Understanding fate and distribution of antimicrobial compounds and antimicrobial resistance genes during wastewater treatment.

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Biology

January 2022

Abstract

Antimicrobial resistance (AMR) is a global health crisis, rendering pharmaceutical action against infections ineffective, threatening lives worldwide. Antibiotics are not fully metabolised by humans or animals, leading to dispersion within sewage streams. Due to this, the final products of wastewater treatment, namely biosolids and final effluent, are considered to be reservoirs for antibiotic compounds (ACs) and antibiotic resistance genes (ARGs). This is important as the final products can be released into the surrounding natural environment, potentially affecting environmental microbial communities.

In light of this, this project aimed to answer the following questions: 1. Can a method be brought together and optimised to detect antibiotic compounds and related genes within complex environmental samples? 2. Which ACs and genes are present in the different stages of wastewater treatment? 3. How do these compounds and genes persist throughout the treatment process? Are there any links that can be made?

The methods resulted in tentative identification of 23 different antibiotic compounds and 119 different genes relating to antimicrobial resistance. These results showed compound and gene persistence throughout the wastewater treatment process and identified areas for further research, especially concerning absolute quantification and longitudinal studies following specific samples throughout.

In conclusion, the methodology resulted in multiple ARGs and ACs across a number of major antibiotic classes being detected at the WWTP on this snapshot study. This project also provides important method development and optimisation for future studies in this area.

Table of Contents

List of Tables

List of Figures

D – Anaerobic Digester Digestate Output; E – Fresh Biosolid; F- Dried Biosolid; G – Final Settlement Tank Distribution Centre.

Figure 5: Mean peak areas from full-scan chromatograms of the different antibiotic compounds. Red indicates the highest peak areas, following the gradient to blue showing the lowest. Grey indicates no data.

Figure 6: Four tetracycline compounds detected in CompoundDiscoverer, plotted with mean peak areas. The compounds are 4-epianhydrotetracycline (a), 4-epitetracycline (b), doxycycline (c) and oxytetracycline (d). The wastewater treatment stages are: A – anaerobic digester feed pump; B – anaerobic digester tank 1; C – anaerobic digester tank 2; D – anaerobic digester tank 3; E – anaerobic digester tank 4; F – anaerobic digester digestate output; G – fresh biosolid; H – dried biosolid and I – quality control conditioner.

Figure 7: Vale of York CCG, data on local antibiotic prescribing, with detail shown for October 2020 (OpenPrescribing.net, 2021).

Acknowledgements

Firstly, I wish to give special thanks to my fantastic supervisors Dr. J Brett Sallach and Prof. James Chong for all their support, patience and feedback. I am extremely appreciative and I am looking forward to continuing working with you both!

I must also give thanks to the Biorenewables Development Centre and Deborah Rathbone for allowing the use of their QIAcube and TissueLyser; to Sarah Forrester and Annabel Cansdale for their help, patience and guidance with me learning how to use R; to Ed Bergstrom in the Centre of Excellence for Mass Spectrometry in the Chemistry Department for technical help using the Orbitrap and to Swen Langer for teaching me how to navigate CompoundDiscoverer, and a massive thanks to the Biology Department for support during this MSc project.

I'd also like to thank the Chong Lab research group, the JBS group in Environment and Geography and the Barilla lab group for their friendship, support and motivation, and a special thanks to Anyi Yuan, Georgia Bateman and Joe McGrory.

A final thanks to my Mum, Dad, George and Jazz, and to Callum for putting up with my stress!

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1.0. Introduction

1.1. Background

Antimicrobial resistance (AMR) is a global health crisis (WHO/FAO/OIE, 2020), responsible for an estimated ~700,000 human deaths annually (O'Neill (Ed), 2016). AMR is the action of active resistance mechanisms against antimicrobial pharmaceuticals, rendering them ineffective, and threatening ability to treat infections (Magiorakos *et al*., 2012; Levin-Reisman *et al*., 2017); this is alarming considering the significant increase in the global antimicrobial consumption rate in recent years (Klein *et al*., 2018).

Many statistics and analyses fail to account for the environmental dimension of the issue, leaving it overlooked and underrepresented in current research, especially in comparison to the clinical context (Ashbolt *et al*., 2013). Although studies have been conducted to bring forth environmental sources into clinical investigations (Cantón, 2009).

A key selective pressure and accelerant of the spread of AMR is unintentional exposure (as well as inappropriate use) (Novo *et al*., 2013); in light of this, intergovernmental organisations proposed the "One Health" approach to encompass the human, animal and environmental sectors (McEwen and Collignon, 2018). In addition, ambition 6 of the UK's 20-year vision for AMR (Department of Health and Social Care, 2019) discusses minimising environmental spread of AMR, including effective wastewater treatment and handling.

Antimicrobial compounds (ACs) and their associated residues (ARs) are classed as micropollutants, and are considered to be contaminants of emerging concern (CECs)

(Alygizakis *et al*., 2020). In addition, several antibiotic compounds, including amoxicillin, ciprofloxacin and azithromycin have previously been recognised as priority active pharmaceutical ingredients (APIs) in terms of environmental and ecotoxicological risk (Guo *et al*., 2016). There have also been antibiotics identified on the “Surface Water Watch List”, these include amoxicillin, ciprofloxacin (Jurado et al. 2022).

Antibiotics are not fully metabolised by humans or animals (Kim *et al*., 2011; Frade *et al*., 2014), leading to notable dispersal within sewage streams (Wang *et al*., 2014). Antibiotic compounds have been found in many environmental matrices (Grenni, Ancona and Barra Caracciolo, 2018), such as wastewater (Chen *et al*., 2020), soil (Kay, Blackwell and Boxall, 2004) and sediment (Gibs *et al*., 2013). Some ACs, for example rifaximin (an antibiotic used to treat local gastrointestinal infections (Gillis and Brogden, 1995)), are so poorly metabolised by the body they a re released virtually unchanged from the parent compound (Rivkin and Gim, 2011) which could result in higher concentrations in wastewater streams.

Sewage streams are of a high importance as they are known to link anthropogenic and natural environments (Li *et al*., 2018). This is an interesting link, especially in terms of the human impact on the environment, associated with AMR.

Removal approaches for pharmaceuticals in general can include conventional practices such as the activated sludge process (Angeles *et al*., 2019), and more advanced processes, like oxidation (specific examples including ozonation or photolysis) (Phoon *et al*., 2020). Adsorption technology can also be used, using carbon-based materials to adsorb to pharmaceutical compounds (Yu *et al*., 2016). Removal percentages of pharmaceuticals in wastewater treatment could potentially

be affected by the concentrations within the wastewater influent. Wang, Fenner and Helbling (2020) stated that there was a positive correlation between removal efficiencies and initial influent concentrations. However, Rios-Miguel, Jetten and Welte (2021) found the opposite, and stated that there was no correlation, although their study excluded purely antibiotic compounds, and instead included antiseptics and other pharmaceuticals. It would be interesting to investigate purely antimicrobial compounds to see whether there is such a correlation. It is also important to note that these findings would vary depending on the treatment process and chemicals investigated.

As well as AMR, there is antimicrobial tolerance (AMT), and there is an important distinction to be made between the two, as AMT is where bacteria exhibit dormancy at high concentrations (Levin-Reisman *et al*., 2017). AMT is usually associated with bacterial biofilms, and is often only temporary, unlike typical resistance mechanisms (Jorge *et al*., 2019).

1.2. Resistance Mechanisms

AMR is a natural occurrence and is known to predate the discovery of antibiotics by thousands of years (D'Costa *et al*., 2011; Bhullar *et al*., 2012). AMR can be broadly classified as either intrinsic or acquired. Intrinsic resistance is due to inherent properties of the bacteria, either structural or functional (Blair *et al*., 2015b), and is normally chromosome-encoded and non-specific (Peterson and Kaur, 2018). An example would be the structural differences between the Gram-positive cell wall and Gram-negative cell membrane (Richter and Hergenrother, 2019), and the targets of specific antimicrobial agents. For example; vancomycin binds to cell wall precursors inhibiting cell wall biosynthesis, and is used against Gram- positive organisms (Wu,

Cameron and Boger, 2020) , or the intrinsic resistance of *Pseudomonas* spp. *(*Murray *et al., 2015)*. Acquired resistance mechanisms are transferred and often plasmid-mediated (Lopatkin *et al*., 2017), this is where horizontal gene transfer (HGT) can be introduced, allowing for the dissemination of AMR across microbial communities.

There are a large number of antimicrobial mechanisms and targets, and for each mechanism of action, there is likely a mechanism of resistance to counteract it. These general mechanisms are illustrated in Figure 1. Examples of broad targets of antimicrobial agents can include synthesis of cell walls, proteins or nucleic acids, as well as metabolites and the cell membrane (Uluseker *et al*., 2021). In addition, examples of generalised methods of antimicrobial resistance include inactivation or modification of the antimicrobial compound, efflux pumps to remove the AC, or by-passing or modifying the target of the antibiotic, so that they no longer work effectively (Uluseker *et al*., 2021).

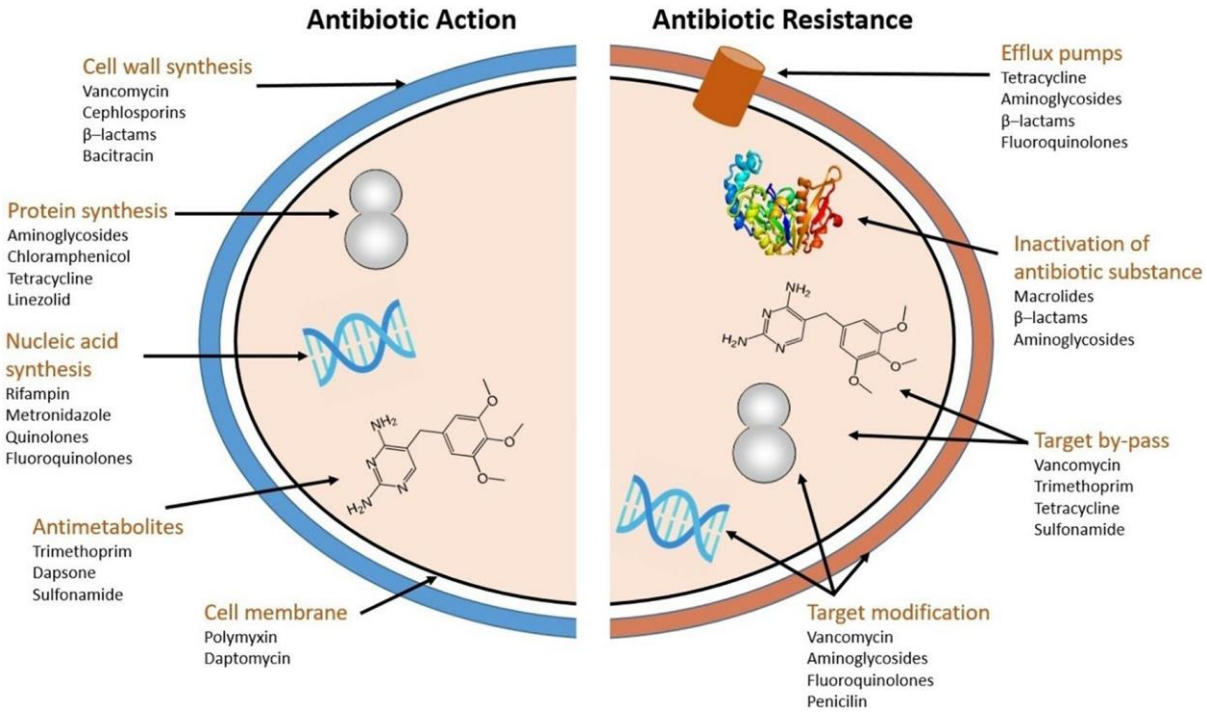


Figure 1: Mechanisms of antibiotics and mechanisms of resistance with examples of antimicrobial classes and individual antibiotics associated, from (Uluseker *et al*., 2021).

A well-known example of AMR would be methicillin-resistant *Staphylococcus aureus* (MRSA). In this case, the main genetic determinant associated is *mecA* (Kim *et al*., 2017), which codes for the transpeptidase penicillin-binding protein 2a (PBP2a) (Zhan and Zhu, 2018).

The mechanism of β-lactam antibiotics (e.g. penicillins, cephalosporins, monobactams and carbapenems (Lenhard and Bulman, 2019)) includes binding to PBPs resulting in the breakdown of peptidoglycan cell wall synthesis (Cho, Uehara and Bernhardt, 2014). PBP2a, however, exhibits decreased affinity for β-lactams, weakening binding and allowing continued cell wall synthesis (Zhan and Zhu, 2018).

Mechanisms of multidrug resistance (MDR) may include efflux proteins, which are membrane transporters, ejecting ACs out of the bacterial cell cytoplasm (Alegre *et al*., 2016). A common efflux pump system in Gram-negative bacteria is the resistance-nodulation-division (RND) transporter system. Examples of RND efflux systems include AcrAB, MdtABC and MdtF (Zhang *et al*., 2011; Blair *et al*., 2015a).

Related to MDR in microbial communities, there is also the possibility for extensive drug resistance (XDR), or even pan-drug resistance (P DR). In the clinical context, although thankfully uncommon, most P DR infections are tuberculosis, and consist of a multitude of resistance mechanisms, and AMT resulting in “persister” cells, furthering the dissemination of AMR (Hameed *et al*., 2018).

1.3. Antibiotic Resistance Genes

As mentioned previously, HGT is a key method of ARG spread within microbial communities (Stevenson *et al*., 2017), and has been evidenced occurring between clinical pathogens and environmental bacteria (Forsberg *et al*., 2012; Chen *et al*., 2016), increasing public health risks associated with AMR. Mobile genetic elements (MGEs) associated with HGT can include plasmids and transposons (Gillings, 2017). Integrons can also be associated with movement as they are gene acquisition systems, although they are not mobile themselves (Gillings, 2014).

Associations of ARGs with MGEs give retrospective evidence of HGT (Miller *et al*., 2016), as it can be difficult to evidence HGT within complex environmental samples (Luby *et al*., 2016). There is also the potential for bacteria to integrate free DNA from the surrounding environment (Gillings, 2017), this is known as transformation, and the free DNA may originate from lysed cells, or during processes such as biofilm formation.

1.4. Antibiotic Compounds

Antimicrobial compounds, like other pharmaceuticals will degrade naturally over a period of time. This degradation process can be exacerbated by abiotic changes such as pH and temperature, or photo-degradation by light. The compounds can also be hydrolysed, or degraded via oxidative processes (Cycoń, Mrozik and Piotrowska-Seget, 2019).

As well as these different degradation processes, there are also different known half-lives for antibiotic compounds, and different susceptibilities to certain methods of degradation. These differences are thought to be dependent on variations in physico-chemical properties of the chemicals and possibly even climactic factors (, or degraded

via oxidative processes (Cycoń, Mrozik and Piotrowska-Seget, 2019). . For example, beta-lactam antibiotics, such as penicillins, are especially susceptible to hydrolysis when exposed to water (Hirte *et al*., 2016), and tetracycline antibiotics are particularly susceptible to photodegradation, although they are relatively persistent in dark environments (Yun *et al*., 2018).

The half-lives of ACs are not synonymous with persistence, explained by Mackay *et al*., (2014). Biodegradation of ACs has been thought to be not a common occurrence during wastewater treatment (Joss *et al*., 2006), although in reality, it is different for different antibiotics with differing properties, although biologically active transformation products (from both metabolism and general chemical degradation) may still be present (Keen and Linden, 2013; Martínez-Piernas, Plaza-Bolaños and Agüera, 2021). The physicochemical properties of ACs (such as water solubility) or surrounding environmental factors (such as temperature or pH) may affect their biodegradation, as well as the presence of microbial communities capable of metabolising them (Barra Caracciolo, Topp and Grenni, 2015).

By-products of metabolisation and chemical degradation are also important factors to consider. For example, sulphonamide antibiotics break down to form the degradation product pterin-sulphonamide. This compound was observed to retain antimicrobial properties (Achermann *et al*., 2018), highlighting the importance of fully investigating transformation pathways, namely metabolisation and chemical degradation, rather than solely looking at the parent compounds. It should be noted that many by-products can be bioactive or exhibit biotoxicity in their own right. For example, the tetracycline degradation by-product 4-epianhydrotetracycline is

associated with severe nephrotoxicity and the development of Fanconi syndrome (Pinto, Campos and Yamamoto, 2002).

Concerning two particular physico-chemical properties that can affect the fate of chemicals within the environment, there are the n-octanol/water partition coefficient (logKow) and the adsorption-desorption coefficients (Kd). logKow is often used in environmental risk assessment as part of the estimation of environmental fate and bioavailability (Hodges *et al*., 2019). It is a good indicator of how hydrophobic a compound is (Gardner *et al*., 2013). It was noted that for antibiotics with a higher logKow, the Kd could be affected, leading to adsorption and partitioning with the solid phases (e.g. suspended solids in process water or sludge) (Li *et al*., 2016; Park *et al*., 2020). Fluoroquinolones and tetracyclines are known to have higher Kds than their other pharmaceutical counterparts (Pan and Chu, 2016), leading to higher adsorption to soil and solid environmental matrices.

1.5. Co-Selectors of AMR

In some resistant bacteria, genes conferring resistance to heavy metal mediated degradation can also result in development of resistance to certain antibiotics. This is known as co-selection (Dickinson *et al*., 2019). Persistent metal contamination, even at small concentrations can exacerbate the spread of AMR as the mechanisms are similar (Kang *et al*., 2018).

In addition, it has been noted that certain other non-antimicrobial pharmaceutical compounds may induce or co-select for AMR, for example, (Jin *et al*., 2018) investigated the antidepressant fluoxetine and the induction of resistance in *Escherichia coli* through efflux pump mechanisms.

Furthermore, broad chemical exposure may also affect selection for AMR, for example, biocides such as triclosan (Lu *et al*., 2018), or disinfection by-products, such as trihalomethanes or haloacetic acids (Lv *et al*., 2014). This is due to the mechanisms of efflux systems and their similarities in ejecting chemically similar compounds from the bacterial cell (Levy, 2002).

The range of chemicals and metals present in the wastewater stream may account for different drivers of the dissemination of AMR (Alexander, Hembach and Schwartz, 2020), and the mitigation for these drivers and removal methods of the chemicals and metals require careful control (Bengtsson-Palme, Kristiansson and Larsson, 2018). More needs to be done in terms of guidance for maximum levels of pharmaceutical contaminants, or more rules need to be implemented surrounding implementation of removal methods.

1.6. Wastewater Treatment

Sewage streams, including wastewater treatment, are thought to contain elevated levels of ACs, antibiotic resistant organisms and ARGs (Guo *et al*., 2017; WHO/FAO/OIE, 2020). Therefore, the final products of treatment, namely biosolids and final effluent , are considered to be reservoirs for these (Wang *et al*., 2014). In addition, the lack of homogeneity in wastewater could provide an indefinite source of ARGs (Alexander, Hembach and Schwartz, 2020). The indefinite source would arise from the constant stream of anthropogenic and animal waste being excreted and arriving in these wastewater streams.

Conventional wastewater treatment includes an initial screen, a primary treatment (forming a sewage sludge), a secondary treatment, and often a tertiary treatment to

remove organic and inorganic pollutants (Camacho-Muñoz *et al*., 2012; Crini and Lichtfouse, 2019). Typically, the primary treatment consists of an initial screen to remove large solid materials, then a primary sedimentation stage, to begin to reduce and remove the suspended solids in the wastewater (Voulvoulis, 2018).

In addition to the treatment of the water stream, there will also need to be treatment of the sewage sludge formed during the primary treatment stage, this can be done by anaerobic digestion (AD) (Zhang and Li, 2018). AD is a biological process, resulting in the production of biogas from biodegradable waste. Microbes metabolise organic compounds such as proteins, lipids and carbohydrates, and methanogens convert the breakdown products into methane (CH4) (Angenent *et al*., 2018; Wang *et al*., 2020).

Following AD, the solid digestate is centrifuged and the solid output is often left to air-dry on outdoor concrete dry beds (Rouch *et al*., 2011). There are other methods of drying, including thermal drying and greenhouse drying in solar plants (Collard, Teychené and Lemée, 2017; Boguniewicz-Zablocka, Klosok-Bazan and Capodaglio, 2021).

T he water stream, from the previous treatments, and the centrifugation stage can be processed through the final stage of wastewater treatment: the final settlement tanks. These allow any remaining solids or persistent heavy metals to be removed from the water before release into the local aquatic environment (e.g. surface water, such as rivers and lakes).

Following total completion of the wastewater treatment processes, the final products must meet certain Environmental Quality Standards and legislation, including minimising biochemical oxygen demand (BOD) and chemical oxygen demand (COD),

correct pH, heavily reducing presence of coliforms and meeting acceptable concentrations of regulated contaminants (The Urban Waste Water Treatment (England and Wales) Regulations 1994). However, there are currently little to no parameters for CECs, such as concentrations of pharmaceutical compounds and any active degradation products.

Wastewater services in the UK collect on average over 11 billion litres each day from a wide range of sources (Department for Environment, Food and Rural Affairs, 2012). Focusing specifically on Yorkshire Water, around 1 billion litres are collected each day, serving 5 million customers with 671 water treatment plants and wastewater treatment plants (WWTPs) (Yorkshire Water, 2021). Naburn is one of ten WWTPs in York and serves a population of >140,000 (City of York Council, 2018). The site produces ~3441 tons of dry raw sludge solids each year and is co-located with a sludge treatment centre. Yorkshire Water have stated that 100% of their sludge is now treated using AD (Yorkshire Water, 2020).

1.7. Current Removal Methods

Typically, removal of ACs and ARGs is defined as the reduction in wastewater influent and final effluent concentrations. However, this does not account for partitioning into the solid stream (sludge) which is also disseminated into the local environment.

As part of the wastewater treatment process, AD can be used to remove organic pollutants and stabilise sludge (Narihiro and Sekiguchi, 2007). AD can occur under either mesophilic or thermophilic conditions, or may adopt both within a two-stage system (Samaras *et al*., 2014).

Mesophilic and thermophilic AD may have an impact on ARG removal (Sun *et al*., 2019), although (Zhang, Yang and Pruden, 2015) found certain ARG concentrations were increased during AD, e.g. *sul1* and *aadA*. This may be due to the genes' persistence and ability to degrade. In addition, thermophilic AD has been shown to be more effective at ARG removal than mesophilic AD (Sun *et al*., 2019), although this was counter argued by (Huang *et al*., 2019), who stated that higher temperatures only seem to have higher rates of ARG removal when any present MGEs are efficiently inhibited. In addition, (Jong *et al*., 2020) noted that HGT and plasmid transfer frequencies were observed to be higher under anaerobic conditions, which could prove interesting for the AD process. However, this was counter argued by (Tian *et al*., 2016) who reported a higher reduction in the abundance of genes investigated, attributed to a decrease in horizontal gene transfer at higher temperatures.

Steps can be taken in terms of sludge pre-treatment, prior to AD (Zhang and Li, 2018). Methods commonly used can include thermal hydrolysis, ultrasonication, ozonation, and microwave and alkaline pre-treatment (Chi *et al*., 2011; Pei *et al*., 2015; Czekalski *et al*., 2016; Sun *et al*., 2019). In addition, there are post-treatment methods for the removal of ARGs, including coagulation treatment (Grehs *et al*., 2019; Yu *et al*., 2021) and membrane filtration (Liang *et al*., 2021).

1.8. Reuse of By- Products

Contaminants within the digestate following AD may be incorporated within the soil environment after dewatering and disposal (Zhang and Li, 2018), this may especially be of concern within the agricultural industry (Calero-Cáceres, Ye and Balcázar, 2019) as biosolids are being increasingly used in the agriculture industry, for example, as organic fertilisers or soil conditioners (Thebo *et al*., 2017).

In the context of crop farming, if the biosolids contain AC residues, these may appear in the plants, resulting in sub-therapeutic exposure to indigenous microbial communities, and potentially affecting a sustainable food supply (Calero-Cáceres, Ye and Balcázar, 2019). Furthermore, it has been suggested by Cerqueira *et al*. (2019) that the use of biosolids on agricultural land drives uptake of ARGs into crops more than reclaimed irrigation water.

(Lau *et al*., 2020) investigated the impact of macrolide exposure in agricultural soils on the antibiotic resistome of the soil's microbial communities. Although it is interesting to note that they found environmentally "realistic" concentrations did not increase ARG abundances.

In terms of ACs, it has been noted that antibiotics can accumulate in soil matrices if biosolids are used as an organic fertiliser. Yang *et al.* (2018) found that fluoroquinolone antibiotics were more persistent in agricultural soils, particularly norfloxacin.

1.9. Aims and Hypothesis

In conclusion, the hypothesis is that wastewater treatment can promote dissemination of AMR, through the spread of sub-therapeutic concentrations of ACs and ARs, resulting in exposure of the microbial populations used within the treatment process, and that the control methods currently used are potentially inadequate for effective removal of these particular CECs.

The research questions to be investigated are:

1. Can a method be developed and optimised to detect antibiotic compounds and related genes within complex environmental samples?
2. Which ACs and ARGs are present in the different stages of wastewater treatment?
3. How do these compounds and genes persist throughout the treatment process? Are there any links that can be made?

2.0. Methods

2.1.1. Sample Collection

Sludge samples were collected from Naburn municipal wastewater treatment plant (WWTP) (Yorkshire Water), York, UK, and were frozen at -20 °C on the day of collection.

The specific sites collected from were the unprocessed influent, activated sludge process tanks and their distribution centre, anaerobic digester tanks, digester feed pump, centrifuge input and output, biosolids dried on concrete, and the final settlement tanks and their distribution centre. These sample sites in relation to the WWTP site are illustrated in Figure 2.

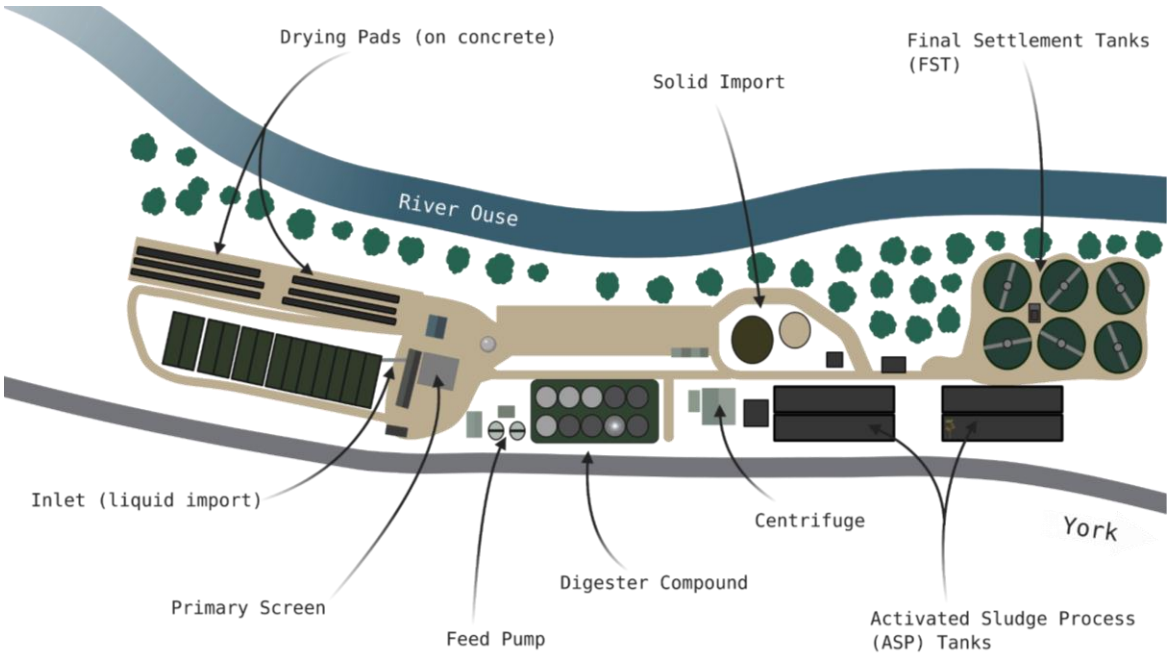


Figure 2: Map of Naburn municipal wastewater treatment plant, including locations of specific sample sites (Created using Biorender.com).

2.1.2. DNA Extraction

Microbial DNA was extracted using QIAGEN DNeasy 96 PowerSoil Pro QIAcube HT Kit (QIAGEN Inc., Manchester, UK) in conjunction with a QIAGEN TissueLyser II for bead-beating.

Before use, the 96-well PowerBead Pro Plate was briefly spun at 1000 rpm for 3 minutes in a bench-top plate centrifuge to agitate and loosen the beads within. 1 mL of each sludge sample was pipetted into a 1.5 mL centrifuge tube, which was spun at 10, 000 rpm for 3 minutes in a bench-top centrifuge, with the supernatant carefully discarded. This was to separate much of the water from the biosolid sample required. Water samples were not as suitable for this method, but were directly pipetted (200 µL volume) into the PowerBead Pro Plate. None of the samples were entirely dry (not even the “dried biosolid”, as some moisture was still present).

~0.2 g biosolid sample was accurately weighed into the PowerBead Pro Plate, noting down sample position and measured weight. The plate was then covered using a sealing film and frozen until bead-beating. The bead-beating was done using the QIAGEN TissueLyser II, according to the manufacturer’s instructions, then the DNA extraction was performed using the QIAcube HT machine according to the manufacturer’s instructions.

Quantification and quality check of the DNA was performed using a NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific Inc.) according to the manufacturer’s instructions. DNA quality and protein contamination was checked for using A260/280 and A260/230 absorbance ratios.Satisfactory ratios were A260/280 >1.8 and <1.96,

and A260/230 >1.85 and <2.35. Extraction repeats were carried out if DNA yield measurement was less than 80 ng μL^{-1}; or if the ratios were out of the specified range.

Satisfactory samples were then diluted to 10 ng μL^{-1} and 100 µL and sent to Resistomap oy (Helsinki, Finland), for SmartChip™ high-throughput real-time quantitative polymerization chain reaction (HT-qPCR) array analysis (Takara Bio Inc., CA, USA) to investigate the presence of 208 different ARGs (See Appendix 1 for a list of these genes and their primer sequences). These genes were chosen to encompass a wide range of resistance to different classes of antimicrobials, as well as related genes including ones associated with gene transfer, metal resistance and multi-drug resistance. The SmartChip methodology was taken from Majlander *et al*. (2021), and the data analysis was adapted from their methods as well.

2.1.3. qPCR Data Analysis

Data was analysed using R (Version 4.1.1) with the “tidyverse” packages (Wickham *et al*., 2019). Following analysis, the graphs were plotted using “ggplot2”.

For the ARG analysis, results were filtered out containing the flags: “Multiple Melt Peaks” or “Curve Fit Failed”, to make sure no false positive results were included. In addition, results appearing in a singular replicate were also removed, to increase reproducibility. The cycle threshold (Ct) values were normalised against 16S rRNA as a “reference gene” and positive control for qPCR measurement, giving a Delta Ct value. These were then normalised against the ASP sample as the earliest treatment point in the sampling campaign to give Delta Delta Ct values.

2.1.4. Mass Spectrometry Sample Preparation

Approximately 8 g of samples from 8 locations throughout the treatment plant were frozen in 50 mL centrifuge tubes. The frozen samples were freeze dried (Department of Chemistry, University of York, York, UK) for 24 hours or until a constant weight was achieved, indicating all moisture was removed. Sample moisture contents (%MC) were calculated using the following formula:

$$\%MC = \frac{Original\,Weight - New\,Weight}{Original\,Weight} \times 100$$

A 200 mg aliquot of each freeze-dried sample was then accurately weighed into 50 mL centrifuge tubes. Then solvent extraction was carried out following the simultaneous extraction method described in (Huang *et al*., 2013).

2.1.5. Stock Solutions

1L of sodium phosphate buffer (SPB) was prepared by mixing 10.56 g monosodium phosphate (NaH_2PO_4) and 0.82 mL phosphoric acid (H_3PO_4) in 1 L ultrapure water. EDTA-SPB (pH 4) was prepared by dissolving 80.0g ethylenediaminetetraacetic acid (EDTA) (Na_2EDTA) in 1 L SPB.

500 mL $Mg(NO_3)_2$-$NH_3{\cdot}H_2O$ solvent was prepared by mixing 50 % magnesium nitrate ($Mg(NO_3)_2{\cdot}6H_2O$) with 2.5 % ammonium hydroxide ($NH_3{\cdot}H_2O$) at a ratio of 24:1 (v/v) (so, 480 mL 50 % magnesium nitrate with 20 mL 2.5 % ammonium hydroxide). This solution was freshly prepared before use.

2 L extraction solvent was prepared by mixing 750 mL EDTA-SPB with 750 mL acetonitrile; then adding 500 mL of the $Mg(NO_3)_2$-$NH_3{\cdot}H_2O$ solvent.

2.1.6. Extraction Method

Solvent extraction was used alongside a solid phase extraction clean-up method. The 200 mg aliquots of freeze-dried sample were roughly ground and 20 mL extraction solvent was added. Samples were then left in the dark overnight.

Samples were shaken for 30 minutes on an orbital shaker at 25 °C, followed by 15 minutes in an ultrasonication bath. Samples were centrifuged for 10 minutes at 4000 rpm, whereby the supernatant was carefully decanted into 500 mL glass jars and wrapped in foil to eliminate photodegradation. The process was repeated twice and the supernatants were combined. The combined supernatants were then diluted to 500 mL using ultrapure water to ensure organic solvent was less than 5 % of the final solution.

