concern into what effects antibiotic pollution has on the ecosystem, and especially on microbial communities (Brandt et al., 2015). The consideration of both public health and the environment is in line with the current shift in focus towards a One Health approach, which considers human, animal, and ecosystem health to be interconnected (*One Health*, 2024).

Antibiotics can enter the environment through multiple pathways (Figure 3). It can be directly from the manufacturer, during improper disposal of the drug, directly from aqua or agricultural use, from treatment of livestock, or after human consumption (Carvalho & Santos, 2016; Cherian et al., 2023; Polianciuc et al., 2020). Following human consumption most of the antibiotics are excreted into the environment, as humans are typically not able to fully metabolise antibiotics (EFSA, 2015). Therefore, they end up in wastewater treatment plants (WWTP), that are not designed to degrade them. As a result, WWTPs are one of the main sources of antibiotics released into the environment (Manaia et al., 2018; Michael et al., 2013).

Antibiotic residues enter the aquatic environment via WWTP discharges (Figure 3). Depending on the chemical properties of each compound, they can remain in the water column or adsorb into solids, ending up in the sediment (Hernando et al., 2006). Once antibiotics have entered the environment, the compounds can either remain or be degraded depending on the physicochemical properties of the antibiotic. For example, some antibiotics are degraded into

transformation products by UV light from the sun (Kumar et al., 2019). However, biodegradation is the main pathway of degrading pharmaceuticals in the environment (Barra Caracciolo et al., 2015).

Ultimately, antibiotics can be found in all places in the environment, from soils, to marine compartments, to freshwater and sediment. In most places, antibiotics are found in mixtures, meaning that multiple antibiotic compounds are found at the same place (Chow et al., 2021; Grenni et al., 2018; Hanna et al., 2023; Larsson & Flach, 2022). Usually, antibiotic concentrations found in environments are in the ng/L - µg/L range, being especially high at the discharge sites of WWTP effluents. In more polluted areas, as in countries such as China and India, antibiotic concentrations can even exceed mg/L (Hanna et al., 2023).

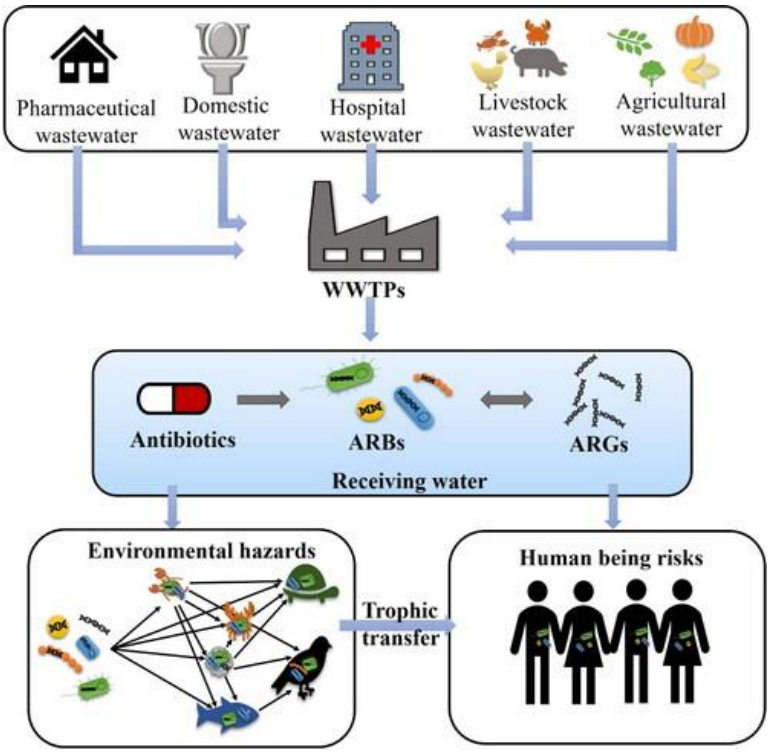

Figure 3. Main processes that lead to discharge of antibiotics in wastewater treatment plant effluent. The schematic also highlights the fact that antibiotic resistant bacteria and antibiotic resistance genes can be spread the same way. Image from (Wang & Chen, 2022).

Once the antibiotics reach the environment, they might affect the structure and function of bacterial communities. In their review article from 2018, Grenni and colleagues summarized the current knowledge about the effects of antibiotics on microbial communities (Grenni et al., 2018). Most commonly, changes in bacterial community diversity were identified, followed by effects of decreased nitrification and denitrification. Effects were also found in pollution-induced community tolerance, decrease bacterial biomass, lower iron reduction capabilities, and lower bacteria to fungi ratio.

Despite the evidence that show there are effects on microbial communities, some scepticism is warranted. For most studies included in the review the test concentrations were in the mg/L or mg/kg range, which is significantly higher than what is usually found throughout the

environment. It is thus difficult to draw direct conclusions from those studies about what effects actually occur in nature.

Additionally, the studies mostly exposed the communities towards a single antibiotic compound. As previously mentioned, antibiotics are often found in mixtures in the environment, and this is of additional importance when it comes to their toxicity. One study investigated the mixture effect of two to five different antibiotics on a cyanobacteria and a green alga (González-Pleiter et al., 2013). In the study it was shown that most mixtures of antibiotics had synergistic effects on both organisms, highlighting the risk organisms experience in reality. This emphasizes the current lack of studies that focus on realistic mixtures of antibiotics at environmentally relevant concentrations, highlighting a need for further research in this area.

Antibiotics notwithstanding, wastewater treatment plants are also hotspots for antibiotic resistant bacteria as they receive discharge from hospitals and municipalities with high concentrations of antibiotics (Bengtsson-Palme et al., 2018) (Figure 3). This results in selective pressure inside the plants, favouring resistant bacteria. Comparatively, in the natural environment antibiotic concentrations are rarely high enough to exert selection pressure favouring the development of resistance. WWTP effluent contains both ARGs and antibiotic-resistant bacteria because of poor removal rates, resulting in the receiving aquatic environment being exposed. However, the factors affecting the spread of antibiotic resistance from WWTPs to the aquatic environment are not well known. Environmental contamination of ARB from WWTPs is likely a factor influencing antibiotic resistance spread, but the mechanism behind their invasion and spread of resistance to natural communities are not known.

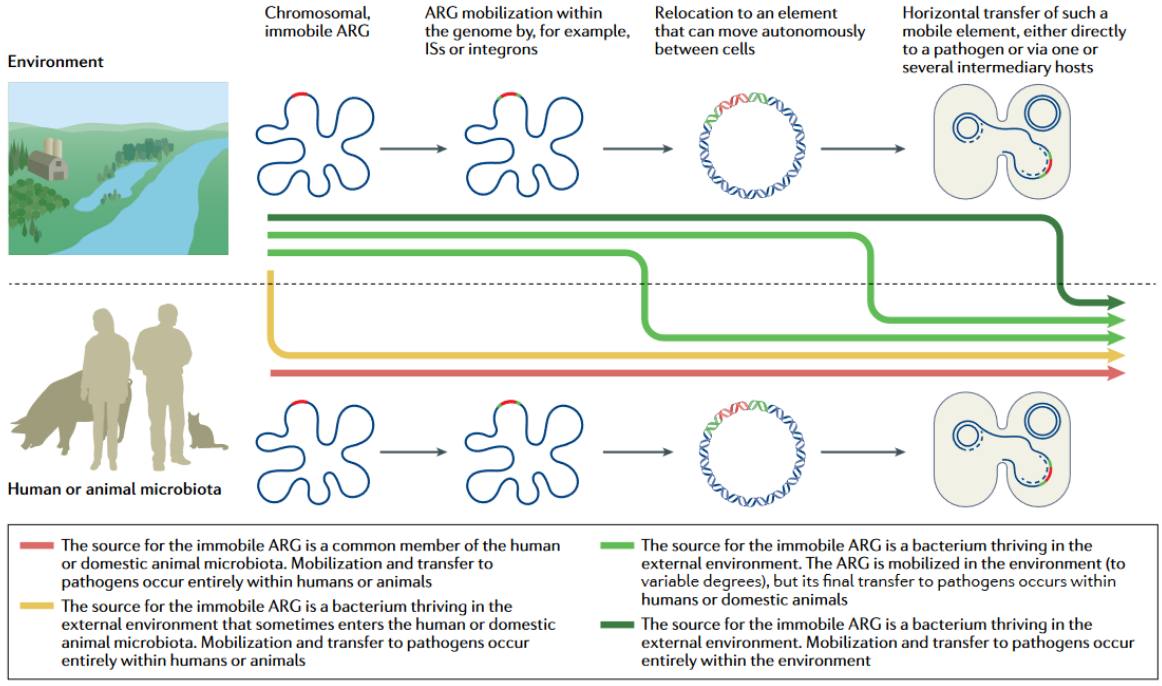

Figure 4. Schematic of how antibiotic resistance genes can be mobilized and spread from the environment to humans or animals. Image from (Larsson & Flach, 2022).

As far as antibiotic resistance spread in the environment is concerned, both horizontal gene transfer and vertical gene transfer (VGT) contribute to its spread (Z. Li et al., 2021) (Figure 4). In their study, Li and colleagues showed that in scenarios where resources are limited VGT is the main driver of ARGs, whereas when resources are unlimited, HGT is the main factor.

Not only does resources play a role in the dissemination of ARGs, but co-selection is another important phenomenon that is involved. Co-selection is the process by which resistance genes towards a second factor, often metals or biocides, are located within a bacteria or MGE that also contains ARGs (Bengtsson-Palme et al., 2018; L. M. Murray et al., 2024). When the second factor is present it can thus lead to the dissemination of ARGs by co-selection, even without the presence of antibiotics. This is known to be a common event that occurs in the presence of metals (Pal et al., 2017). Ultimately, both the environment, and human and veterinary medicine affect the spread of ARGs.

The fact that ARGs and ARB are discharged in the sediment downstream of WWTPs, combined with the fact that antibiotics frequently accumulate within the sediment, makes it possible that the sediment plays a hitherto underestimated role in the spread of antibiotic resistance.

As of yet, it has not been well-established if ARB from WWTPs can establish themselves in natural sediment communities once they enter the aquatic environment. Generally, well-established and stable bacterial communities are considered resistant towards invading species of bacteria (Bagra et al., 2023). On the other hand, bacterial communities downstream of WWTPs, where the expected invasion might occur, frequently experience disturbance and stress leading to unstable and poorly established bacterial communities. As a matter of fact, Bagra and colleagues has shown that during stressful conditions antibiotic resistant *E. coli* is able to establish itself in bacterial communities on glass slides collected from aquatic rivers, but not under normal conditions. Nonetheless, research is lacking as to whether this can occur in sediment bacteria, and whether those communities can acquire ARGs should the ARB be able to establish themselves. Ultimately, more considerations of microbial communities are required when it comes to risk assessment of antibiotics (Brandt et al., 2015; Grenni et al., 2018; A. K. Murray et al., 2021).

1.5 Adaptation and fitness

It is known that bacterial adaptation towards an antibiotic stressor can reduce the fitness of the organisms once the stressor has been removed (Dawan & Ahn, 2022; Dunai et al., 2019). Yet, what factors influence the cost in fitness has not been fully elucidated (Andersson & Hughes, 2010; Humphrey et al., 2012). Current evidence shows that a fitness cost is commonly associated with antibiotic resistance based on chromosomal mutations (Andersson & Hughes, 2010). Such mutations are often accompanied by lower physiological activity in the targeted site, resulting in lower fitness for the organism (Andersson & Hughes, 2010; Andersson & Levin, 1999).

Comparatively, the fitness cost associated with MGEs are less clear than with chromosomal mutations. Some studies find reduced fitness with MGEs containing ARGs (McDermott et al., 1993; Valenzuela et al., 1996), while others find increased fitness (Enne et al., 2004; Yates et al., 2006). As previously stated, the spread of antibiotic resistance is mostly affiliated with

MGEs. The difference in fitness costs have not been fully established but one explanation is that there are more mechanisms to counteract potential costs of MGEs than with chromosomal mutations. Loss of the MGE, reduced gene expression, or compensatory mutations are available mechanisms for bacteria to compensate for reduced fitness from a MGE, while compensatory mutations is the main mechanism available to compensate for chromosomal mutations (Andersson & Hughes, 2010; Humphrey et al., 2012; Maisnier-Patin & Andersson, 2004). In the end, fitness changes associated with MGEs seem to depend on the specific organism, the type of MGE, and the environment that the organism exists in (Andersson & Hughes, 2010; Humphrey et al., 2012).

It is important to highlight that most of the studies have focused on single bacterial species and used growth as a measure of fitness. As a result, little knowledge exists on whether there is a fitness cost or reduction in the function of bacterial communities that acquire resistance through MGEs, as this has not been investigated yet.

1.6 Aim of the study

The aim of this study is to assess, in microcosms conditions, the potential effects of a mixture of antibiotics and the invasion of a multidrug-resistant *E. coli* on natural communities of sediment bacteria. The specific objectives were to:

1. Optimize a method to inoculate *E. coli* in a microcosm system composed by two compartments, sediment and water, and methods to track the potential invasion of the *E. coli* in the sediment.
2. Assess whether *E. coli* is able to establish itself and for how long in the natural bacterial community.
3. Assess the potential antibiotic resistance gene transmission from *E. coli* to the natural bacterial community.
4. Determine the role of antibiotic pollution in *E. coli* invasion and establishment.
5. Assess if a mixture of antibiotics, or *E. coli* invasion, affects the native bacterial community in abundance, and functions of nitrification and carbon metabolism.

2 Methods and materials

During this study two experiments were run, one initial pilot study between December 2024 to January 2025 covering 28 days, and then a full-scale study spanning a total of 14 days between February to March in 2025. The experiments were conducted indoors in a climate room at the facilities of the Department of Biology and Environmental Sciences, in Natrium. Independent microcosms made by rectangular glass vessels with two compartments, sediment and water, were used for the experiments (see section 2.2.2). The test system was static, which means that no renewal of the water or the sediment occurred during the duration of the exposure.

2.1 Pilot study

The pilot study was conducted with the purpose to test methods and assess an optimal workflow. Furthermore, the goal was to assess two different ways to prepare the two compartments of the microcosms (water and sediment), while inoculating the *E. coli*. Two different concentrations of *E. coli* were also tested. One concentration at 10^7 cells/ml, which has been used in similar studies before (Bagra et al., 2023) but is considered high and not environmental realistic, and another concentration at 6000 cells/ml based on what wastewater treatment plants along Göta Älv release in their effluent into the river (Emelie Grubbström at Ryaverket, Katarina Enbom at Holmängens wastewater treatment plant, personal communication, 11th November, 2024). Similar concentrations of *E. coli* have also been found in wastewater treatment plant effluent in other parts of Europe (Bréchet et al., 2014; Kotlarska et al., 2015). Additionally, the survival time of the invading *E. coli* on the test system was investigated.

2.1.1 Sediment and water sampling

Sediment and water were collected from Vänern, upstream of the river Göta Älv (58°23'4.8"N 12°19'57.3"E, Figure 5, B) on the 10th of December 2024. The collected sediment was rich in clay, and was collected next to reeds (Figure 5, A). The collected sediment contained a large amount of root-like material. The sediment was placed in a cold box and transported to the lab, where it was stored at 4°C until the start of the experiment. The water was collected in a 10 L plastic container and brought to the lab where it was first filtered through GF/C, then GF/F filters before being stored at 4°C until the start of the experiment.

Figure 5. A) shows the sampling site. Aquatic vegetation can be seen. B) shows the sampling site on a map, located on the south side of Vänern, upstream of the river Göta Älv. 250 m east of the sampling site is the local wastewater treatment plant.

2.1.2 Microcosm setup

A total of six microcosms were set up for the test, which are described in Table 2. The sediment was prepared by manually removing large debris, such as roots and rocks using tweezers and hands. The same type of microcosm, table-top shaker, and thermoconstant room settings as described in 2.2.2. was used. Into each vessel 400 ml of sediment was added, as well as 400 ml of the filtered water, containing *E. coli* in the four exposed vessel, and only containing filtered water in the two control vessels (Table 2).

Table 2. Treatments during the small-scale microcosm setup, with the exposed E. coli concentrations in the 400 ml of filtered water added, and the inoculation technique.

Treatment name	E. coli concentration (cells/ml)	Inoculation technique
Control MIX	0	Mixing
Control LAYER	0	Layering
High MIX	10^7	Mixing
High LAYER	10^7	Layering
Low MIX	6000	Mixing
Low LAYER	6000	Layering

2.1.3 *E. coli* inoculation technique

E. coli CV601 (described in 2.2.3) was incubated in lysogeny broth (LB) medium overnight, the OD_{600} was then measured and dilutions applied according to the same principles described in 2.2.5. The *E. coli* was diluted to either 6000 cells/ml or 10^7 cells/ml, in volumes of 400 ml for each corresponding treatment.

Two techniques were assessed to test different ways of inoculating the microcosms with the *E. coli*. The technique referred to as "mixing" consisted of putting 400 ml of sediment (600 g) and 300 ml of water with *E. coli* in a 1 L Pyrex bottle and manually shaking the bottle for 1 minute. The slurry was then poured directly into the vessel evenly, 100 ml of water was added into the bottle to wash down residues inside and then poured into the vessel. The second technique referred to as "layering" involved directly adding 400 ml of sediment evenly into the vessel. The 400 ml of water with *E. coli* was then poured carefully into the vessel, onto the back of a spoon to not disturb the sediment (Figure 6). In total 400 ml of sediment and 400 ml of water was added to each vessel for both techniques (Figure 7). Into the control vessels water without *E. coli* was added using the same methods as described above.