Solid phase extraction (SPE) was performed to provide sample clean-up and compound pre-concentration. Samples were run through Oasis® Hydrophilic Lipophilic Balance (HLB) SPE cartridges (Waters Corp., MA, USA) affixed to a vacuum manifold to achieve a flow rate of approximately 3 ml min^{-1}. The cartridges were pre-conditioned by sequential washing of 10 mL methanol then 10 mL ultrapure water. Following the run-through, the cartridges were dried for 20 minutes, then frozen at -20 °C. Once run, the samples were eluted using 10 mL of the elution solution (methanol + 0.1 % formic acid), and left to run through via gravity into glass collection tubes. The collection tubes were then completely dried using a constant gentle stream of nitrogen gas (~30-40 psi), followed by a 1mL reconstitution using the mobile phase of the HPLC method (60% H2O + 40% methanol + 0.1 % formic acid). These 1 mL samples were vortexed to mix and then pipetted into 1.5 mL chromatography vials, before being frozen at -20 °C.

Validation of the method was carried out via the preparation of an antibiotic mixture. The antibiotics used can be found in Table 1. For the antibiotic validation mix, 5 mL stock solutions of 1 mg mL^{-1} concentration were made for each antibiotic in amber glass vials. 15 µL of each stock solution was mixed with methanol and made up to 30 mL for a 0.5 µL mL^{-1} concentration. This mixture was then diluted to make 500 ng L^{-1}, 50 ng L^{-1} and 5 ng L^{-1} concentrations for SPE filtration.

Antibiotic Name	**Antibiotic Class**
Cephalexin	Beta-Lactam (Cephalosporin)
Chloramphenicol	Other (Anaerobic)
Ciprofloxacin	Quinolone
Doxycycline Hyclate	Tetracycline
Erythromycin	Macrolide (MLSB)
Gentamicin	Aminoglycoside
Metronidazole	Other (Nitroimidazole)
Oxytetracycline	Tetracycline
Phenoxymethylpenicillin (Penicillin V)	Beta-Lactam(Penicillin)
Polymyxin B	Other (Polypeptide)
Puromycin	Other (Aminonucleoside)
Rifampicin (Rifampin)	Other (Mycobacteria)
Sulfadiazine	Sulphonamide
Trimethoprim	Other

Table 1: Names and classes of antibiotic compounds used for solid phase extraction (SPE) method validation in a methanol mixture.

2.1.7. HPLC-MSMS Analysis

The HPLC method was adapted from Wilkinson, Boxall and Kolpin (2019). For liquid chromatography separation, t he mobile phase used was methanol, LCMS-grade water + 0.1% formic acid and this was used with a Waters™ T3 column. The HPLC was coupled with a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific Inc.), with positive electrospray ionisation. The samples were introduced to the analyser via direct injection of 100-µL volume.

In contrast to the ARG method, the replicates in the AC method were sample replicates rather than technical, although there were technical replicates for the QC samples.

2.1.8. CompoundDiscoverer™ Software and Data Analysis

A workflow was designed in Thermo Scientific™ CompoundDiscoverer™ software (Thermo Fisher Scientific Inc.), incorporating an alignment of retention times, detecting compounds against different databases (e.g. mzcloud, DrugBank, ChemSpider), assigning compound annotations, marking background compounds and applying QC correction. . The primary screen for compounds was searched against the ITN Antibiotic List from the NORMAN suspect list exchange (Alygizakis, 2016).

The detection and annotation of compounds for the AC investigation was done within the CompoundDiscoverer™ software and data was extracted from the initial CompoundDiscoverer™ tables and was analysed and plotted in R to produce graphs using the package“ggplot2” (Wickham 2016).

3.0. Results

The methods described were able to tentatively identify 23 antibiotic compounds and 119 different genes. Compounds and genes associated with all the antibiotic classes investigated were found at each stage of wastewater treatment with results pointing towards varying concentrations and abundances.

The DNA extraction and SmartChip analysis method was chosen as the technology allows for the rapid quantitative analysis of a wide range of targeted genes. ARGs were detected across a range of target antibiotic classes and individual genes have been plotted and split according to their associated target antibiotic class (this information was provided by Resistomap Oy, Helsinki, Finland). The solvent and solid phase extraction was chosen due to the high recovery yields for a wide range of antibiotic compounds as reported by (Huang *et al*., 2013).

Over the course of the treatment system, 119 different genes were detected. Out of the 208 genes investigated, this accounts for 57.21%. A heatmap plotting the mean gene expression calculations is shown in Figure 3.

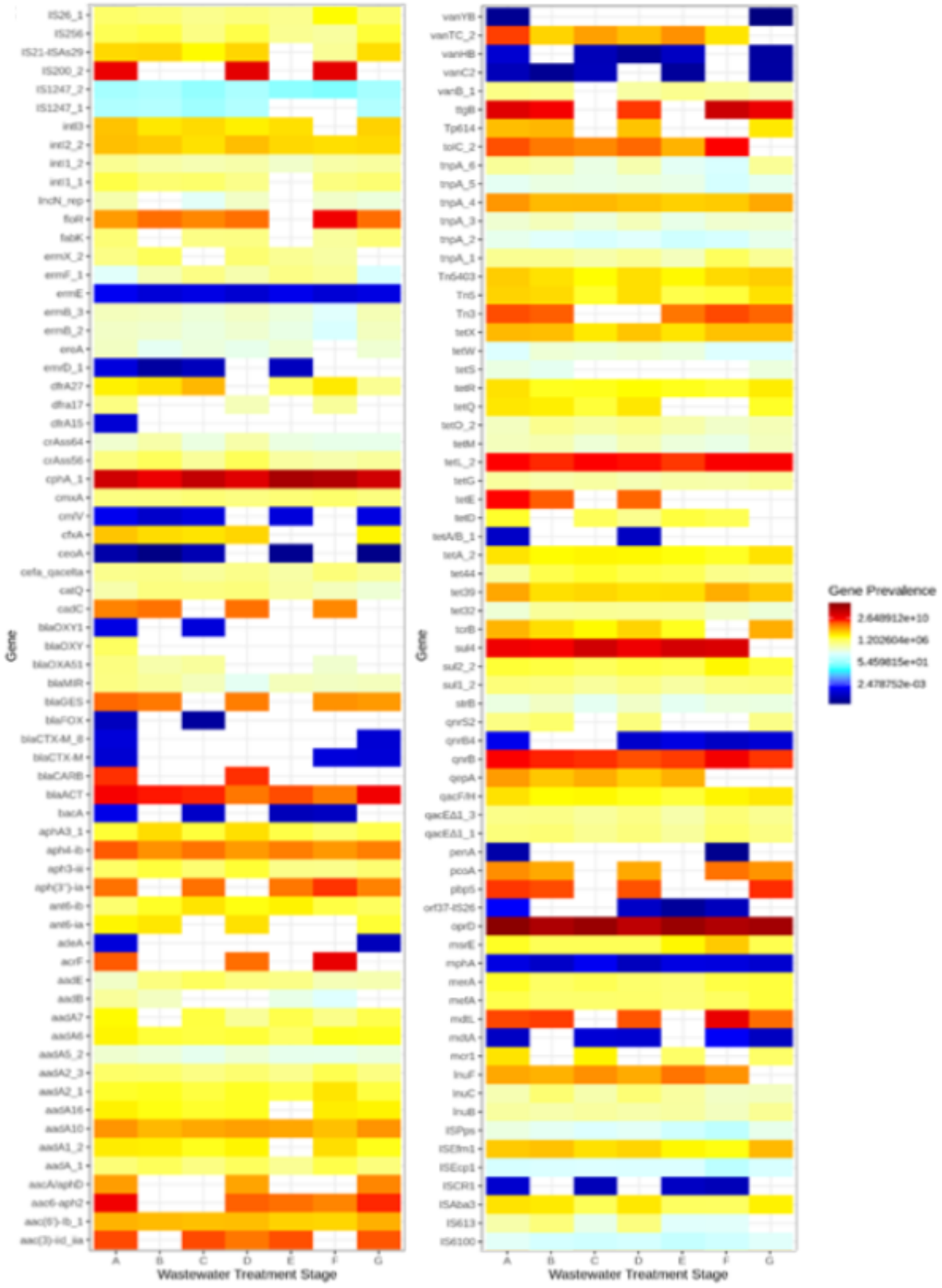


Figure 3: Mean normalised expressions for each individual gene. Red indicates the highest $2^{-\Delta\Delta Ct}$ value, normalised to the 16S rRNA gene and to the activated sludge process sample (as the earliest sample), decreasing to yellow, cyan and finally navy

for the lowest calculated values. Missing/not detected values have no colour, and a grey cross. Classes of genes can be found in Appendix 1. For the wastewater treatment stages: A – Anaerobic Digester Feed Pump; B – Anaerobic Digester Tank 2; C – Anaerobic Digester Tank 4; D – Anaerobic Digester Digestate Output; E – Fresh Biosolid; F- Dried Biosolid; G – Final Settlement Tank Distribution Centre.

Across all treatment stages, there does not seem to be a trend or pattern, although this will depend entirely on the contents of the wastewater influent.

Taking a focus on the genes conferring resistance to the tetracycline class of antibiotics, the mean relative gene expression was plotted for each individual gene, which can be found in Figure 4. 6 of these genes were chosen to show a wide range of differences throughout the wastewater treatment system. Those chosen were *tet39*, *tet44*, *tetA2*, *tetL2*, *tetR*, and *tetX*.

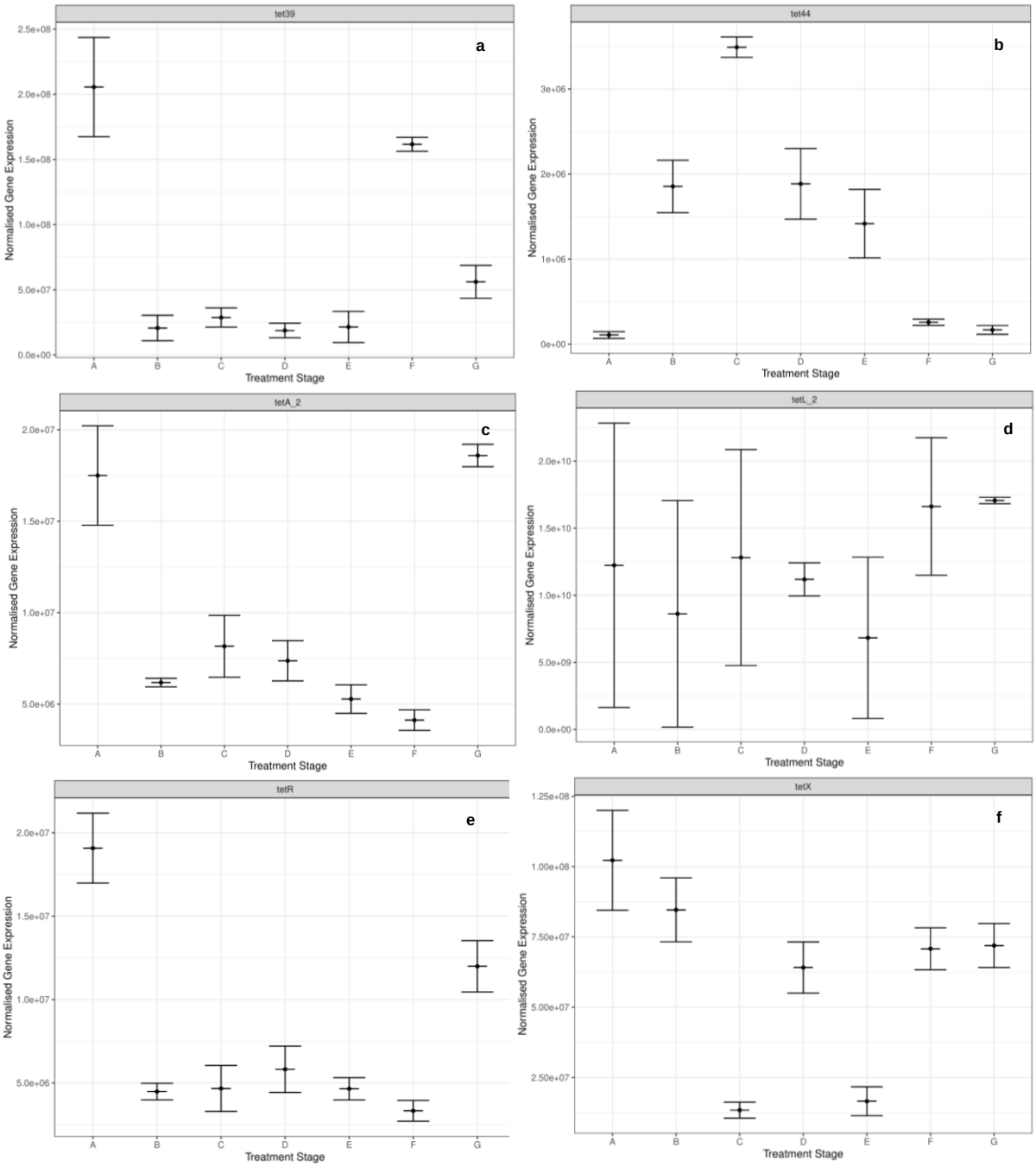


Figure 4: Mean normalised abundances for each individual gene. Plotted are the $2^{-\Delta\Delta Ct}$ values, normalised to the 16S rRNA gene and to the activated sludge process sample (as the earliest sample). The genes plotted are *tet39* (a), *tet44* (b), *tetA2* (c), *tetL2* (d), *tetR* (e), and *tetX* (f). For the wastewater treatment stages: A – Anaerobic Digester Feed Pump; B – Anaerobic Digester Tank 2; C – Anaerobic Digester Tank 4;

D – Anaerobic Digester Digestate Output; E – Fresh Biosolid; F- Dried Biosolid; G – Final Settlement Tank Distribution Centre.

Each of these genes exhibited a variety of patterns between the different stages of wastewater treatment. For example, *tetR* (Figure 4e) indicated higher gene abundance in the final settlement tank distribution centre (sample G), whereas *tet44* (Figure 4b) illustrated a lower value. Interestingly, *tetX* (Figure 4f) pointed towards significant differences in abundance between the different anaerobic digester tanks (samples B and C), yet for *tet39* (Figure 4a) there was no significant difference seen.

In addition to the genes, the antibiotic compounds were also plotted via heatmap, shown below in Figure 5. Compound results were filtered to remove compounds tagged as “background” and to only include compounds that had a match with the ITN mass list (Alygizakis, 2016), to detect the following analytes, shown in Figure 5. 23 different compounds were detected using the CompoundDiscoverer™ software. For this graph, red indicates higher peak areas compared to blue for lower values.

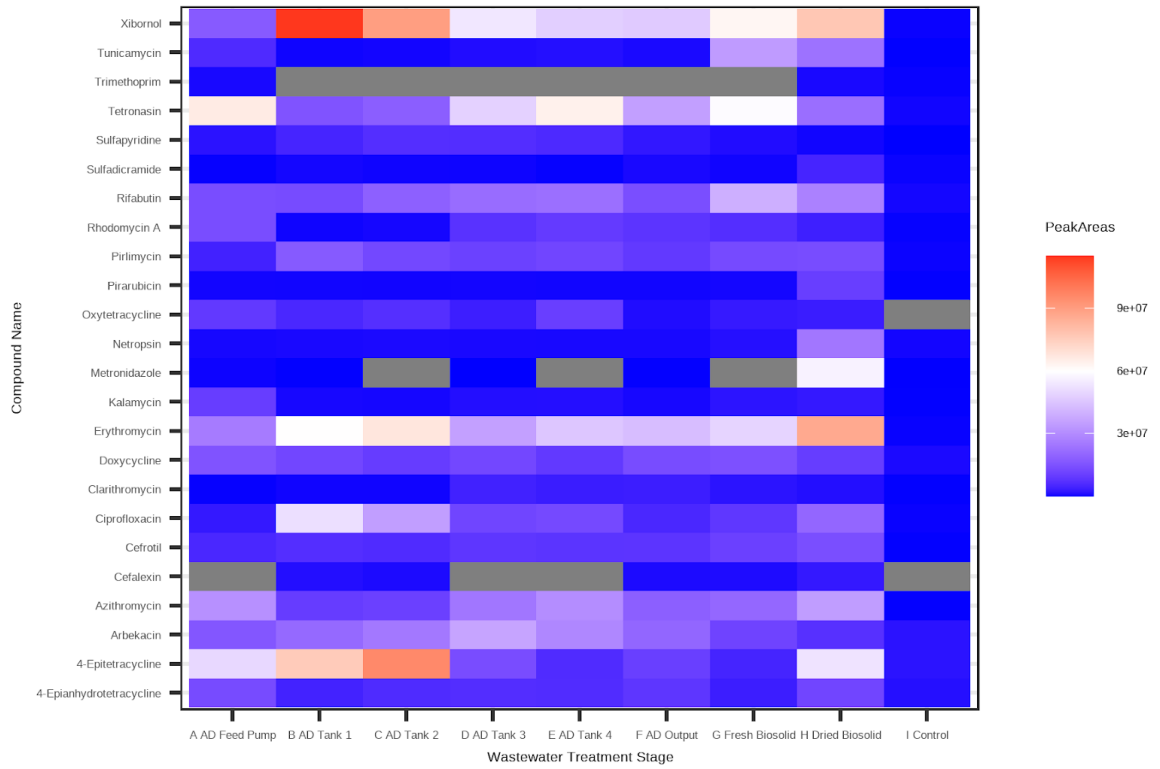


Figure 5: Mean peak areas from full-scan chromatograms of the different antibiotic compounds. Red indicates the highest peak areas, following the gradient to blue showing the lowest. Grey indicates no data.

It is interesting to note from Figure 5, that the majority of compounds were detected in each sample, and the increased size of the peak areas for compounds such as xibornol. It is also interesting to note how many of the compounds, especially erythromycin, exhibited increased peak areas for the dried biosolid sample in comparison to the rest of the wastewater treatment stages.

As previously with the gene investigation, focusing on compounds falling under the category of tetracyclines, the methods tentatively identified 4 compounds, two parent compounds and two common degradation products. These are illustrated below in Figure 6.

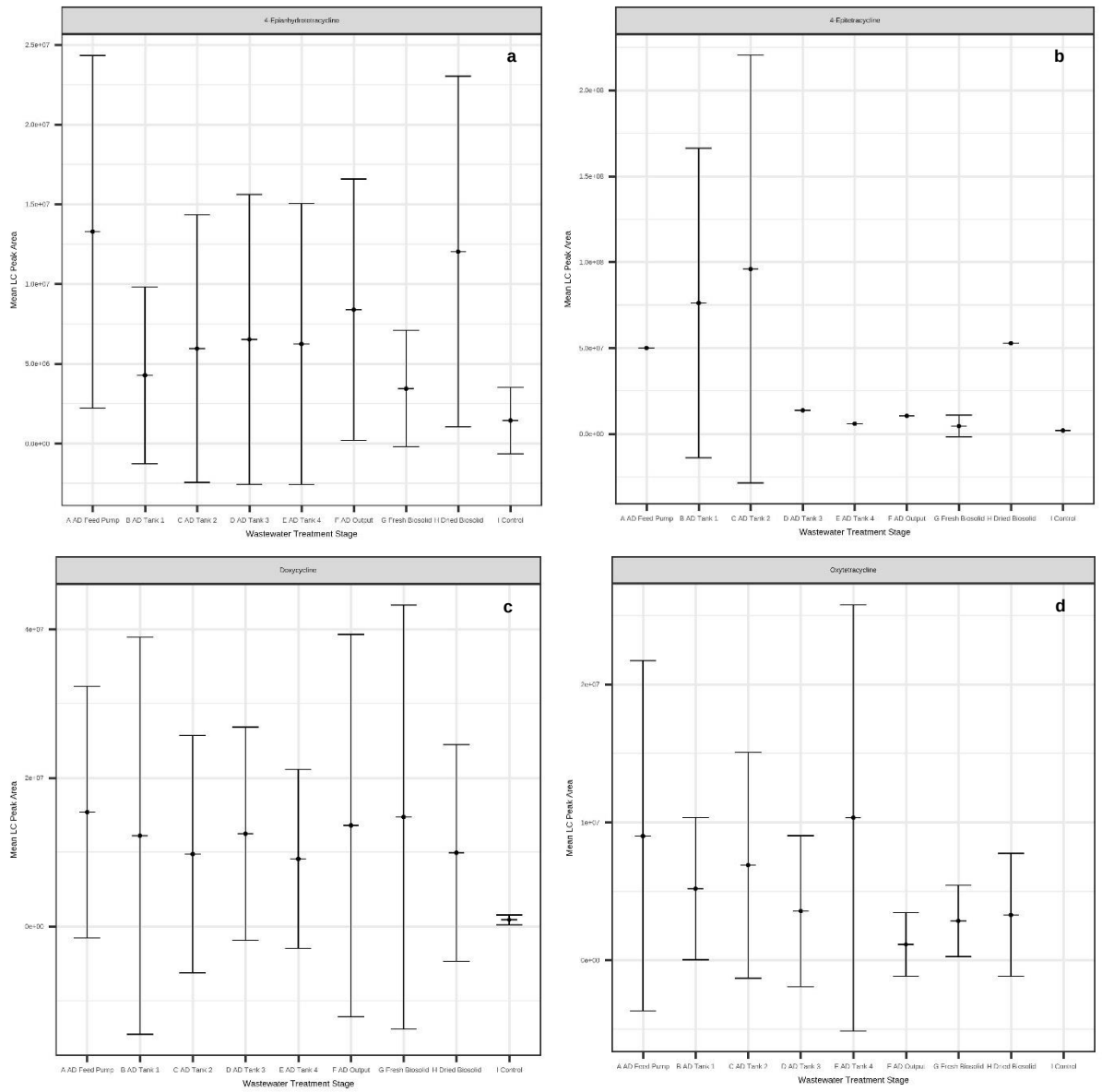


Figure 6: Four tetracycline compounds detected in CompoundDiscoverer, plotted with mean peak areas. The compounds are 4-epianhydrotetracycline (a), 4-epitetracycline (b), doxycycline (c) and oxytetracycline (d). The wastewater treatment stages are: A – anaerobic digester feed pump; B – anaerobic digester tank 1; C – anaerobic digester tank 2; D – anaerobic digester tank 3; E – anaerobic digester tank 4; F – anaerobic digester digestate output; G – fresh biosolid; H – dried biosolid and I – quality control conditioner.

The error bars seen in Figure 6 may at first look abnormally large. Due to the nature of this experiment, the data is only of the mean chromatogram peak area with standard deviations. It is unfortunately not possible to calculate relative or absolute concentrations, as there is an absence of calibration curves. The important message to take away from these graphs is deciphering the presence or absence of compounds in each stage of wastewater treatment.

The data shown in Figure 6 does not seem to show significant patterns between the different sampling sites, although the presence and detection of degradation products shows some stability/persistence. There is variation shown in all 4 compounds between the 4 different anaerobic digestion tanks.

4.0. Discussion

This study aimed to develop and optimise a method for extracting and detecting antibiotic compounds and resistance genes, as well as other related genes of interest. Due to the volume of data gathered, this aim has been met with some success. In addition, there has been both genes and compounds seen throughout the stages of wastewater treatment, indicating persistence if not inadequate methods of removal.

4.1. Antibiotic Resistance Genes

Of the 208 genes associated with AMR targeted in this analysis, 119 genes were detected in the panel. That does not mean that the 89 ARGs not detected were not present, as they may have been at undetectable levels, or may have only been at detectable levels in one of the sample replicates so were filtered out. In addition, the undetected genes could also have simply not been present in the wastewater influent at that particular time, but they may be more concentrated in other locations, or at other times.

It i s also interesting to note that throughout most of the genes detected, there is a difference between the relative abundances calculated in the different AD tanks. This may point towards the inherent heterogeneity of wastewater, as the processes themselves are identical, so one might expect the outcome of the dissemination of ARGs to also be equal. The fact that the results are so different may be an area of further research, because the heterogeneity may make it more difficult to control and regulate the levels of ARGs and biological CECs. It could also be due to the fact that the anaerobic digester tanks are fed at different rates with sludge as the waste passes through the treatment system. They will all be at different stages of their retention

times, and may not be mixing at the same rates. If the digester tanks were to be fed with identical feeds at the same time, the results would be expected to even out.

For the *tet* genes, many of these code for efflux pump proteins to eject the compound out of the bacterial cell (e.g. *tetA*, *tetD*, *tetL* and *tet39*), but there are also genes that code for protective proteins for the bacterial ribosomes (e.g. *tetM*, *tetS* and *tetW*), and for enzymes to deactivate the tetracycline compounds (e.g. *tetX*) (Roberts and Schwarz, 2016).

Concerning one of the genes marked “other” (see Appendix.1) , the detection of crAssphage may be associated with faecal contamination, and has been used as a biological marker for this in certain surface water studies (Sala-Comorera *et al*., 2021; Kongprajug *et al*., 2021). This is interesting, considering that *crAss56* and *crAss64* were detected in both the dried biosolid sample, and the final settlement tank distribution centre. As mentioned previously, these samples can be indicative of what is being released into the surrounding environment, as the dried biosolid can be reused in agriculture (e.g. as an organic fertiliser (Thebo *et al*., 2017)) and the final effluent from the settlement tanks is subsequently released into the local river adjacent to the WWTP.

Considering release into the surrounding environment, it could be an interesting further study to sample downstream, either from the surface water that the effluent is released to, or to the agricultural land on which the biosolid is being spread. This could provide a good image of what is occurring after this process is finished.

4.2. Antibiotic Compounds

The mean peak area was used as the y-axis, as due to the untargeted nature of the study, there were no calibration curves available to be able to accurately predict concentrations. This would be a limitation to address in future study. Especially as there is now a tentative idea of which antibiotic compounds are present, persistent and detectable within municipal wastewater treatment. All of the compounds identified and annotated also had the correct MS2 data, therefore giving a confidence level of 3 according to Schymanski *et al*. (2014).

Hydrolysis and degradation may affect whether compounds are detected; for example, even though the QC samples were spiked with phenoxymethylpenicillin, this was not detected through the methods described. Also, it must be noted that no calibration curves for these QCs were available, so accurate concentrations of compounds detected could unfortunately not be calculated. Although generally, degradation of some pharmaceuticals during wastewater treatment is known to be inefficient (Joss *et al*., 2006), it has been noted in other studies (Keen and Linden, 2013; Achermann *et al*., 2018; Martínez-Piernas, Plaza-Bolaños and Agüera, 2021).

An example: 4-epianhydrotetracycline is a key tetracycline degradation product (Wang *et al*., 2021), and has been detected in the samples (see Figure 6). As mentioned previously, this by-product is nephrotoxic (Pinto *et al*., 2002). There may be similar by-products that are also toxic in their own right that haven't been fully characterised.

The freeze-drying sample preparation would remove any low-to-high vapour pressure compounds. This would potentially remove volatile compounds, although the compounds of interest for this study are relatively non-volatile. Also, in terms of

physico-chemical properties of the various compounds, tetracycline compounds would be expected to partition into the solid stream due to their Kd values. The Kds of tetracyclines and fluoroquinolones are both much higher than of other pharmaceuticals, leading to low mobility in soil matrices and higher accumulation (Wang and Wang, 2015), explaining the expectation towards solid partition.

Local prescribing and antibiotic stewardship may have an impact on the levels of ACs being detected. Data from the Vale of York Clinical Commissioning Group (CCG) compared to national medians and other CCGs, shown in Figure 7, seems to indicate that antibiotic prescribing is following a decreasing pattern. The Vale of York CCG is also well below the national median. This is generally positive from an antimicrobial stewardship perspective, and may mean if we investigated other WWTPs in areas with higher levels of local prescribing, there may be a relationship in AC signals detected, especially with the use of more quantifiable methods. If prescription levels are decreasing, this would mean in a long-term study we would expect to see a decrease in the concentrations of antibiotic compounds throughout wastewater streams and treatment processes.

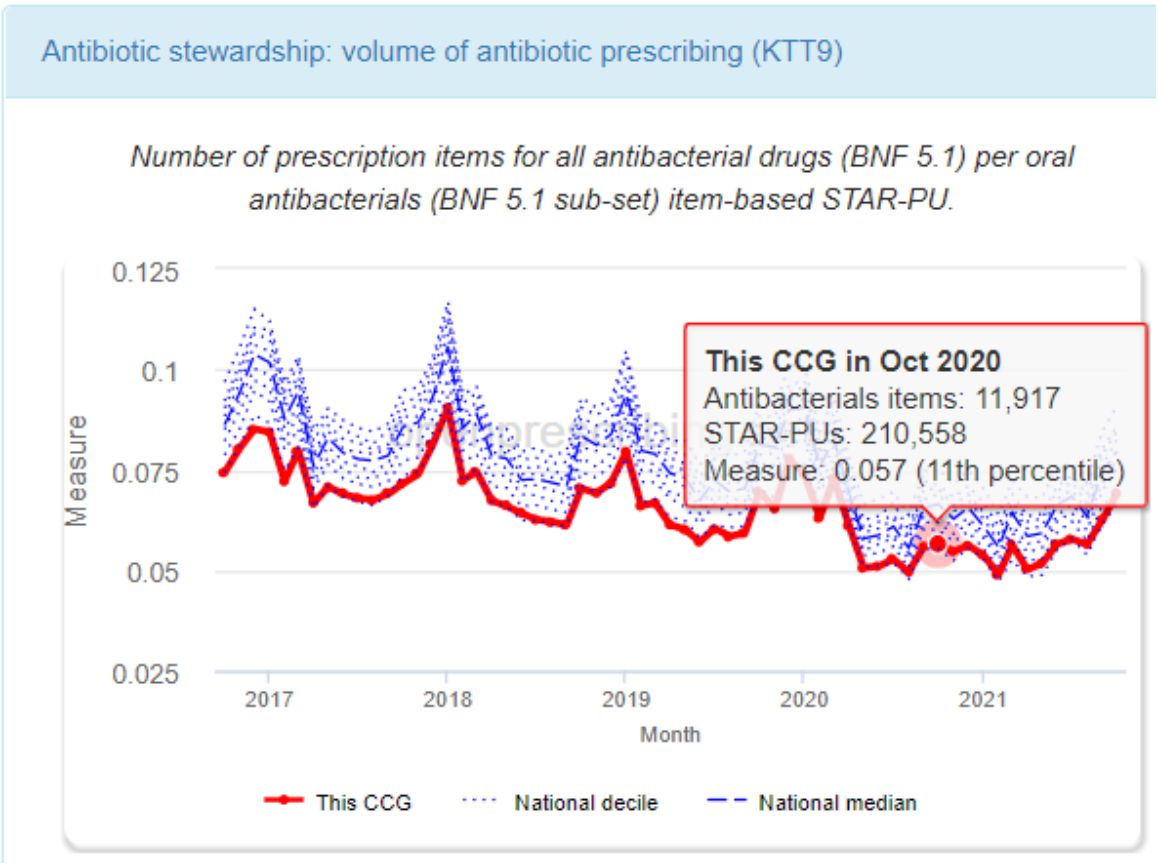


Figure 7: Vale of York CCG, data on local antibiotic prescribing, with detail shown for October 2020 (OpenPrescribing.net, 2021).

However, local prescribing data does not always give a complete picture of antibiotic compounds taken and released into local sewage systems, as not all antibiotics taken locally will have been prescribed locally. Some will have been prescribed in other areas of the UK, and some even abroad. For example, xibornol is mainly found in Italy and Spain (Cirri, Mura and Mora, 2007), and has been detected in the Naburn samples, as seen in Figure 5. In addition, not everyone who is prescribed antibiotics takes them, and not everyone who takes antibiotics has been prescribed them (e.g. self-prescribing with old, or another person's medication).

It is also important to recognise that the COVID-19 pandemic may have had potential impact on the levels of antimicrobials measured. It is thought that there would be an increase in concentrations of biocides in the environment, due to an increase in use of products such as disinfectants and domestic cleaners (Murray, 2020). Figure 7 did show a dip in antibiotic prescribing in 2020, but it is unknown whether this is entirely down to the pandemic or better local prescribing practices, and prescription data fails to include self-medication and over-the-counter products (although over-the-counter antibiotics are not available in the UK). A penultimate note to make is that not all ACs taken are by humans, and that veterinary and agricultural antibiotics will also make up a significant proportion of AC use in the local area (Manzetti and Ghisi, 2014).

Finally, wastewater is inherently heterogeneous, and is susceptible to change even over a 24-hour period, so it is important to note that the results shown here are only a snapshot of a single point in time. This is a key limitation of the study, samples taken over a period of time, following a particular influent, would be more representative of the contents, in terms of ARGs and ACs. For example, the anaerobic digestion tanks will have all been fed at different times with different feeds, and will have been at various points of respective retention times. This could be accounted for by developing a longitudinal study with these methods, and by increasing the WWTP sites investigated, possibly including sites with differing treatment techniques, and by considering both smaller rural sites and larger sites treating urban municipal wastewater. To link both the genes and compounds together, there did not seem to be any patterns. This again, could be due to the heterogeneity of the samples, and the fact that the influent for each sample taken would probably have had different concentrations of genes, compounds and maybe even resistant organisms. There may

also have been co-selectors present, such as biocides or metals that were not looked for. All this considered would point towards areas of further research.

The main aim of the study was to gain an idea of the composition of genes and compounds in wastewater treatment, but not really to see what impact the treatment processes have. It would be sufficient to say that the genes and compounds between the treatment stages are highly variable, and this is important for drawing conclusions on the stability and persistence of compounds.

Finally, since patterns cannot be deduced from this particular set of data, and there is a clear idea of what needs to be studied next, it would be interesting to see if there is a link between concentrations of antibiotics and abundances of genes. More quantitative work needs to be carried out. Although a link has not been ruled out by this study as both genes and compounds of matching classes, such as the tetracyclines, have been detected and tentatively identified.

4.3. Conclusions

This snapshot study sampled different points of the treatment system at different individual process stages, so it is difficult to interpret the mechanism and fate of the genes and related compounds. However, where the results are indicating higher concentrations, this says something about either the stability and persistence of the genes/compounds or the speed of transition through the system to the different stages. Linking both the genes and the compounds together, it is difficult to identify patterns between the two, as the heterogeneity is clear.

In conclusion, the methodology resulted in multiple ARGs and ACs across a number of major antibiotic classes being detected at the WWTP on this snapshot day. This

project has been important for method development and optimisation for future studies in this area, as it has brought together many methods from different areas. A targeted extraction method has been taken and proven to work successfully in a non-targeted way and detect more compounds than initially designed for. Many genes and related antibiotic compounds across a variety of classes have been successfully detected, although only semi-quantified.

Future work needs to focus on following samples through the treatment system, considering retention times and specific process lengths. There is also scope to focus on the drying methods of biosolids prior to application on agricultural lands, and investigating whether there is anything that can be changed to limit ARG and AC levels.

In addition, further research could be done to fully quantify levels of ARGs and ACs by using longitudinal studies, possibly to develop an environmental risk model for potential use in WWTP regulations. It could also be interesting to discover whether there are any seasonal patterns in levels of ARGs and ACs in local sewage systems, for example, are there higher levels in the winter when infection levels are typically increased?

5.0. Appendices

5.1. Primer Information

Information provided by Resistomap Oy (Helsinki, Finland).

Gene	Target antibiotics (major)	Forward Primer	Reverse Primer
16S rRNA	16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG
aacC2	Aminoglycoside	ACGGCATTCTCGATTGCTTT	CCGAGCTTCACGTAAGCATTT
aacA/aphD	Aminoglycoside	AGAGCCTTGGGAAGATGAAGTTT	TTGATCCATACCATAGACTATCTCATCA
aac(6')-II	Aminoglycoside	CGACCCGACTCCGAACAA	GCACGAATCCTGCCTTCTCA
aphA3_1	Aminoglycoside	AAAAGCCCGAAGAGGAACTTG	CATCTTTCACAAAGATGTTGCTGTCT
aac(6')-Ib_1	Aminoglycoside	CGTCGCCGAGCAACTTG	CGGTACCTTGCCTCTCAAACC
aadA2_1	Aminoglycoside	ACGGCTCCGCAGTGGAT	GGCCACAGTAACCAACAAATCA
aadA_1	Aminoglycoside	GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA
aadD	Aminoglycoside	CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA
aphA1/7	Aminoglycoside	TGAACAAGTCTGGAAAGAAATGCA	CCTATTAATTTCCCCTCGTCAAAAA

aadE	Aminoglycoside	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT
str	Aminoglycoside	AATGAGTTTTGGAGTGTCTCAACGTA	AATCAAAACCCCTATTAAAGCCAAT
strB	Aminoglycoside	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT
aadA5_2	Aminoglycoside	ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG
aadA2_3	Aminoglycoside	CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG
aac(3)-iid_iia	Aminoglycoside	CGATGGTCGCGGTTGGTC	TCGGCGTAGTGCAATGCG
aac(6)-im	Aminoglycoside	CGTGAGCATTATACAGAGCAATGG	CCATTTCCGTTCGTAGATATTGGC
aac6-aph2	Aminoglycoside	CCAAGAGCAATAAGGGCATACCAA	GCCACACTATCATAACCACTACCG
aadA10	Aminoglycoside	ACAGGCACTCAACGTCATCG	CGCGGAGAACTCTGCTTTGA
aadA16	Aminoglycoside	ACGGTGGCCTGAAGCC	GAATTGCAGTTCCCGTCTGG
aadA1_2	Aminoglycoside	TGTACGGCTCCGCAGTG	CACGGAATGATGTCGTCGTG
aadA6	Aminoglycoside	CCATCGAGCGTCATCTGGAA	CCCGTCTGGCCGGATAAC
aadA7	Aminoglycoside	CACTCCGCGCCTTGGA	TGTGGCGGGCTCGAAG
aadB	Aminoglycoside	CCTGCTTGGTGGGCAGAC	CGGCACGCAAGACCTCAA
ant6-ia	Aminoglycoside	TCGCCATGAGCTGCTGA	CCTATCATACTCCGGATAGGCATA

ant6-ib	Aminoglycoside	AGAACATCCGACAGCACGTTC	CCAACCTTCCATGAAATCATTCGC
aph(3")-ia	Aminoglycoside	TAACAGCGATCGCGTATTTCG	TCCGACTCGTCCAACATCAATA
aph3-iii	Aminoglycoside	CAGAAGGCAATGTCATACCACTTG	GACAGCCGCTTAGCCGAA
aph4-ib	Aminoglycoside	GGGAACACCGTGCTCACC	GTTGGTCCCGTGCAGGTC
cfiA	Beta Lactam	GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT
blaMOX/blaCMY	Beta Lactam	CTATGTCAATGTGCCGAAGCA	GGCTTGTCCTCTTTCGAATAGC
blaVEB	Beta Lactam	CCCGATGCAAAGCGTTATG	GAAAGATTCCCTTTATCTATCTCAGACAA
blaOXY	Beta Lactam	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT
blaPSE	Beta Lactam	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC
cphA_1	Beta Lactam	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC
cfxA	Beta Lactam	TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT
cepA	Beta Lactam	AGTTGCGCAGAACAGTCCTCTT	TCGTATCTTGCCCGTCGATAAT
blaGES	Beta Lactam	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG
blaSFO	Beta Lactam	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT

blaTLA	Beta Lactam	ACACTTTGCCATTGCTGTTTATGT	TGCAAATTTCGGCAATAATCTTT
blaVIM	Beta Lactam	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT
pbp5	Beta Lactam	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT
blaCTX-M_5	Beta Lactam	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT
penA	Beta Lactam	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG
blaCTX-M_8	Beta Lactam	CGTCACGCTGTTGTTAGGAA	CGCTCATCAGCACGATAAAG
blaNDM	Beta Lactam	GGCCACACCAGTGACAATATCA	CAGGCAGCCACCAAAAGC
blaCMY_2	Beta Lactam	AAAGCCTCAT GGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA
blaCTX-M	Beta Lactam	CGTACCGAGCCGACGTTAA	CAACCCAGGAAGCAGGCA
blaFOX	Beta Lactam	CCTACGGCTATTCGAAGGAAGATAAG	CCGGATTGGCCTGGAAGC
blaOXA51	Beta Lactam	CGACCGAGTATGTACCTGCTTC	TCAAGTCCAATACGACGAGCTA
blaOXY1	Beta Lactam	AAAGGTGACCGCATTCGC	CCAGCGTCAGCTTGCG
blaSHV11	Beta Lactam	TTGACCGCTGGGAAACGG	TCCGGTCTTATCGGCGATAAAC
blaTEM	Beta Lactam	CGCCGCATACACTATTCTCAG	GCTTCATTCAGCTCCGGTTC
blaKPC	Beta Lactam	GCCGCCAATTTGTTGCTGAA	GCCGGTCGTGTTTCCCTTT
beta_B2	Beta Lactam	GTAACGCCTACTGGAAGTCCA	CAGCTTCTCCTTGAGAATGCAG

blaACT	Beta Lactam	AAGCCGCTCAAGCTGGA	GCCATATCCTGCACGTTGG
blaCARB	Beta Lactam	TGATTTGAGGGATACGACAACTCC	CTGTAATACTCCGAGCACCAA
blaLEN	Beta Lactam	TGTTCGCCTGTGTGTTATCTCC	GCAGCACTTTAAAGGTGCTCAC
blaMIR	Beta Lactam	CGGTCTGCCGTTACAGGTG	AAAGACCCGCGTCGTCATG
blaBEL-nonmobile	Beta Lactam	ATGTCCATGGCACAGACTGTG	CCTGTCTTGTCACCCGTTACC
intI1_2	Integrons	CGAAGTCGAGGCATTTCTGTC	GCCTTCCAGAAAACCGAGGA
intI1_1	Integrons	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA
intl3	Integrons	CAGGTGCTGGGCATGGA	CCTGGGCAGCATCACCA
acrB_1	MDR	AGTCGGTGTTCGCCGTTAAC	CAAGGAAACGAACGCAATACC
acrF	MDR	GCGGCCAGGCACAAAA	TACGCTCTTCCCACGGTTTC
adeA	MDR	CAGTTCGAGCGCCTATTTCTG	CGCCCTGACCGACCAAT
acrA_1	MDR	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC
emrD_1	MDR	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC
mdtE	MDR	CGTCGGCGCACTCGTT	TCCAGACGTTGTACGGTAACCA
oprD	MDR	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA
ttgA	MDR	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA

mexE	MDR	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC
tolC_2	MDR	CAGGCAGAGAACCTGATGCA	CGCAATTCCGGGTTGCT
bexA/norM	MDR	TCGGGCATCCCGTTTATGATC	GTAGGCTGCGCATAATACCCA
mdtA	MDR	ACAAGCCCAGGGCCAAC	CCTTAATGGTGCCTTCGGTTTC
mdtH	MDR	ATGCTGGCTGTACAAGTGATG	CACTCCAGCGGGCGATA
cefa_qacelta	MDR	TAGTTGGCGAAGTAATCGCAAC	TGCGATGCCATAACCGATTATG
qacF/H	MDR	CTGAAGTCTAGCCATGGATTCACTAG	CAAGCAATAGCTGCCACAAGC
arsA	MDR	CAGGTCAGCCGCATCAACC	GCCTGAAACACGGCAATTTCTTC
cadC	MDR	CGCTCTGTGTCAGGATGAAGAG	CTTTCTTATGTGCTAGGGCGATCA
pcoA	MDR	TGGCGTATGGAGTTTCAATGC	GAATAATGCCGTGCCAGTGAA
sugE	MDR	CTTAGTTATTGCTGGTCTGCTGGA	GCATCGGGTTAGCGGACTC
tcrB	MDR	GTGCCGGAACTCAAGTAGCA	GCACCGACTGCTGGACTTAA
Tp614	MGE	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT
IS613	MGE	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT
tnpA_1	MGE	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT
tnpA_2	MGE	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC

tnpA_3	MGE	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT
tnpA_4	MGE	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT
tnpA_5	MGE	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG
tnpA_6	MGE	TGCAGATGGTTTAACCTTGGATATTT	TCGGTTCATCAAACTGCTTCAC
orf37-IS26	MGE	GCCGGGTTGTGCAAATAGAC	TGGCAATCTGTCGCTGCTG
ISPps	MGE	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC
IS1247_2	MGE	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT
ISAba3	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
ISEfm1	MGE	AGGTGTCCATGACGTGAAAGTG	TCCTTTGTCCCCTAGGATATTGG
Tn5	MGE	TCAGAGGCAGCGGTATACGA	GGTTGATTCAGTTAAAGTACGTAAAACTTT
IncN_rep	MGE	AGTTCACCACCTACTCGCTCCG	CAAGTTCTTCTGTTGGGATTCCG
IncP_oriT	MGE	CAGCCTCGCAGAGCAGGAT	CAGCCGGGCAGGATAGGTGAAGT
IS1247_1	MGE	CGGCCGTCACTGACCAA	TCGGCAGGTTGGTGACG
IS200_2	MGE	GCACACCCGATGGAACTGTAAA	TCGGCGGGATCTCCAGAAG

IS21-ISAs29	MGE	GGTCCGTCAGGCACAAGTC	GGGATCGTATCGGCAAGCC
IS256	MGE	CTTGCGCATCATTGGATGATGG	AAGAACGGCTCCAATTAAGCGA
IS26_1	MGE	ATGGATGAAACCTACGTGAAGGTC	CGGTACTTAATCTGTCGGTGTTCA
IS3	MGE	CGGTCTGAGCTTCGGGAA	AGAACTGTCACTCCGGTCTG
IS6100	MGE	CGCACCGGCTTGATCAGTA	CTGCCACGCTCAATACCGA
ISCR1	MGE	ATGGTTTCATGCGGGTT	CTGAGGGTGTGAGCGAG
ISEcp1	MGE	CATGCTCTGCGGTCACTTC	GACGCACCTTCTTGATGACC
Tn3	MGE	GCTGAGGTGTTCAGCTACATCC	GCTGAGGTAGTCACAGGCATTC
Tn5403	MGE	AAGCGAATGGCGCGAAC	CGCGCAGGGTAAACTGC
trbC	MGE	CGGYATWCCGSCSACRCTGCG	GCCACCTGYSBGCAGTCMCC
ermF_1	MLSB	CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA
ereA	MLSB	GATAATTCTGCTGGCGCACA	GCAGGCGTGGTCACAAC
ermB_2	MLSB	GAACACTAGGGTTGTTCTTGCA	CTGGAACATCTGTGGTATGGC
lnuB	MLSB	GGATCGTTTACCAAAGGAGAAGG	AGCATAGCCTTCGTATCAGGAA
lnuC	MLSB	GGGTGTAGATGCTCTTCTTGGA	CTTTACCCGAAAGAGTTTCTACCG

mefA	MLSB	TAATTATCGCAGCAGCTGGTTC	GTTCCCAAACGGAGTATAAGAGTG
mphA	MLSB	TCAGCGGGATGATCGACTG	GAGGGCGTAGAGGGCGTA
ermE	MLSB	GTCACGCAGCTGGAGTTCG	CGGTGAAGCACAGCTCGAC
ermX_2	MLSB	TGATGACGGCTCAGTGG	GTGCACCAGCGCCTGA
ermB_3	MLSB	TGAAAGCCATGCGTCTGAC	TTCAGCTGGCAGCTTAAGC
lnuF	MLSB	ATACCGGTCATTTCCACTTGGC	GCATCAGGCTGATGAGGTTCAA
mefB	MLSB	CCGATAGGCTTACTTGTTGCAG	AGTCCACTTGCGGTTTCATTG
msrE	MLSB	CGGCAGATGGTCTGAGCTTAAA	CGCACTCTTCCTGCATAAAGGA
ttgB	Other	TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC
nisB_1	Other	GGGAGAGTTGCCGATGTTGTA	AGCCACTCGTTAAAGGGCAAT
nimE	Other	TGCGCCAAGATAGGGCATA	GTCGTGAATTCGGCAGGTTTA
merA	Other	GTGCCGTCCAAGATCATG	GGTGGAAGTCCAGTAGGGTGA
crAss56	Other	CAGAAGTACAAACTCCTAAAAAACGTAGAG	GATGACCAATAAACAAGCCATTAGC
crAss64	Other	TGTATAGATGCTGCTGCAACTGTACTC	CGTTGTTTTCATCTTTATCTTGTCCAT
sat4	Other	GAATGGGCAAAGCATAAAAACTTG	CCGATTTTGAAACCACAATTATGATA

qacEΔ1_1	Other	TCGCAACATCCGCATTAAAA	ATGGATTTCAGAACCAGAGAAAGAAA
qacEΔ1_3	Other	GTCGGTGTTGCTTATGCAGTCT	CAACCAGGCAATGGCTGTAA
bacA	Other	ATCCGCGGCACCCTGA	CCTGCTTGATGGACTTGATGAAGA
mcr1	Other	CACATCGACGGCGTATTCTG	CAACGAGCATACCGACATCG
arr2	Other	TTGGCGATTGGTGACTTGCTAA	ATCGTCTTCGAACGGTCCTG
fabK	Other	CAGGAGCAGGAAATCCAAGC	CCAGCTTCCATTCCTTCTGC
catB3	Phenicol	GCACTCGATGCCTTCCAAAA	AGAGCCGATCCAAACGTCAT
catB8	Phenicol	CACTCGACGCCTTCCAAAG	CCGAGCCTATCCAGACATCATT
ceoA	Phenicol	ATCAACACGGACCAGGACAAG	GGAAAGTCCGCTCACGATGA
cmlA_2	Phenicol	TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG
cmxA	Phenicol	GCGATCGCCATCCTCTGT	TCGACACGGAGCCTTGGT
catA1	Phenicol	GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA
catB2	Phenicol	GCTACTATTCCGGCTATTACCATG	GGGCTCCTCGTTCATGTAGA
catP	Phenicol	CCTTTGGACTGAGTGTAAGTCTGA	TAAAGCCATCGAAGGTTGACCA

catQ	Phenicol	AGGTGCACTTACAGTATGACTGC	AACGTGGGAAGTTCTCGTCATAC
cmlV	Phenicol	GCCCTCATCACCGTCTTCG	GGACGTTGGCGATGGAGAG
floR	Phenicol	AACCCGCCCTCTGGATCA	GCCGTCGAGAAGAAGACGAA
qnrA	Quinolone	AGGATTTCTCACGCCAGGATT	CCGCTTTCAATGAAACTGCAA
qnrB	Quinolone	GCGACGTTCAGTGGTTCAGA	GCTGCTCGCCAGTCGAA
qepA	Quinolone	GGGCATCGCGCTGTTC	GCGCATCGGTGAAGCC
qnrB4	Quinolone	TCACCACCCGCACCTG	GGATATCTAAATCGCCCAGTTCC
qnrB_2	Quinolone	CGACGTTCAGTGGTTCAGATCTC	GCCAAGCCGCTCCATGAG
qnrD	Quinolone	CGCTGGAATGGCACTGTGA	GCTCTCCATCCAACTTCACTCC
qnrS2	Quinolone	TCCCGAGCAAACTTTGCCAA	GGTGAGTCCCTATCCAGCGA
qnrVC_2	Quinolone	TTCCTTTAAACGGGCAAACCTC	CGATACCTGATTCATGAAGCTAGC
sul4	Sulphonamide	TCAACGTCACTCCAGACAGC	TGGAAATAACGACGTCCACA
sul1_2	Sulphonamide	GCCGATGAGATCAGACGTATTG	CGCATAGCGCTGGGTTTC
sul2_2	Sulphonamide	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT
tet36_1	Tetracycline	AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT

tet32	Tetracycline	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA
tetA_2	Tetracycline	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG
tetA/B_1	Tetracycline	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA
tetQ	Tetracycline	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT
tetH	Tetracycline	TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA
tetW	Tetracycline	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC
tetO_2	Tetracycline	CAACATTAACGGAAAGTTTATTGTATACCA	TTGACGCTCCAAATTCATTGTATC
tetX	Tetracycline	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT
tetC_2	Tetracycline	ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG
tetS	Tetracycline	TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA
tetE	Tetracycline	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA

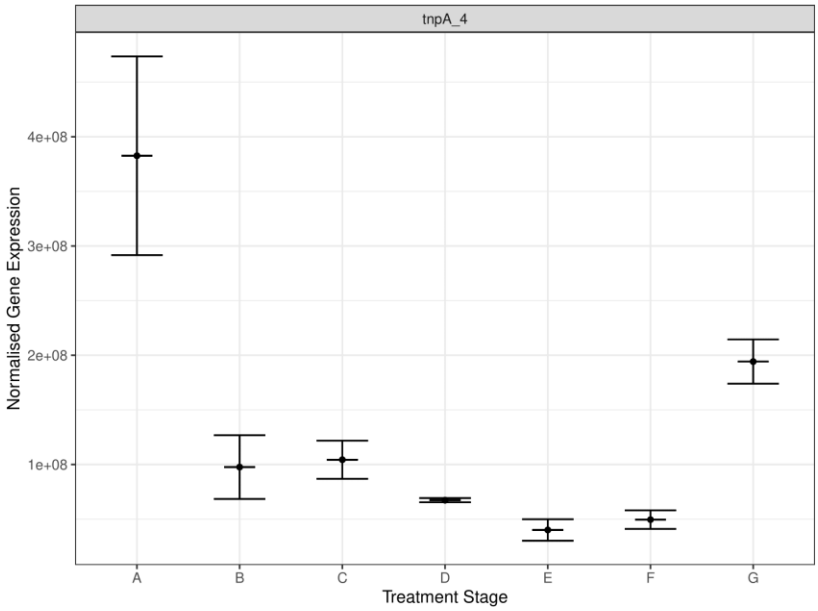
tetT	Tetracycline	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG
tetL_2	Tetracycline	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT
tet39	Tetracycline	TATAGCGGGTCCGGTAATAGGTG	CCATAACGATCCTGCCCATAGATAAC
tetD	Tetracycline	AATTGCACTGCCTGCATTGC	GACAGATTGCCAGCAGCAGA
tetG	Tetracycline	TCGCGTTCCTGCTTGCC	CCGCGAGCGACAAACCA
tetM	Tetracycline	GGAGCGATTACAGAATTAGGAAGC	TCCATATGTCCTGGCGTGTC
tet44	Tetracycline	CTCATGTAGATGCAGGAAAGACG	GTAACTGCTGCCTGAATTGTGA
tetR	Tetracycline	CCGTCAATGCGCTGATGAC	GCCAATCCATCGACAATCACC
dfrA1_1	Trimethoprim	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG
dfrA12	Trimethoprim	CCTCTACCGAACCGTCACACA	GCGACAGCGTTGAAACAACTAC
dfrA15	Trimethoprim	AGGCCGAAAGACTTTCGAGTC	TCACCTTCTGGCTCAATGTCG
dfra17	Trimethoprim	CGGGAACGGCCCTGATATTCC	CGTGTTGCGACCGCATACTTTC
dfrA27	Trimethoprim	GCCGCTCAGGATCGGTA	GTCGAGATATGTAGCGTGTCG
dfrAB4	Trimethoprim	CGGTTCGCATTCCCATCAAA	CGCAGTCATGGGATAAATCTGG
dfrG	Trimethoprim	TCAATCGGAAGAGCCTTACCTGA	TGGGCAAATACCTCATTCCATTCC

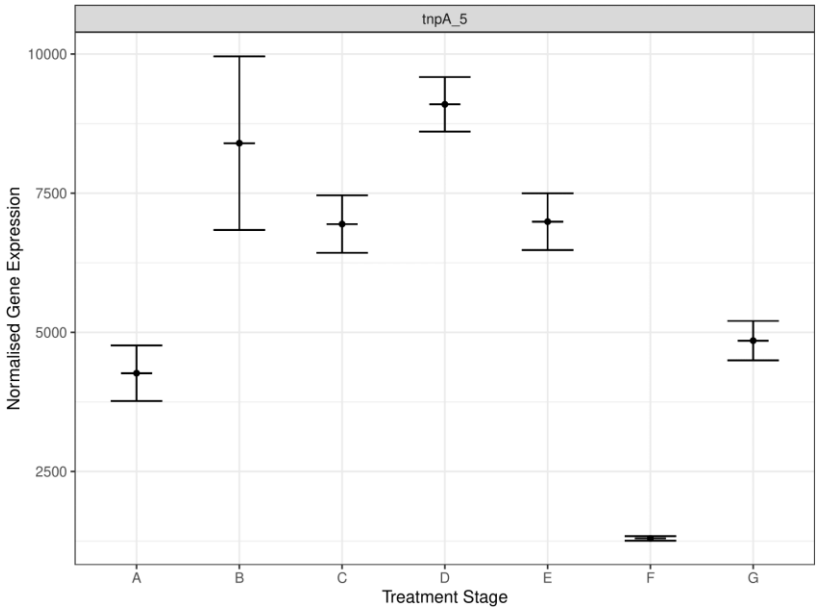
vanC_2	Vancomycin	CCTGCCACAATCGATCGTT	CGGCTTCATTCGGCTTGATA
vanB_1	Vancomycin	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT
vanD	Vancomycin	CAGAGGAACATAATGTTTCGATAAAATCT	GCCGGATTTTGTGATTCCAA
vanHB	Vancomycin	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT
vanXB	Vancomycin	AGGCACAAAATCGAAGATGCTT	GGGTATGGCTCATCAATCAACTT
vanRB	Vancomycin	GCCCTGTCGGATGACGAA	TTACATAGTCGTCTGCCTCTGCAT
vanTG	Vancomycin	CGTGTAGCCGTTCCGTTCTT	CGGCATTACAGGTATATCTGGAAA
vanYB	Vancomycin	GGCTAAAGCGGAAGCAGAAA	GATATCCACAGCAAGACCAAGCT
vanTC_2	Vancomycin	ACAGTTGCCGCTGGTGAAG	CGTGGCTGGTCGATCAAAA
vanA	Vancomycin	GGGCTGTGAGGTCGGTTG	TTCAGTACAATGCGGCCGTTA
vanC2	Vancomycin	TGACTGTCGGTGCTTGTGA	GATAGAGCAGCTGAGCTTGTTC
vanG	Vancomycin	TGTTTCGCAGAACCGTGTCAA	CCCTGCACTGTTCCATCTTCTC
vanSB	Vancomycin	GAAGATAAAGAGGGAAGCGTACTC	CCGAATTGTCAGCCCTTGATAA
intI2_2	Integrons	TGCTTTTCCCACCCTTACC	GACGGCTACCCTCTGTTATCTC
ermD_1	MLSB	GGACTCGGCAATGGTCAGAA	CCCCGAAACGCAATATAATGTT

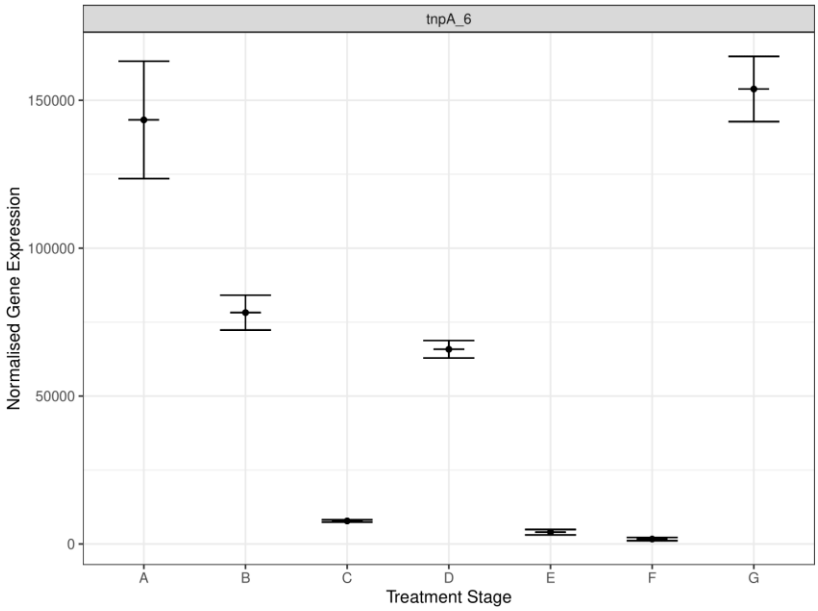
vgaA_1	MLSB	CGAGTATTGTGGAAAGCAGCTAGTT	CCCGTACCGTTAGAGCCGATA
yidY/mdtL	Phenicol	GCAGTTGCATATCGCCTTCTC	CTTCCCGGCAAACAGCAT
mdtL	Phenicol	TGCTGATCGGGATTCTGATTG	CAGGCGCGACGAACATAAT
sul3_1	Sulphonamide	TCCGTTCAGCGAATTGGTGCAG	TTCGTTCACGCCTTACACCAGC
blaOXA48	Beta Lactam	TGTTTTTGGTGGCATCGAT	GTAAMRATGCTTGGTTCGC
armA_2	Aminoglycoside	TGCATCAAATATGGGGGTCT	TGAAGCCACAACCAAAATCT
rmtB	Aminoglycoside	GCTGTGATATCCACCAGGGA	AAGCTTAAAAATCAGCGCCA

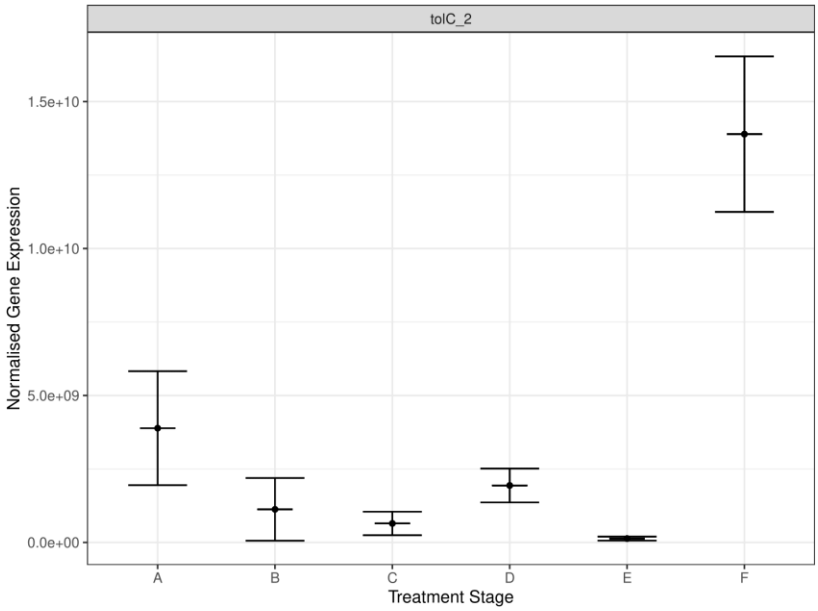
5.2. qPCR Relative Abundance Data

Attached below are all of the produced relative abundance graphs from the qPCR study.