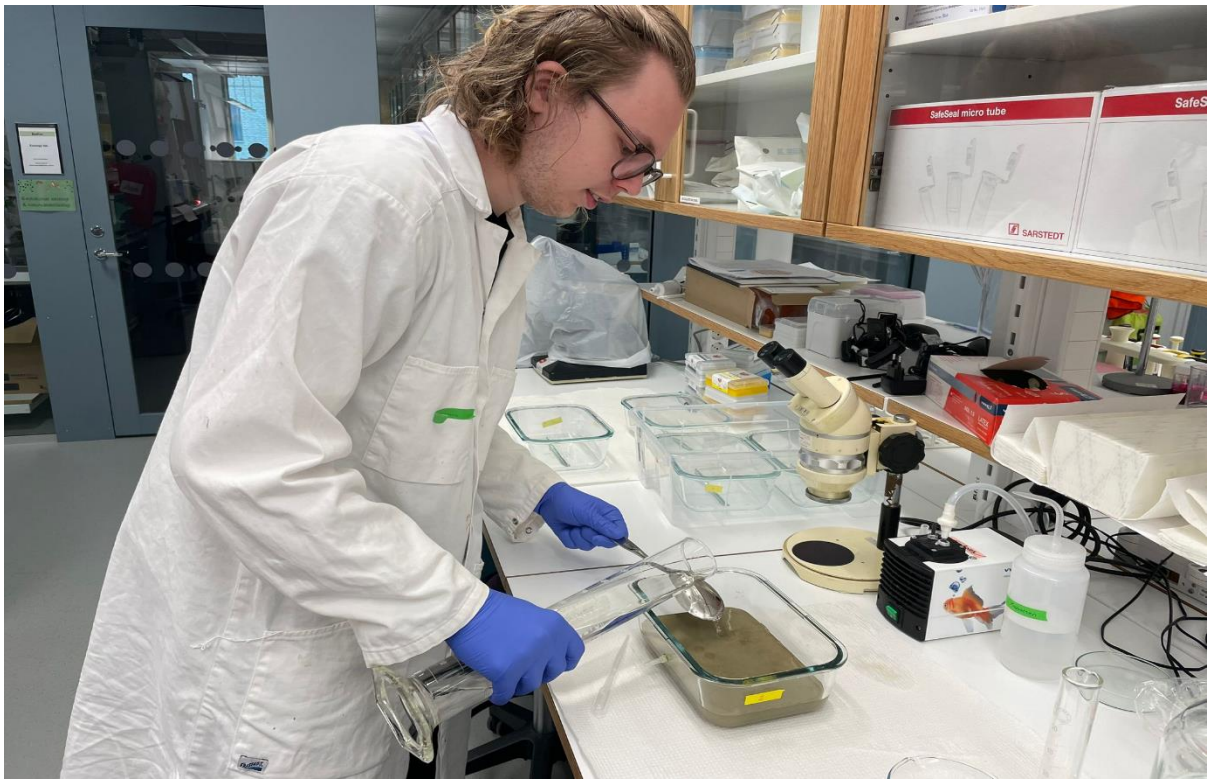

Figure 6. Work with setting up the microcosms. The images show the method of water being added by pouring on to the back of a spoon to evenly distribute the water and reduce the resuspension of sediment.

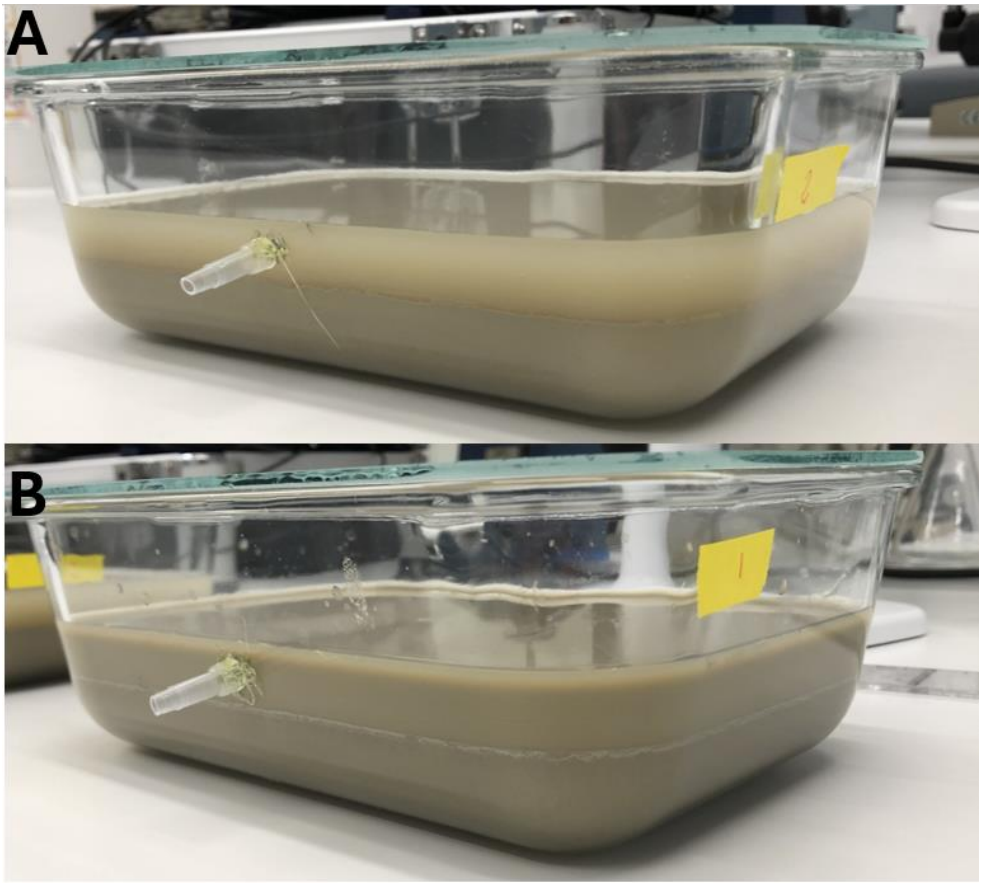

Figure 7. A) shows a vessel prepared using the "layering" style a few minutes after the preparation of the vessel. B) shows a vessel prepared using the "mixing" style a few minutes after preparation. A murkier water column can be noticed in the "mixing" style vessel.

2.1.4 *E. coli* establishment in sediment using CFU

The number of *E. coli* established in sediments was assessed using an agar plating technique in which colony forming units (CFUs) are used as proxy of live cell abundance. A total of 2 ml of sediment was collected from each vessel at each sampling time using a 5 ml pipette. The sediment was placed in 50 ml Falcon tubes, weighed, and 2 ml of river water was added for each gram of sediment. The tubes were then vortexed for 10 minutes, after which they were centrifuged at 1800xg for 5 minutes. 100 µL of the supernatant was spread onto one LB agar plate (Kanamycin + Rifampicin 50 µg/mL) per treatment and then incubated overnight at 37°C. The plates were then photographed and colonies counted using ImageJ. Sampling was done 3, 6, and 28 days after the start of the experiment. Samples were also collected from the control plates and used as negative controls.

2.2 Full-scale study

2.2.1 Sediment and water sampling

Sediment and water used for the experiment were collected from the lake Vänern, upstream of the river Göta Älv (58°23'07.3"N 12°19'17.3"E, Figure 8, B) on the 7th of February 2025. The site is a public swimming place called “Hamnbad” with relatively sandy sediment, and no visible aquatic plants in the vicinity (Figure 8, A). Vänern is the largest lake in Sweden, and is drained through the most water-rich river in Sweden, Göta Älv (*Vänerns vattenvårdsförbund*, 2025). The site used is located upstream of the WWTP in Vänersborg and was chosen because a previous study in the group had shown low levels of antibiotics in the sediment nearby (unpublished). However, Vänern has historically been polluted with chemicals such as mercury and PCB, many of which are still found in the lake (*Vänerns vattenvårdsförbund*, 2025). Vänersborg is a medium sized town in Sweden, with 24,731 inhabitants in the town centre (*SCB*, 2024). Metal processing, electronic manufacturing, and technology companies are some of the largest companies found in the town (*Fakta om näringslivet*, 2023).

Figure 8. A) shows the sampling site located at a local swimming area, with an artificial beach. Little nearby vegetation can be seen. B) shows the sampling site on a map, located on the south side of Vänern, upstream of the river Göta Älv. 250 m west of the sampling site is a local dock, that has space for about 200 small boats.

The sediment was collected using a shovel, with the top 5 cm collected manually. Once the sediment was collected it was stored in a cold box and transported to the lab where it was

sieved through 2 mm pore-size metal woven mesh sieves (Endecotts), homogenized, and stored at 4°C until the start of the experiment. The sediment was also further characterised by sieving using metal woven mesh sieves (Endecotts), through 1 mm and 0.5 mm pore-sized sieves to determine the particle size composition in the sample. The composition was 29.5% particles > 2 mm, 16.6% of particles were between 1-2 mm, and 33.9% had a size < 1 mm (Figure 9). The organic matter content of the sediment was 0.59% and was determined by burning the sediment in a muffle oven at 550°C for 5 hours.

Figure 9. Particle size composition of the sediment, A) shows particles >2 mm, B) shows particles between 1-2 mm, and C) shows particles smaller than 1 mm in size.

In situ physicochemical parameters, pH (6.64), temperature (0.26°C) and conductivity (206 µS/cm), were collected using a multiparameter instrument (HANNA Instruments, Italy). The water was collected in 10 L plastic containers and brought to the lab where it was first filtered through GF/C, then GF/F filters before being stored at 4°C until the start of the experiment.

2.2.2 Microcosm setup

The microcosms were confined inside glass vessels with a volume of 1 L (length=21 cm, width=15 cm, depth=7 cm)(Figure 10). The vessels were covered with a glass lid during the entire period and put on tabletop side-to-side shakers at 26 rpm (Figure 10). The experiment took place in 18°C, 18-20 PAR, 12/12 day/night cycle and lasted for 10 days.

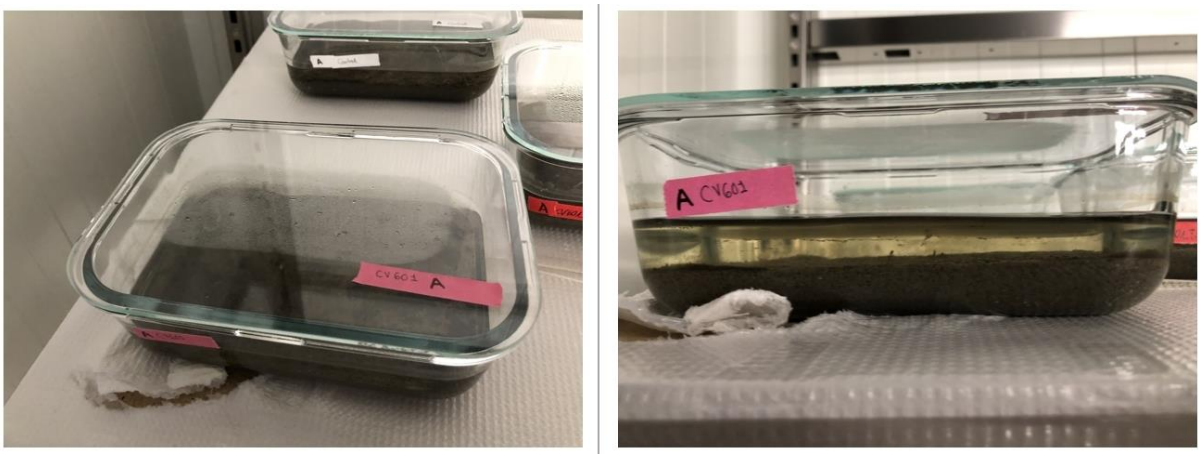

Figure 10. Setup of vessel. Left image shows and overhead view of the vessel with the glass lid covering the top. The right image shows a sideview of the vessel, with a layer of sediment in the bottom and a layer of water on top.

Before the start of the experiment (0 days), an acclimation period of 4 days was conducted. A total of 12 glass vessels (individual microcosms) were used in this study (Figure 11). Inside each glass vessel 400 ml (735 gram) of sediment was added evenly, and 300 ml filtered water with added nutrients (P and N in the form of KH_2PO_4 and NH_4Cl, nominal concentration of P-PO_4^{3-} at 50 µg/L and N-NH_4^+ at 40 µg/L) was added on top of the sediment using the "layering" technique. Water was not renewed within the whole experiment (static system). General water physicochemical parameters, i.e pH, oxygen dissolved, temperature and conductivity, were measured during some days of the experiment in the Control vessels (see section 8.4) using a multiparameter instrument (HANNA Instruments, Italy). In average, with standard deviations written, the pH was 6.64 ± 0.24, dissolved oxygen was 7.4 ± 0.82 mg/L, temperature was 19.5 ± 0.65 °C, and conductivity was 132.4 ± 27.7 µS/cm, n=13.

Figure 11. Setup of the microcosm vessels inside the thermoconstant room. The vessels are placed on top of the tabletop side-to-side shakers that were used during the experiment.

Five different treatments were setup for the experiment. All treatments contained sediment and river water. The treatments were Control (no exposure), Abx (exposed to a mixture of antibiotics, CV601-T (exposed to CV601-T bacteria), and CV601-T+Abx (exposed to CV601-T and a mixture of antibiotics (Table 6). A treatment with CV601 was also made, but has been excluded from the rest of the thesis for clarity, as it did not differ noticeably from CV601-T. All treatments were performed in triplicate.

2.2.3 *E. coli* strains

The *E. coli* used were kindly donated by Åsa Sjöling, Professor at the University of Gothenburg. The *E. coli* strain CV601 was used in this study, as a modified 18CV601 transconjugant version referred to as CV601-T in this thesis. CV601 is a frequently used strain identified by its expression of the green fluorescent protein (GFP) see Figure 12, as well as its resistance towards Kanamycin and Rifampicin (Heuer et al., 2002). The CV601-T was created as a result of conjugation between an environmental *E. coli,* SE-HDI-W-EcE1 and CV601 reported in a master's thesis (Sheikh, 2024). SE-HDI-W-EcE1 was isolated from Henriksdal WWTP in Nacka, Stockholm, in 2024. It contains an IncN conjugative plasmid (plasmid 18), that gives resistance towards multiple antibiotics seen in Table 3. The CV601-T also has additional resistances that can be seen in the table. The IncN plasmid was found have high rates of conjugation as a result of high expression levels of its transfer genes. The plasmid encodes a rigid, but flexible pilus that was found to be optimal at conjugation in solid medium, such as on agar plates. In the thesis it was theorized that conjugation could be limited in liquid medium, because movements are known to be able to break similar pili (Bradley et al., 1980). This study focused on the ARGs located in the plasmid, as mobile ARGs with clinical interest. These are *blaCTX-M-15* that give resistance to β-lactams, *qnrA1* that provides resistance to Ciprofloxacin and *drfA14* that provides resistance to Trimethoprim.

Table 3. Resistance genes contained in the CV601-T used in the study. (Åsa Sjöling, personal communication, 7th November 2024). Resistance mechanisms of each gene are shown in the table, and their respective references.

Gene	Resistance mechanism	Reference	Antibiotics target	Gene location
blaCTX-M-15	Hydrolysis of β-lactams	(Mazumder et al., 2020)	β-lactams	Plasmid 18
qnrA1	Protein modification	(Tran et al., 2005)	Ciprofloxacin	Plasmid 18
drfA14	Enzyme substitution	(Miranda et al., 2016)	Trimethoprim	Plasmid 18
blaTEM	Hydrolysis of amide bond in β-lactams	(Muhammad et al., 2014)	β-lactams	Unknown
tetA	Efflux antiporter pump	(Bannam & Rood, 1999)	Tetracycline	Unknown
mdfA	Multidrug efflux pump	(Edgar & Bibi, 1997)	General	Chromosomal

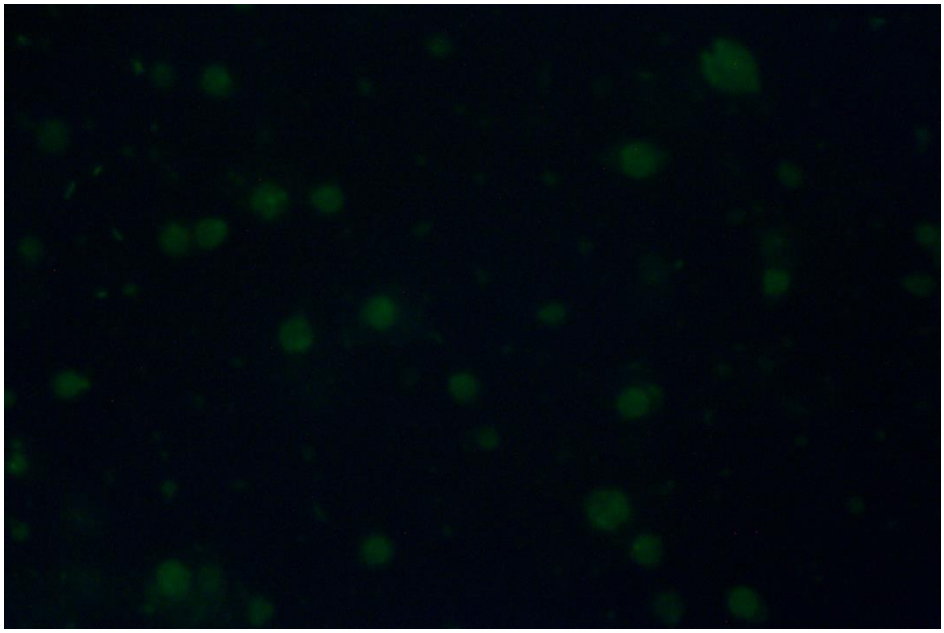

Figure 12. Image of the E. coli strain CV601 under a fluorescent microscope.

2.2.4 Antibiotic mixture

The antibiotic mixture used in the experiment contained three different antibiotics: Cefotaxime (CAS 64485-93-4), Ciprofloxacin (CAS 85721-33-1), and Trimethoprim (CAS 738-70-5). All three antibiotics were purchased from Sigma-Aldrich. Antibiotic stock solutions were prepared by dissolving Cefotaxime in distilled water (0.5 mg/mL), Ciprofloxacin in 0.1 N HCl (1 mg/mL), and Trimethoprim in DMSO (10 mg/mL). The solutions were aliquoted and stored at -80°C until use. Prior to the experiment, the stocks were diluted with distilled water to prepare an intermediate solution containing Cefotaxime (8 µg/mL), Ciprofloxacin (2 µg/mL), and Trimethoprim (20 µg/mL). An aliquot of this intermediate solution was then mixed with river water to achieve the nominal concentrations listed in Table 4.

The choice of the antibiotic mixture was based upon the mobile ARG genes within the CV601-T transconjugant made them suitable. Ciprofloxacin and Trimethoprim were also selected based on their frequent use, and consequently their occurrence in the environment. They have also been added to the European Surface Water Watch List under the EU Water Framework Directive (Decision 2020/1161). Additionally, all three compounds can be found on WHO's "List of Essential Medicine" (WHO, 2023).

The test concentrations used were based on the lowest concentration tested that showed effects in other studies (Table 5). The decision to use the lowest concentration that had an effect was based on it being the more environmentally probable concentrations for each antibiotic. As cefotaxime is generally readily degraded in the environment and is thus difficult to detect in the environment with current methods. The measured environmental concentrations (MEC) for the chemical were based concentrations found in wastewater treatment plant effluent. For ciprofloxacin and trimethoprim, the MEC reported in Table 5 refers to the highest concentration found in surface waters in Europe.

Table 4. Antibiotic mixture used in the microcosms, with the nominal concentration added to each microcosm.