tnpA_4
Normalised Gene Expression
4e+08
3e+08
2e+08
1e+08
A
B
C
D
E
F
G
Treatment Stage


tnpA_5
Normalised Gene Expression
10000
7500
5000
2500
A
B
C
D
E
F
G
Treatment Stage


tnpA_6
Normalised Gene Expression
150000
100000
50000
0
A
B
C
D
E
F
G
Treatment Stage


tolC_2
Normalised Gene Expression
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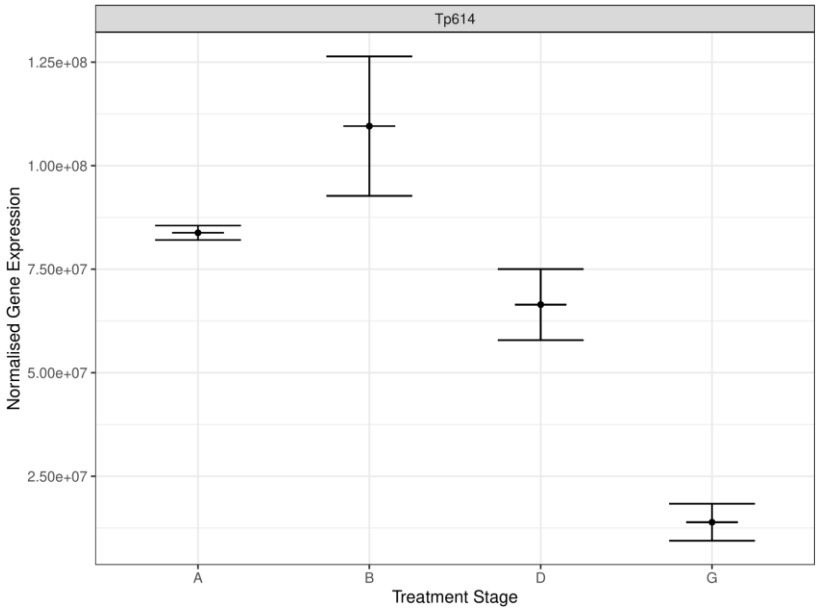
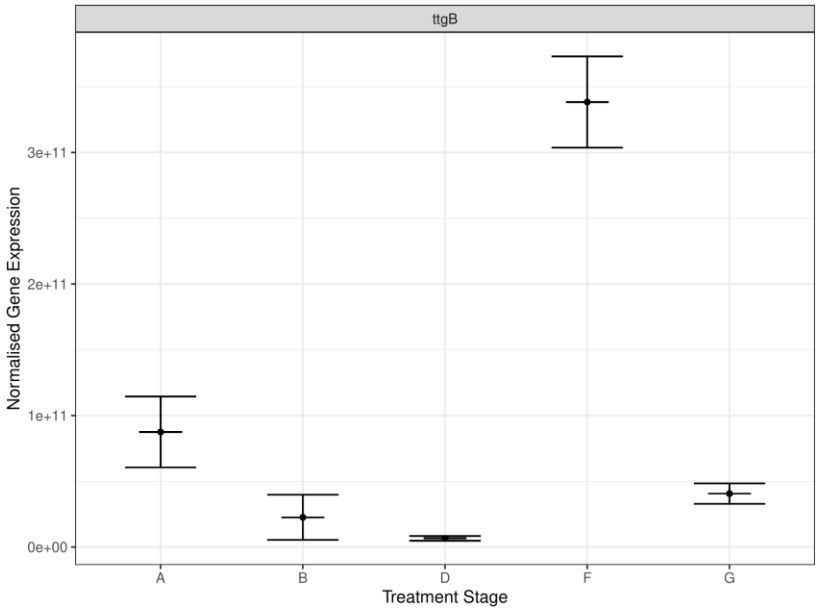
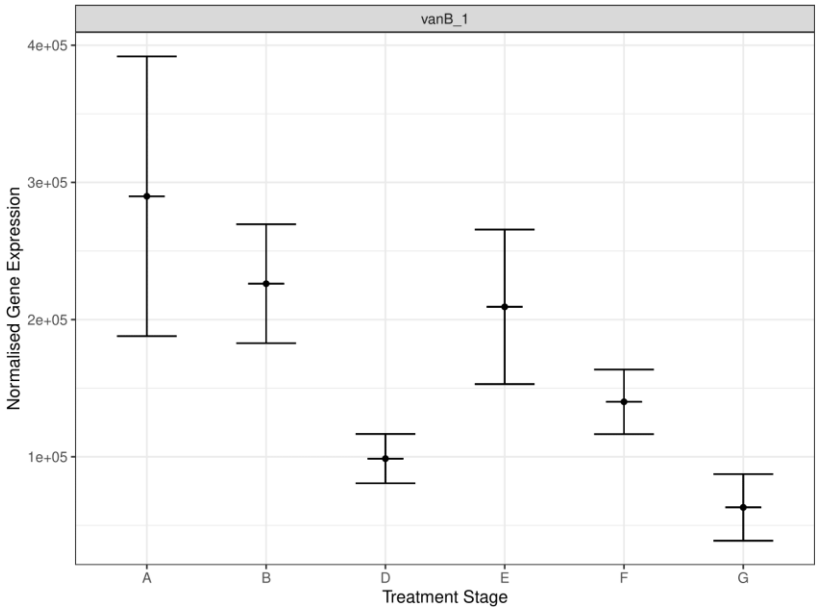
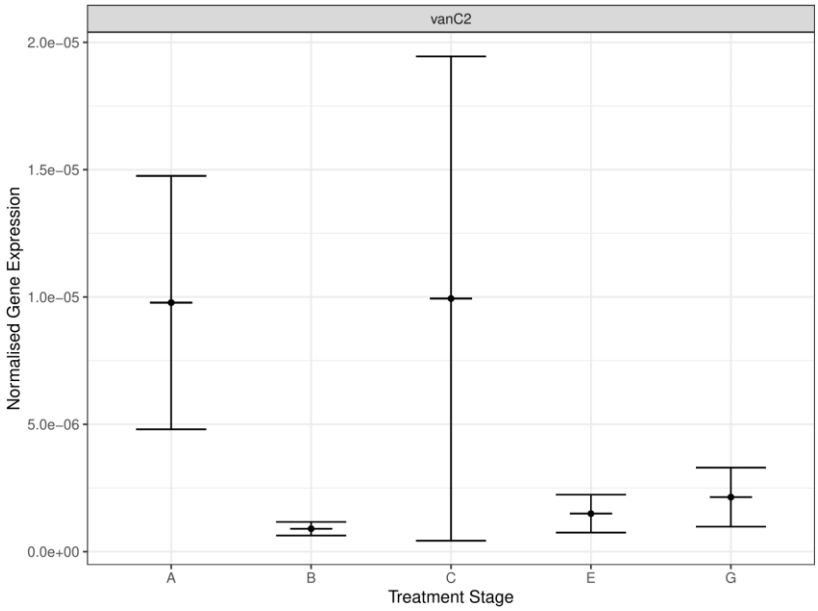
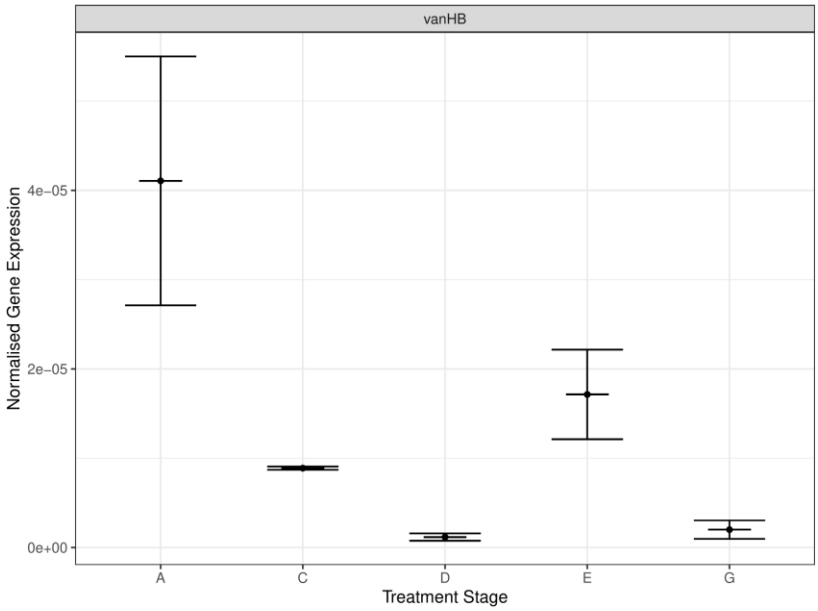
1.0e+10
5.0e+09
0.0e+00
A
B
C
D
E
F
Treatment Stage

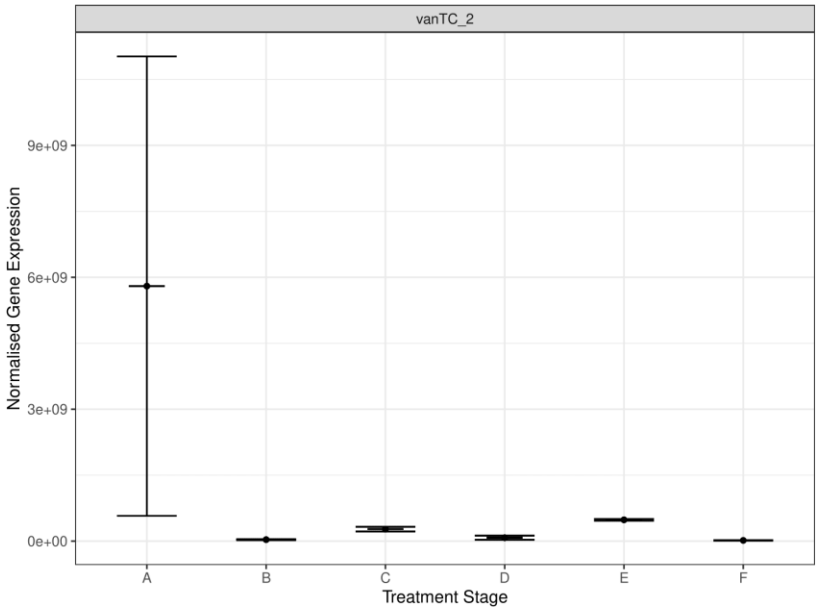

Tp614
1.25e+08
1.00e+08
7.50e+07
5.00e+07
2.50e+07
Normalised Gene Expression
A
B
D
G
Treatment Stage

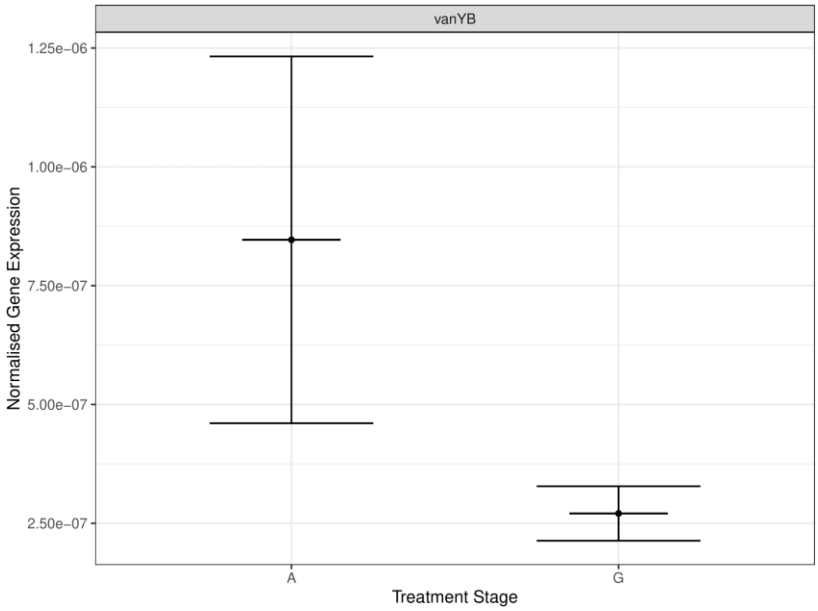

ttgB
3e+11
2e+11
1e+11
0e+00
Normalised Gene Expression
A
B
D
F
G
Treatment Stage

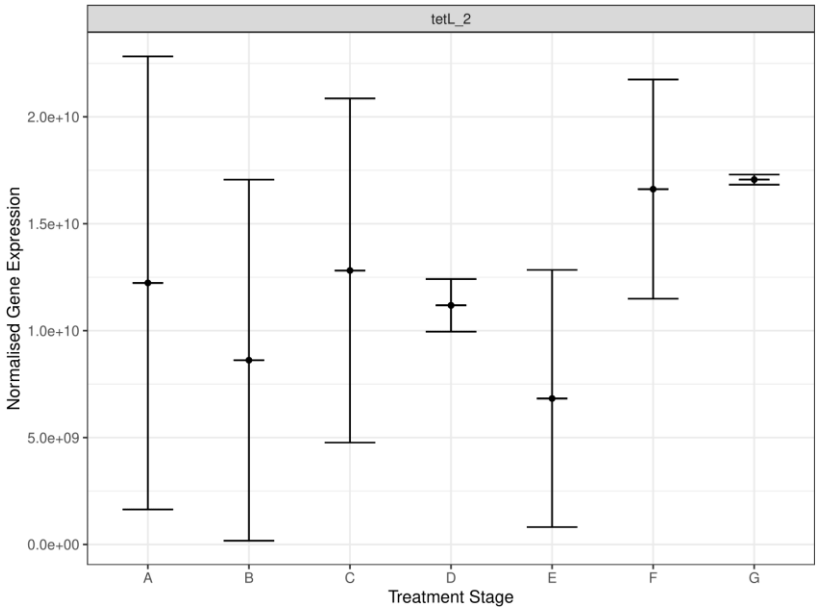

vanB_1
Normalised Gene Expression
4e+05
3e+05
2e+05
1e+05
A
B
D
E
F
G
Treatment Stage

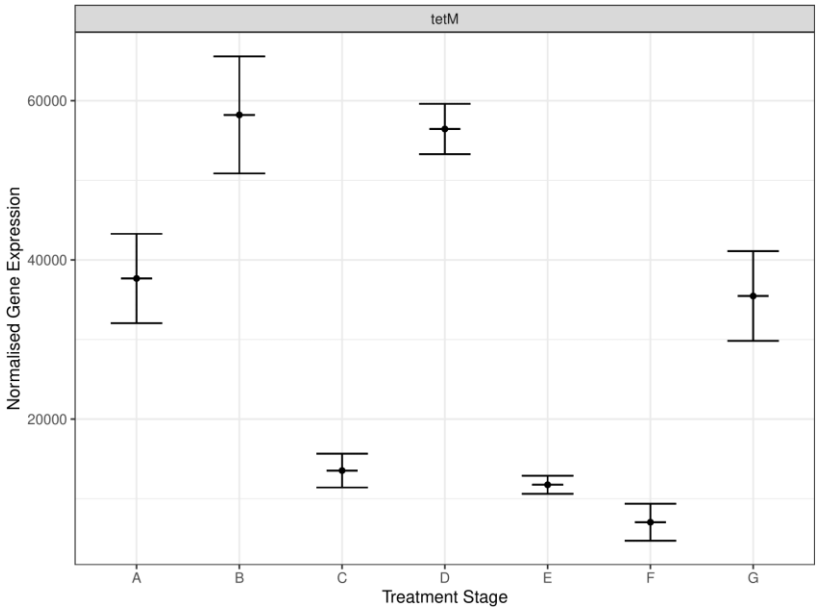

vanC2
Normalised Gene Expression
2.0e−05
1.5e−05
1.0e−05
5.0e−06
0.0e+00
A
B
C
E
G
Treatment Stage

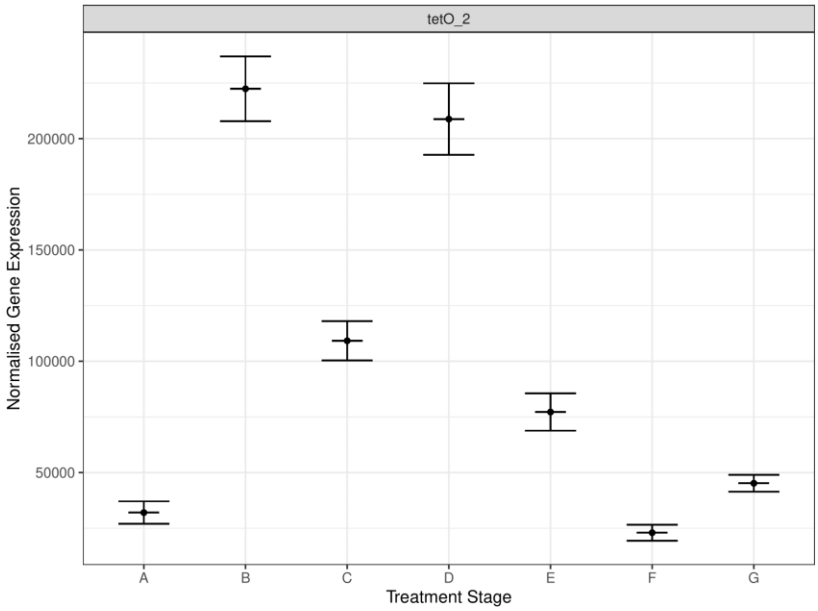

vanHB
Normalised Gene Expression
4e−05
2e−05
0e+00
A
C
D
E
G
Treatment Stage

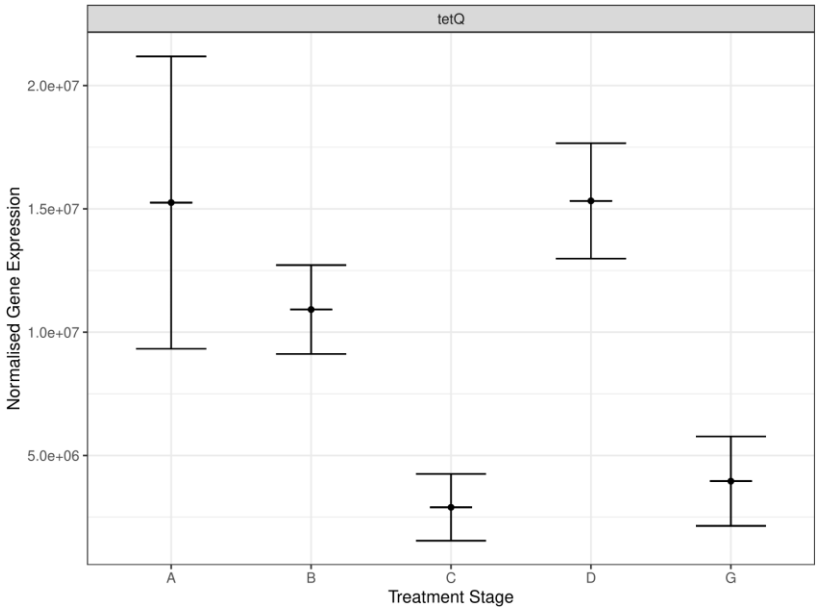

vanTC_2
Normalised Gene Expression
9e+09
6e+09
3e+09
0e+00
A
B
C
D
E
F
Treatment Stage

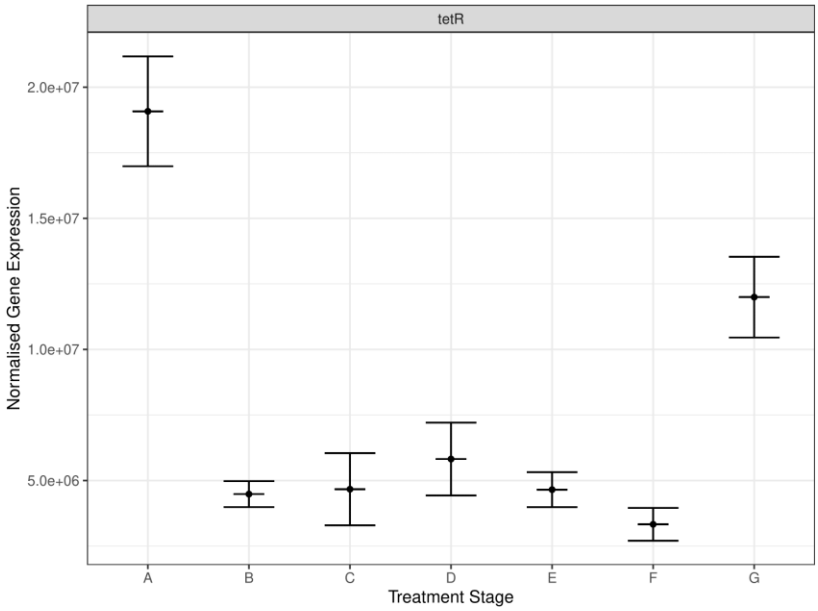

vanYB
1.25e−06
1.00e−06
7.50e−07
5.00e−07
2.50e−07
Normalised Gene Expression
A
G
Treatment Stage

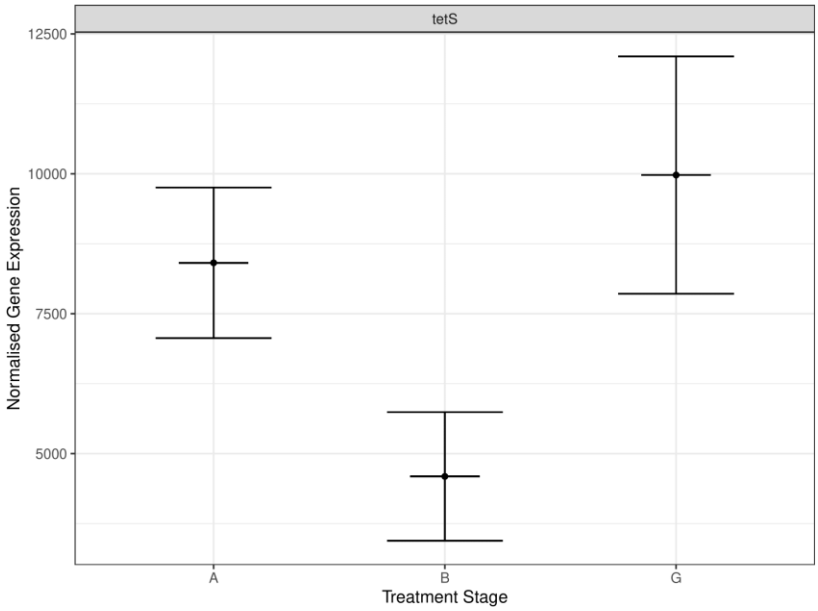

tetL_2
Normalised Gene Expression
2.0e+10
1.5e+10
1.0e+10
5.0e+09
0.0e+00
A
B
C
D
E
F
G
Treatment Stage

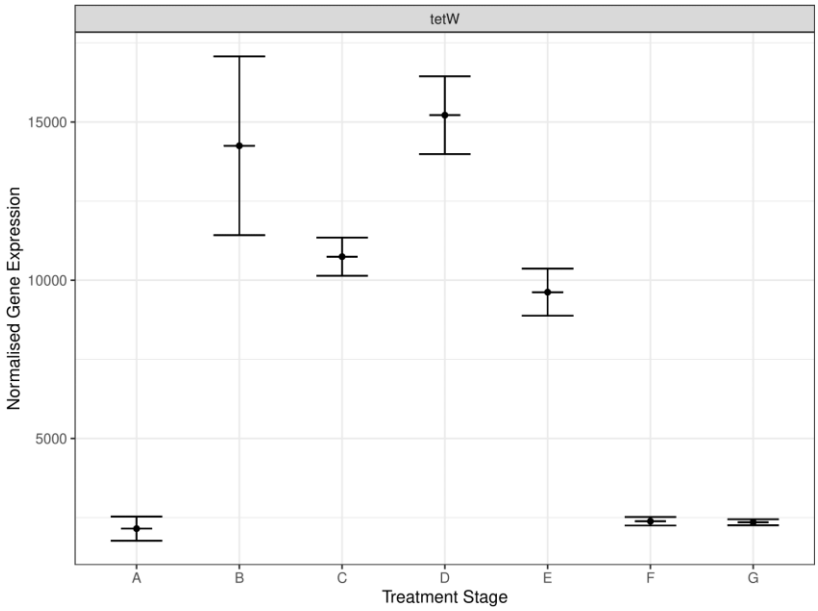

tetM
Normalised Gene Expression
60000
40000
20000
A
B
C
D
E
F
G
Treatment Stage

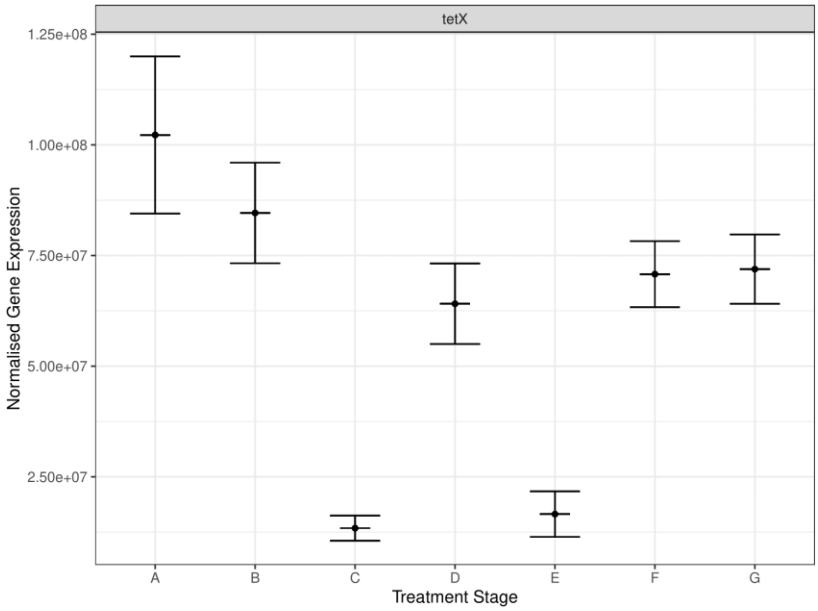

tetO_2
Normalised Gene Expression
200000
150000
100000
50000
A
B
C
D
E
F
G
Treatment Stage

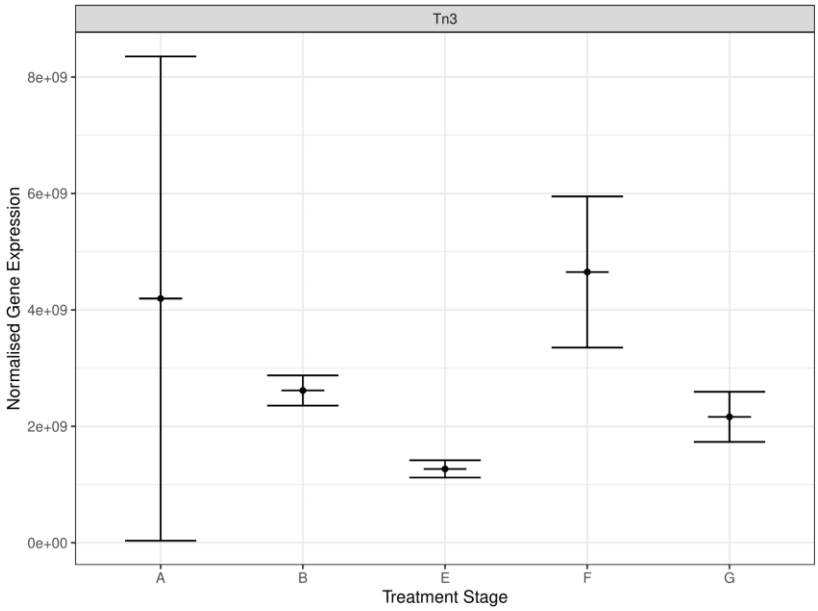

tetQ
Normalised Gene Expression
2.0e+07
1.5e+07
1.0e+07
5.0e+06
A
B
C
D
G
Treatment Stage

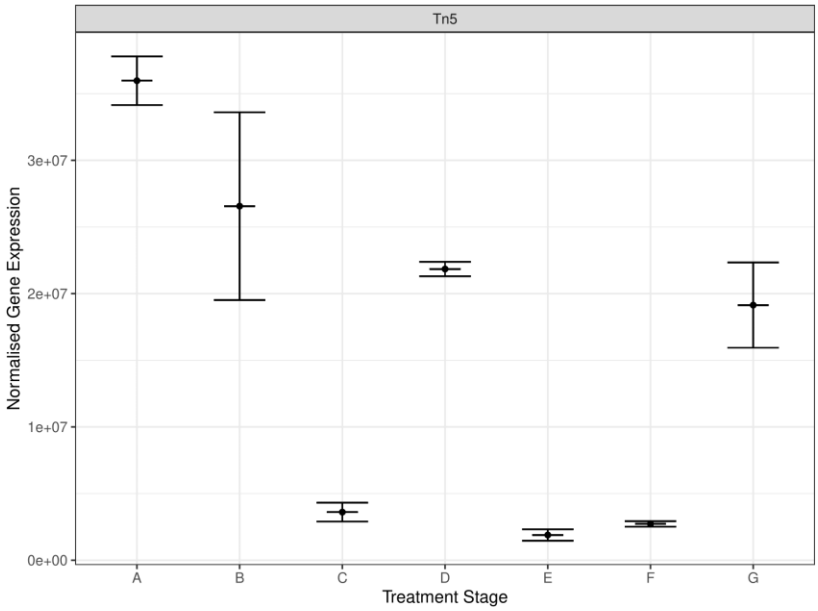

tetR
2.0e+07
1.5e+07
1.0e+07
5.0e+06
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

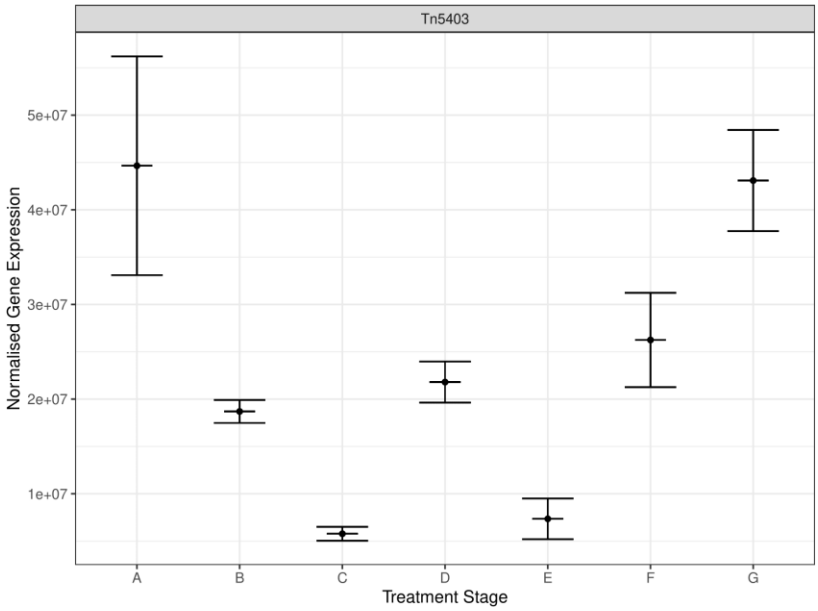

tetS
12500
10000
7500
5000
Normalised Gene Expression
A
B
G
Treatment Stage


tetW
15000
10000
5000
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage


tetX
1.25e+08
1.00e+08
7.50e+07
5.00e+07
2.50e+07
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage


Tn3
Normalised Gene Expression
8e+09
6e+09
4e+09
2e+09
0e+00
A
B
E
F
G
Treatment Stage


Tn5
Normalised Gene Expression
3e+07
2e+07
1e+07
0e+00
A
B
C
D
E
F
G
Treatment Stage


Tn5403
Normalised Gene Expression
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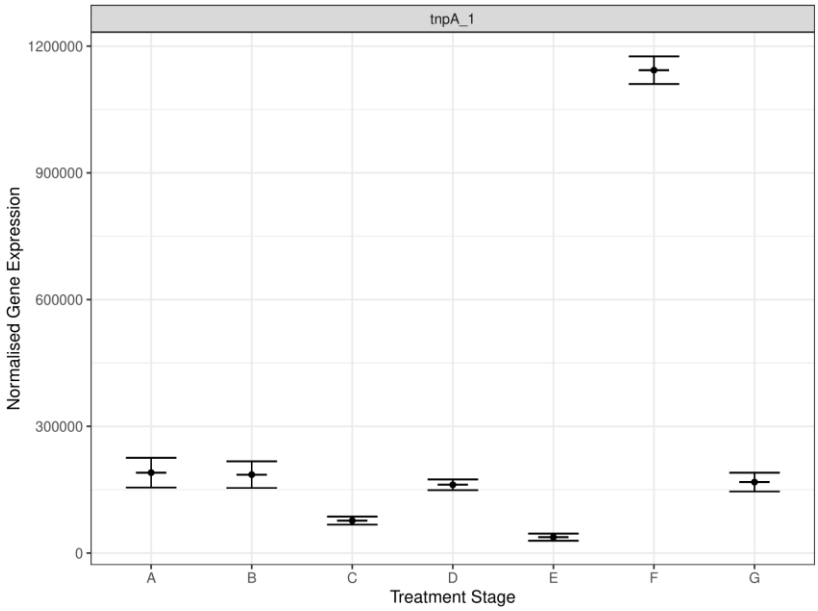
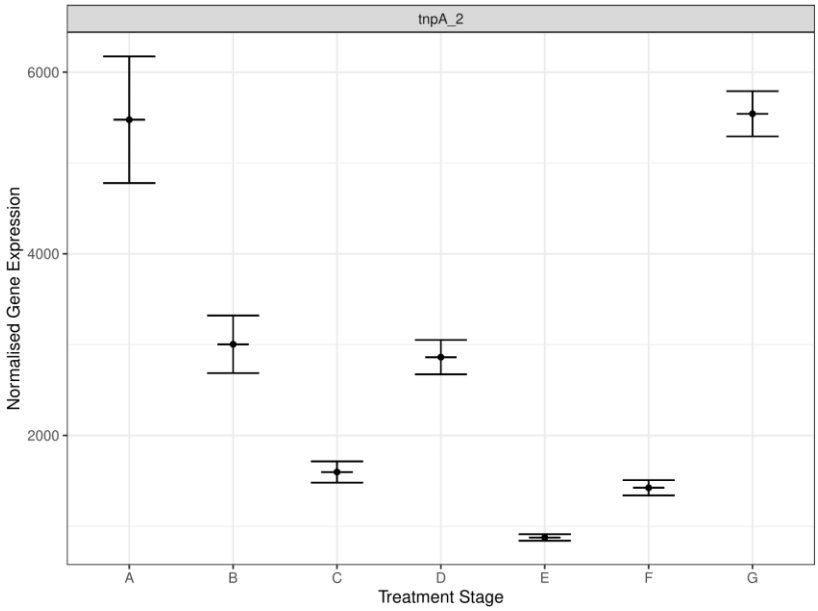
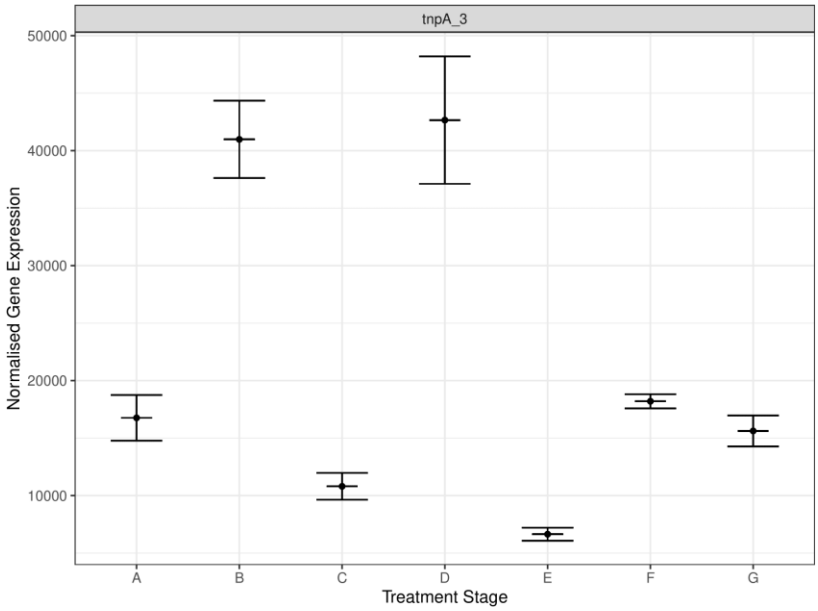
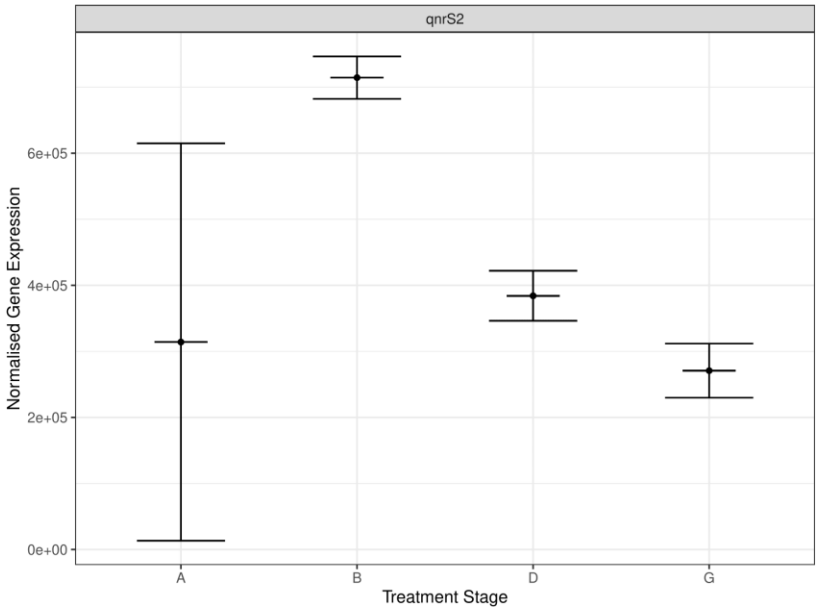
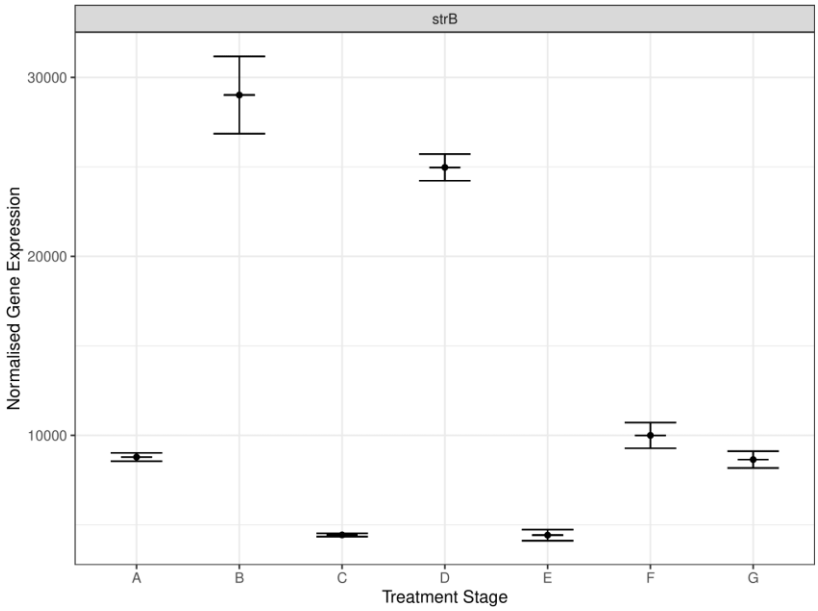
4e+07
3e+07
2e+07
1e+07
A
B
C
D
E
F
G
Treatment Stage

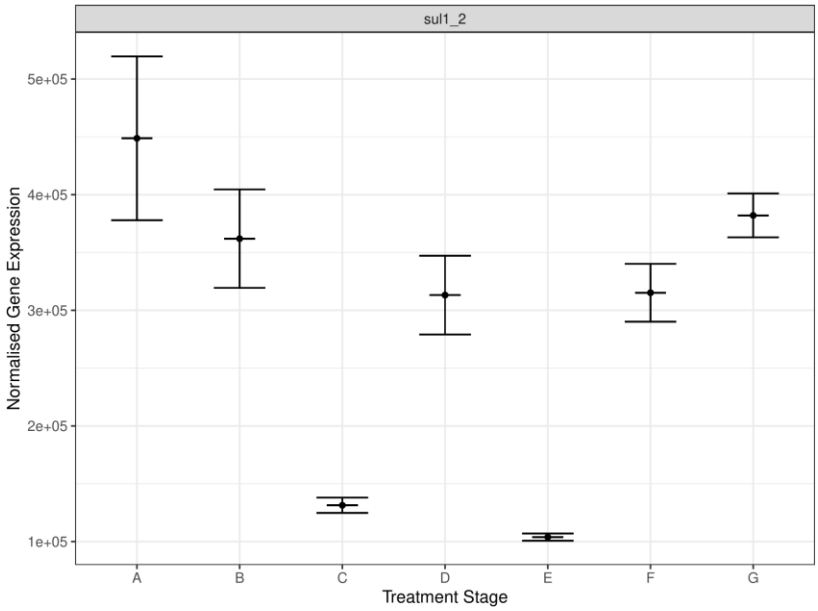

tnpA_1
Normalised Gene Expression
1200000
900000
600000
300000
0
A
B
C
D
E
F
G
Treatment Stage

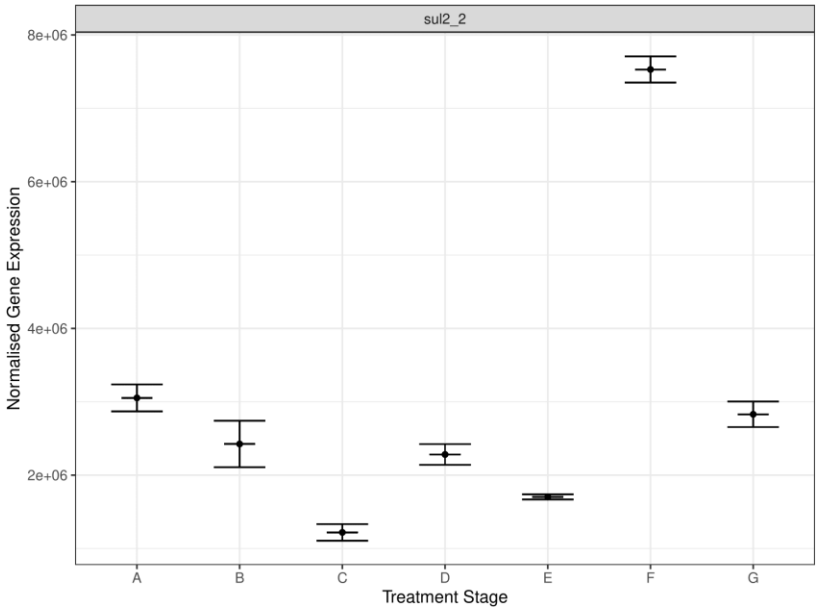

tnpA_2
Normalised Gene Expression
6000
4000
2000
A
B
C
D
E
F
G
Treatment Stage

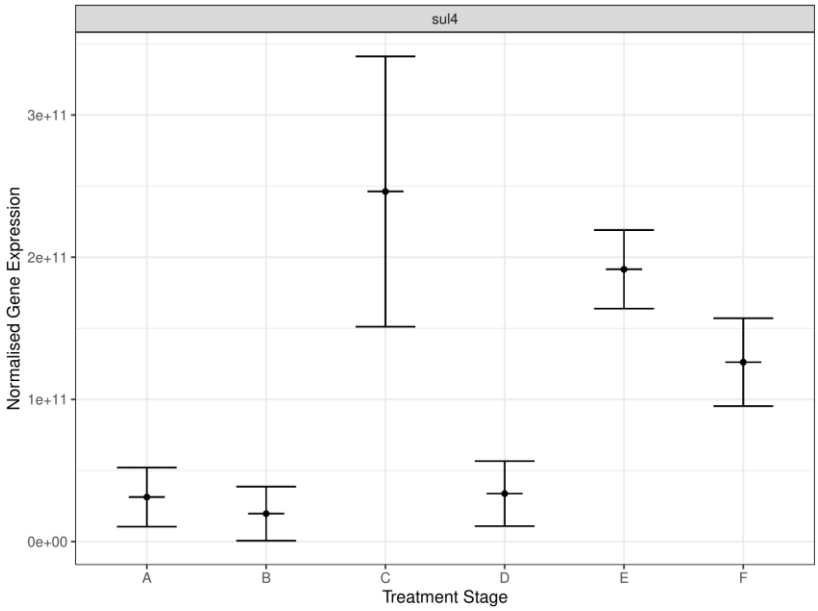

tnpA_3
Normalised Gene Expression
50000
40000
30000
20000
10000
A
B
C
D
E
F
G
Treatment Stage

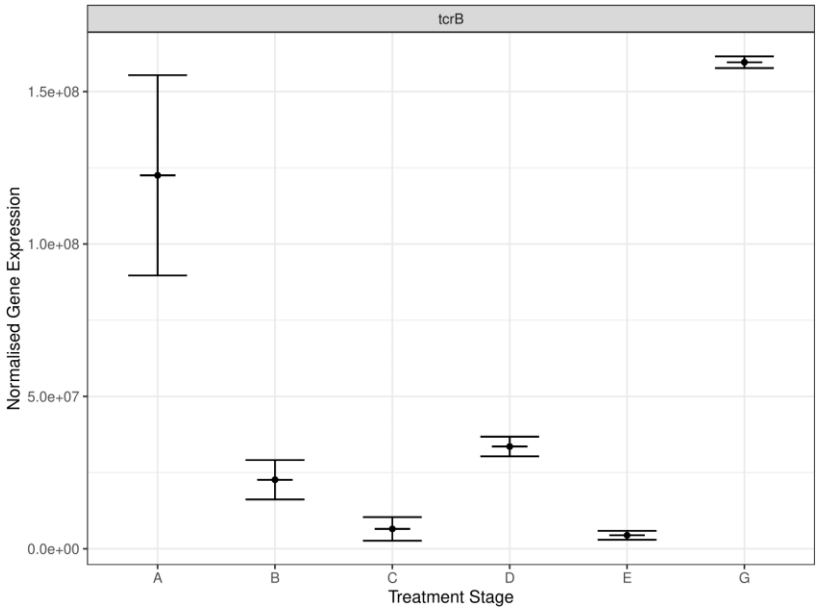

qnrS2
Normalised Gene Expression
6e+05
4e+05
2e+05
0e+00
A
B
D
G
Treatment Stage

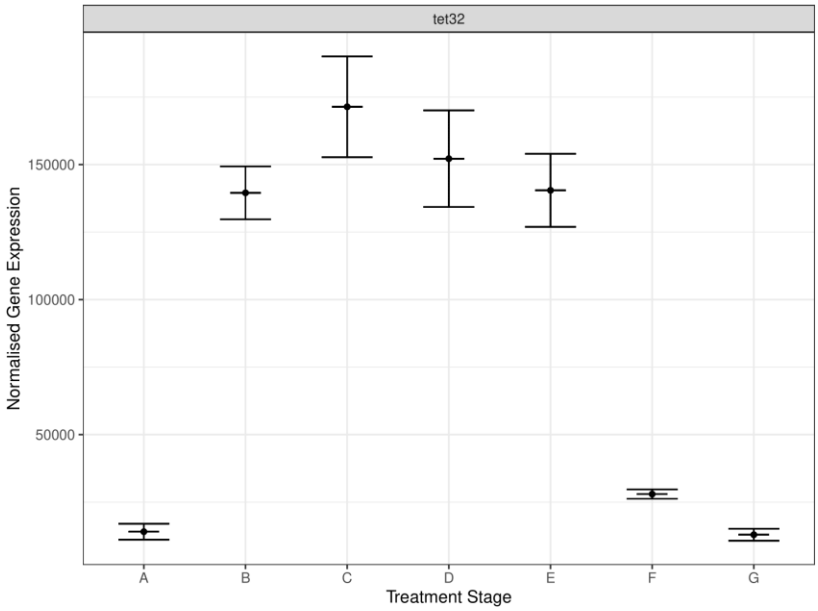

strB
Normalised Gene Expression
30000
20000
10000
A
B
C
D
E
F
G
Treatment Stage

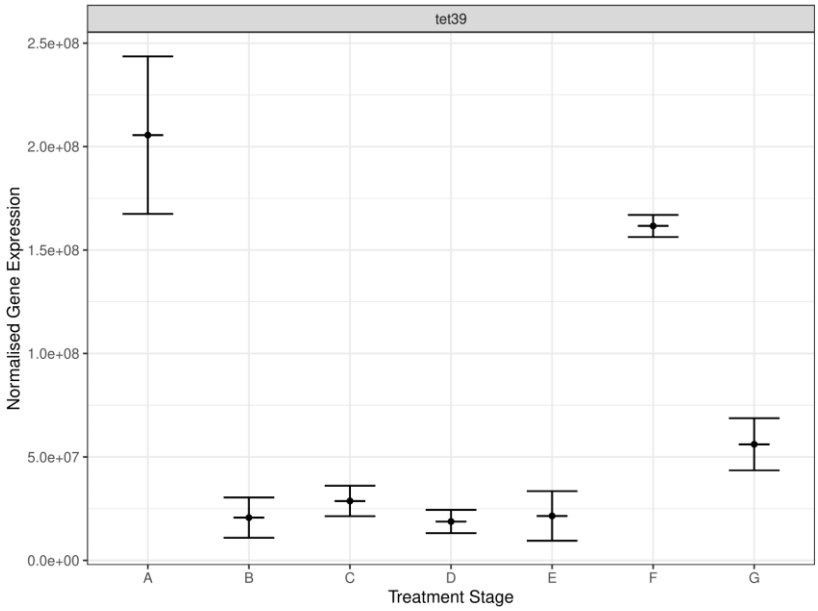

sul1_2
Normalised Gene Expression
5e+05
4e+05
3e+05
2e+05
1e+05
A
B
C
D
E
F
G
Treatment Stage

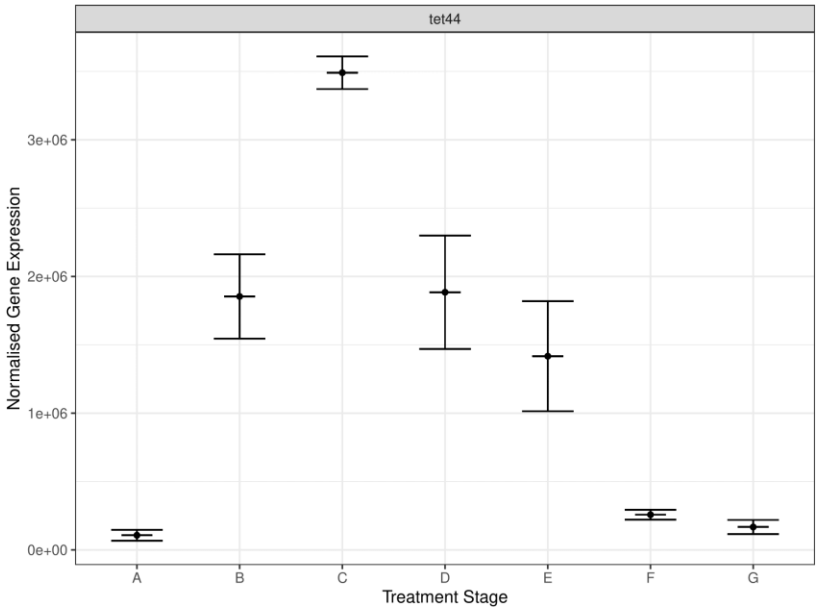

sul2_2
Normalised Gene Expression
8e+06
6e+06
4e+06
2e+06
A
B
C
D
E
F
G
Treatment Stage

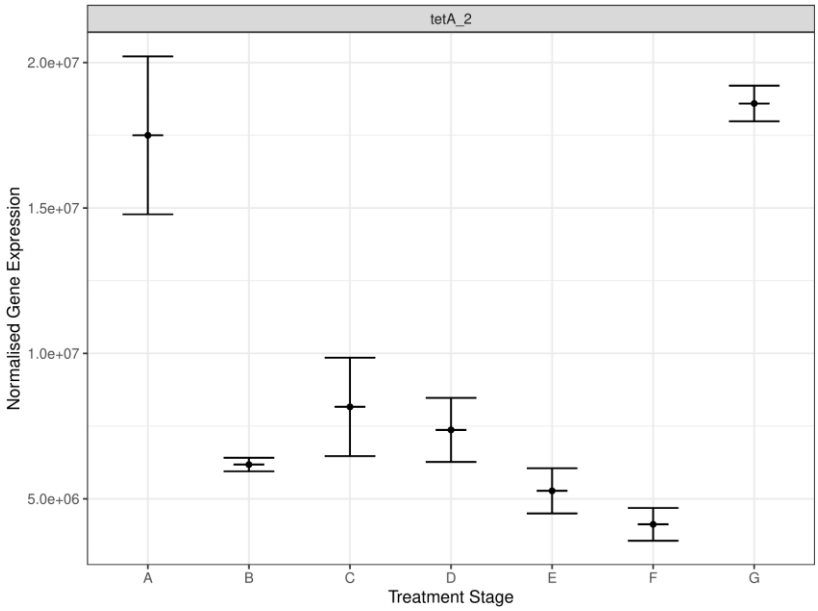

sul4
Normalised Gene Expression
3e+11
2e+11
1e+11
0e+00
A
B
C
D
E
F
Treatment Stage

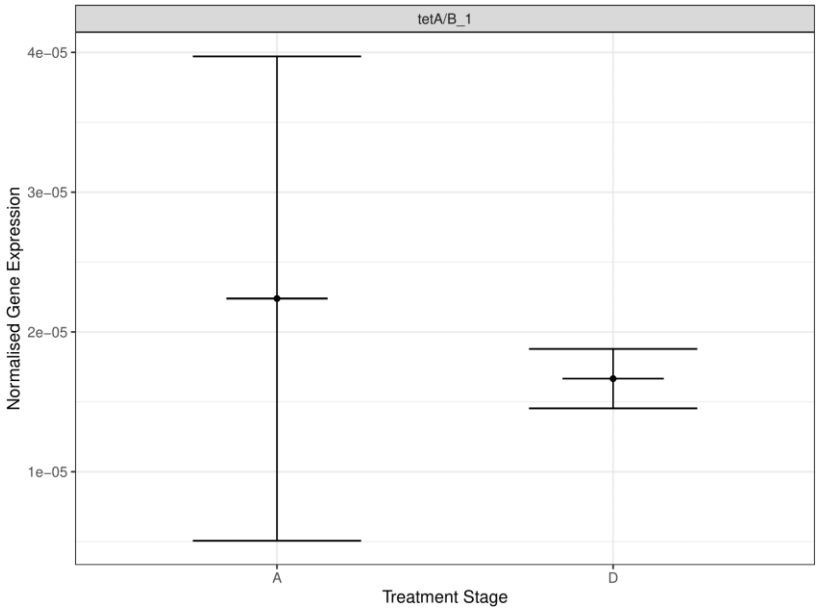

tcrB
Normalised Gene Expression
1.5e+08
1.0e+08
5.0e+07
0.0e+00
A
B
C
D
E
G
Treatment Stage

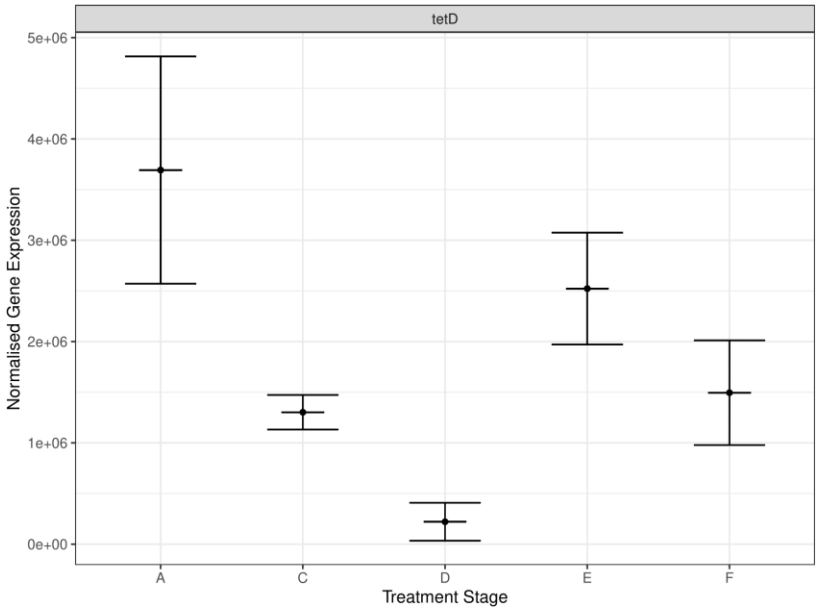

tet32
Normalised Gene Expression
150000
100000
50000
A
B
C
D
E
F
G
Treatment Stage

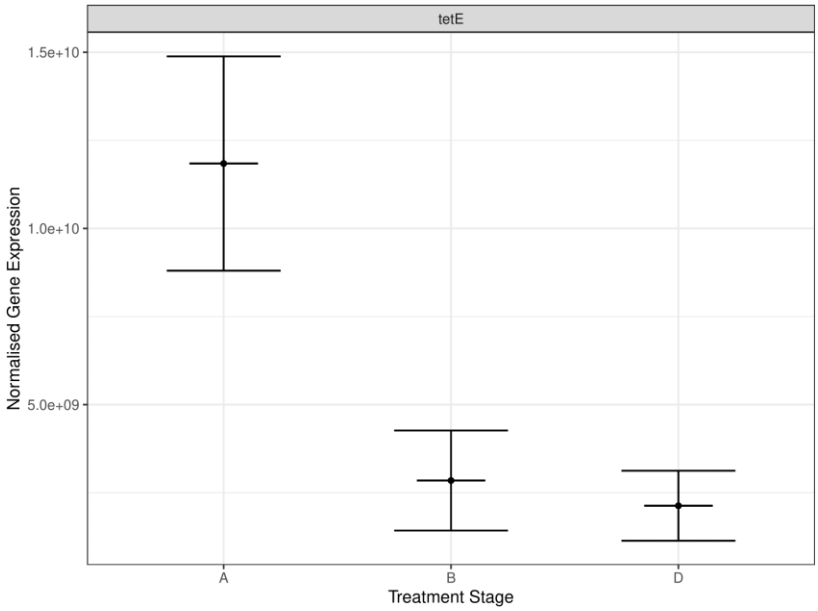

tet39
Normalised Gene Expression
2.5e+08
2.0e+08
1.5e+08
1.0e+08
5.0e+07
0.0e+00
A
B
C
D
E
F
G
Treatment Stage

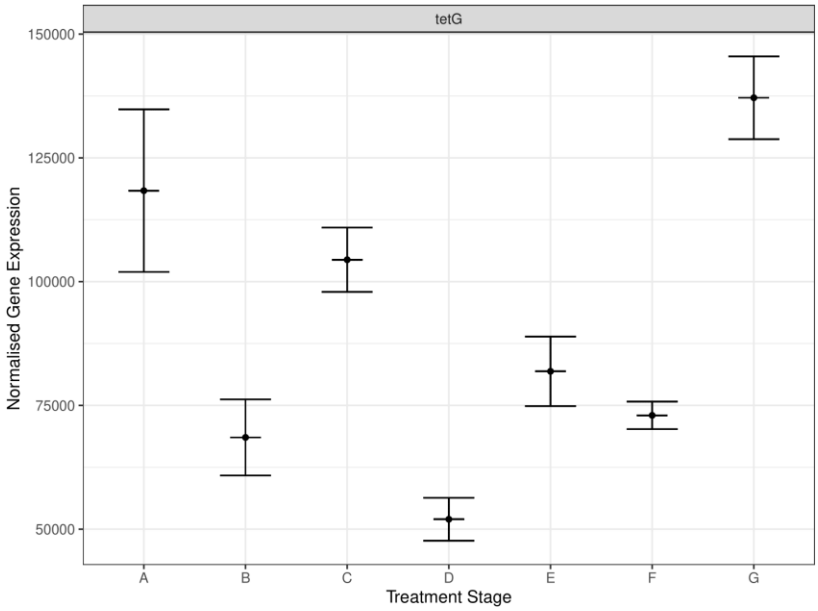

tet44
Normalised Gene Expression
3e+06
2e+06
1e+06
0e+00
A
B
C
D
E
F
G
Treatment Stage

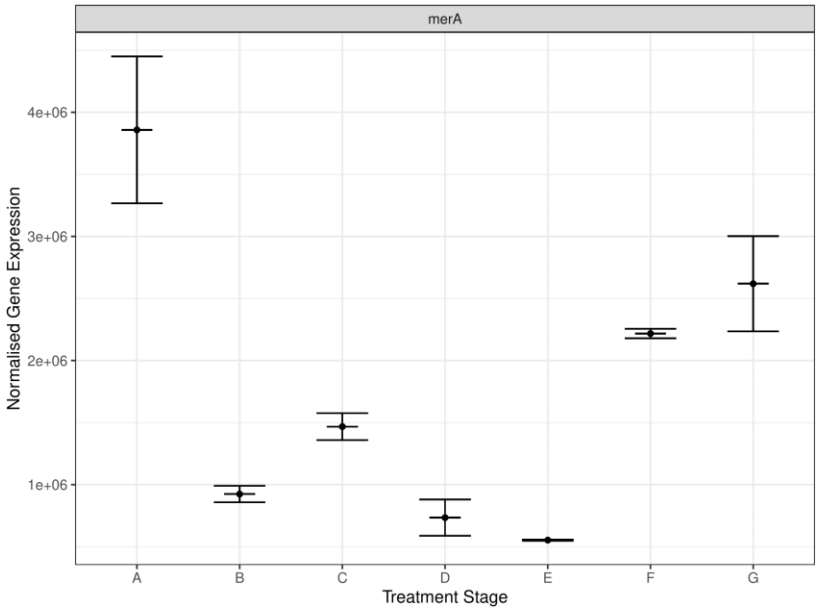

tetA_2
Normalised Gene Expression
2.0e+07
1.5e+07
1.0e+07
5.0e+06
A
B
C
D
E
F
G
Treatment Stage

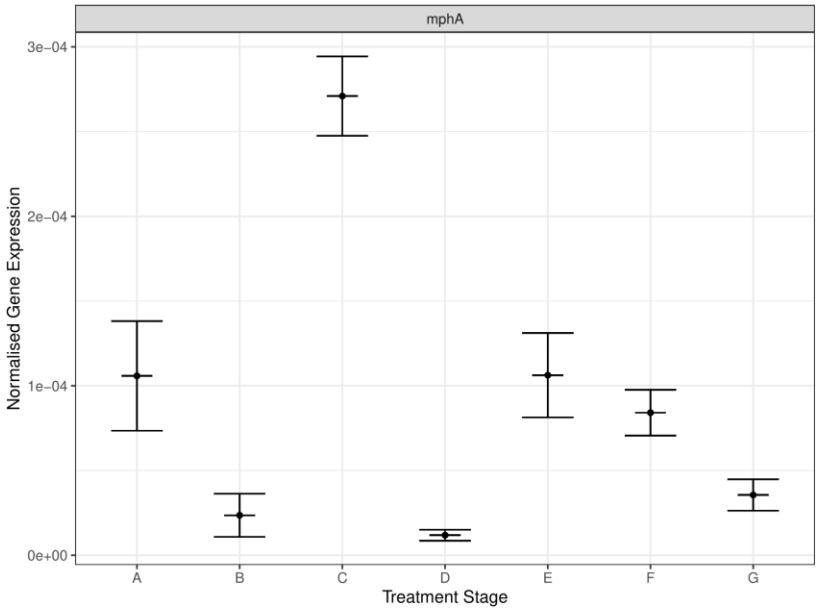

tetA/B_1
4e−05
3e−05
2e−05
1e−05
Normalised Gene Expression
A
D
Treatment Stage

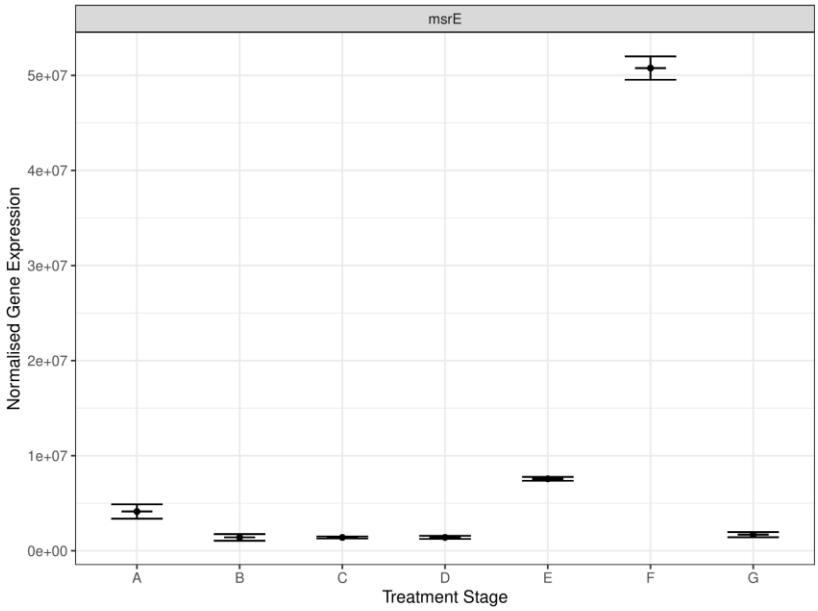

tetD
5e+06
4e+06
3e+06
2e+06
1e+06
0e+00
Normalised Gene Expression
A
C
D
E
F
Treatment Stage

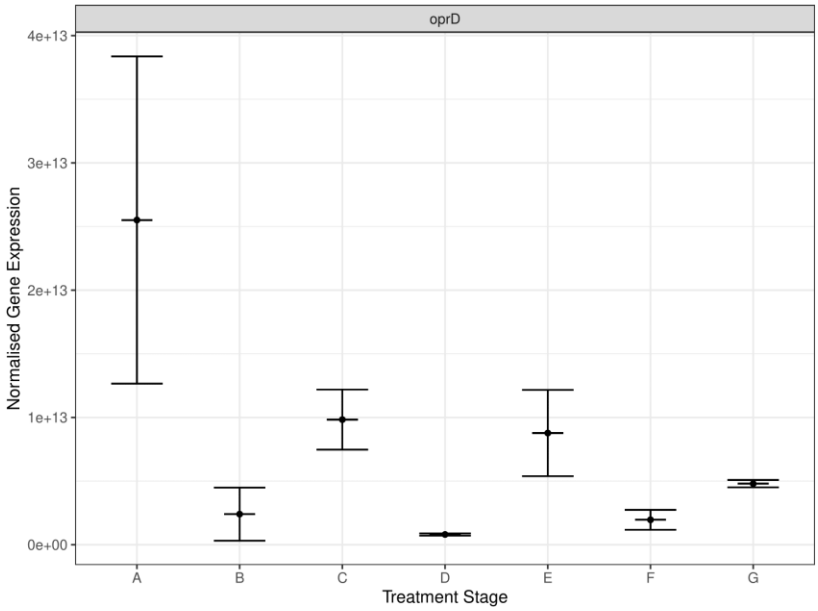

tetE
1.5e+10
1.0e+10
5.0e+09
Normalised Gene Expression
A
B
D
Treatment Stage

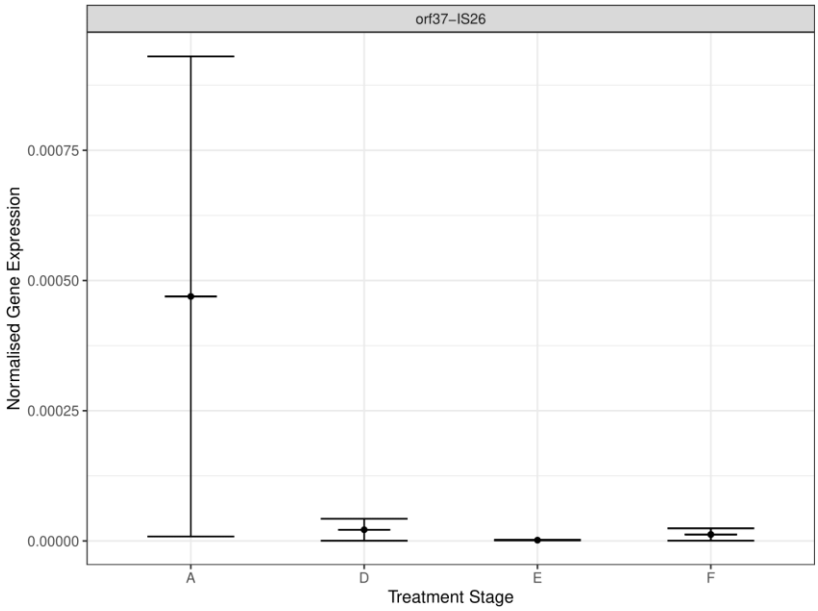

tetG
150000
125000
100000
75000
50000
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

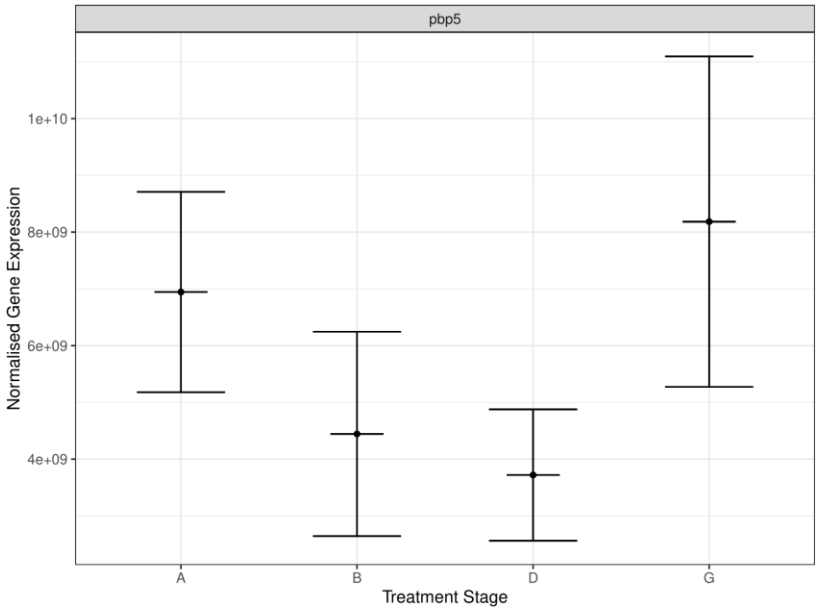

merA
Normalised Gene Expression
4e+06
3e+06
2e+06
1e+06
A
B
C
D
E
F
G
Treatment Stage