Compound	Antibiotic class	Nominal concentration (µg/L)
Cefotaxime	β-lactam	4
Ciprofloxacin	Quinolones	1
Trimethoprim	Diaminopyrimidine	10

Table 5. Overview of antibiotic compounds used in the mixture. The minimum inhibitory concentration (MIC) represents the lowest concentration of an antibiotic that inhibits visible growth of a microorganism. According to EUCAST guidelines, Enterobacterales are classified as 'Susceptible' when the MIC is at or below the defined breakpoint written in the table, for a given antibiotic, based on standardized testing methods. (EUCAST, 2025)*. The MEC refers to the highest measured environmental concentration within Europe in surface waters or WWTP effluent. Effect conc. is the lowest concentration that found an effect in the referenced study.*

Compound	MIC (mg/L)	MEC (µg/L)	Ref.	Effect conc. (µg/L)	Ref.
Cefotaxime	1	0.87	(Rodriguez-Mozaz et al., 2020)	4	(A. K. Murray et al., 2020)
Ciprofloxacin	0.25	59.3	*NORMAN EMPODAT Database - Chemical Occurrence Data*, 2024	1	(Kraupner et al., 2018)
Trimethoprim	4	65	*NORMAN EMPODAT Database - Chemical Occurrence Data*, 2024	10	(Kraupner et al., 2020)

2.2.5 E. coli invasion

E. coli CV601-T was incubated overnight in 30 ml of LB media inside a 50 ml Falcon tube at 220 rpm, 37°C. The overnight culture was measured on a spectrophotometer, measuring OD_{600}. Based on previous reports the concentrations of bacteria at OD_{600}=1 was assumed to be 10^9 cells/ml (Beal et al., 2020; Sezonov et al., 2007; Volkmer & Heinemann, 2011). The Falcon tube was then centrifuged at 4600xg for 5 minutes, and the supernatant removed. The pellet was resuspended in filtered water and then diluted to a concentration of 6000 cells/mL in the 300 mL of water prepared in step 2.2.6.

2.2.6 Exposure

The water from the acclimation period was removed from the vessels after 4 days using 50 ml plastic syringes. Into each vessel 300 ml of fresh filtered river water with added nutrients (nominal concentration of $P\text{-}PO_4^{3-}$ at 50 µg/L and $N\text{-}NH_4^+$ at 40 µg/L from KH_2PO_4 and NH_4Cl)

as well as *E. coli* and antibiotics corresponding to each treatment using the "layering" technique, Table 6. The antibiotic mixture used can be found in Table 4 (see section 2.2.4.).

Table 6. Experimental setup for each treatment.

Treatment	E. coli	Antibiotics	Nutrients
Control	-	-	KH_2PO_4 and NH_4Cl
Abx	-	Mixture	KH_2PO_4 and NH_4Cl
CV601-T	CV601-T	-	KH_2PO_4 and NH_4Cl
CV601-T + Abx	CV601-T	Mixture	KH_2PO_4 and NH_4Cl

2.2.7 Sampling

Samples were collected by hand with a plastic syringe (Codan Luer 5 ml, REF62.5202) with the tip cut off (Figure 13,A). The upper centimetre of sediment of the syringe cores were used as samples (Figure 13, B). In total, 5 cores per sampling day were collected, one for the β-glucosidase assay, one for the nitrification assay, one for qPCR and 16S sequencing, and one for the bacterial community in glycerol stocks, and stored at -80°C. The sampling was done before the initial exposure (t0, 0 days, 9 am) as well as at 9 am on day 1, 3, 7 and 10 following the exposure.

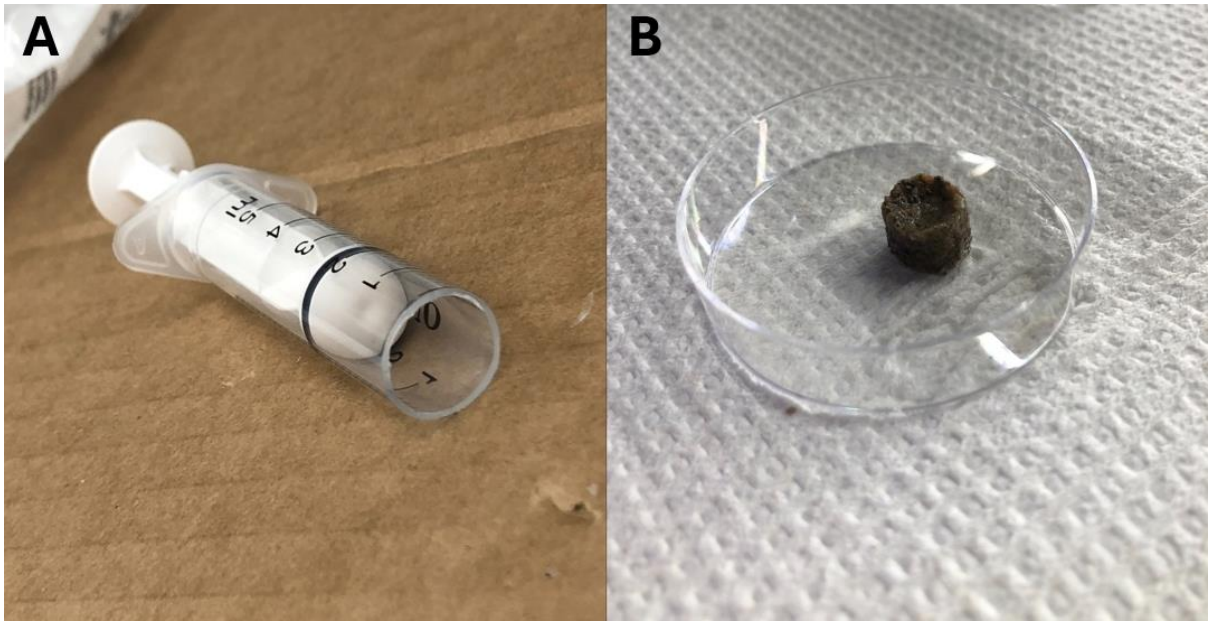

Figure 13. A) shows the sampling syringes used to collect sample cores with the tip cut off. B) shows a collected sample core in a petri dish.

2.2.8 *E. coli* establishment in sediment

The survival and establishment of the invading *E. coli* CV601-T was assessed by multiple methods. Initially, the cores collected for glycerol storage of bacterial communities were placed inside scintillation vials, 1 ml river water was added and the vials shaken for 1 hour at

600 rpm. The supernatant was put into 15 ml Falcon tubes and centrifuged at 4560xg for 5 minutes. The supernatant was removed, and the pellet resuspended in 250 µL PBS solution (NaCl 8g/L, KCl 0.2g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24g/L) and put into cryo-vials. 250 µL glycerol solution (50%) was then added and the bacteria stored at -80°C.

At first, fluorescent microscopy was attempted to be used as a method to track the GFP-tagged CV601-T, but because of background fluorescence in the sediment, coming from either anthropogenic contamination, organic sources, or both, the method proved unusable. Next, plating was attempted to track the CV601-T. 50 µL of each glycerol stock from the CV601-T and CV601-T+Abx treatments were plated onto ChromoSelect Agar B (Sigma Aldrich, 70722), a selective medium for *E. coli*, containing 50 µg/mL of Kanamycin and Rifampicin. Duplicates for all CV601-T exposed treatments were made, as well as positive and negative controls with duplicates. The plates were incubated at 37°C overnight. Finally, qPCR was used to track the resistant bacteria by targeting the *Gfp*-gene, which is further described in section 2.2.11.

2.2.9 β-glucosidase assay

The assay is based on β-glucosidase enzyme activity and works by incubating samples with Methylumbelliferyl β-D-glucopyranoside (MUD), which is metabolised to β-D-Glucose and 4-methylumbelliferone (MUF) by the enzyme (Figure 14). The method was adapted from (Corcoll et al., 2014). During sampling one collected core from each vessel was weighed and added to separate 15 ml Falcon tubes. Into each tube 4 ml of 0.22 µm filtered river water was added. 4-Methylumbelliferyl β-D-Glucopyranoside (Sigma Aldrich M3633) was added to each tube to a concentration of 0.3 mM and all samples incubated for 1 hour in the dark while shaking, 150 rpm, at room temperature. A calibration curve with 4-methylumbelliferone was made each sampling day and incubated together with the samples. After incubation, 4 ml of Glycine buffer (0.2 M, pH 10.4) was added to each sample tube. The results were then read using a plate reader at 365/455 nm excitation/emission (Varioskan Flash, Cat. No. 5250030) . Results were standardized to the weight of wet sediment collected as well as the dilution with river water.

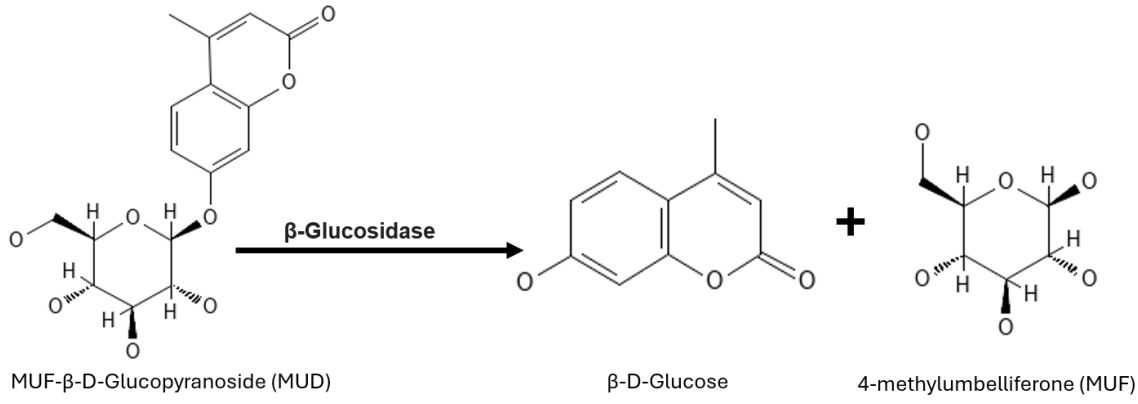

Figure 14. Chemical reaction of the β-Glycosidase enzyme on the MUD substrate. Reaction products are β-D-Glucose and MUF, the MUF is fluorescent and can therefore be detected.

2.2.10 Nitrification

The cores were collected in 15 ml Falcon tubes, into which 10 ml of NH4-N (5 mg/L in PBS) was added. The tubes were then incubated in the dark for 150 minutes while shaking on a tabletop 150 rpm. The solution from each tube was filtered through a 0.45 µm PES filter (Avantor, Cat. No. 514-1261). The samples were then stored at -20°C and afterwards sent for analysis of NO_2^- levels at Kristineberg Center for Marine Research and Innovation following the Grasshoff, Methods of Seawater Analysis,1999 with an QuAAtro, XY-3 Sampler, Seal Analytical 2015. The nitrite was analysed spectrophotometrically by the SS-EN 26777 (quantification range: 0.05 – 35 µmol/L).

2.2.11 Antibiotic resistance in the sediment

DNA was extracted from the cores using the DNeasy PowerSoil Pro Kit (Qiagen, ID 47014) with the provided manual, and stored at -20°C. The concentrations of DNA was measure using a Qubit fluorometer 3.0 (Life Technologies, USA), and the purity was measured using a NanoDrop spectrophotometer. Quantitative Polymerase Chain Reaction (qPCR) was used to determine the levels of antibiotic resistance genes in the samples. The *rpoB* gene was used as a reference gene to be able to normalize the levels of ARGs in the samples. Primers used for qPCR are shown in Table 7. The SYBR Green Supermix (BIO-RAD, Cat. 1725270) was used for the qPCR reactions. Each reaction contained 5 µL 2X SYBR Green qPCR Master mix (BIO-RAD), 500 nM of each primer, and 4 µL of extracted DNA at 2.5 ng/µL for a total reaction volume of 10 µL. The qPCR cycle settings for each gene can be found in Table 8.

Table 7. Primers used for qPCR are shown for each respective gene and sequence. The sequences are arranged with forward primers written on top, and reverse sequences on the bottom for each gene. References for the sequences are shown on the right.

Gene	Target type	Sequences	Reference
rpoB	Bacterial RNA polymerase	5'-AACATCGGTTTGATCAAC-3' 5'-CGTTGCATGTTGGTACCCAT-3'	(Dahllöf et al., 2000)
blaCTX-M-15	B-lactam degradation	5'-CTATGGCACCACCAACGATA-3' 5'-ACGGCTTTCTGCCTTAGGTT-3'	(Sorel Ngou, 2023)
Gfp	Green-fluorescent protein	5'-CTGTCGACACAATCTGCCCT-3' 5'-CCACATCGGCCAGATCGTTA-3'	(Jutkina et al., 2016)

Table 8. qPCR protocol for each gene that was amplified. Settings described inside parenthesis were repeated in cycles which is written within each parenthesis.

Gene	qPCR protocol	Reference
rpoB	98°C 3 min, (98°C 15 sec, 50°C 90 sec, 72°C 90 sec, 30 cycles), 72°C 10 min	(Sorel Ngou, 2023)

blaCTX-M	98°C 3 min, (98°C 15 sec, 60°C 30 sec, 40 cycles), 95°C 5 sec	(Sorel Ngou, 2023)
Gfp	94°C 3 min, (94°C 30 sec, 55°C 30 sec, 72°C 40 sec, 35 cycles), 72°C 5 min	(Jutkina et al., 2016)

2.2.12 Data analysis

Data analysis for means and standard deviations were calculated in R Studio. The data was analysed using Leven's test for homogeneity of variance and Shapiro-Wilk's test for normality in R Studio. Two-way repeated ANOVA, Post-hoc pairwise Tukey test and plots were created in R Studio. R Studio version 4.3.3 was used throughout the entire data analysis, and scripts can be provided upon request. All treatments were standardized per gram of sediment in wet weight.

3 Results

3.1 Pilot study – E. coli survival and inoculation techniques

The number of surviving *E. coli* was substantially higher at the highest tested concentration (10^7 cells/mL) compared to the lowest concentration (6,000 cells/mL), as shown in Figure 15 and Table 9. At 10^7 cells/mL, no significant difference was observed between the "mixing" and "layering" methods at day 3 or day 6. However, by day 28, the "mixing" treatment still had an uncountable number of *E. coli* colonies, whereas the "layering" plate only had seven countable colonies. In contrast, at 6,000 cells/mL, all days yielded countable colony numbers. After 3 days, the "mixing" treatment produced 214 colonies, approximately 35% more than the 158 observed in the "layering" treatment. By day 6, the "layering" treatment yielded a higher colony count, and by day 28, only a single colony was visible on the "mixing" plate.

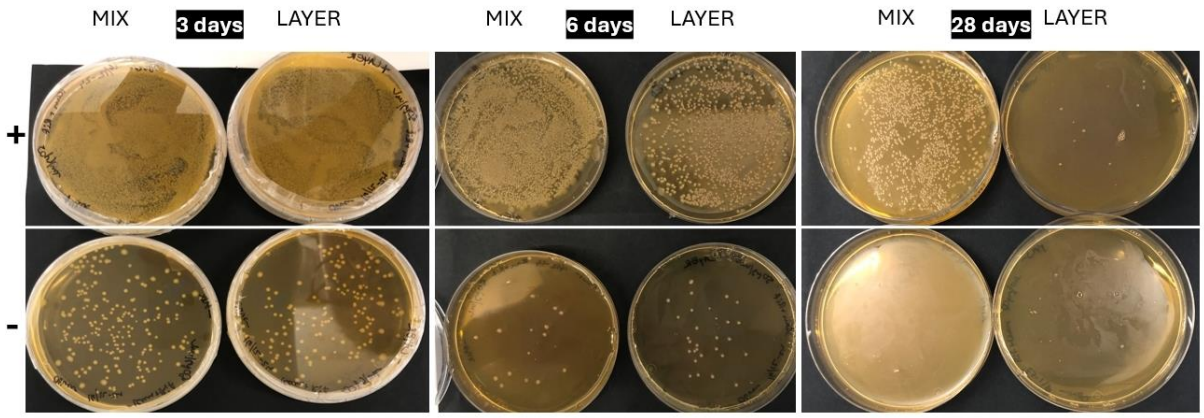

Figure 15. Image of plates each sampling day. MIX refers to the mixing technique, and LAY refers to the layering technique described in the methods and materials section. (+) refers to 10^7 cells/ml concentration of E. coli, and (-) refers to 6000 cells/ml of E. coli.

Table 9. Results from the ImageJ colony count. When plates contained more than 250 colonies these were regarded as too numerous to count, because it was not possible to differentiate between separate colonies. No colonies grew on plates from either control vessel.

Inoculation method	E. coli concentration (cells/ml)	3 days	6 days	28 days
Mixing	**10^7**	>250	>250	>250
Mixing	**6000**	214	17	1
Layering	**10^7**	>250	>250	7
Layering	**6000**	158	29	0

3.2 Full-scale study

3.2.1 Bacterial abundance

The general trend of all treatments showed a decline in *rpoB* copies per gram of sediment throughout the experiment (Figure 16). Overall, the treatments decreased from day 0 to day 10 with a factor of about 10. Initial *rpoB* gene copy numbers were approximately 2–3 × 10^8 copies per gram of sediment, decreasing to around 2–3 × 10^7 copies per gram by the end of the experiment. The treatments show differing trends between day 0 and day 1. Both the Control and the CV601-T treatment increased in relative rpoB copy numbers between those days, while both treatments exposed to the antibiotic mixture decreased in relative *rpoB* copy numbers the first day.

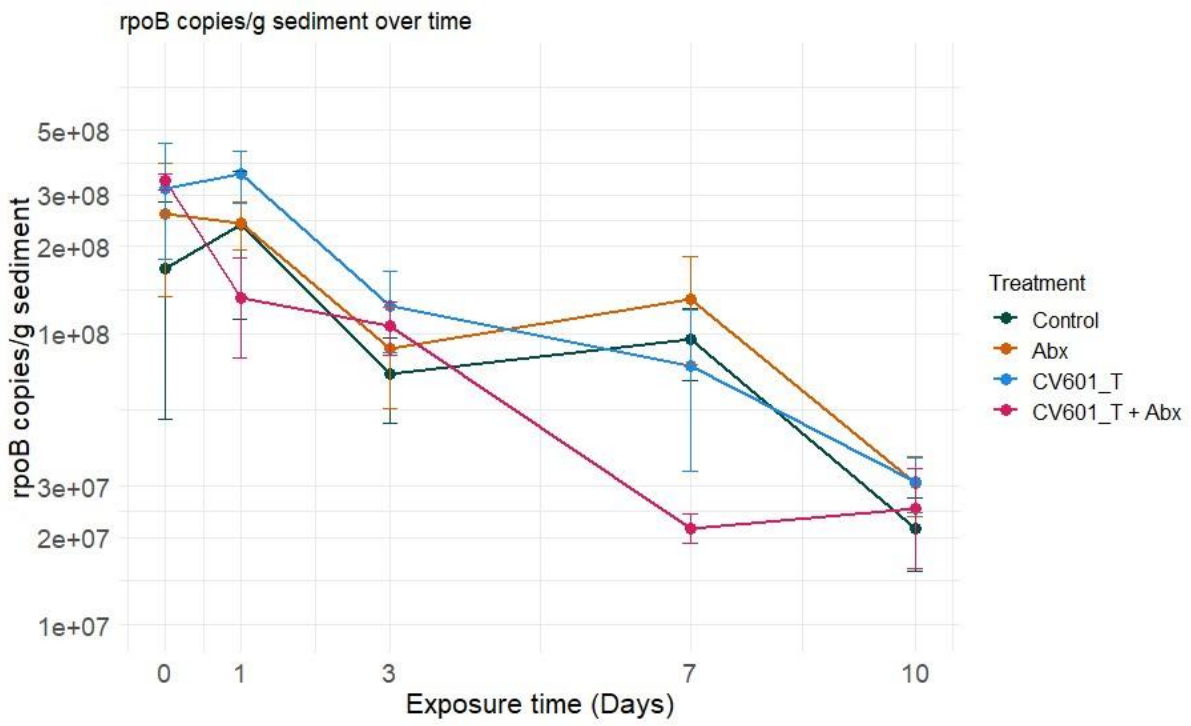

Figure 16. Abundance of the rpoB gene (in copy numbers per gram of sediment) over the exposure time (0, 1, 3, 7 and 10 days) for all treatments. Dots represent the mean, and error bars represent the standard deviation (n=3).

The ANOVA analysis showed that there was no significant difference between treatments over the exposure time (Table 10). Similarly, there was no significant effect on the interaction between how treatments varied over time (Treatment x Day). However, the analysis showed that time had a significant effect on the *rpoB* copy numbers. Post-hoc Tukey test to comparing treatments within each day found no significant difference either. However, comparing days within each treatment showed that all treatments changed significantly throughout time (Table 11).

Table 10. Two-way ANOVA of repeated measurements results from the rpoB gene copies/g sediment.

Factor	p-value
Treatment	0.529
Day	<.001
Treatment x Day	0.094

Table 11. Post-hoc Tukey test from the rpoB gene copies/g sediment comparing differences between days within each treatment. Only significant differences are shown, were p < 0.05. No significant difference between treatments for any day was found.

Treatment	Day	p-value
Control	1-3	0.0393
	1-10	0.0263
Abx	1-10	0.0311
	7-10	0.0225
CV601-T	0-7	0.0310
	0-10	0.0305
	1-3	0.0066
	1-7	0.0094
	1-10	0.0025
	3-10	0.0113
CV601-T + Abx	0-7	0.0069
	0-10	0.0201
	3-10	0.0241

3.2.2 E. coli establishment in sediment

Quantifying the *E. coli* CV601-T using fluorescent microscopy was not possible because of background levels of fluorescence. Plating yielded no colonies on any plates expected the positive controls and could therefore not be used to quantify CV601-T. qPCR of the *Gfp*-gene was then attempted. A trial was done with DNA extracted from culture containing only CV601-T, as well as with DNA extracted directly from the field sediment, and a negative control (Figure 17). Amplification occurred for both the CV601-T sample (green) and field sample (blue), indicating that there was natural occurrence of a similar amplicon. However, the melt peaks were distinctly different between the samples (Figure 18). CV601-T had a clear melt peak close to 85°C, while the field sample had a melt peak around 90°C.

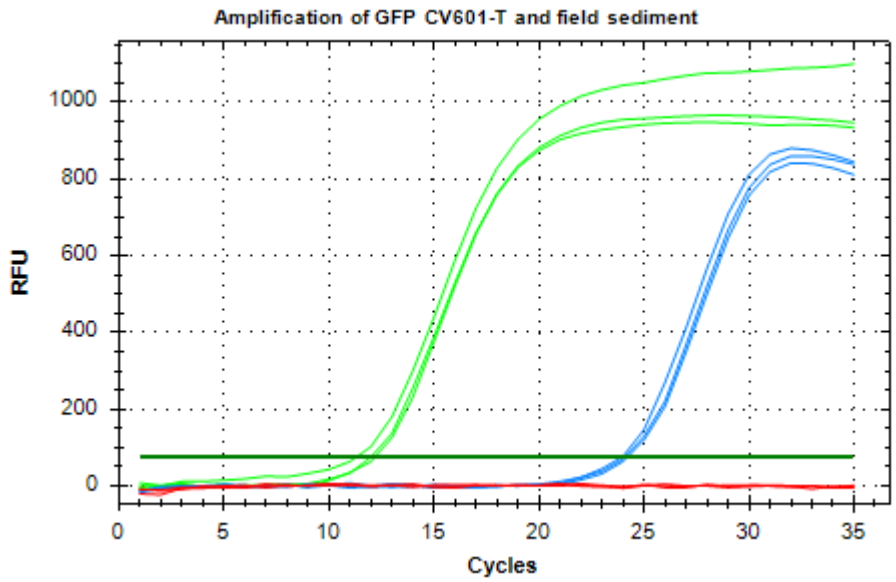

Figure 17. Amplification curves of the Gfp-gene using CV601-T culture DNA (green curves), and field sample DNA (blue curves). Negative controls are shown in red.

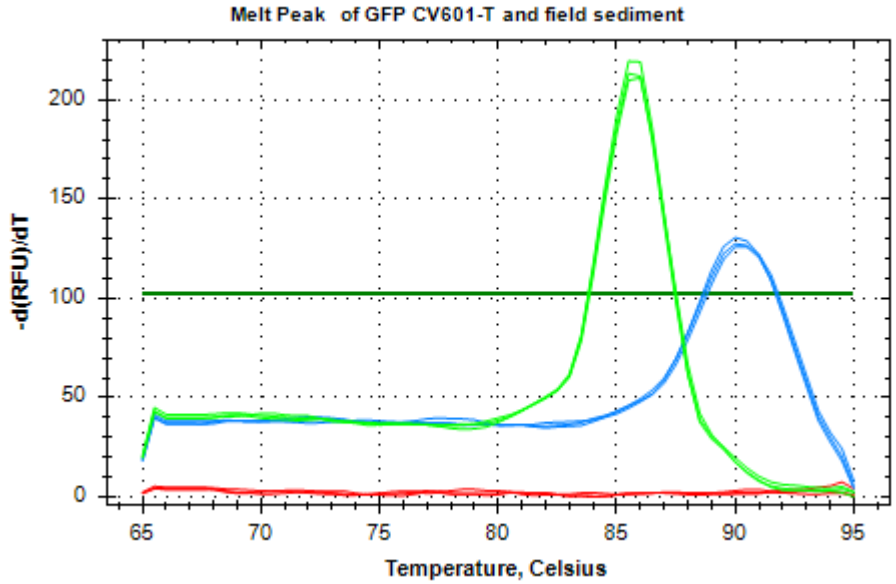

Figure 18. Melt peak curves of the Gfp-gene using CV601-T culture DNA (green curves), and field sample DNA (blue curves). Negative controls are shown in red.

When qPCR of *Gfp*-gene was attempted using the DNA extracts from the samples cores during the experiment there was no noticeable difference between amplification curves for any of the treatments (see Figure 19, and Appendix Figures S6, S8, and S10). All treatment samples yielded a single melt curve peak at around 90°C, while the positive controls with CV601-T had single peaks around 85°C (see Figure 20, and Appendix Figures S7, S9, and S11). Therefore, no qPCR amplification of the *Gfp*-gene from CV601-T could be identified in any of the treatments.

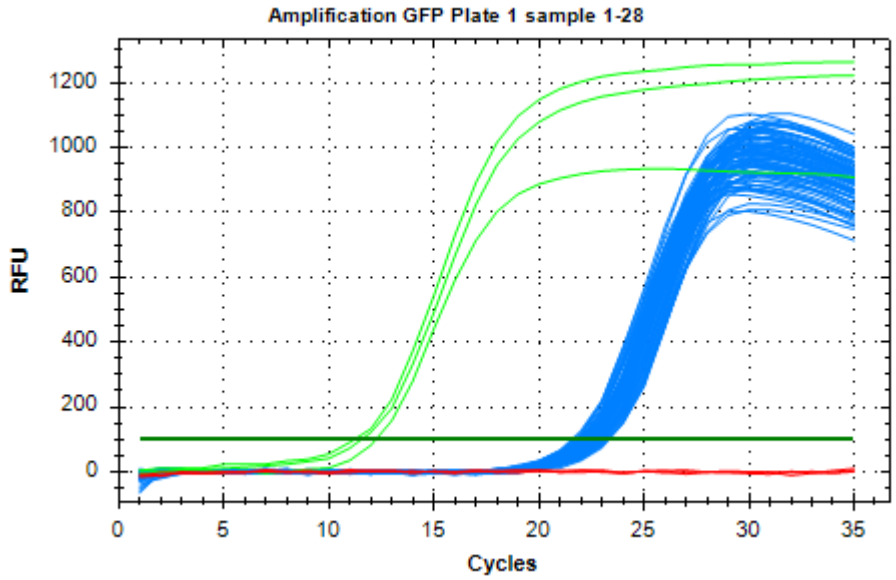

Figure 19. Amplification curves of the Gfp-gene using CV601-T culture DNA (green curves) and DNA extracted from the cores (blue curves). This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

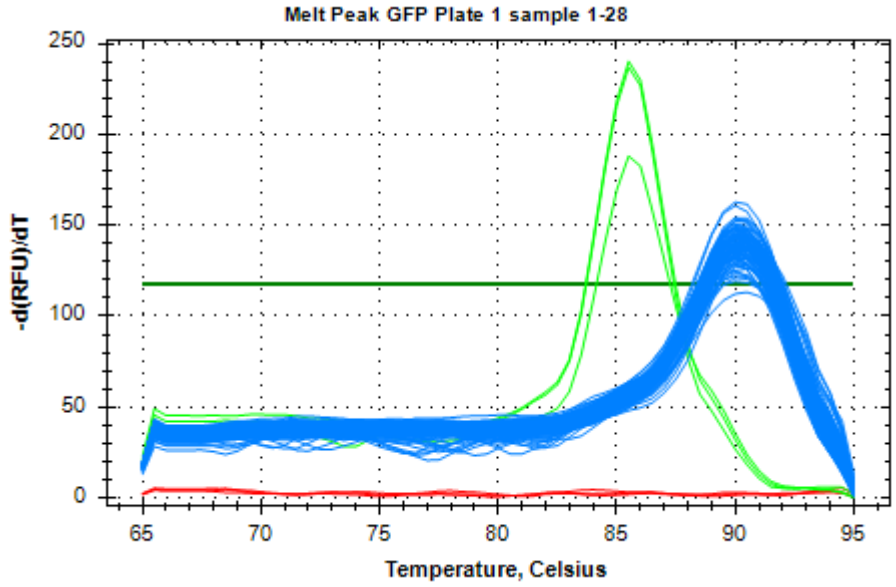

Figure 20. Melt peak curves of the Gfp-gene using CV601-T culture DNA (green curves) and DNA extracted from the cores (blue curves). This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

3.2.3 Antibiotic resistance genes

The gene *blaCTX-M* was detected in all treatments. At the start of the experiment (t0), the level of *blaCTX-M* genes was comparable between treatments (Figure 21). Notably, there were background levels of the gene in the sediment from the beginning. Both CV601-T exposed treatments increased significantly between day 0 to day 1 and then declined at day 3. Treatment Abx increased through days 1 and 3, while the Control treatment initially declined the first day, then increased the rest of the experiment. After 3 days all treatments remained mostly stable for the remaining duration of the experiment. At the end of the experiment the treatments exposed to the *E. coli* remained higher compared to the Control and Abx treatments.

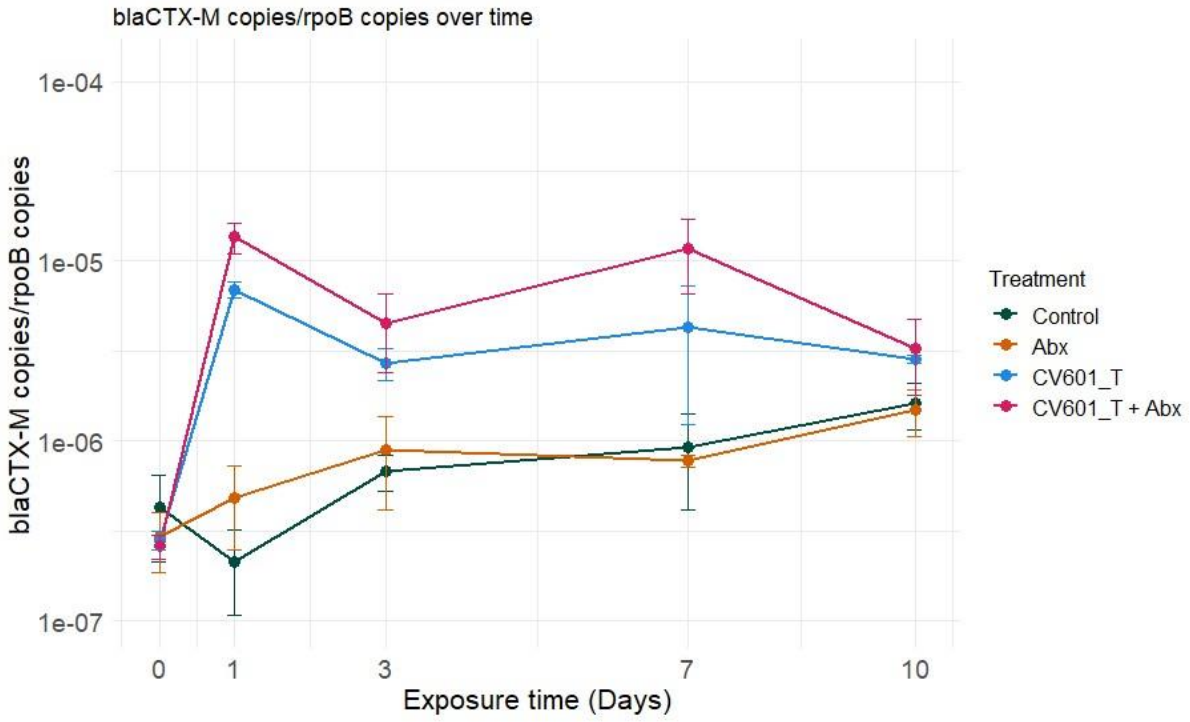

Figure 21. Abundance of the blaCTX-M gene (in copies relative to the rpoB gene copies) for all treatments over the exposure time (0, 1. 3 , 7 and 10 days). Dots represent the mean, and error bars represent the standard deviation (n=3).

The ANOVA showed that all factors have significant effects (Table 12). Tukey test showed that both treatments with CV601-T changed significantly over time. There were also significant differences between treatments on day 1, day 3, and day 7 (Table 13). Notably, there were significant differences between CV601-T and CV601-T+Abx on day 1 (p=0.0055).

Table 12. Two-way ANOVA of repeated measurements results from the blaCTX-M /rpoB gene copies.

Factor	p-value
Treatment	<.001

Day	<.001
Treatment x Day	<.001

Table 13. Summary of the post-hoc Tukey test results for the blaCTX-M/rpoB copy numbers, that show a significant difference were p < 0.05. On the left side the differences between days within each treatment are shown. On the right side the differences between treatments for each day are shown.

Treatment	Day	p-value	Day	Treatment	p-value
CV601-T	0-1	0.0010	**1**	Control – CV601-T	0.0053
	0-10	0.0138		Control – CV601-T+Abx	<.0001
	1-3	0.0082		Abx – CV601-T	0.0067
CV601-T + Abx	0-1	<.0001		Abx – CV601-T+Abx	0.0001
	0-3	0.0046		CV601-T – CV601-T+Abx	0.0055
	0-7	0.0044	**3**	Control – CV601-T+Abx	0.0355
	0-10	0.0053		Abx – CV601-T+Abx	0.0462
	1-3	<.0001	**7**	Control – CV601-T+Abx	0.0291
	1-10	0.0004		Abx – CV601-T+Abx	0.0272

3.2.4 β-glucosidase assay

The MUF degradation increased over time for all treatments (Figure 22). The trend was stable for most of the treatments on most days, except for Control that declined on day 1 and day 7, and Abx that declined on day 7. The MUF degradation of CV601-T+Abx had the highest increase after the first day of the experiment. At day 10 all treatments had higher degradations rates relative to the *rpoB* copy number than they had at the start. The Control treatments started at higher MUF degradation rates than the other treatments, with significant differences found on the Tukey test (Table 15). Notably, the Control and Abx treatments follow very similar patterns over time.

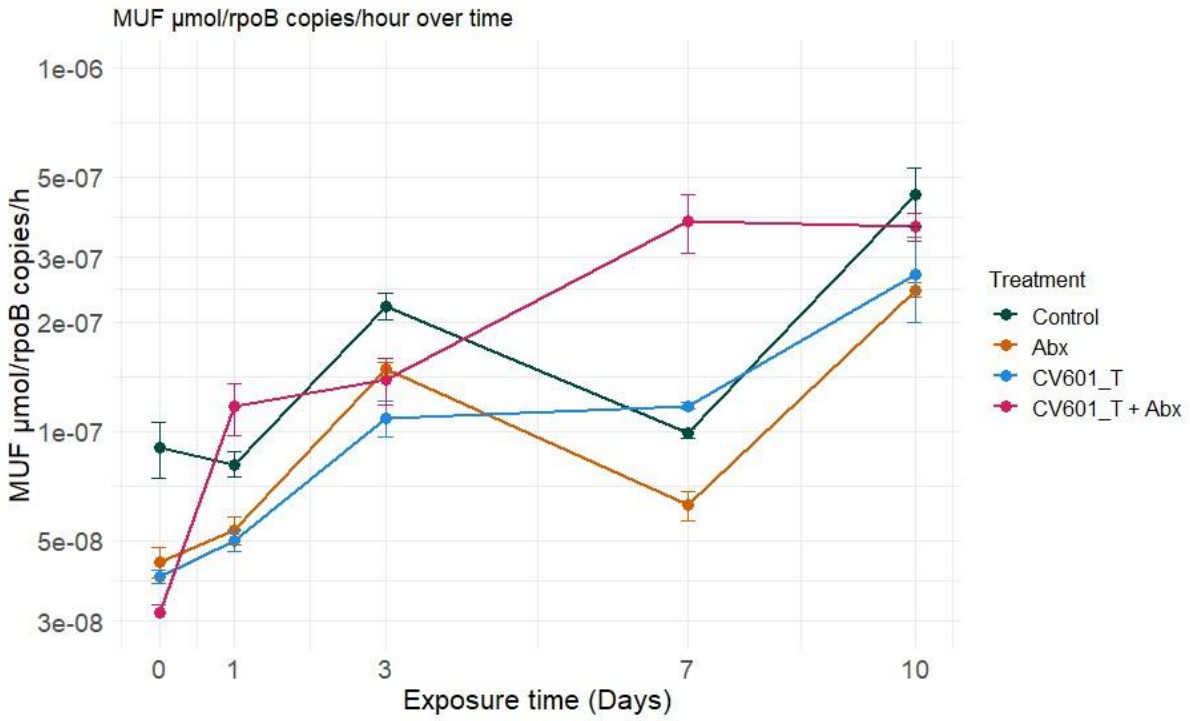

Figure 22. Average values of MUF degradation relative to the rpoB copy numbers for each treatment over time (0, 1. 3, 7 and 10 days). Dots represent the mean, and error bars represent the standard deviation (n=3).