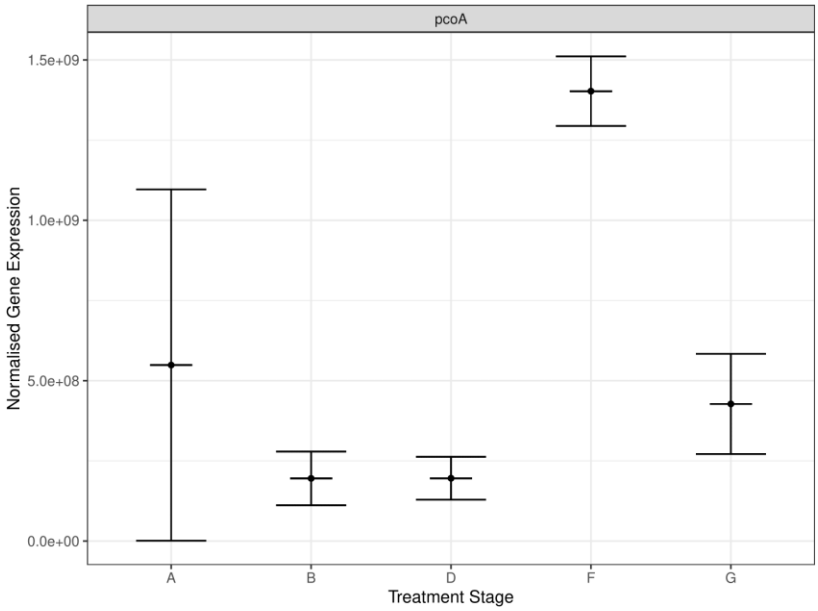

mphA
Normalised Gene Expression
3e−04
2e−04
1e−04
0e+00
A
B
C
D
E
F
G
Treatment Stage

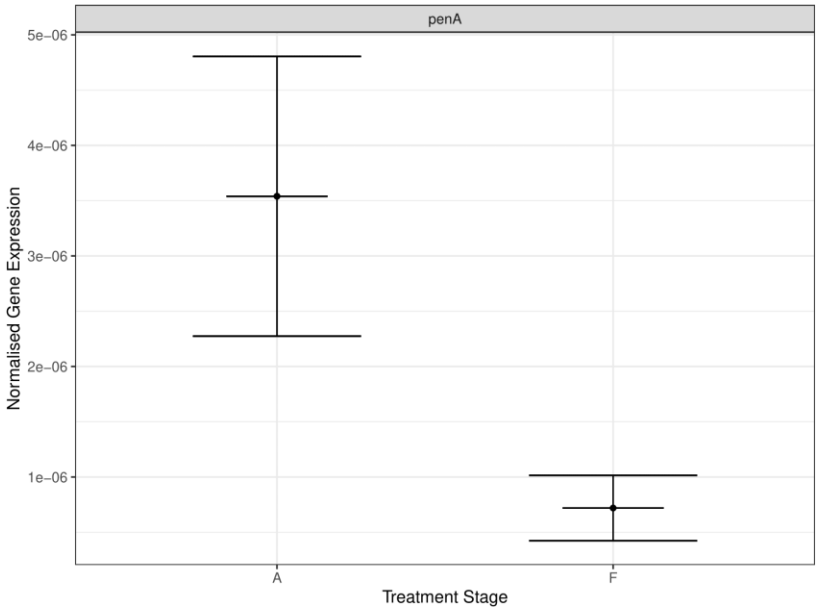

msrE
Normalised Gene Expression
5e+07
4e+07
3e+07
2e+07
1e+07
0e+00
A
B
C
D
E
F
G
Treatment Stage

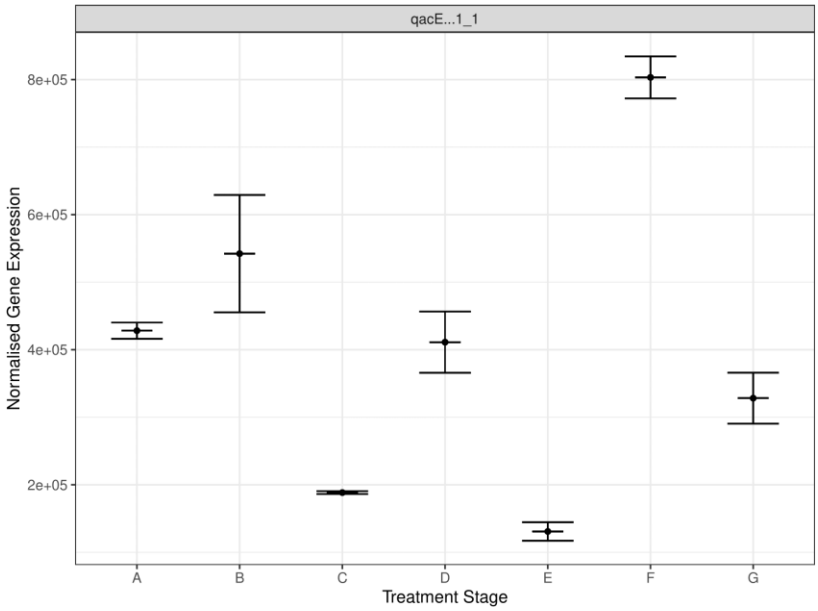

oprD
Normalised Gene Expression
4e+13
3e+13
2e+13
1e+13
0e+00
A
B
C
D
E
F
G
Treatment Stage

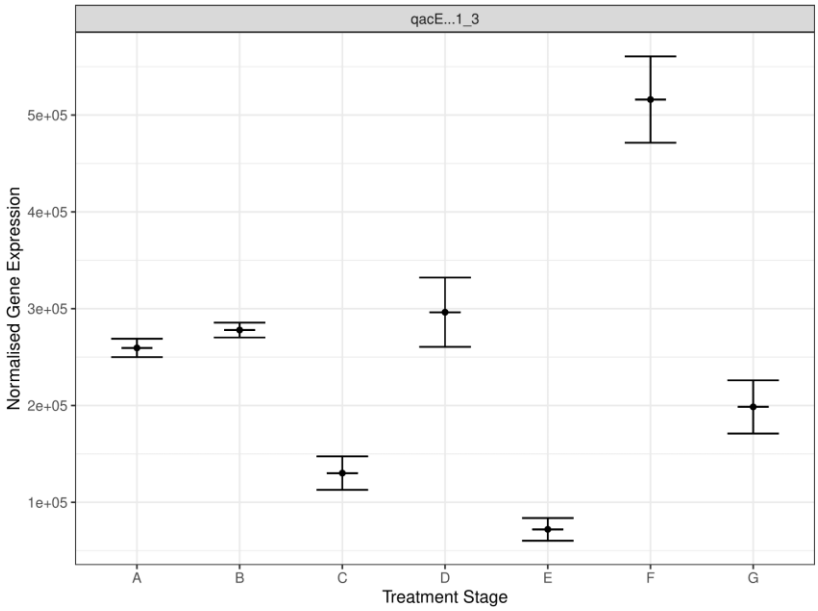

orf37−IS26
Normalised Gene Expression
0.00075
0.00050
0.00025
0.00000
A
D
E
F
Treatment Stage

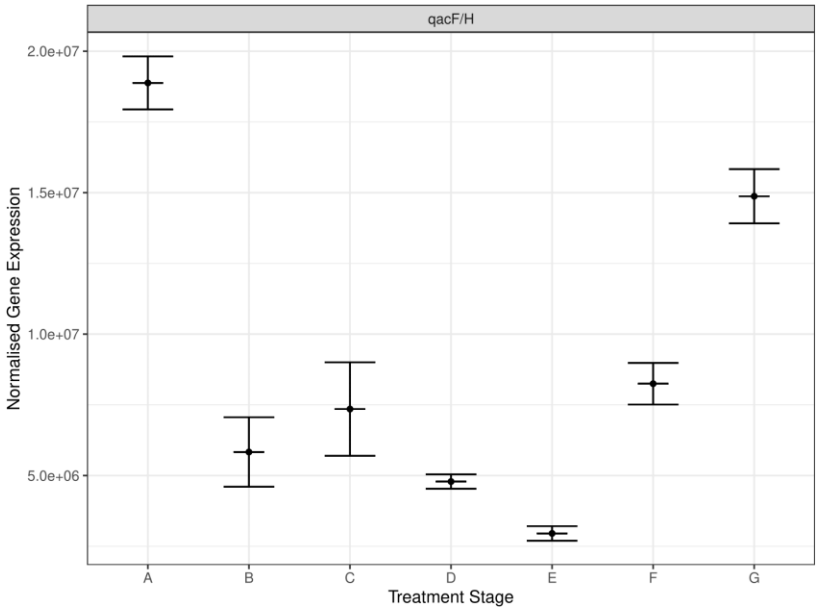

pbp5
Normalised Gene Expression
1e+10
8e+09
6e+09
4e+09
A
B
D
G
Treatment Stage

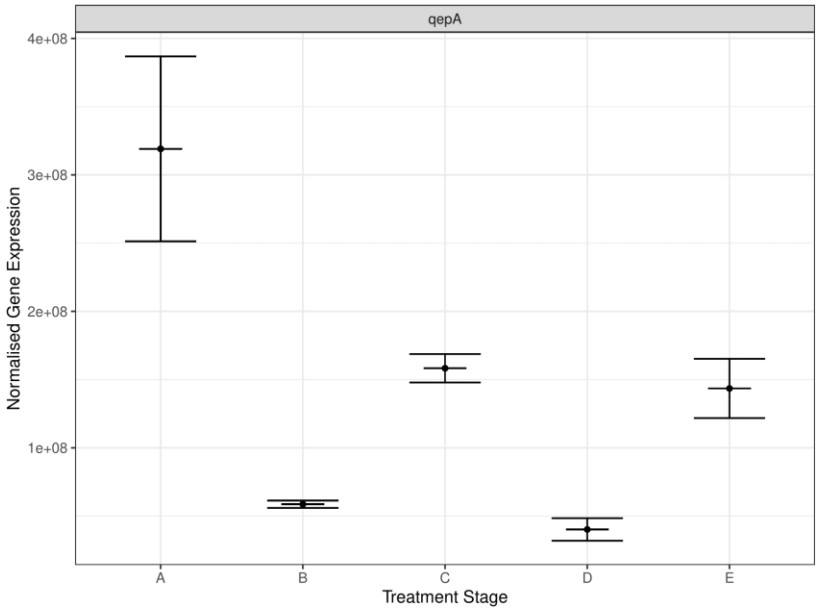

pcoA
1.5e+09
1.0e+09
5.0e+08
0.0e+00
Normalised Gene Expression
A
B
D
F
G
Treatment Stage

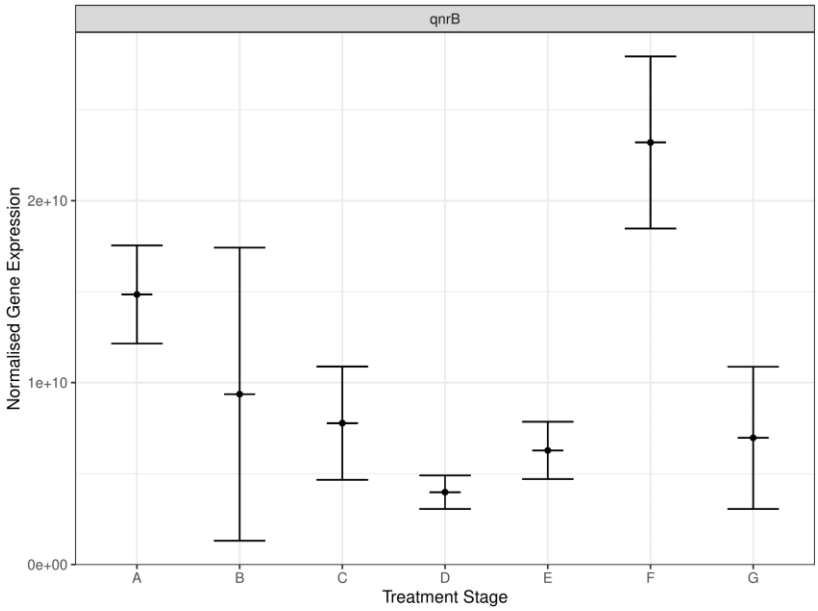

penA
5e−06
4e−06
3e−06
2e−06
1e−06
Normalised Gene Expression
A
F
Treatment Stage

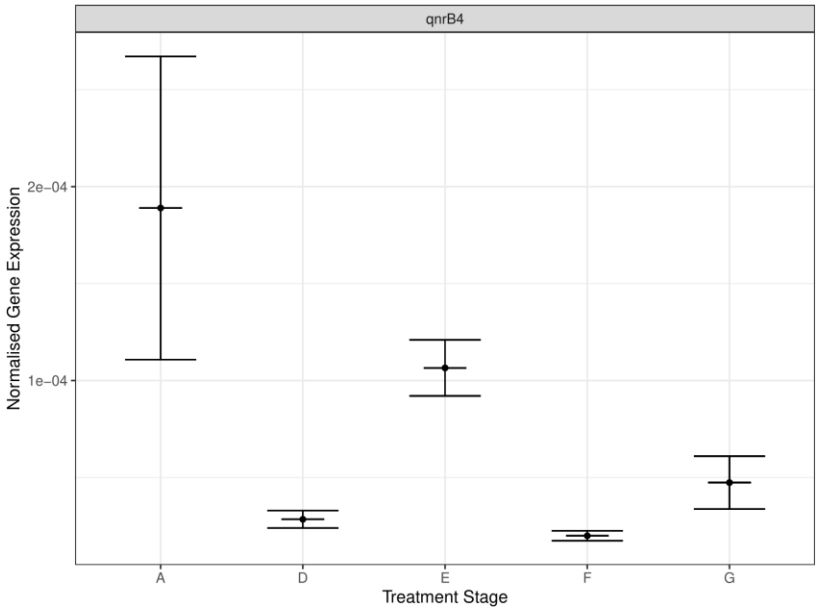

qacE...1_1
Normalised Gene Expression
8e+05
6e+05
4e+05
2e+05
A
B
C
D
E
F
G
Treatment Stage

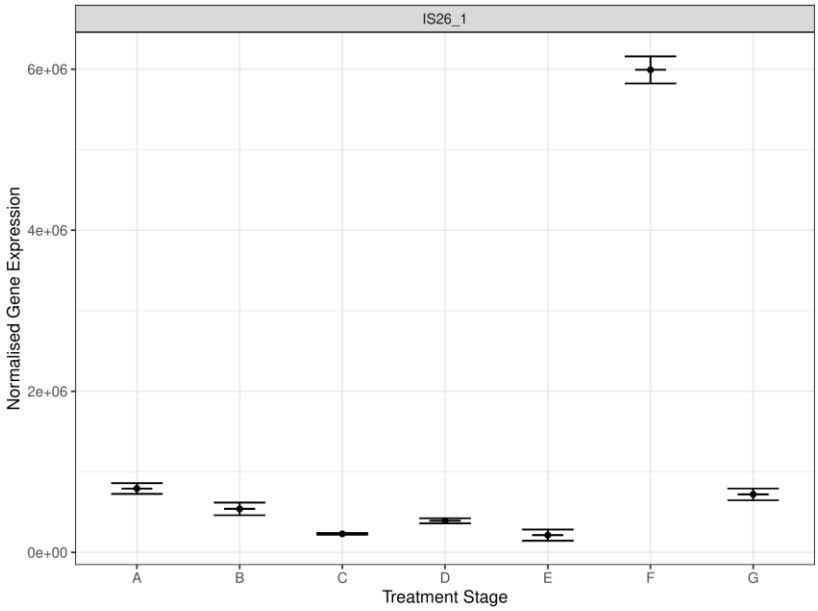

qacE...1_3
Normalised Gene Expression
5e+05
4e+05
3e+05
2e+05
1e+05
A
B
C
D
E
F
G
Treatment Stage

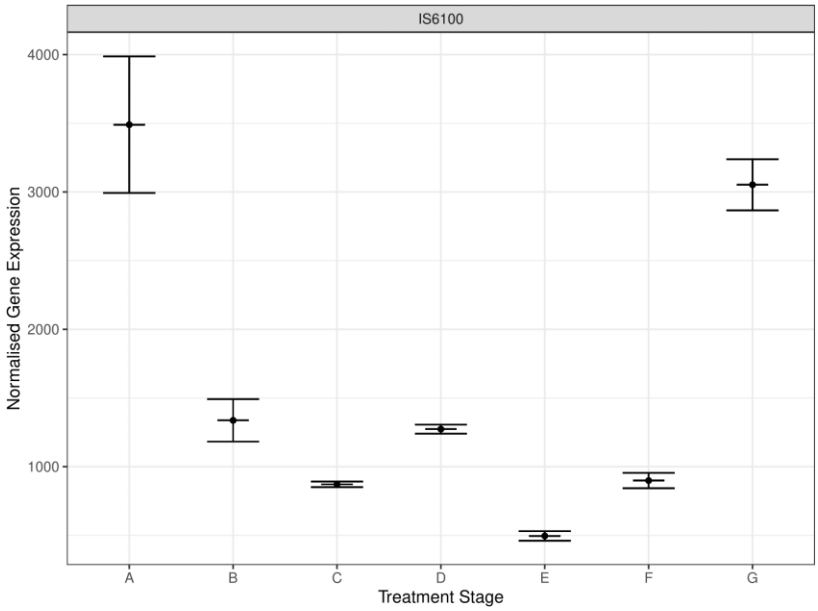

qacF/H
2.0e+07
1.5e+07
1.0e+07
5.0e+06
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

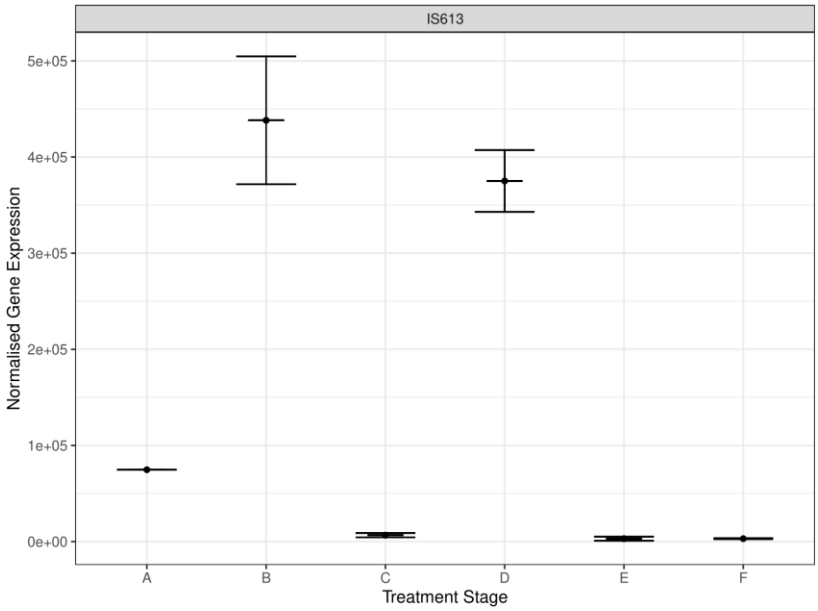

qepA
4e+08
3e+08
2e+08
1e+08
Normalised Gene Expression
A
B
C
D
E
Treatment Stage

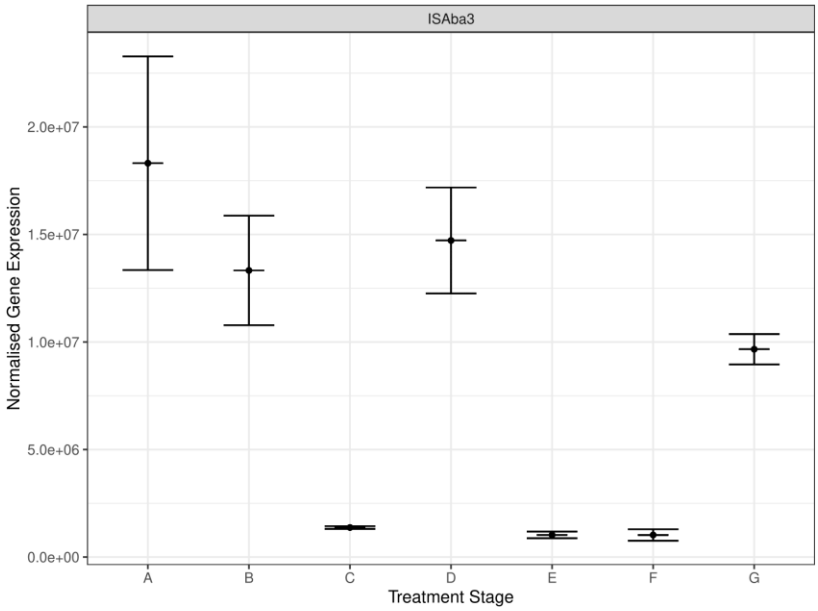

qnrB
Normalised Gene Expression
2e+10
1e+10
0e+00
A
B
C
D
E
F
G
Treatment Stage

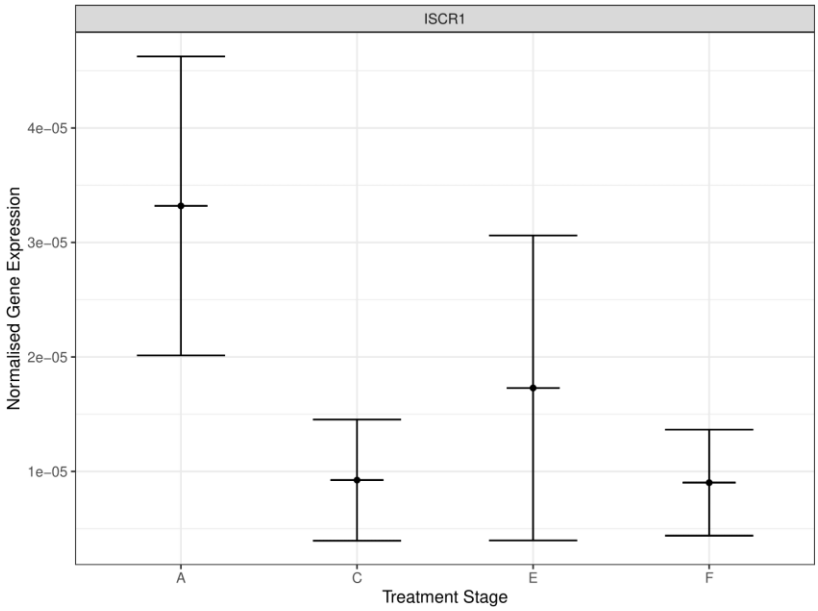

qnrB4
Normalised Gene Expression
2e−04
1e−04
A
D
E
F
G
Treatment Stage

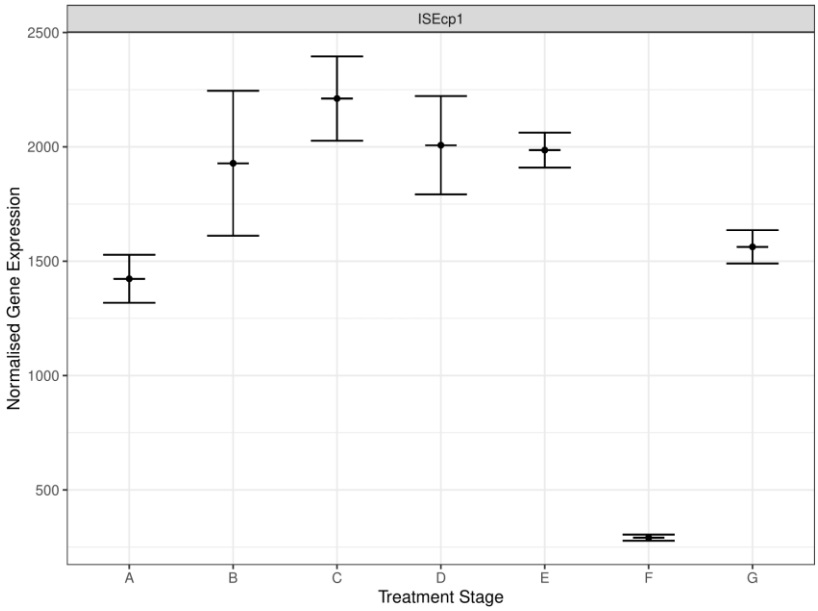

IS26_1
Normalised Gene Expression
6e+06
4e+06
2e+06
0e+00
A
B
C
D
E
F
G
Treatment Stage

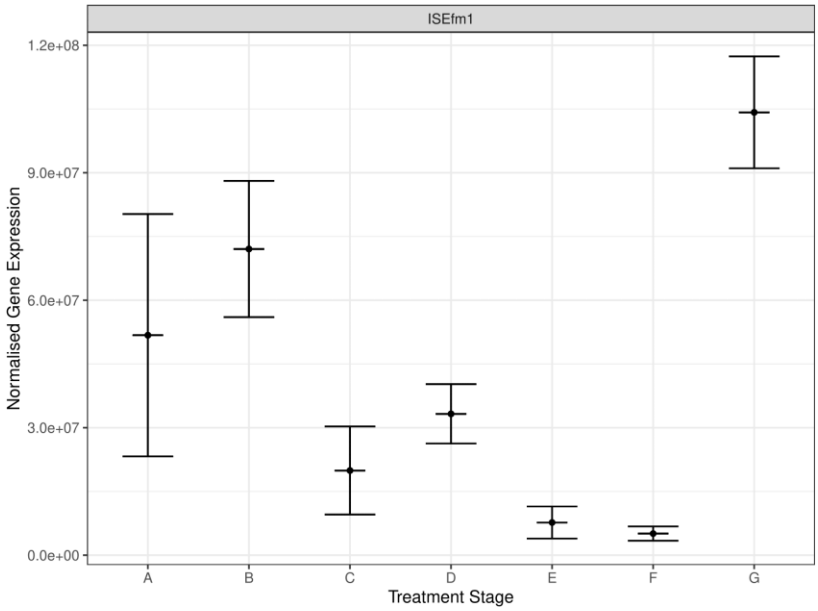

IS6100
Normalised Gene Expression
4000
3000
2000
1000
A
B
C
D
E
F
G
Treatment Stage

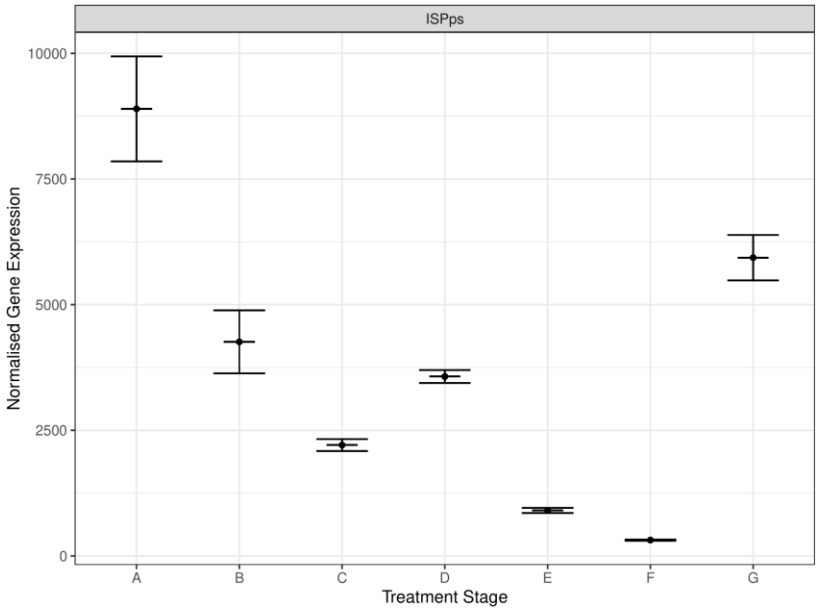

IS613
Normalised Gene Expression
5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
A
B
C
D
E
F
Treatment Stage

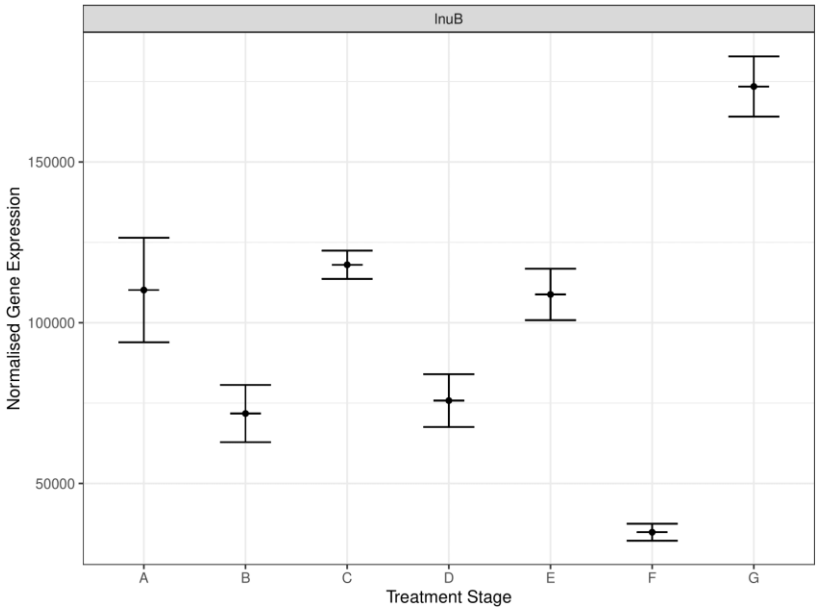

ISAba3
Normalised Gene Expression
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
A
B
C
D
E
F
G
Treatment Stage

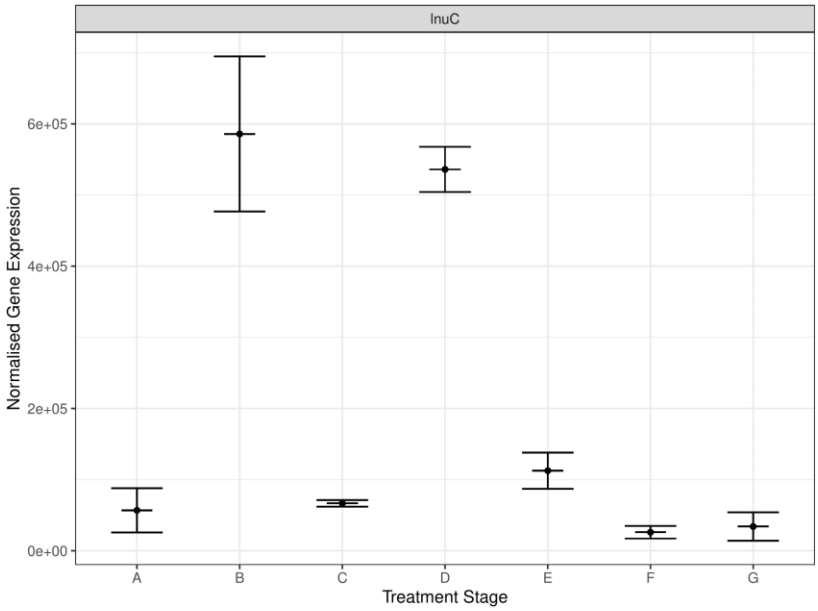

ISCR1
Normalised Gene Expression
4e−05
3e−05
2e−05
1e−05
A
C
E
F
Treatment Stage

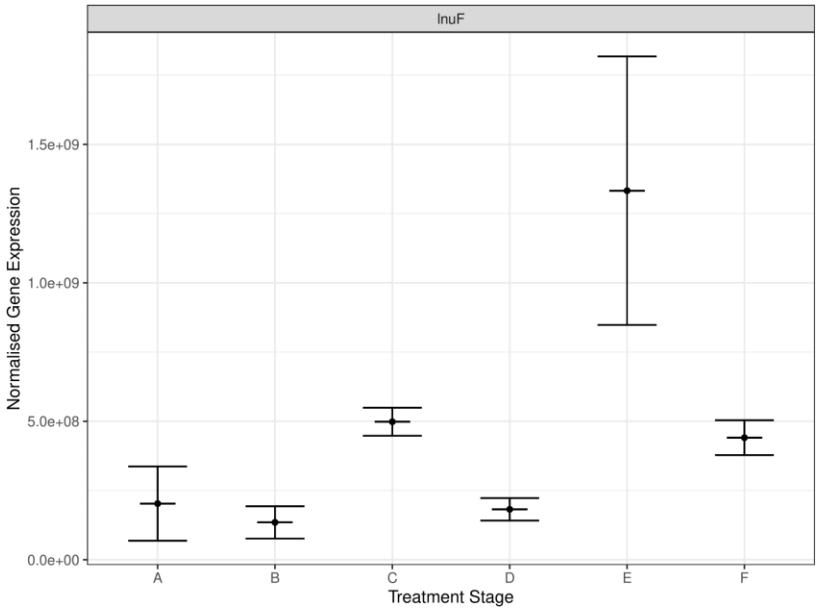

ISEcp1
Normalised Gene Expression
2500
2000
1500
1000
500
A
B
C
D
E
F
G
Treatment Stage