The ANOVA identified all factors as significant for the MUF degradation relative to *rpoB* copy numbers (Table 14). There were significant differences from the Tukey test between treatments for all days expect the final day (Table 15). Similarly, the test identified that all treatments had significant changes throughout time, with multiple timepoints being significantly different for all treatments.

Table 14. Two-way ANOVA of repeated measurements results from the MUF degradation relative to the rpoB copy numbers for each treatment.

Factor	p-value
Treatment	<.001
Day	<.001
Treatment x Day	<.001

Table 15. Summary of the post-hoc Tukey test results for the MUF degradation relative to the rpoB copy number, that show a significant difference were p < 0.05. On the left side the differences between days within each treatment are shown. On the right side the differences between treatments for each day are shown.

Treatment	Day	p-value	Day	Treatment	p-value
Control	0-3	0.0001	**0**	Control – Abx	0.0020
	0-10	0.0001		Control – CV601-T	0.0012
	1-3	<.0001		Control – CV601-T + Abx	0.0004

	1-10	0.0001	**1**	Control – CV601-T + Abx	0.0378
	3-7	0.0066		Abx – CV601-T + Abx	0.0014
	3-10	0.0037		CV601-T – CV601-T + Abx	0.0010
	7-10	0.0005	**3**	Control – Abx	0.0065
Abx	0-3	0.0004		Control – CV601-T	0.0004
	0-10	0.0045		Control – CV601-T + Abx	0.0030
	1-3	<.0001	**7**	Control – CV601-T + Abx	0.0002
	1-10	0.0103		Abx – CV601-T + Abx	0.0001
	3-7	0.0451		CV601-T – CV601-T + Abx	0.0003
	7-10	0.0300			
CV601-T	0-3	0.0059			
	0-10	0.0019			
	1-3	0.0008			
	1-10	0.0044			
	3-10	0.0266			
CV601-T + Abx	0-1	0.0003			
	0-3	0.0003			
	0-7	<.0001			
	0-10	0.0001			
	1-7	0.0001			
	1-10	0.0020			
	3-7	0.0001			
	3-10	0.0037			

3.2.5 Nitrification

The nitrification (measured as nitrite per *rpoB* copy numbers) increased over time for all treatments (Figure 23). Both treatments exposed to the antibiotic mixture increase in relative nitrite levels the first three days, after which Abx declined on day 7, while CV601-T+Abx continued to increase. On day 7 both treatments exposed to the *E. coli* show higher nitrite levels compared to the other two treatments, but after 10 days there is no noticeable difference between any treatment.

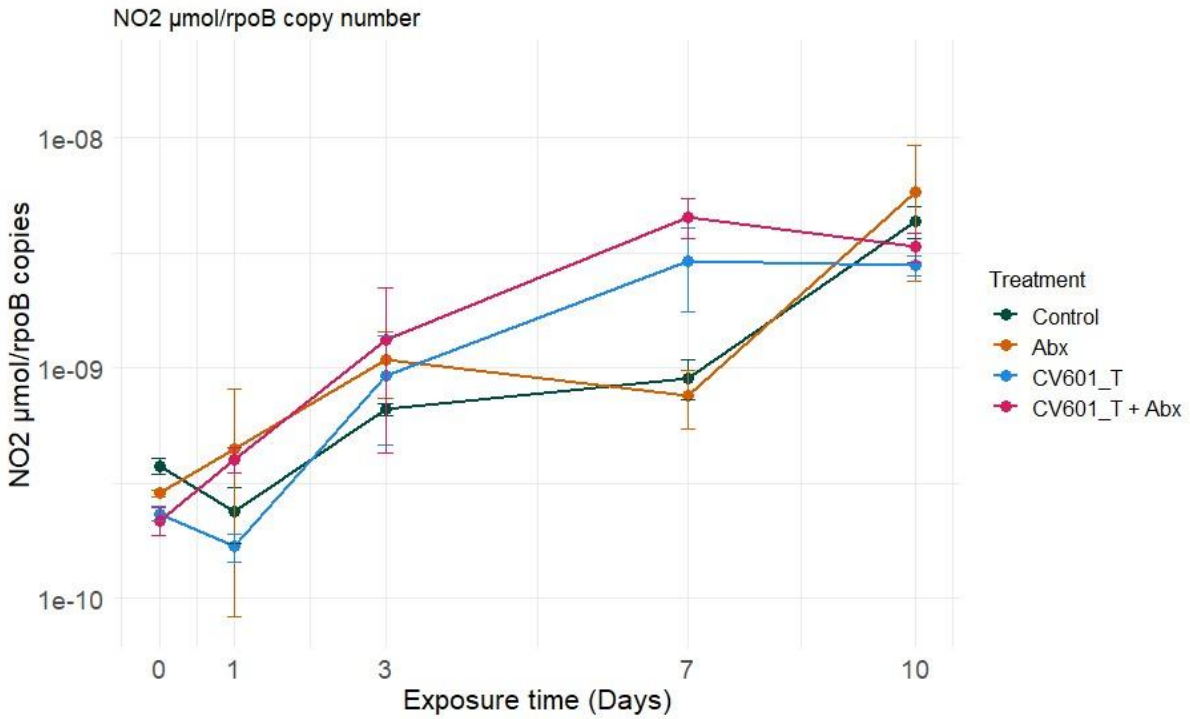

Figure 23. Nitrification rates (of NO_2^- µmol/rpoB copies) over time for each treatment. Dots represent the mean, and error bars represent the standard deviation (n=3).

According to the ANOVA test, no significant effects among treatments were observed (Table 16). However, there were significant effects over time, as well as significant effects for the combined factors (Treatment x Day). This indicates that the pattern of change throughout the experiment depend on the type of treatment. The post hoc Tukey test showed that Abx, CV601-T, and CV601-T+Abx had significant changes throughout time (Table 17). Furthermore, on day 0 there was significant differences between Control and the other three treatments, as well as between Abx and CV601-T+Abx on day 7.

Table 16. Two-way ANOVA of repeated measurements results from the nitrite levels to levels of rpoB gene copy numbers for all treatments.

Factor	p-value
Treatment	0.415
Day	<.001
Treatment x Day	0.007

Table 17. Summary of the post-hoc Tukey test results for nitrite levels relative to the rpoB copy number, that show a significant difference were $p < 0.05$. On the left side the differences between days within each treatment are shown. On the right side the differences between treatments for each day are shown.

Treatment	Day	p-value	Day	Treatment	p-value
Abx	0-10	0.0138	**0**	Control – Abx	0.0245
	1-10	0.0220		Control – CV601-T	0.0016
	3-10	0.0301		Control – CV601-T + Abx	0.0008
	7-10	0.0270	**7**	Abx – CV601-T + Abx	0.0040
CV601-T	0-7	0.0058			
	1-7	0.0075			
CV601-T + Abx	0-7	0.0002			
	1-7	0.0005			
	3-7	0.0110			

4 Discussion

This study investigates the potential effects of environmentally relevant concentrations of a mixture of antibiotics, and the establishment of multidrug-resistant *E. coli* on natural communities of sediment bacteria collected from the lake Vänern. In addition, the study evaluates how the potential E.coli establishment was affected by the occurrence of antibiotics. Two experiments were conducted, one initial pilot study between December 2024 to January 2025 covering 28 days, and then a full-scale study spanning a total of 14 days between February to March in 2025. The establishment success of the invading bacterial species was analysed, as well as bacterial abundance by levels of *rpoB* gene copies, antibiotic resistance gene dissemination by measurement of *blaCTX-M* gene copies, and bacterial community function by measuring carbon metabolism and nitrification.

Effect on the bacterial abundance

Throughout the experiment, bacterial abundance, estimated by *rpoB* gene copy numbers, decreased by approximately one order of magnitude across all treatments (Figure 16). This decline was statistically significant over time but not between treatments, suggesting that environmental conditions inside the microcosms, rather than the presence of antibiotics or invading *E. coli*, were the dominant drivers of community-level bacterial loss. A likely explanation for the loss of bacterial abundance can be resource depletion. This can be supported to the analysis of nutrients, which saw depletions of ammonium and phosphate by the end of the experiment (see Appendix Table S7) and the low content of organic matter (<1%) in the tested sediment. Notably, treatments exposed to antibiotics (Abx and CV601-T+Abx) showed a sharper decrease in *rpoB* copy numbers during the first day, which could reflect an initial decline of susceptible bacteria due to antibiotic stress. However, the absence of significant treatment effects overall implies that the bacterial community was either partially resistant to the antibiotics used, or that recovery and adaptation occurred quickly. It is also possible that the levels of antibiotics were not enough to have significant effects on the bacterial community in the timeframe of the study, or did not penetrate into the sediment deep enough to affect more than the absolute surface level bacteria. Further chemical analysis on the fate of the antibiotics on the test system might help to elucidate these hypotheses. Comparatively, previous studies have indicated loss of bacterial biomass following exposure to a single antibiotic (Awad et al., 2016; Hammesfahr et al., 2011; Hou et al., 2015), however the studies employed concentrations of mg/L, higher than what is environmentally realistic. Therefore, the results in this thesis suggest that bacterial abundance is not significantly affected by pulse-exposure to realistic concentrations of antibiotics. Nevertheless, the environment receives continuous antibiotic pollution from WWTPs and other sources, and it is possible that more significant effects take place in reality.

Invading multidrug-resistant bacteria establishment in sediment

The invasion success of multidrug-resistant *E. coli* was investigated in both the pilot study and the full-scale study, although the two experiments used different types of *E. coli*, CV601 or

CV601-T respectively. The pilot study assessed two different nominal concentrations of invading bacteria, one environmentally realistic concentration at 6000 cells/mL, and a higher one at 10^7 cells/mL. It is important to note that the exact concentration of *E. coli* added is not known. Calculations were based on literary values based of OD_{600} measurements, however, research has shown that this is not the most reliable method to estimate bacterial cell density (Beal et al., 2020).

CV601 was identified in sediments at the two tested cell densities for the full duration of the pilot study (28 days). However, at the lowest tested density (i.e 6000 cells/mL) after 28 days only a colony was detected on the agar plates. These results can be supported by a similar study from Bagra and colleagues who found that *E. coli* was able to establish and survive on biofilms for at least 14 days (Bagra et al., 2023). Additionally, a positive dependency between the inoculation density and the *E. coli* detection in the sediment bacteria was observed, which highlights the importance of WWTPs to limit the release of bacteria through removal steps to reduce the invasion of alien bacteria on natural bacterial communities.

In the full-scale study it was not possible to directly track the *Gfp*-tagged CV601-T bacteria using fluorescent microscopy, likely because of autofluorescence in the sediment. Plating was not successful either. It is likely that the process of collecting and freezing the community in glycerol reduced the culturability of the bacteria, as directly plating the bacteria worked well during the pilot study. It is also plausible that the medium had an effect, as LB agar was used in the pilot study, while ChromoSelect Agar B was used during the full-scale study. Finally, qPCR of the *Gfp*-gene could not be used as a way to track the *E. coli* either. Because of the clear differences in melt curve peaks between the positive control at 85°C and the samples at 90°C, it seems to indicate that there existed a gene that yielded a similar, but distinctly different amplicon, at higher abundance in the sediment than the *Gfp*-gene from CV601-T. However, invasion success can be inferred from the levels of *blaCTX-M* in the sediment, that increased in both CV601-T treatments, looking at *blaCTX-M* copy numbers relative to *rpoB* copy numbers (Figure 21). Both treatments had higher *blaCTX-M* until the end of the experiment, indicating that either CV601-T survived the duration of the experiment, or that horizontal gene transfer (HGT) took place. Nevertheless, since the CV601-T treatment without antibiotics remained at higher *blaCTX-M* than both the Control and Abx treatment, it is probable that CV601-T survived and established in the sediment. Comparing treatment CV601-T+Abx to CV601-T there were significant differences after the first day of exposure with CV601-T+Abx having higher *blaCTX-M* levels (Table 13). This trend continued the whole exposure time (Figure 21). This indicates that the antibiotic mixture either increased the establishment and survival of CV601-T or increased the rate of horizontal gene transfer of the ARG. Both ideas are supported by previous research. Bagra and colleagues found that stressful conditions of copper exposure increased the establishment of invading *E. coli* (Bagra et al., 2023). It is possible that in this experiment the antibiotic mixture was a stress inducer which increased the establishment of *E. coli.* On the other hand, Bengtsson-Palme and colleagues highlights research that show HGT upregulated during exposure to antibiotics

(Bengtsson-Palme et al., 2018). It is conceivable that both processes occur in the experiment, and without concrete methods of tracking CV601-T it is not possible to differentiate the contribution of either factor. It is important to note that the total amount of *E. coli* was less in the full-scale study than in the pilot study in each vessel. This is a result of the full-scale study only having 300 ml of *E. coli* spiked water added to each vessel, while the pilot study had 400 ml. In total there were therefore 25% less *E. coli* in the full-scale study in each vessel. Additionally, it is important to highlight while in both experiments in this study the sediments were exposed to a pulse of invading *E. coli*, in natural environments this exposure is expected to be continuous because of continuous discharges from WWTP into recipient freshwater bodies. It is therefore likely that natural bacterial communities experience much greater exposure to invading bacteria, which most likely will increase the chance of invasion over time. This can be supported by research that suggests that even failed invasions could increase the success of future invasions (Mallon et al., 2018).

As it is presented in this study it was not possible to definitively track the establishment of CV601-T. But there are still potential ways of analysing this, either using qPCR of another unique gene, or by amending the glycerol stocks to boost bacterial growth before plating. However, due to time constraints this was not done in this thesis. Ultimately, both the concentration of *E coli* and antibiotic mixture likely play important roles in the possible invasion of *E. coli* into bacterial sediment communities. In the end it was possible to show that invading *E. coli* could establish and survive for up to four weeks in natural bacterial communities from freshwater sediment.

Antibiotic resistance gene spread within the bacterial community

Investigating the possible dissemination of antibiotic resistance genes is not directly possible within this thesis as distinguishing between horizontal gene transfer of plasmid 18, or the multidrug-resistant *E. coli* CV601-T, is not possible. Conducting qPCR with additional resistance genes present on the plasmid, or in other locations within the *E. coli,* could help elucidate many of these remaining questions. However, it was not possible to do within the timespan of this thesis.

One important discovery was the fact that *blaCTX-M* resistance genes existed within the natural sediment from the start. This highlights the near omnipresence of antibiotic resistance genes in the environment. Although the site was upstream of any WWTP, it is possible that the site experienced elevated contamination of antibiotics and bacteria due to recreational activities such as swimming. Additionally, an interesting trend was found in all treatments as the relative levels of *blaCTX-M* increased during the exposure period. This suggests that environmental conditions inside the microcosms favoured bacteria that carried the resistance genes. It is not clear what this cause could be, but one explanation would be that such bacteria carry additional genes to deal with other stressors, and that the conditions inside the microcosms exerted stress on the community. There could then be co-selection for the resistance genes, even without any direct antibiotic exposure.

Changes in bacterial community function

There were no obvious patterns between treatments for the β-glucosidase assay. The similarity between treatments can also be noted when plotting the relative change for each treatment to its starting activity (t0), (see Appendix Figure S2). Notably, the Control vessel started with higher activity than the other treatments and ended with the highest activity.

There were significant changes over the duration of the exposure for all treatments. All treatments had a similar effect with relative activity increasing over time. This could have happened as a result of the bacterial community having more efficient carbon degraders over time. It is possible that due to the low organic matter content in the sediment (0.59%) the bacterial communities contained bacteria more capable, or efficient, of degrading carbon molecules. These bacteria could have had adaptive advantages overtime as the nutrient levels decreased. Another explanation could be related to the temperature. As the water temperature in the field was close to 0°C, while in the microcosms the temperature was around 20°C, it seems probable that the bacterial community shifted towards species more adapted to higher temperatures. This can be supported by research that show bacterial metabolic rates increase significantly at higher temperatures (Kritzberg et al., 2010; Price & Sowers, 2004).

The pattern is similar when looking at nitrification. There are no obvious differences between treatments, but all treatments showed increasing trends throughout the exposure period. This could be explained by research that show nitrification processes increase by temperatures, with optimal ranges between 20-40°C in soils and wetlands (Jones & Hood, 1980; Saad & Conrad, 1993). It is therefore possible that the bacterial community shifted towards more nitrifiers during the experiment. It was not possible during the time frame of this thesis but conducting qPCR of genes for nitrifiers could help investigating these potential changes. Similarly, there was not enough time for 16S rRNA amplicon sequencing, but such results could elucidate potential changes in bacterial community composition overtime and between treatments in the experiment. Nitrite is also an intermediate in denitrification, and it is possible that this process contributed to the measured levels of nitrite. It is thus not guaranteed that increased nitrite levels directly correspond to increased nitrification. To better understand this, analysis of nitrate levels was conducted. However, the data did not arrive in time for it to be included in the thesis.

There were surprisingly no signification effects of the antibiotic mixture on bacterial community functions. It is possible that the concentrations of each antibiotic were not high enough to induce significant changes in the communities. It is also possible that enough of the antibiotic compounds degraded to prevent significant effects from taking place. Another hypothesis is functional redundancy in bacterial communities. Even if some bacterial taxa might have been inhibited because of the antibiotic mixture exposure, more tolerant taxa could perform the same activity. It is important to highlight that environmental bacteria are exposed to constant streams of antibiotics, in contrast to the pulse exposure that was done

in the experiment. Therefore, it is expected that bacterial community functions are more strongly affected during continuous exposure than what was found here.

Similarly, there were no noticeable effects for either nitrification or β-glucosidase activity for treatments with elevated *blaCTX-M* levels. This could suggest that fitness costs are negligible for the gene. This could be supported by the fact that the *blaCTX-M* gene is located on a conjugative plasmid. As previously stated, genes located on plasmids are less associated with fitness costs than chromosomic ones (Andersson & Hughes, 2010; Enne et al., 2004; Humphrey et al., 2012). From the results in this thesis, it was not possible to identify any fitness costs of the acquisition of the ARG gene.

Future outlook

Ultimately, while total bacterial abundance declined over the exposure time, the observed maintenance or increase in enzymatic activity per unit *rpoB*, as seen in the carbon metabolism and nitrification results, suggests potential functional compensation or community restructuring during the experiment. Future studies should incorporate longer exposure periods, specific molecular tracking tools to fully assess factors influencing the invasion and establishment of invading bacteria, and community sequencing approaches to further elucidate the ecological consequences of antibiotic resistance dissemination in aquatic environments.

5 Conclusion

In this thesis, the effects of antibiotics and invasion of a multidrug resistant bacteria on natural communities of sediment bacteria in microcosms conditions was assessed. The results support the hypothesis that multidrug-resistant *E. coli*, especially when introduced alongside antibiotics, can influence sediment microbial communities, albeit transiently, in concentration dependent ways. Overall, these findings contribute to the understanding of the spread of ARGs in receiving environments. These findings highlight the need for continued assessment of microbial ecosystem health in the context of antibiotic pollution and support the One Health framework, which recognizes the interconnectedness of environmental, human, and animal health.

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8 Appendix – Supplementary materials

8.1 Additional plots

8.1.1 Antibiotic resistance genes

The levels of the genes stayed relatively stable throughout the experiment for the Control treatment. After one day, the treatments Abx, CV601-T, and CV601-T+Abx all increased in *blaCTX-M* copy concentration. *E. coli* containing treatments declined sharply after day one but ended up with higher concentration than the Control and Abx treatments at day 10. Abx remained relatively stable after day 1 until it declined at day 10. After all treatments had very small changes between day 3 and 7, they had a similarly sharp decrease in blaCTX-M levels between day 7 and 10.

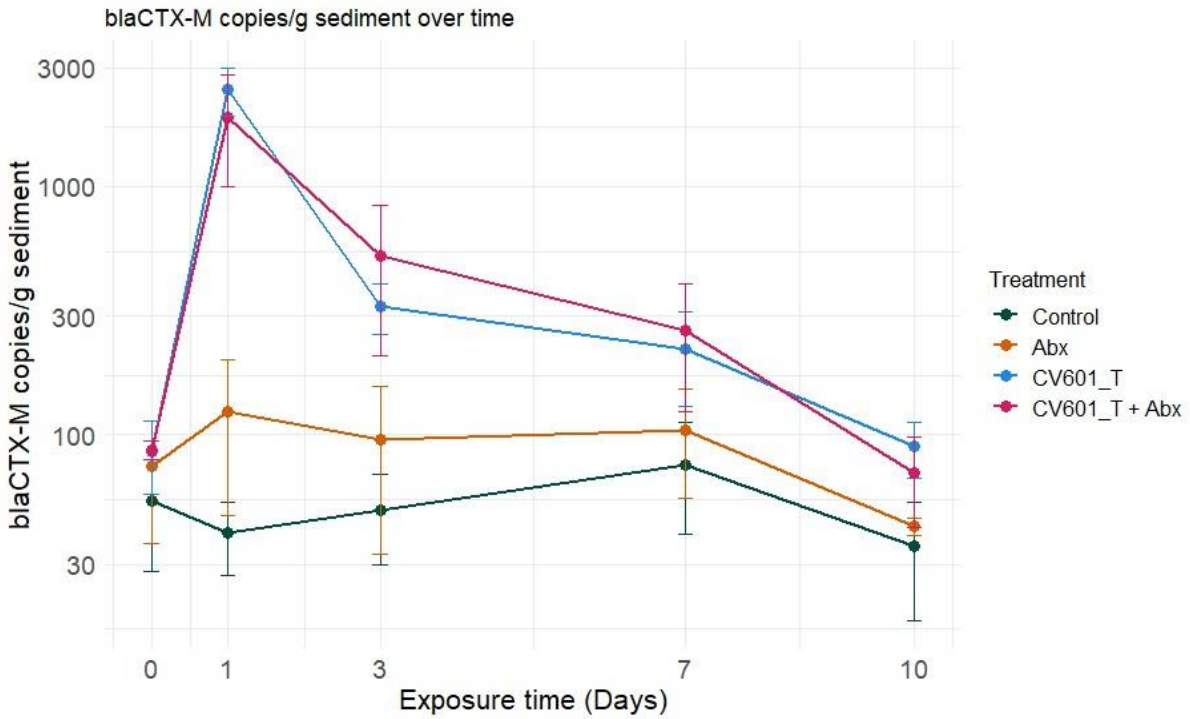

Figure S1. Line plot showing the levels of blaCTX-M gene copy numbers per gram of sediment for all treatments throughout the experiment. Dots represent the mean, and error bars represent the standard deviation, (N=3).

Table S1. Two-way ANOVA of repeated measurements results from the blaCTX-M copies/g sediment.

Factor	p-value
Treatment	0.005
Day	<.001
Treatment x Day	0.003

Table S2. Summary of the post-hoc Tukey test results for the blaCTX-M/g sediment calculations, that show a significant difference were p < 0.05. On the left side the differences

between days within each treatment are shown. On the right side the differences between treatments for each day are shown.

Treatment	Day	p-value	Day	Treatment	p-value
CV601-T	0-1	0.0014	**1**	Control - CV601-T	0.0080
	1-3	0.0004		Control - CV601-T+Abx	0.0317
	1-7	0.0021		Abx - CV601-T	0.0098
	1-10	0.0016		Abx - CV601-T+Abx	0.0395
CV601-T + Abx	0-1	0.0076			
	0-3	0.0364			
	1-3	0.0066			
	1-7	0.0137			
	1-10	0.0079			
	3-10	0.0356			

8.1.2 β-glucosidase assay

When it comes to the β-glucosidase assay all treatments had similar trends in the relative change of metabolism (Figure S2). There was an increase in the metabolism of MUF during the first day, where CV601-T+Abx had the biggest increase. Between day 1 and day 3 all treatments except Abx declined slightly, and between day 3 and day 7 all treatments had similar relative declines in the metabolism of MUF. The treatments then remained quite stable for the rest of the experiment. Overall, treatments had declined in relative MUF degradation at the end of the experiment. For Control, Abx, and CV601-T the mean relative MUF degradation on day 10 was between 63-67%, while for CV601-T+Abx the mean relative activity was at 87% on day 10.

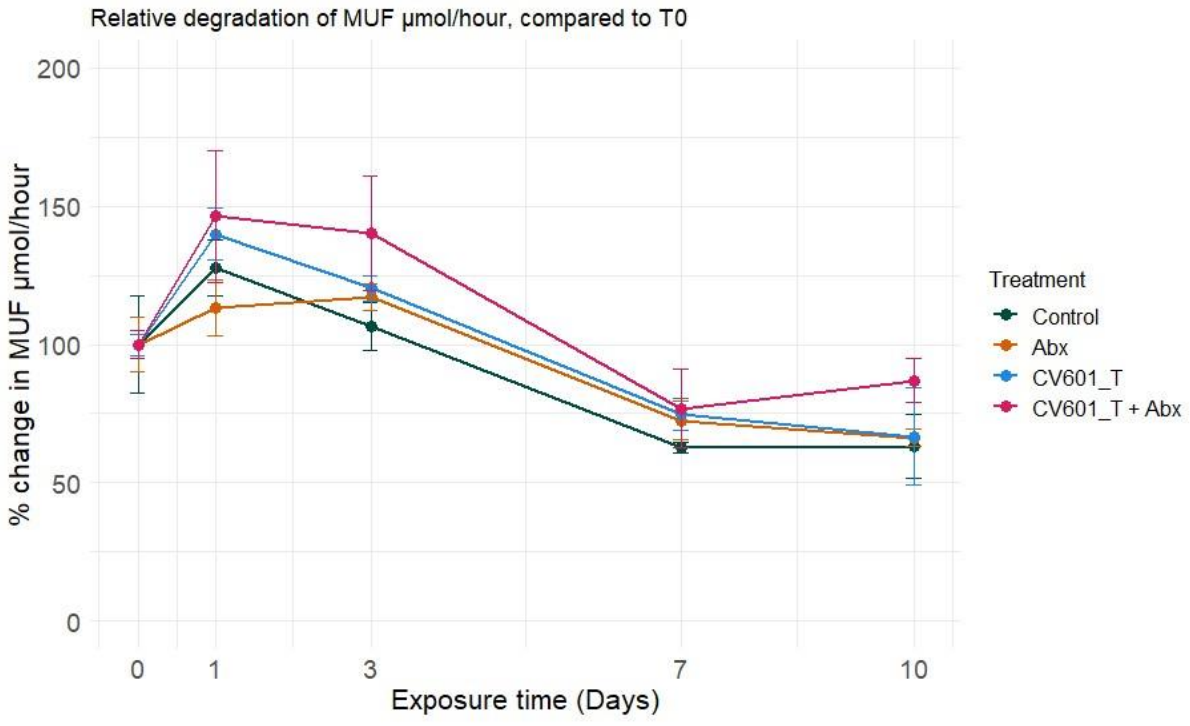

Figure S2. Line plot showing relative change in MUF degradation compared to T0 for all treatments. Dots represent the mean, and error bars represent the standard deviation, (N=3).

The ANOVA found close to, but no significant effect between treatments. However, there was a significant effect between the days of the experiment. Similarly, the Tukey test showed that all treatments changed significantly throughout days of the experiment but there was no significant difference between the treatments for any day (Appendix Table S4).

Table S3. Two-way ANOVA of repeated measurements results from the relative MUF degradation in the β-glucosidase assay.

Factor	p-value
Treatment	0.055
Day	<.001
Treatment x Day	0.487

Table S4. Summary of the post-hoc Tukey test results for the relative MUF degradation in the β-glucosidase assay compared to T0, that show a significant difference were p < 0.05. Shown in the table is differences between days within each treatment. No significant difference between any treatments for at any day was found.

Treatment	Day	p-value
Control	0-7	0.0215
	0-10	0.0184
	1-7	0.0094
	1-10	0.0103
	3-7	0.0073
	3-10	0.0183
Abx	0-10	0.0283
	3-7	0.0066
	3-10	0.0071
CV601-T	0-10	0.0311
	1-7	0.0092
	1-10	0.0047
	3-7	0.0058
	3-10	0.0054
CV601-T + Abx	1-7	0.0062
	1-10	0.0160
	3-7	0.0007
	3-10	0.0053

Table S5. Summary of the post-hoc Tukey test results for the MUF degradation relative to the rpoB copy number, that show a significant difference were p < 0.05. On the left side the

differences between days within each treatment are shown. On the right side the differences between treatments for each day are shown.

Treatment	Day	p-value	Day	Treatment	p-value
Control	0-3	0.0001	**0**	Control – Abx	0.0020
	0-10	0.0001		Control – CV601-T	0.0012
	1-3	<.0001		Control – CV601-T + Abx	0.0004
	1-10	0.0001	**1**	Control – CV601-T + Abx	0.0378
	3-7	0.0066		Abx – CV601-T + Abx	0.0014
	3-10	0.0037		CV601-T – CV601-T + Abx	0.0010
	7-10	0.0005	**3**	Control – Abx	0.0065
Abx	0-3	0.0004		Control – CV601-T	0.0004
	0-10	0.0045		Control – CV601-T + Abx	0.0030
	1-3	<.0001	**7**	Control – CV601-T + Abx	0.0002
	1-10	0.0103		Abx – CV601-T + Abx	0.0001
	3-7	0.0451		CV601-T – CV601-T + Abx	0.0003
	7-10	0.0300			
CV601-T	0-3	0.0059			
	0-10	0.0019			
	1-3	0.0008			
	1-10	0.0044			
	3-10	0.0266			
CV601-T + Abx	0-1	0.0003			
	0-3	0.0003			
	0-7	<.0001			
	0-10	0.0001			
	1-7	0.0001			
	1-10	0.0020			
	3-7	0.0001			
	3-10	0.0037			

8.1.3 Nitrite levels

Nitrite (NO_2^-) levels per gram of sediment did not change notably when comparing day 0 to day 10 for any treatment except Abx, which had higher mean levels of nitrite but also a very large standard deviation on day 10. For the other days there was no noticeable difference between treatments except for Abx on day 1, and CV601-T on day 7, however those treatments also had a large standard deviation on those days.

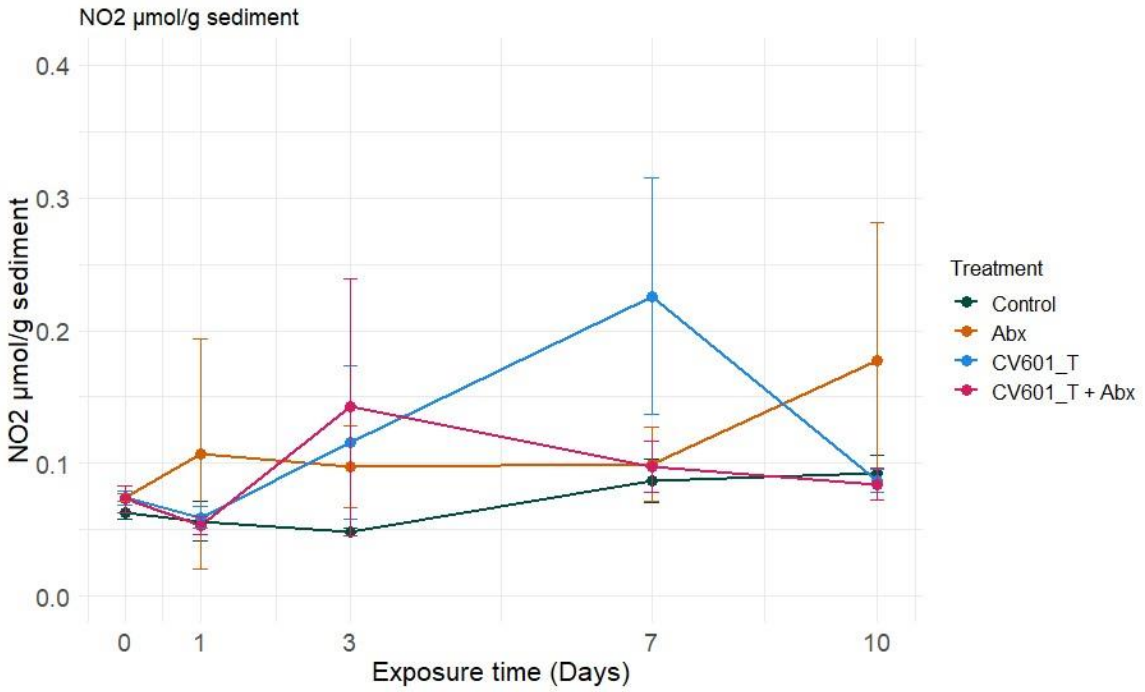

Figure S3. Line plot containing the levels of NO_2^- found per gram of sediment for all treatments. Dots represent the mean, and error bars represent the standard deviation, (N=3).

ANOVA found no significant effects for any of the factors (Table S6). Similarly, the post hoc Tukey test could not identify any significant effects (data not shown).

Table S6. Two-way ANOVA of repeated measurements results from the nitrite levels relative to grams of sediment.

Factor	p-value
Treatment	0.206
Day	0.093
Treatment x Day	0.193

8.2 Post-hoc Tukey bar plot comparison of treatments within days for blaCTX-M

8.2.1 *blaCTX-M* copies per gram of sediment

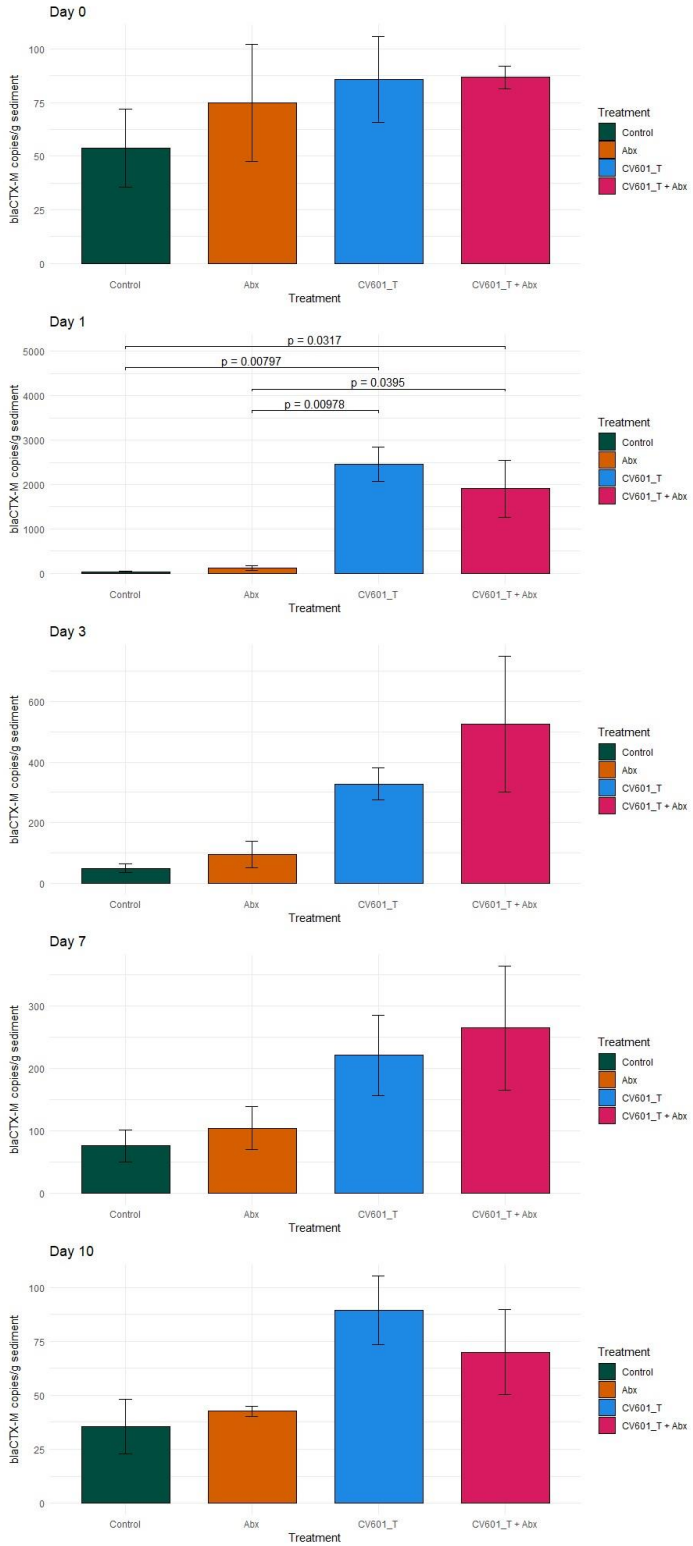

Figure S4. Bar plots of blaCTX-M per gram of sediment, separated per day, with significant differences between treatments at each day shown as bracket p-values, using Post-hoc Tukey test.