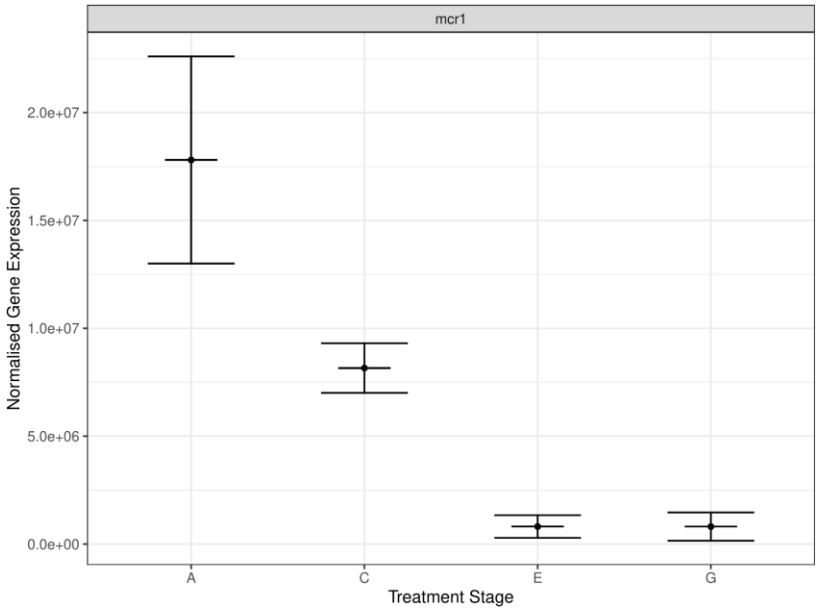

ISEfm1
1.2e+08
9.0e+07
6.0e+07
3.0e+07
0.0e+00
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

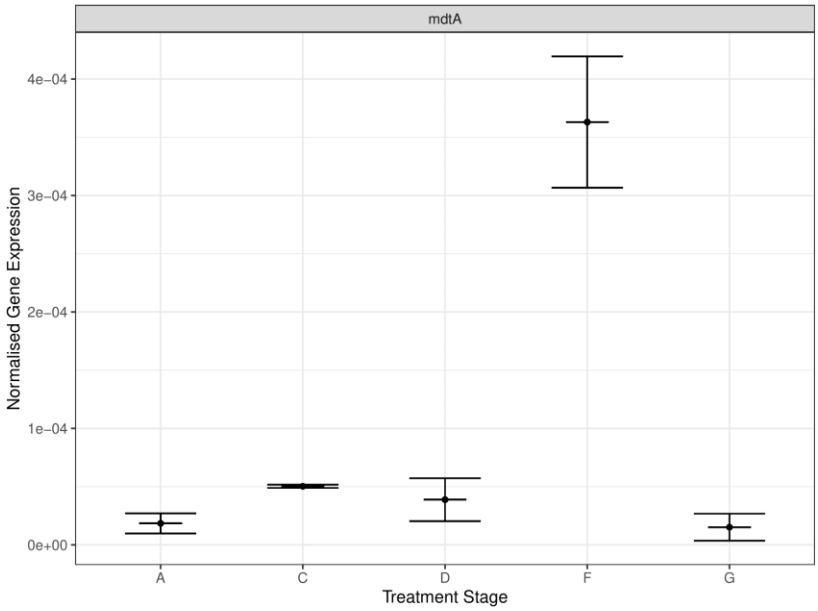

ISPps
10000
7500
5000
2500
0
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

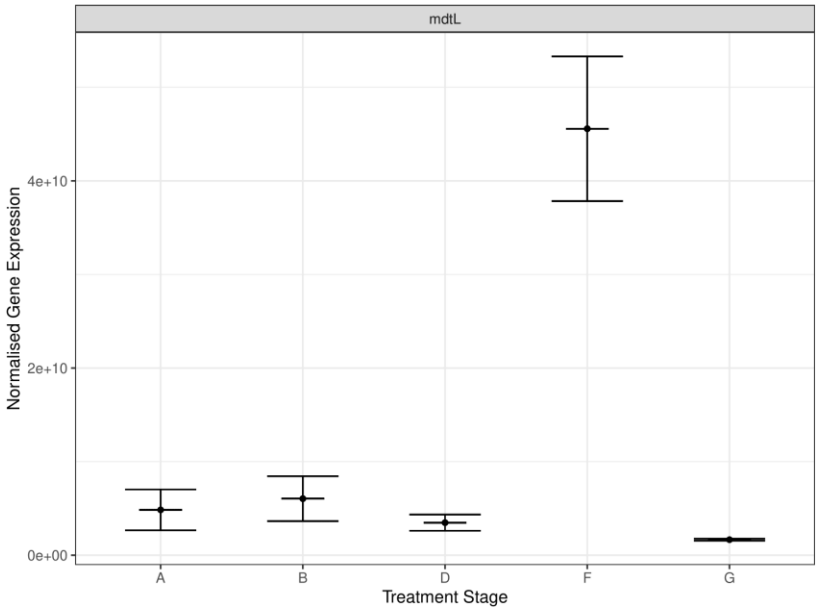

InuB
Normalised Gene Expression
150000
100000
50000
A
B
C
D
E
F
G
Treatment Stage

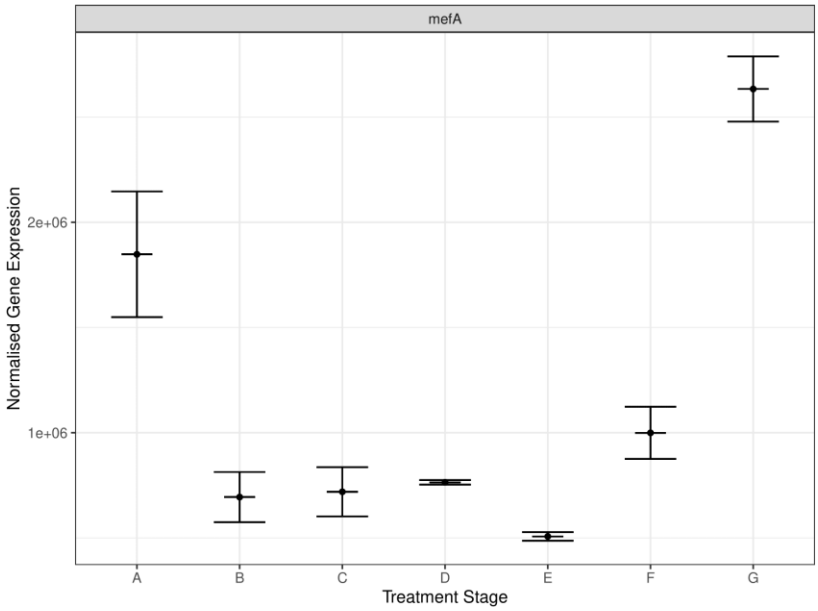

InuC
Normalised Gene Expression
6e+05
4e+05
2e+05
0e+00
A
B
C
D
E
F
G
Treatment Stage

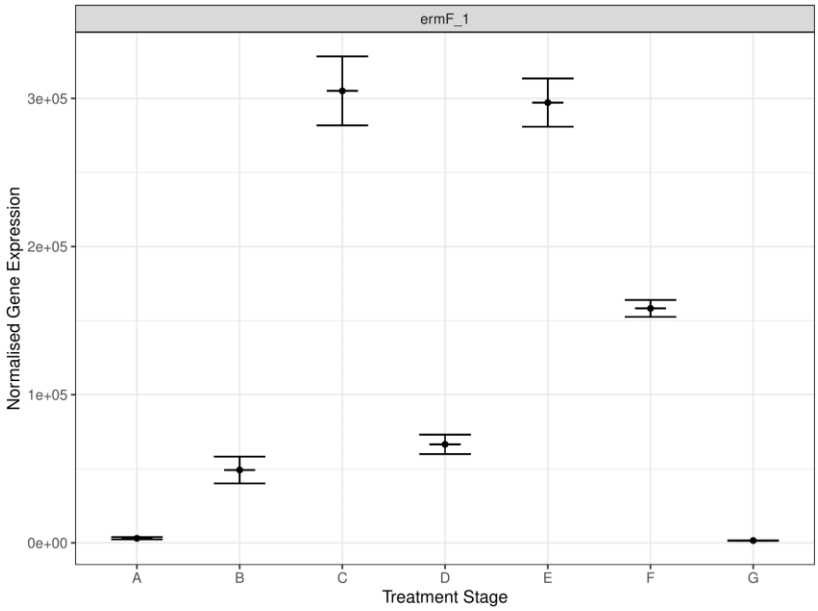

lnuF
Normalised Gene Expression
1.5e+09
1.0e+09
5.0e+08
0.0e+00
A
B
C
D
E
F
Treatment Stage

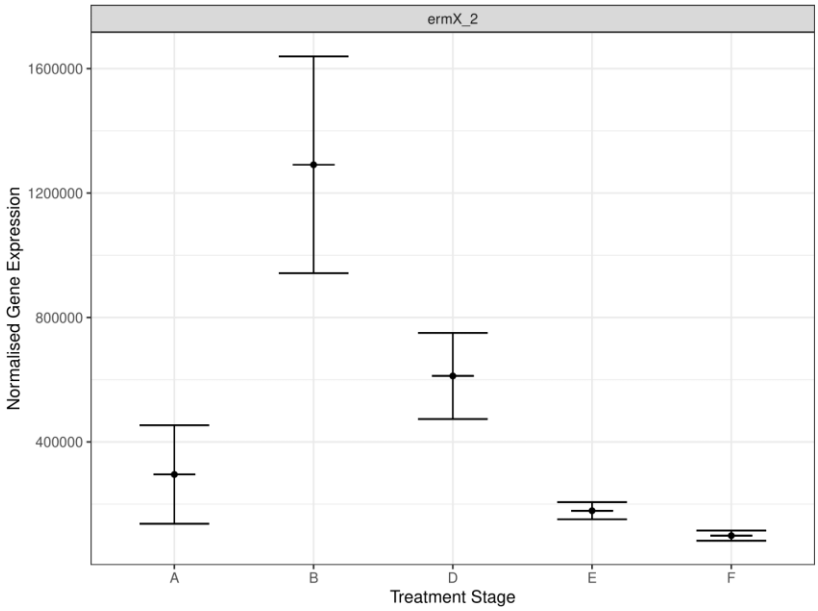

mcr1
Normalised Gene Expression
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
A
C
E
G
Treatment Stage

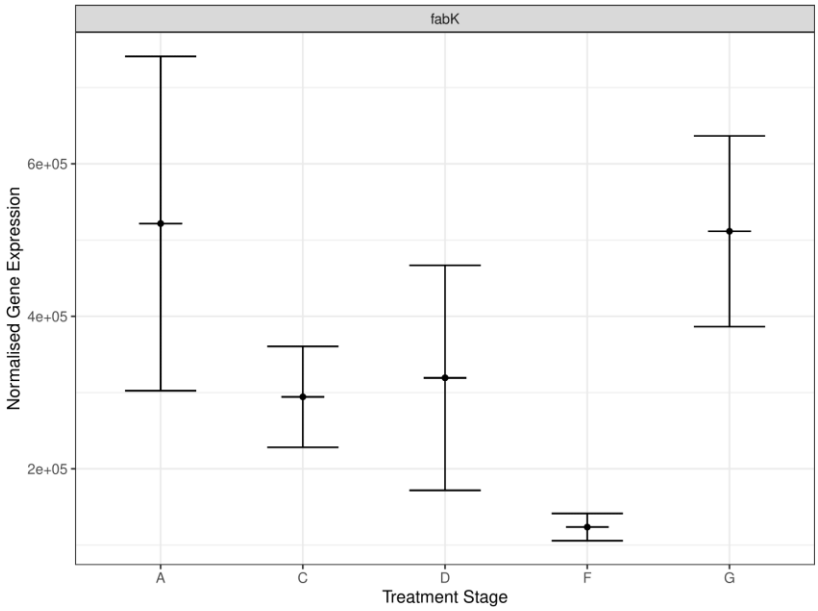

mdtA
Normalised Gene Expression
4e−04
3e−04
2e−04
1e−04
0e+00
A
C
D
F
G
Treatment Stage

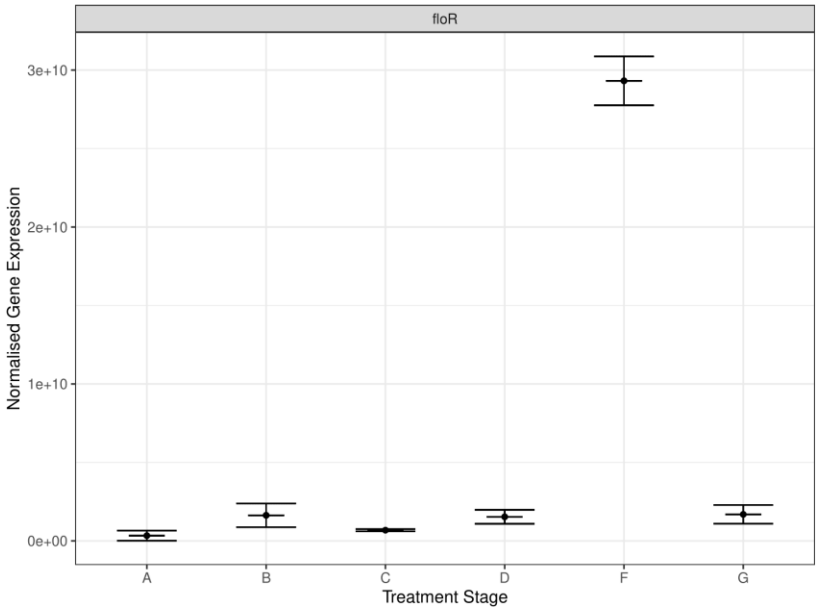

mdtL
Normalised Gene Expression
4e+10
2e+10
0e+00
A
B
D
F
G
Treatment Stage

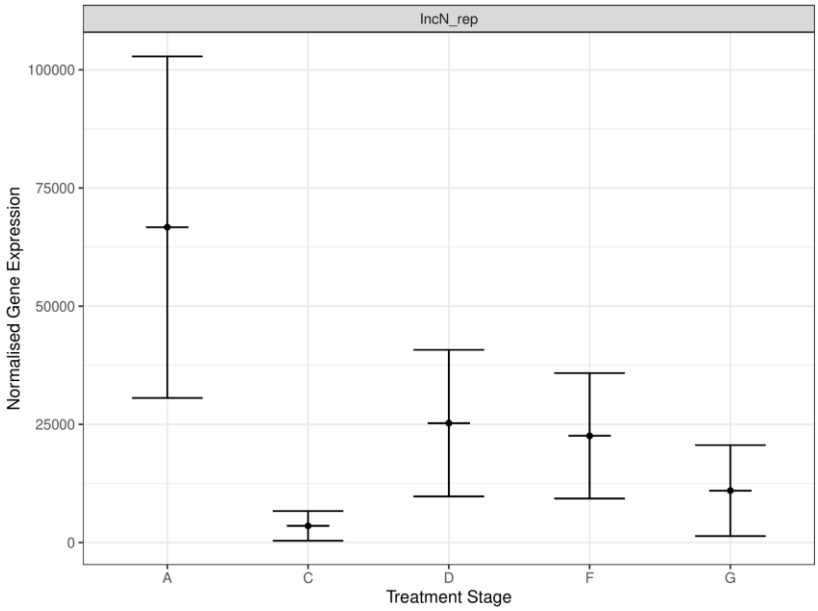

mefA
Normalised Gene Expression
2e+06
1e+06
A
B
C
D
E
F
G
Treatment Stage

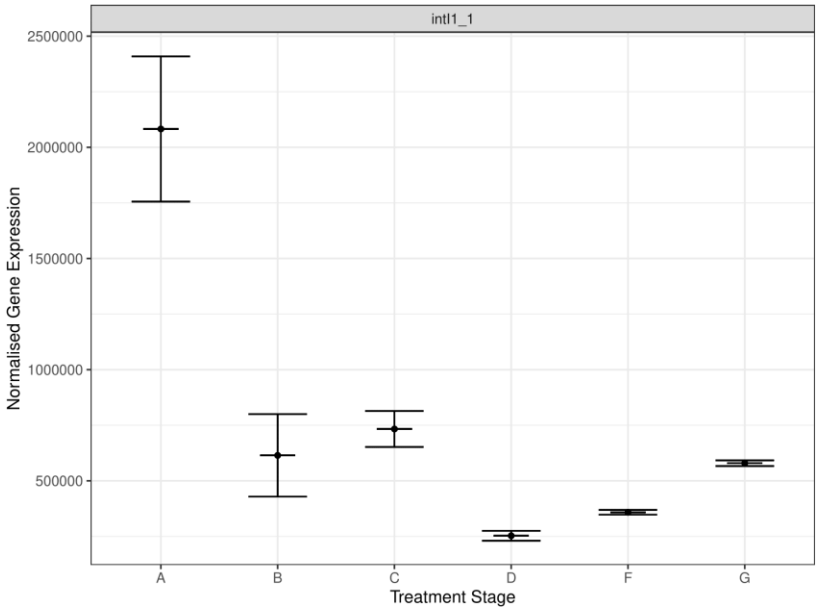

ermF_1
Normalised Gene Expression
3e+05
2e+05
1e+05
0e+00
A
B
C
D
E
F
G
Treatment Stage

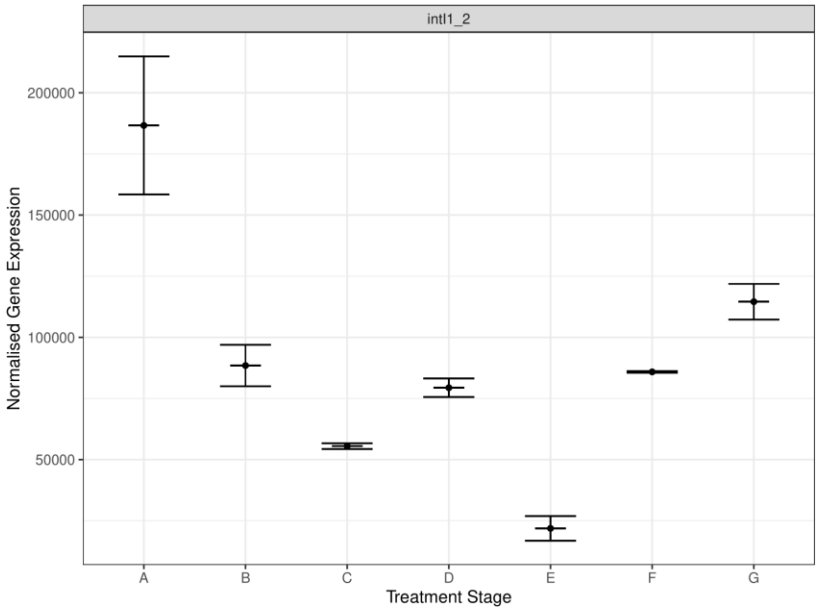

ermX_2
1600000
1200000
800000
400000
Normalised Gene Expression
A
B
D
E
F
Treatment Stage

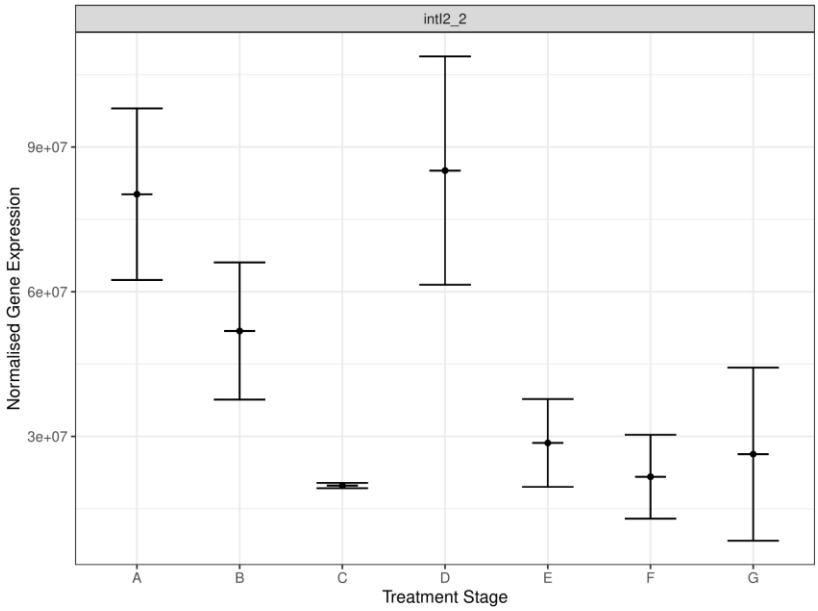

fabK
6e+05
4e+05
2e+05
Normalised Gene Expression
A
C
D
F
G
Treatment Stage

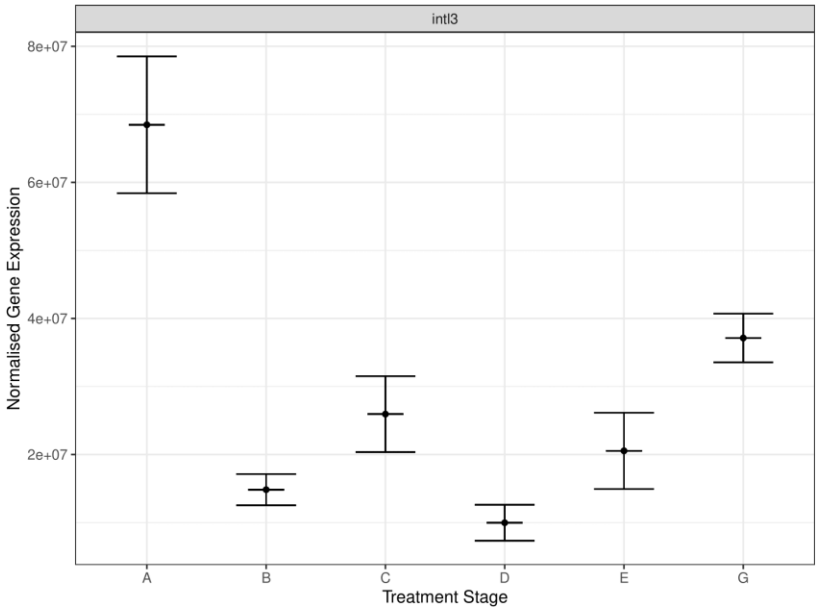

floR
3e+10
2e+10
1e+10
0e+00
Normalised Gene Expression
A
B
C
D
F
G
Treatment Stage

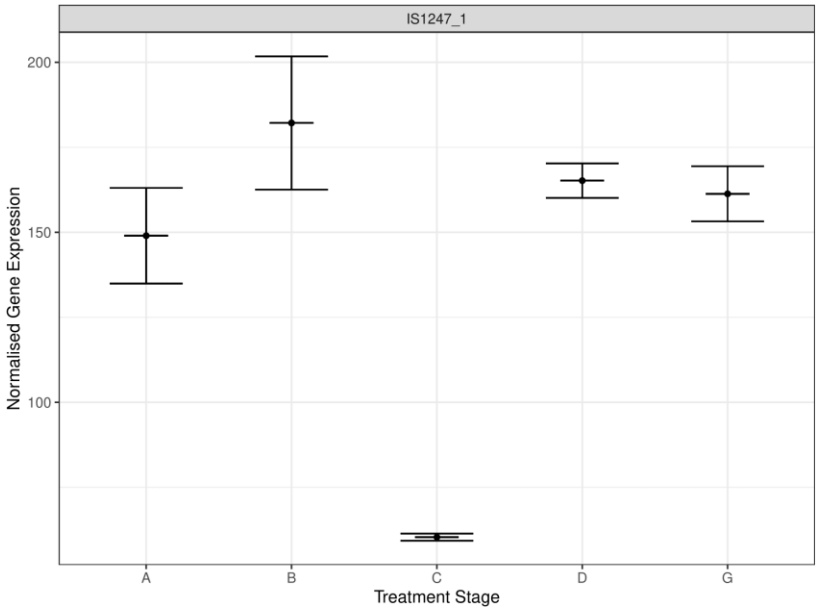

IncN_rep
100000
75000
50000
25000
0
Normalised Gene Expression
A
C
D
F
G
Treatment Stage

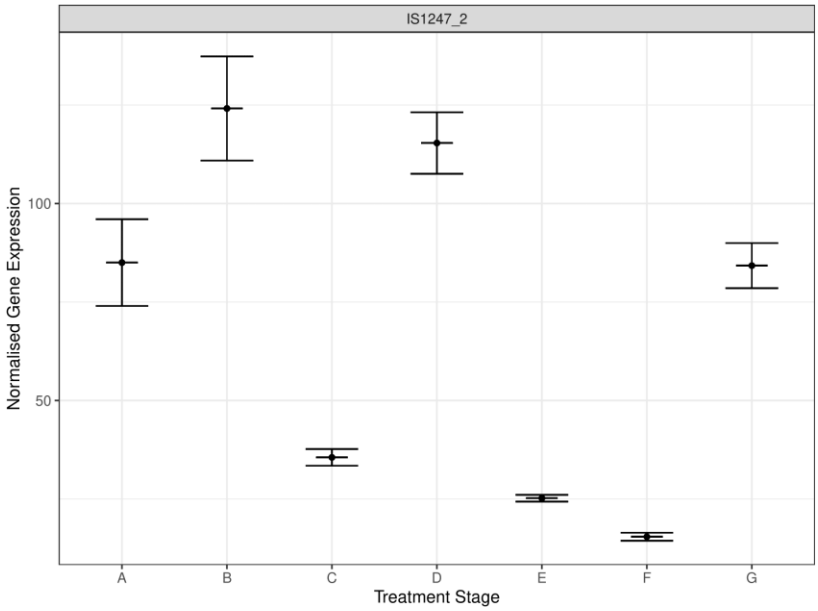

intl1_1
2500000
2000000
1500000
1000000
500000
Normalised Gene Expression
A
B
C
D
F
G
Treatment Stage

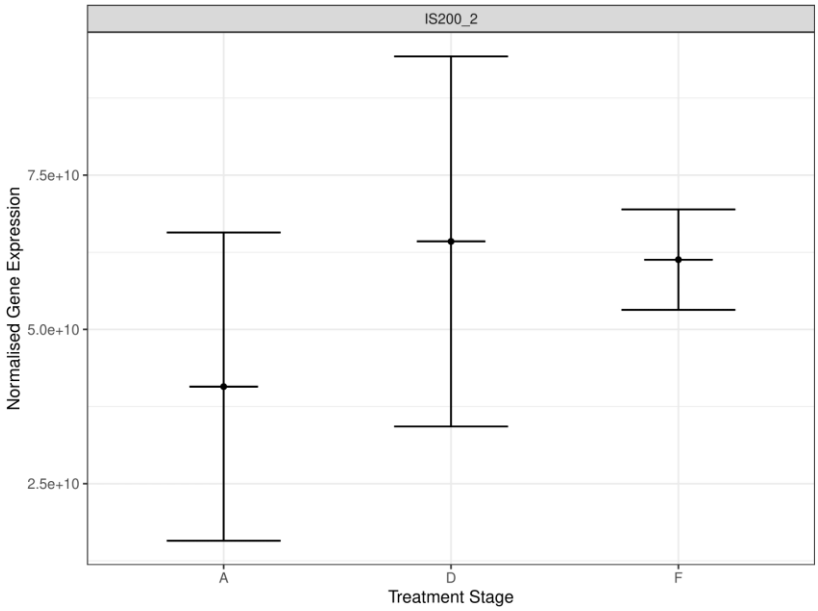

intl1_2
200000
150000
100000
50000
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

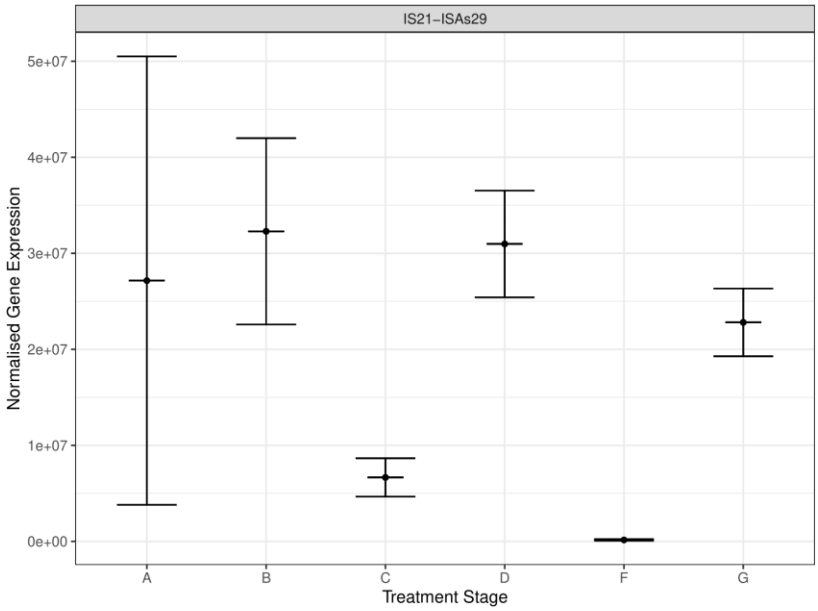

intI2_2
Normalised Gene Expression
9e+07
6e+07
3e+07
A
B
C
D
E
F
G
Treatment Stage

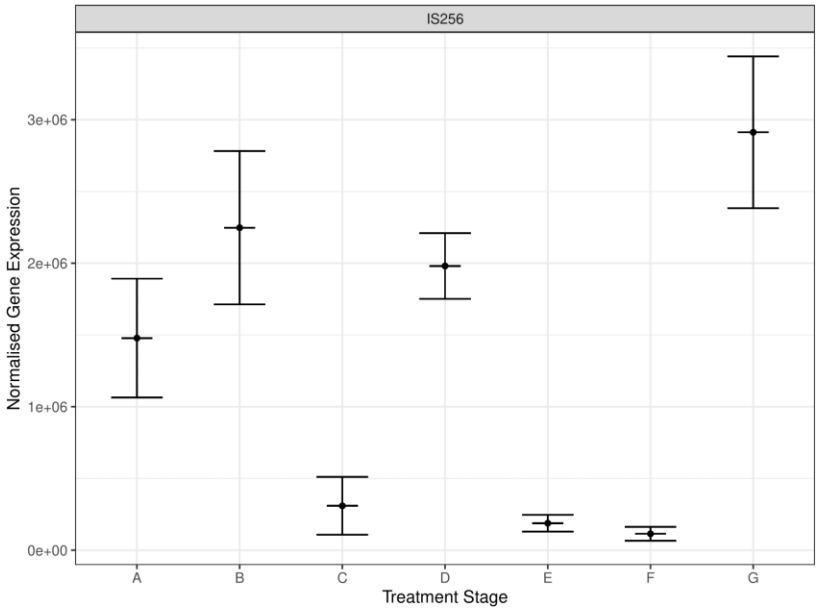

intI3
Normalised Gene Expression
8e+07
6e+07
4e+07
2e+07
A
B
C
D
E
G
Treatment Stage