8.2.2 *blaCTX-M* copies per *rpoB* copies

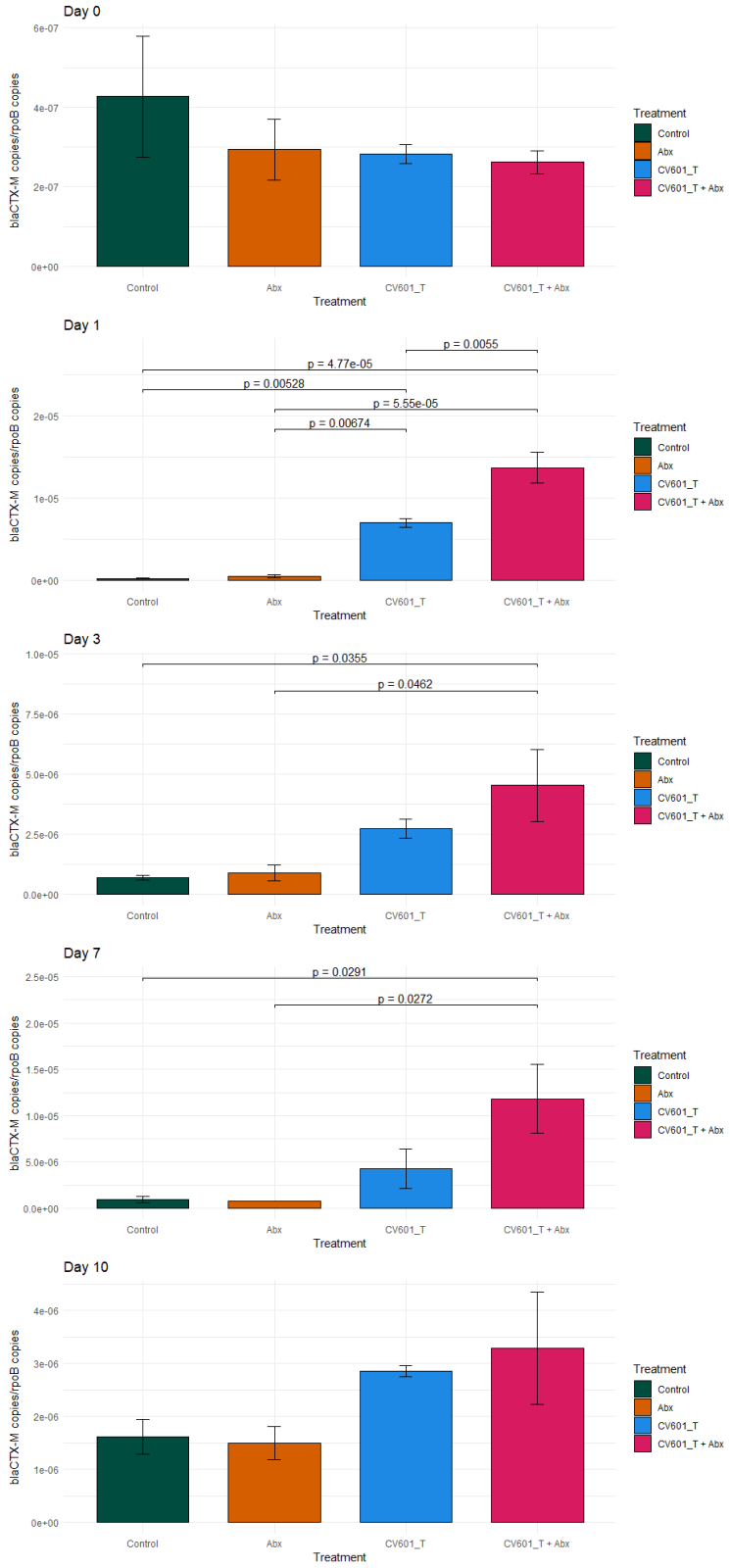

Figure S5. Bar plots of blaCTX-M copies/rpoB copies separated per day, with significant differences between treatments at each day shown as bracket p-values, using Post-hoc Tukey test.

8.3 Nutrient analysis

Nutrients analysis was conducted in the same using the same method as nitrite analysis, described in section 2.2.10. The lab at Kristineberg analysed nitrate, ammonium, and phosphate levels (Table S7).

Table S7. Analysis of nutrient levels in the microcosm vessels before exposure (at T0), and at the end of the exposure period (T10). Nutrient levels in the field were also analysed from the day of sampling, 7th February 2025.

Treatment	Day	NO3 (µmol/L)	NH4 (µmol/L)	PO4 (µmol/L)
Control	T0	29.37	3.89	1.85
Abx	T0	30.46	3.92	1.46
CV601_T	T0	30.27	4.13	1.5
CV601_T + Abx	T0	30.11	3.97	1.46
Control	T10	31.41	2.23	2.06
Abx	T10	35.2	0.84	0.13
CV601_T	T10	39.62	0.51	0.07
CV601_T + Abx	T10	32.19	0.39	< 0,07
Water Vänersborg	07-02-2025	29.75	1.2	0.07
Water Vänersborg	07-02-2025	28.04	0.49	17.34
Water Vänersborg	07-02-2025	30.06	0.46	15.77

8.4 Physiochemical measurements

Table S8. Time refers to days after exposure.

Treatment	Time (days)	pH	Temperature (°C)	Conductivity (µS/cm)	Oxygen (mg/L)
Control A	0	6.64	18.4	164	6.2
Control B	0	6.66	18.5	161	6.0
Control C	0	6.64	18.6	163	6.3
Control A	1	6.66	20.5	158	7.2
Control B	1	6.67	20.3	160	7.0
Control C	1	6.65	19.5	159	7.3
Control A	3	5.91	20.0	118	8.0
Control A	7	6.96	19.7	109	7.3
Control B	7	6.64	19.7	109	7.6
Control C	7	6.58	19.8	104	7.6
Control A	10	6.85	19.4	104	8.2
Control B	10	6.75	19.6	107	8.5
Control C	10	6.68	19.7	105	8.4

8.5 Melt curves from qPCR

8.5.1 GFP

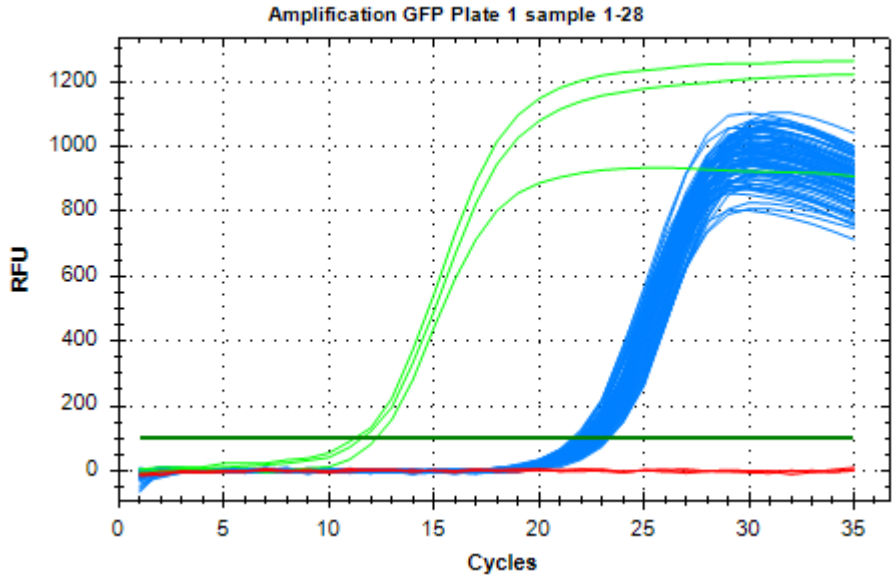

Figure S6. Amplification curves of the Gfp-gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

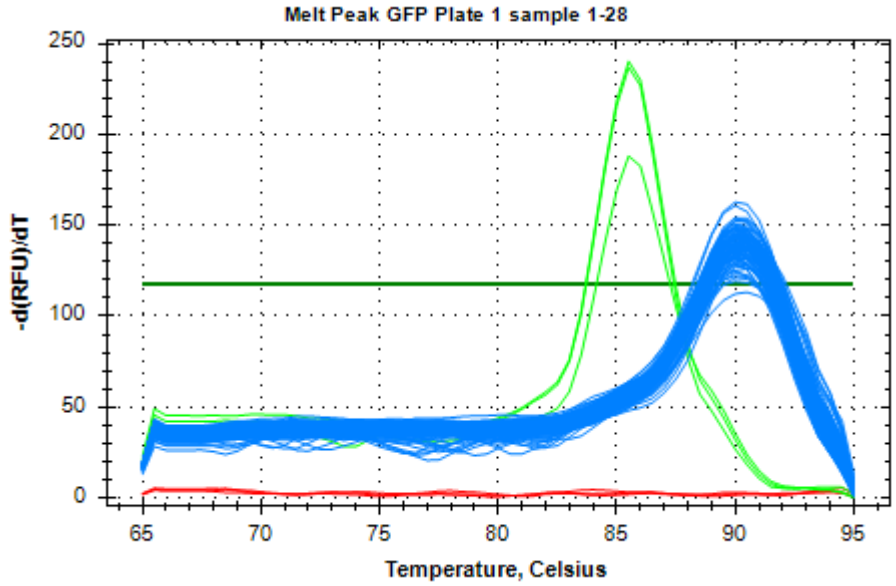

Figure S7. Melt peak curves of the Gfp-gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

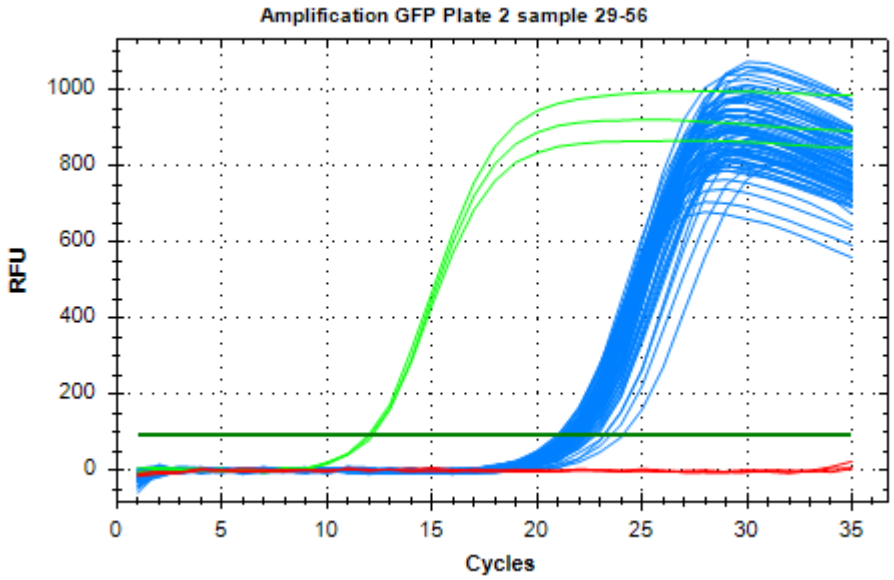

Figure S8. Amplification curves of the Gfp-gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

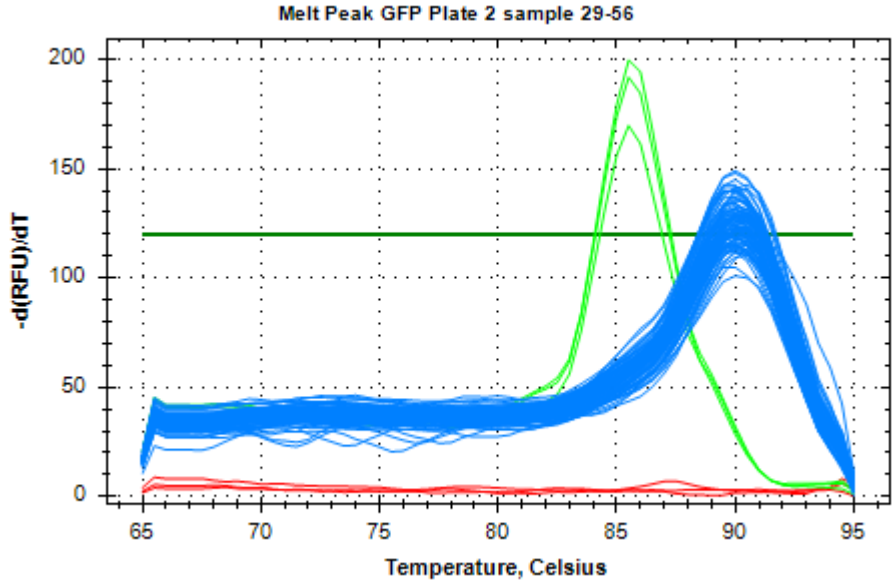

Figure S9. Melt peak curves of the Gfp-gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

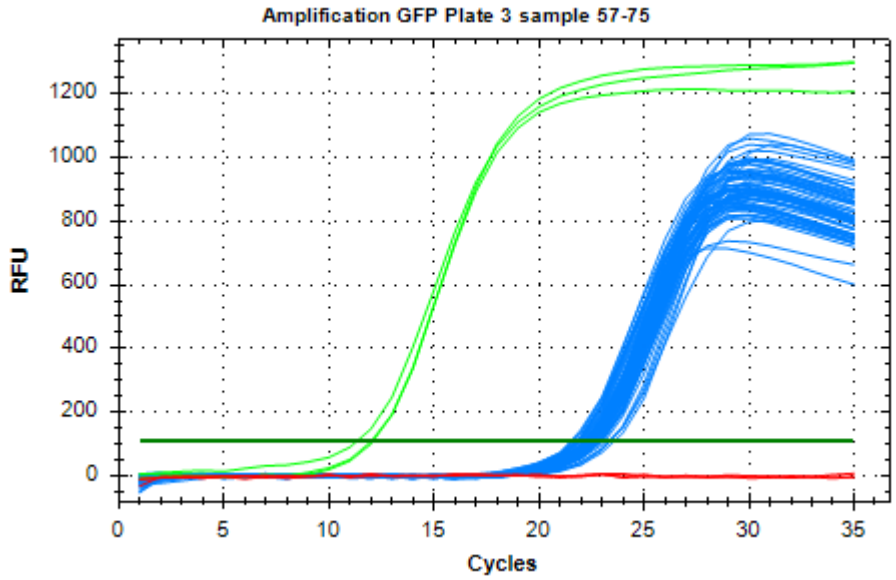

Figure S10. Amplification curves of the Gfp-gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.

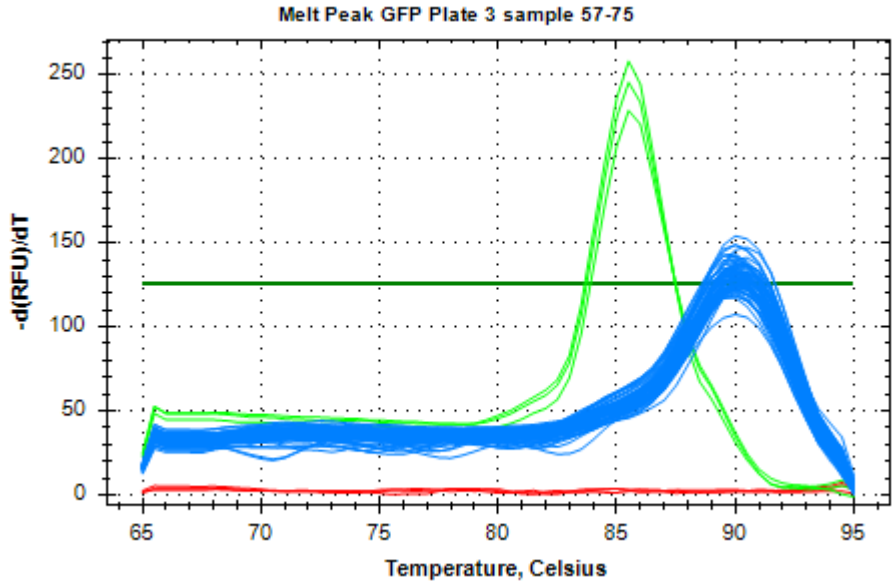

Figure S11. Melt peak curves of the Gfp-gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.

8.5.2 rpoB

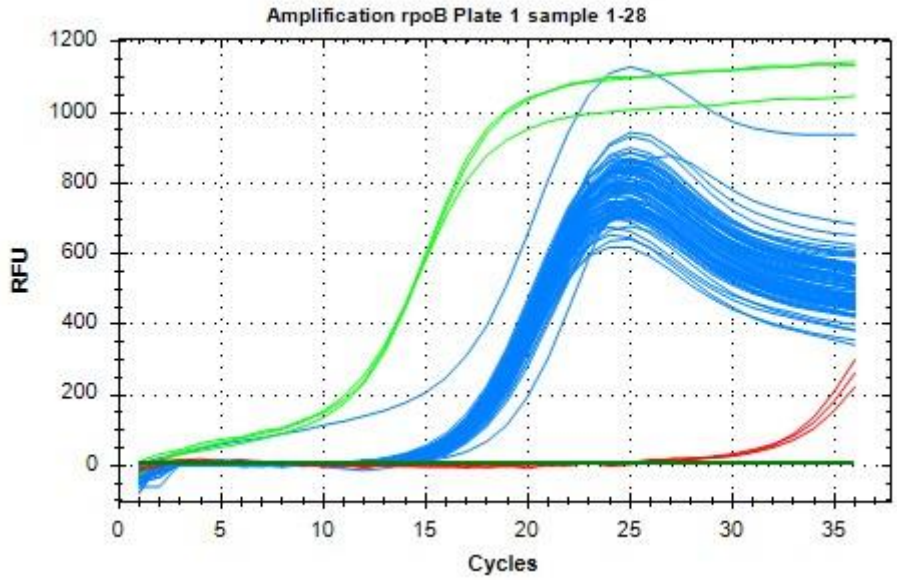

Figure S12. Amplification curves of the rpoB gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

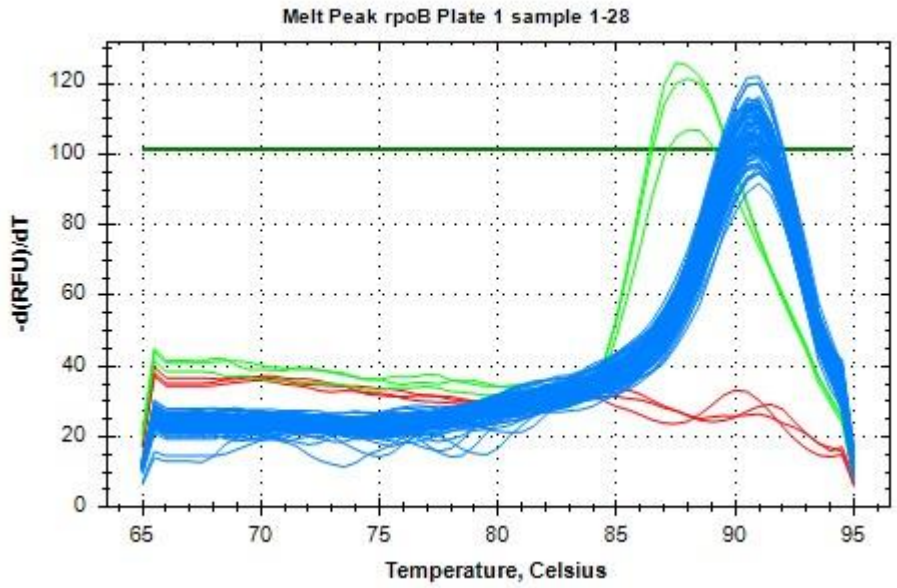

Figure S13. Melt peak curves of the rpoB gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

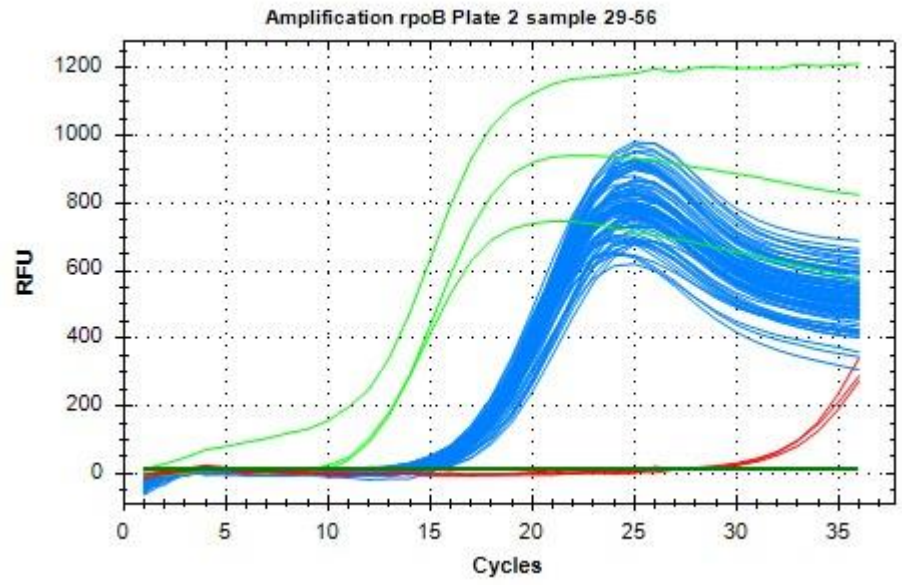

Figure S14. Amplification curves of the rpoB gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

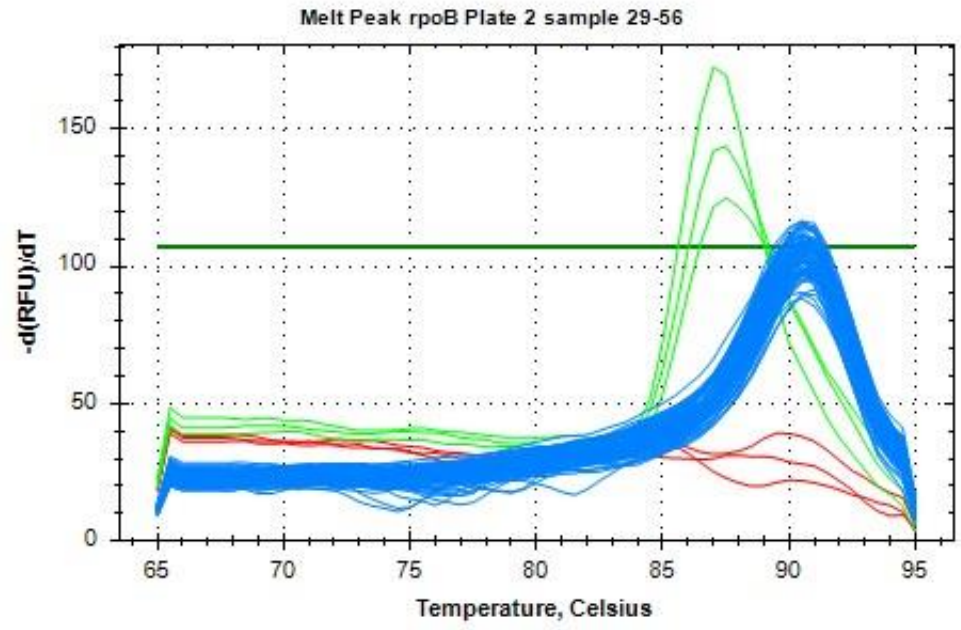

Figure S15. Melt peak curves of the rpoB gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

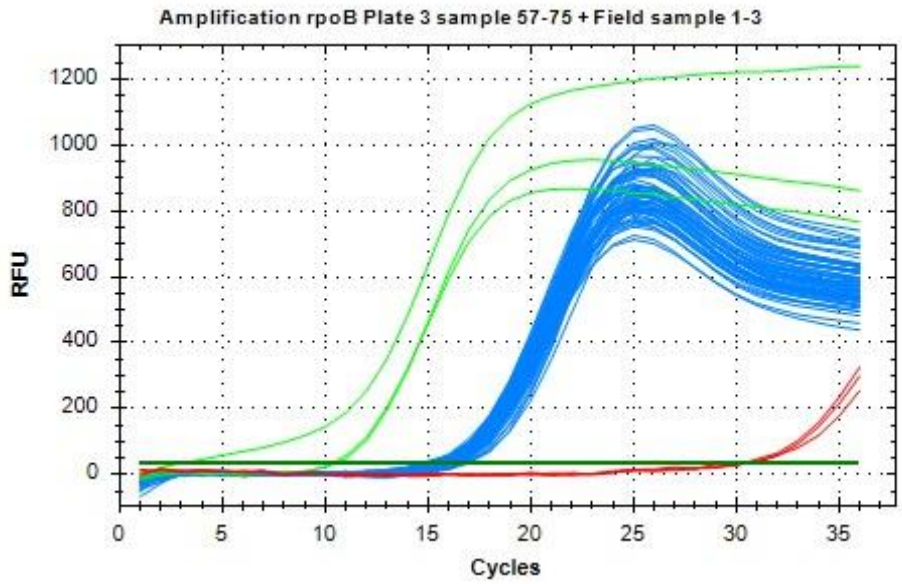

Figure S16. Amplification curves of the rpoB gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.

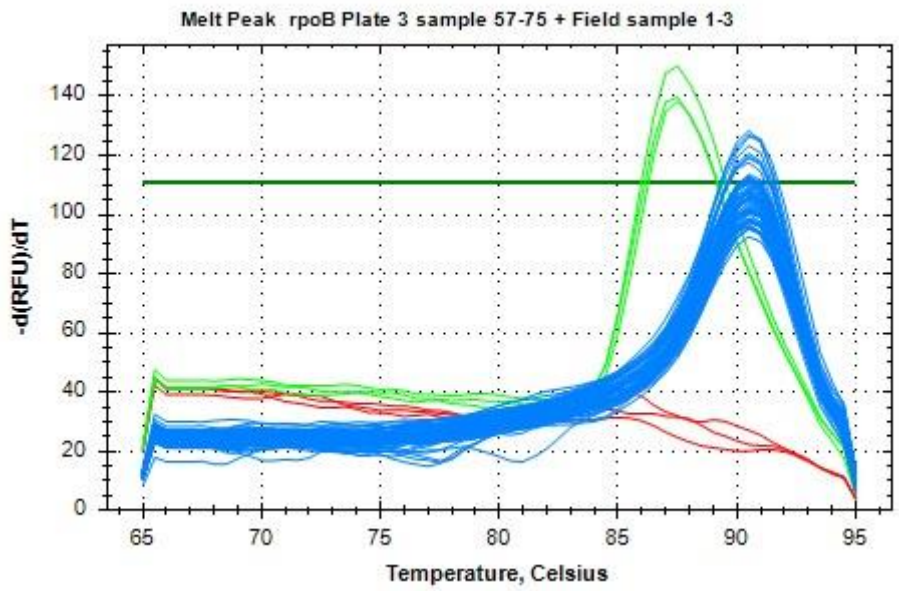

Figure S17. Melt peak curves of the rpoB gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.

8.5.3 blaCTX-M

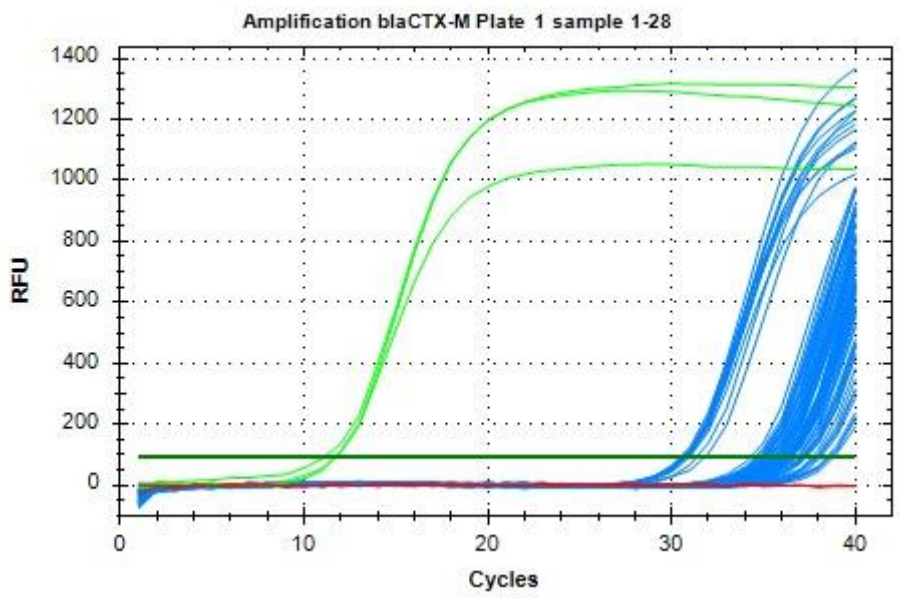

Figure S18. Amplification curves of the blaCTX-M gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

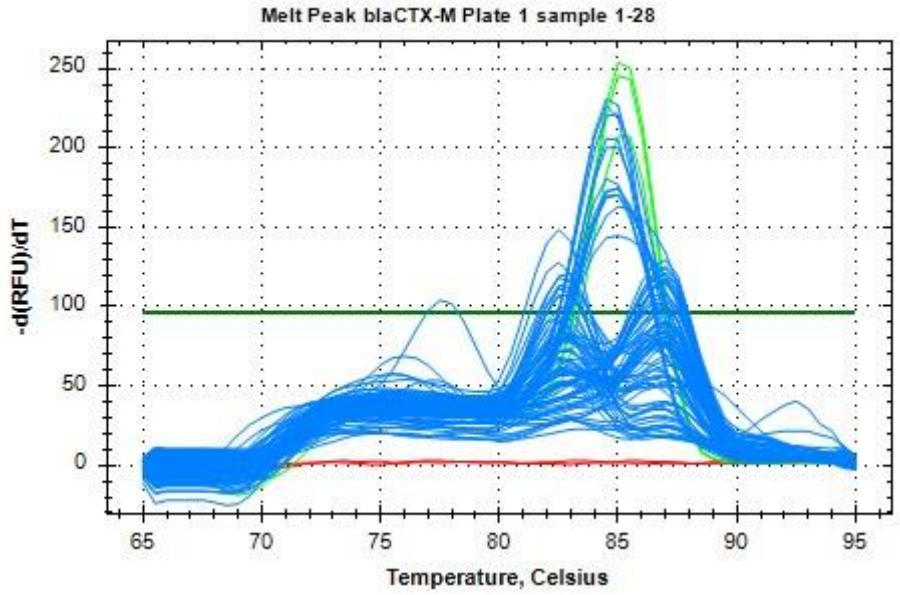

Figure S19. Melt peak curves of the blaCTX-M gene. This shows the curve for samples 1-28 which corresponds to all treatments for day 0, as well as most treatments for day 1 except two of the CV601-T+Abx vessels. Negative controls are shown in red.

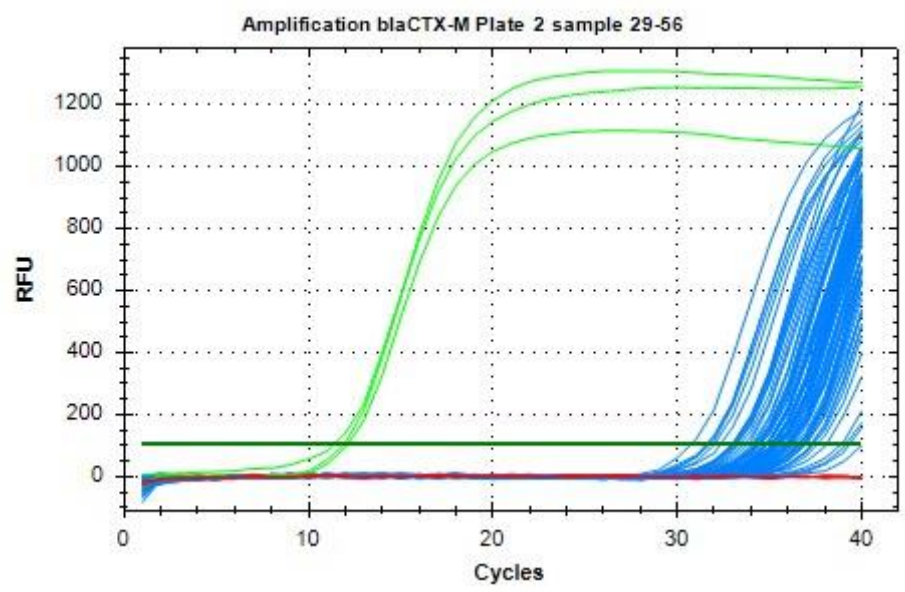

Figure S20. Amplification curves of the blaCTX-M gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

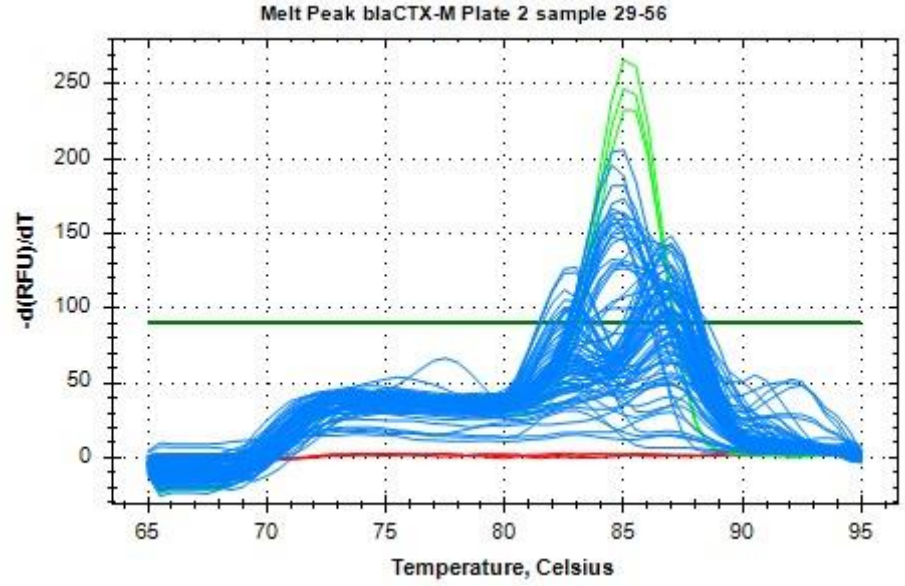

Figure S21. Melt peak curves of the blaCTX-M gene. This shows the curve for samples 29-56 which corresponds to the final two vessels of CV601-T+Abx on day 1, all treatments for day 3, as well as most treatments for day 7 except one of the CV601-T vessels and all three of the CV601-T+Abx vessels. Negative controls are shown in red.

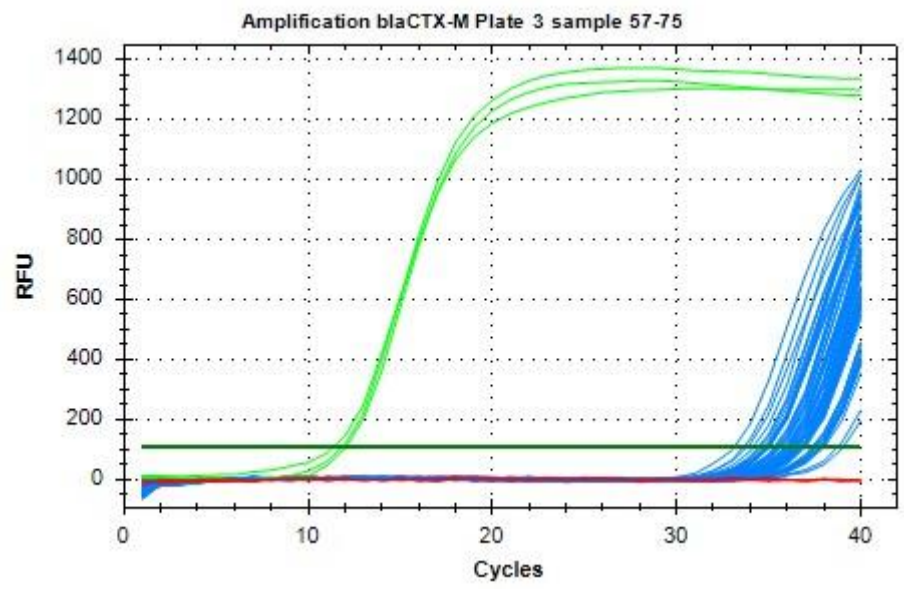

Figure S22. Amplification curves of the blaCTX-M gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.

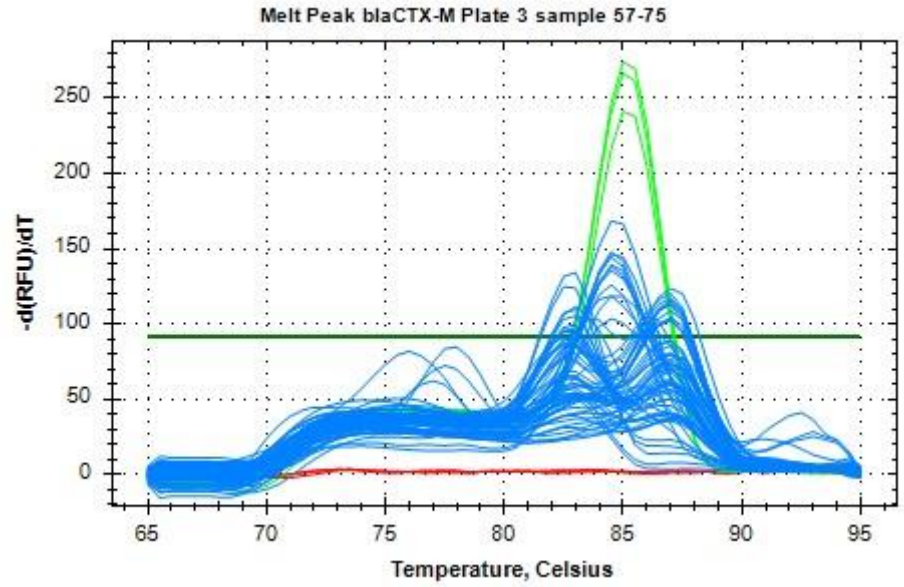

Figure S23. Melt peak curves of the blaCTX-M gene. This shows the curve for samples 56-75 which corresponds to the final two vessels on day 7, all treatments for day 10. Negative controls are shown in red.