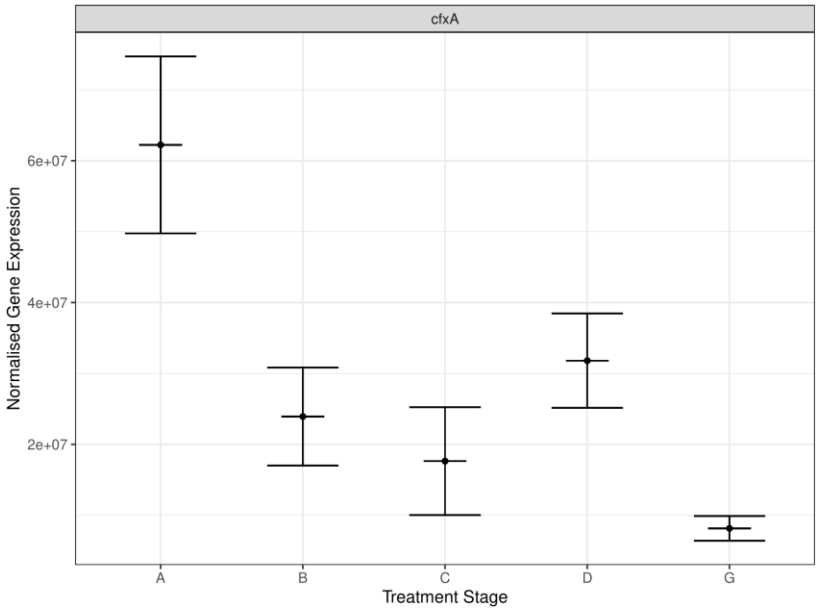

IS1247_1
200
150
100
Normalised Gene Expression
A
B
C
D
G
Treatment Stage

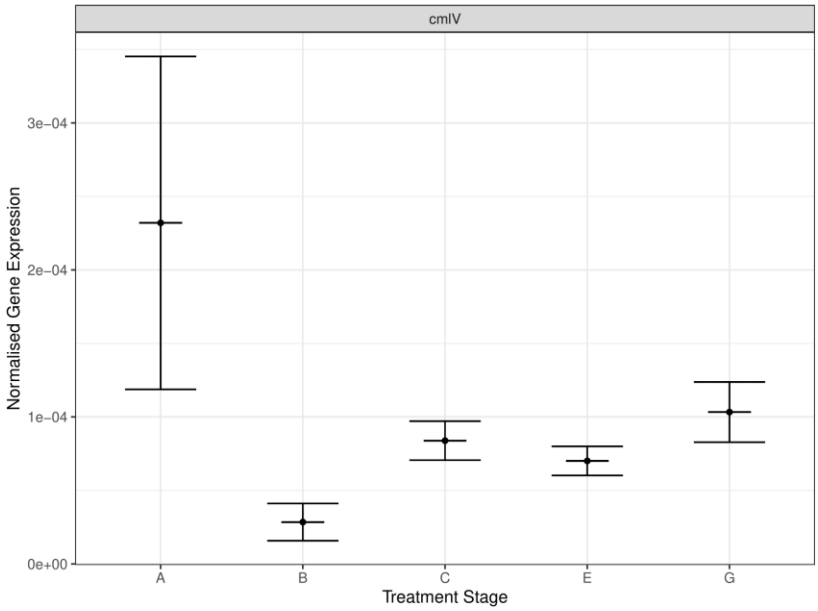

IS1247_2
100
50
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

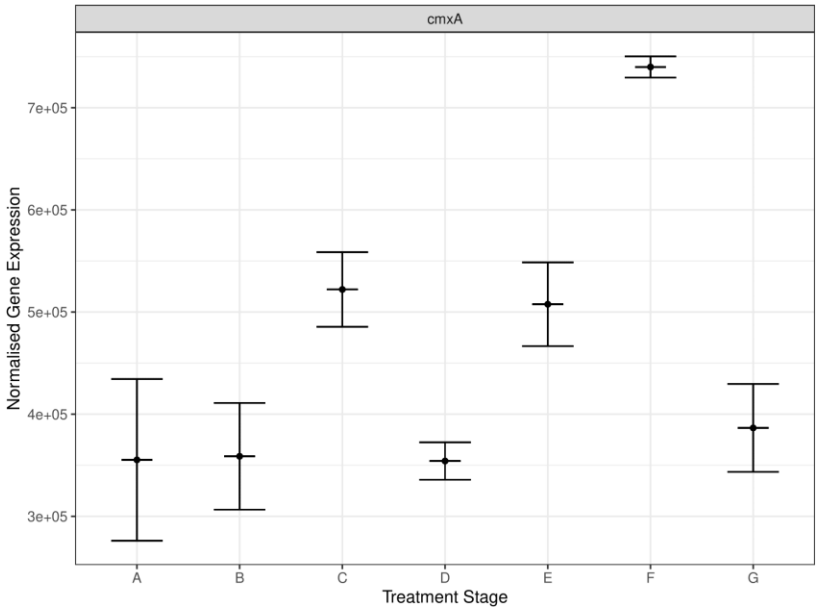

IS200_2
Normalised Gene Expression
7.5e+10
5.0e+10
2.5e+10
A
D
F
Treatment Stage

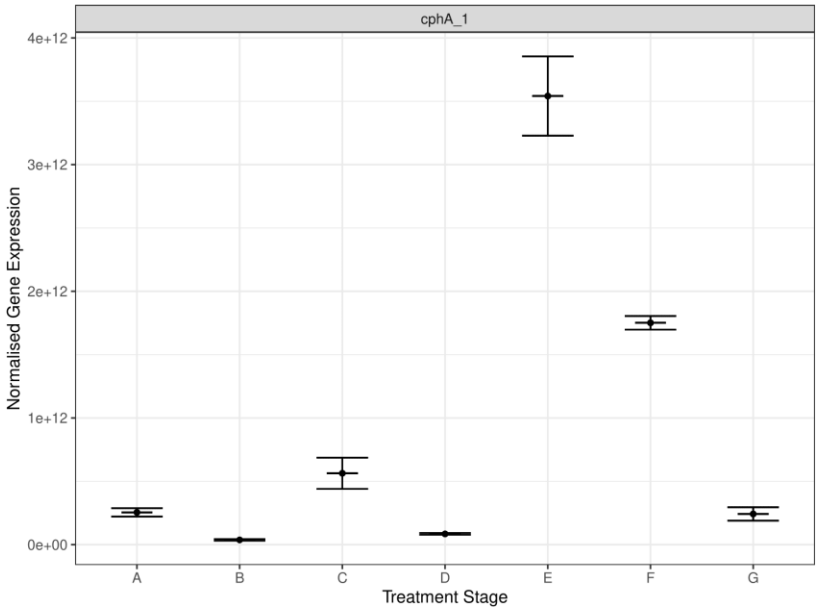

IS21−ISAs29
Normalised Gene Expression
5e+07
4e+07
3e+07
2e+07
1e+07
0e+00
A
B
C
D
F
G
Treatment Stage

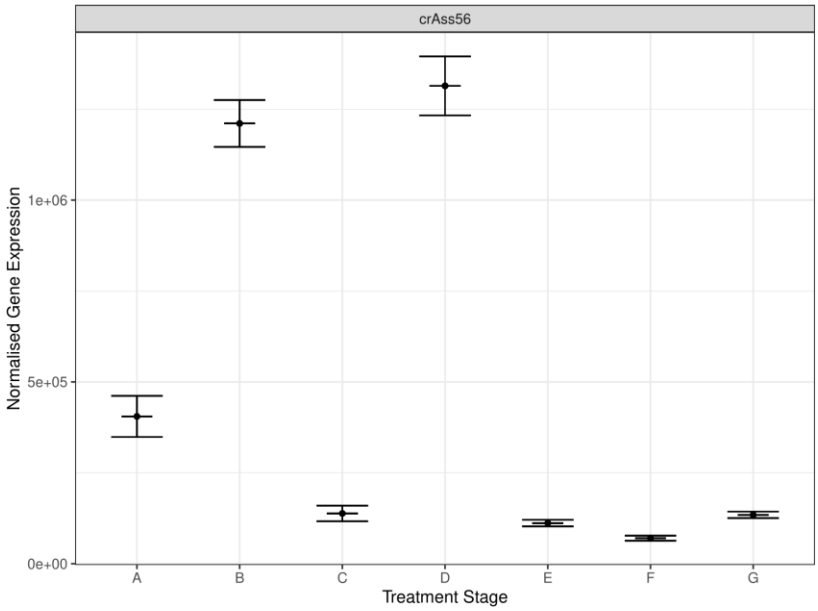

IS256
Normalised Gene Expression
3e+06
2e+06
1e+06
0e+00
A
B
C
D
E
F
G
Treatment Stage

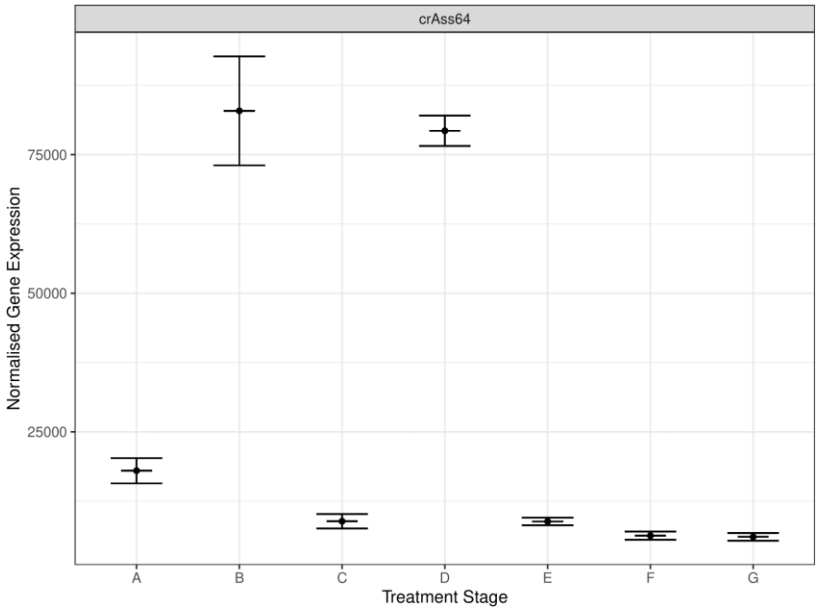

cfxA
Normalised Gene Expression
6e+07
4e+07
2e+07
A
B
C
D
G
Treatment Stage

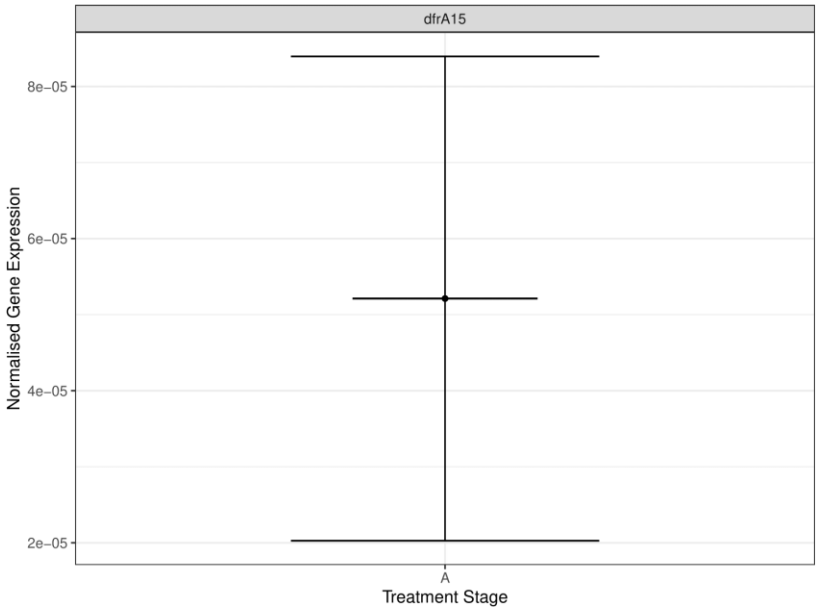

cmlV
Normalised Gene Expression
3e−04
2e−04
1e−04
0e+00
A
B
C
E
G
Treatment Stage

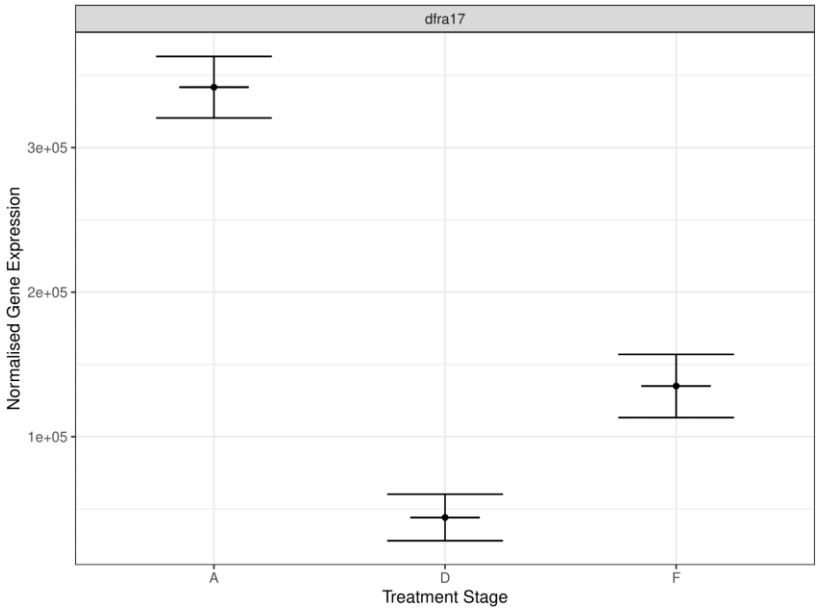

cmxA
Normalised Gene Expression
7e+05
6e+05
5e+05
4e+05
3e+05
A
B
C
D
E
F
G
Treatment Stage

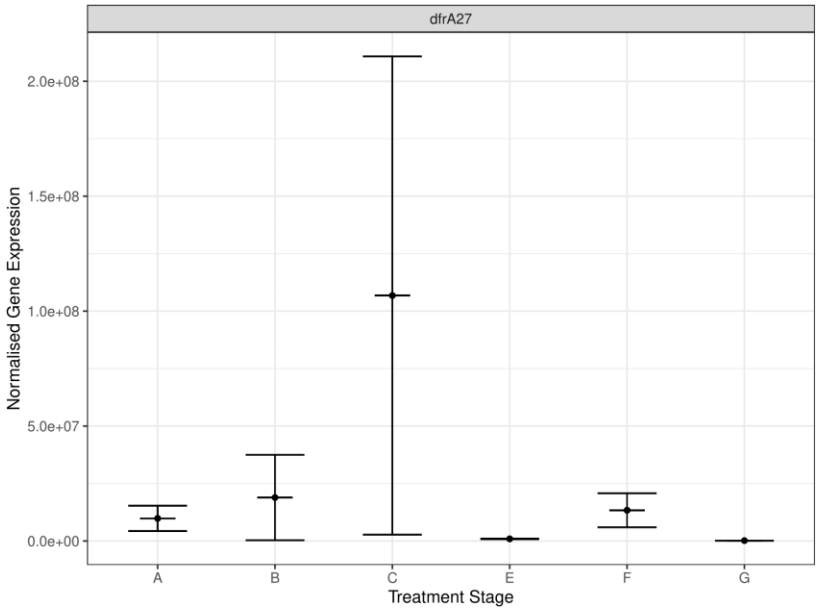

cphA_1
4e+12
3e+12
2e+12
1e+12
0e+00
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

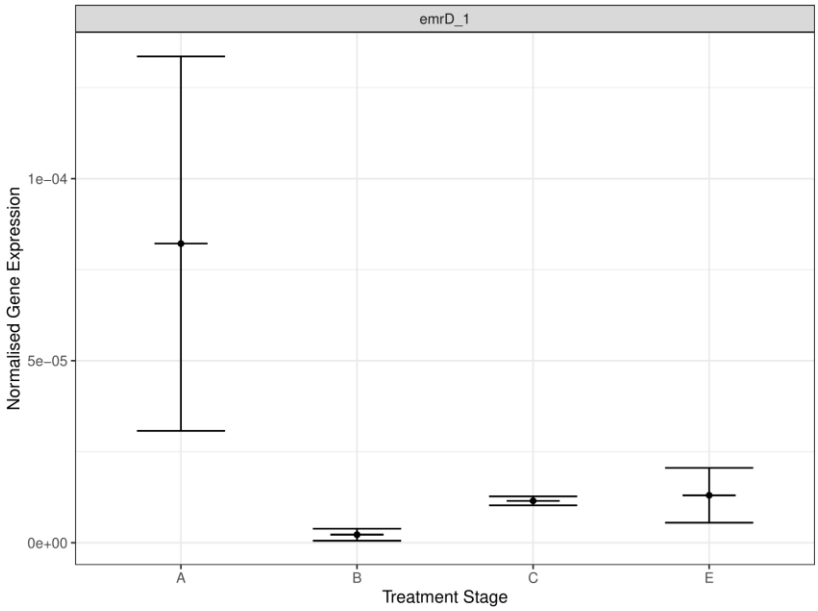

crAss56
1e+06
5e+05
0e+00
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

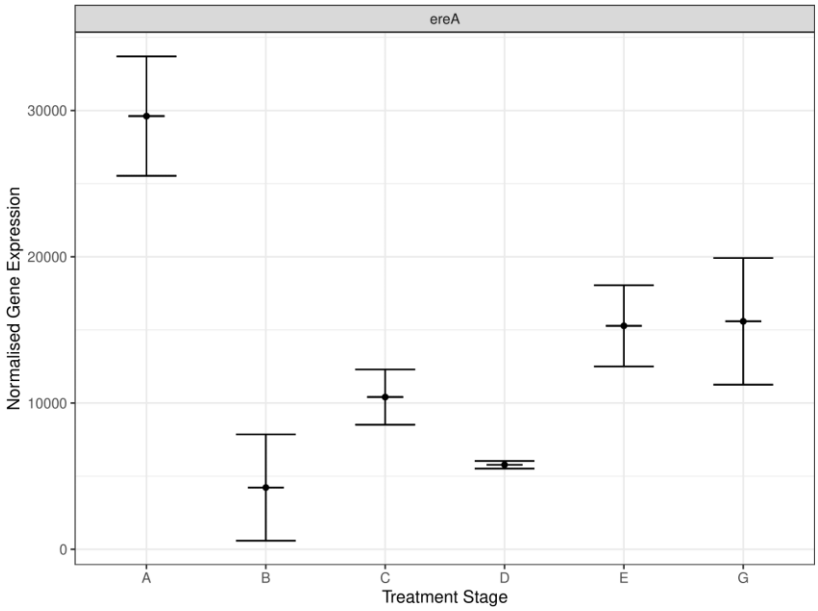

crAss64
Normalised Gene Expression
75000
50000
25000
A
B
C
D
E
F
G
Treatment Stage

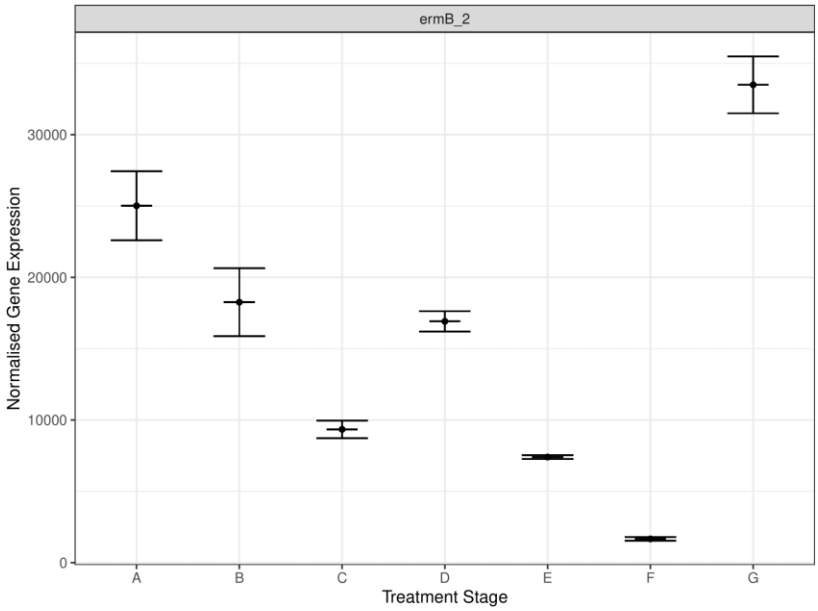

dfrA15
Normalised Gene Expression
8e−05
6e−05
4e−05
2e−05
A
Treatment Stage

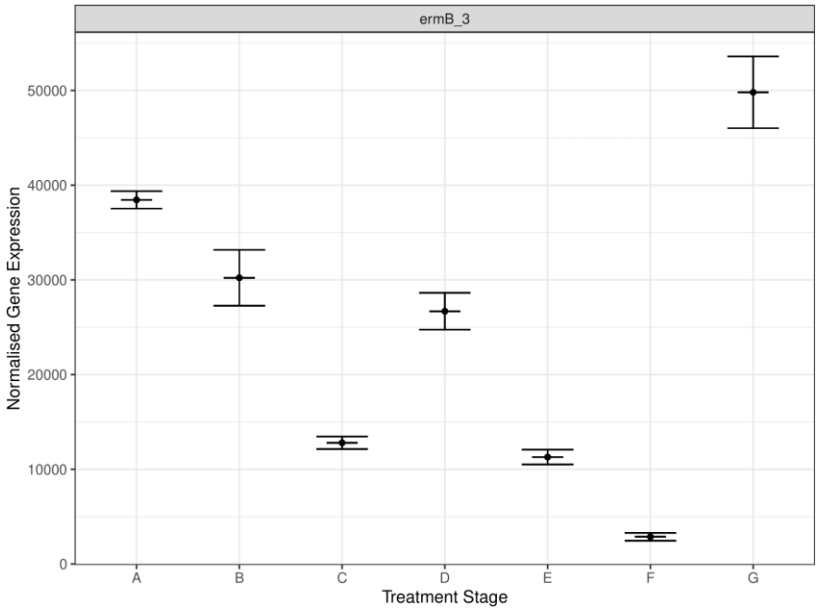

dfra17
Normalised Gene Expression
3e+05
2e+05
1e+05
A
D
F
Treatment Stage

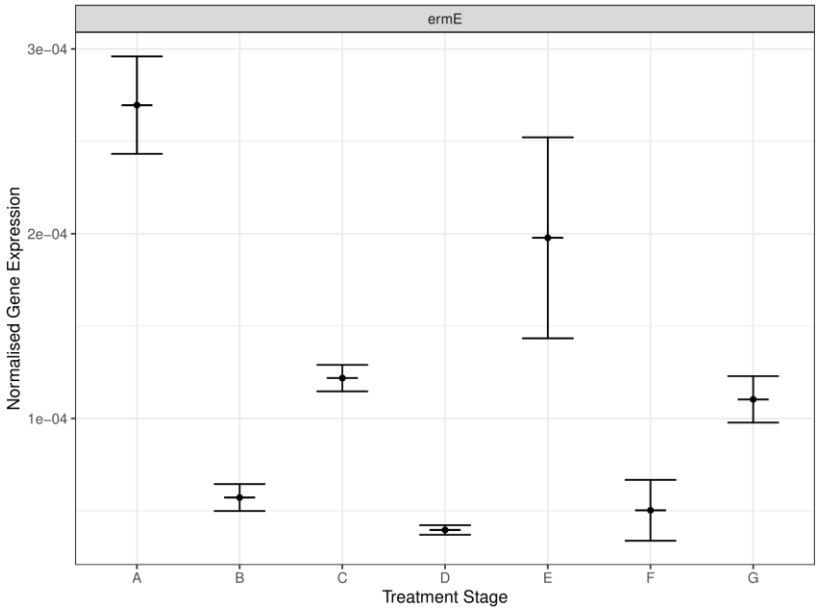

dfrA27
Normalised Gene Expression
2.0e+08
1.5e+08
1.0e+08
5.0e+07
0.0e+00
A
B
C
E
F
G
Treatment Stage

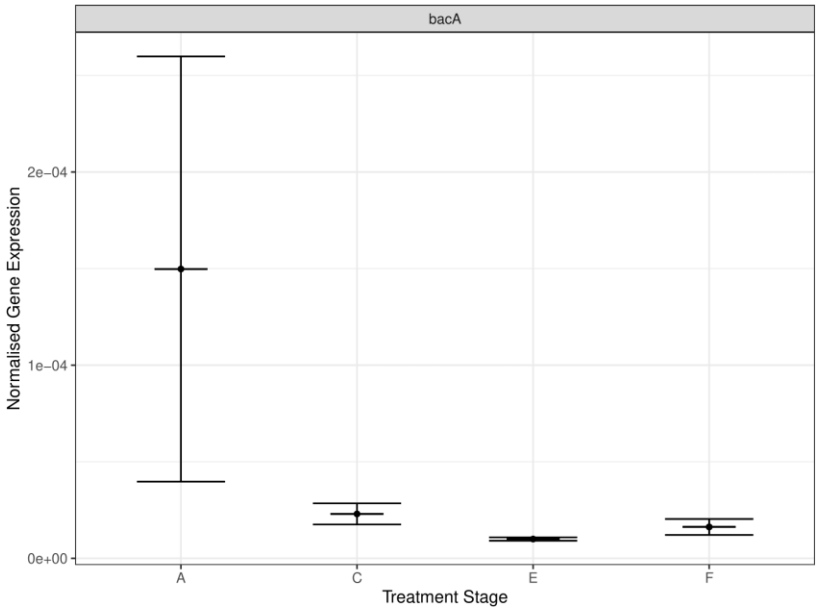

emrD_1
Normalised Gene Expression
1e−04
5e−05
0e+00
A
B
C
E
Treatment Stage

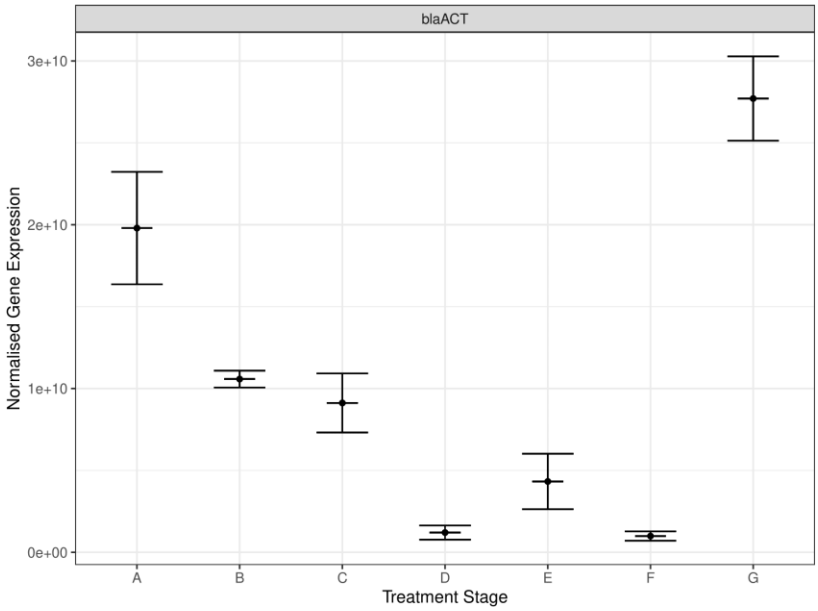

ereA
Normalised Gene Expression
30000
20000
10000
0
A
B
C
D
E
G
Treatment Stage

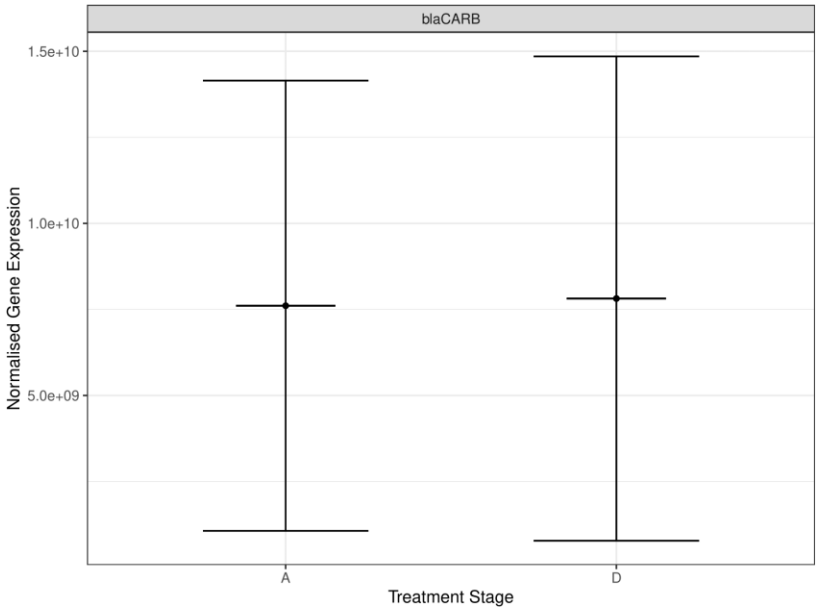

ermB_2
Normalised Gene Expression
30000
20000
10000
0
A
B
C
D
E
F
G
Treatment Stage

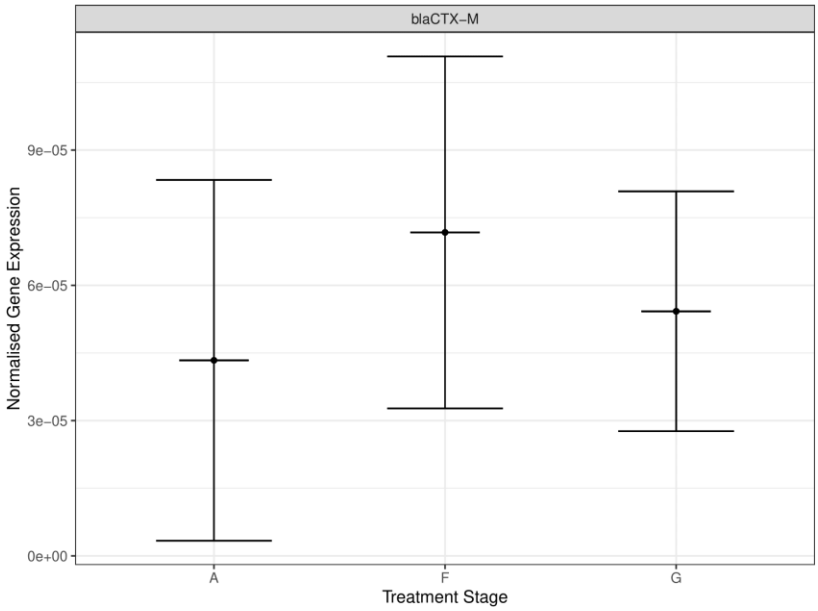

ermB_3
Normalised Gene Expression
50000
40000
30000
20000
10000
0
A
B
C
D
E
F
G
Treatment Stage

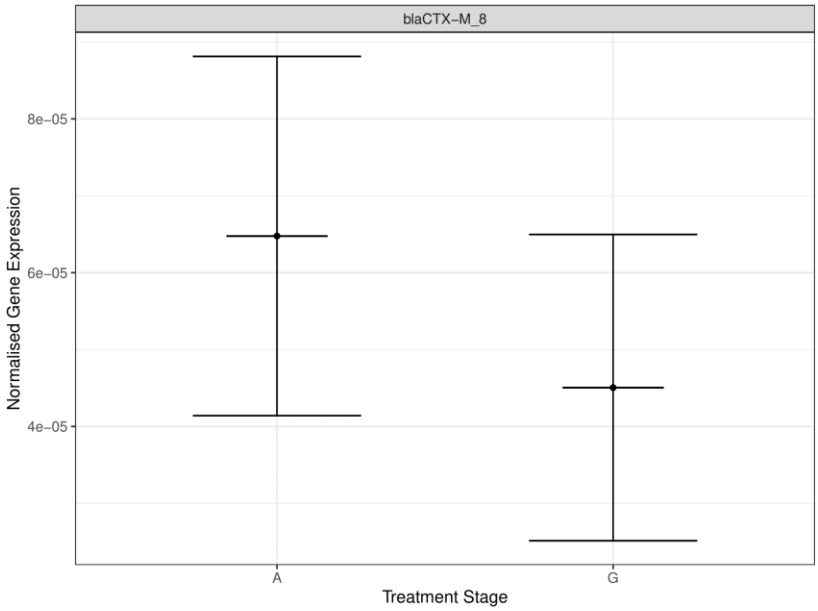

ermE
3e−04
2e−04
1e−04
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

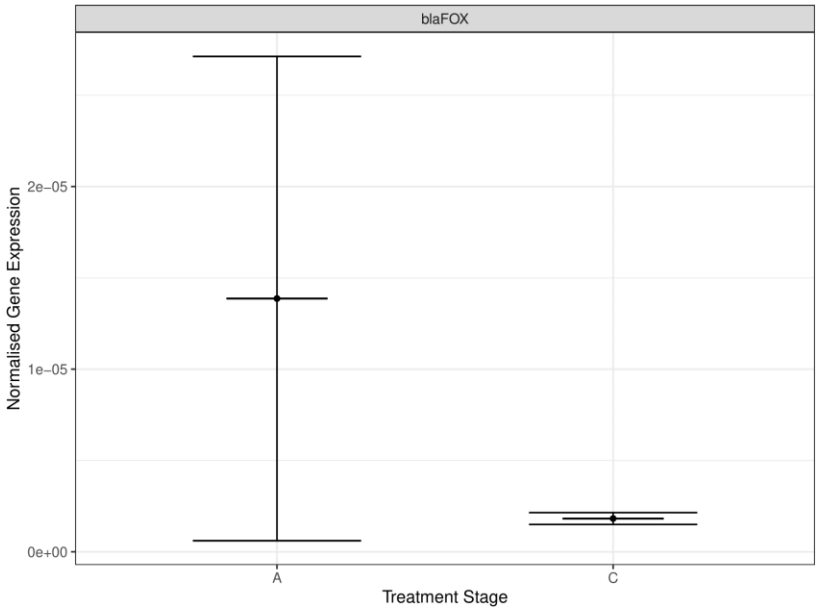

bacA
2e−04
1e−04
0e+00
Normalised Gene Expression
A
C
E
F
Treatment Stage

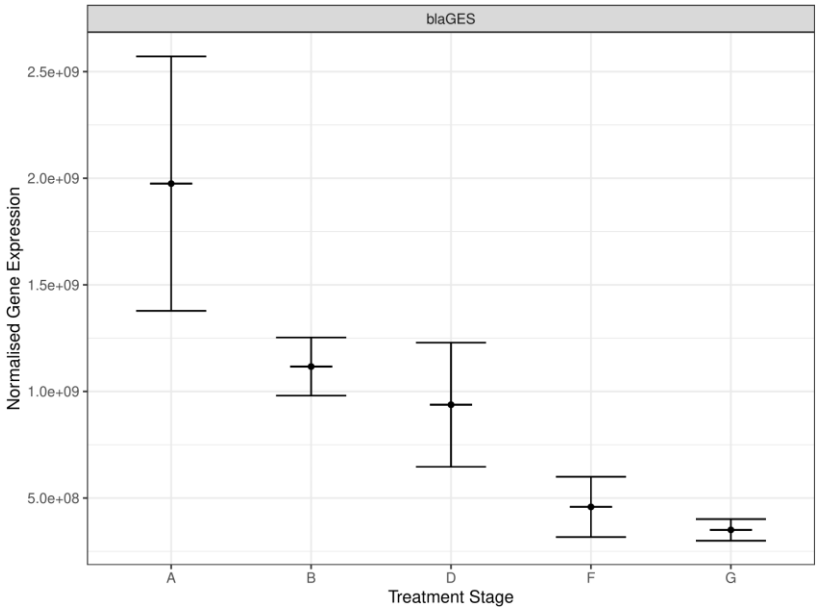

blaACT
3e+10
2e+10
1e+10
0e+00
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

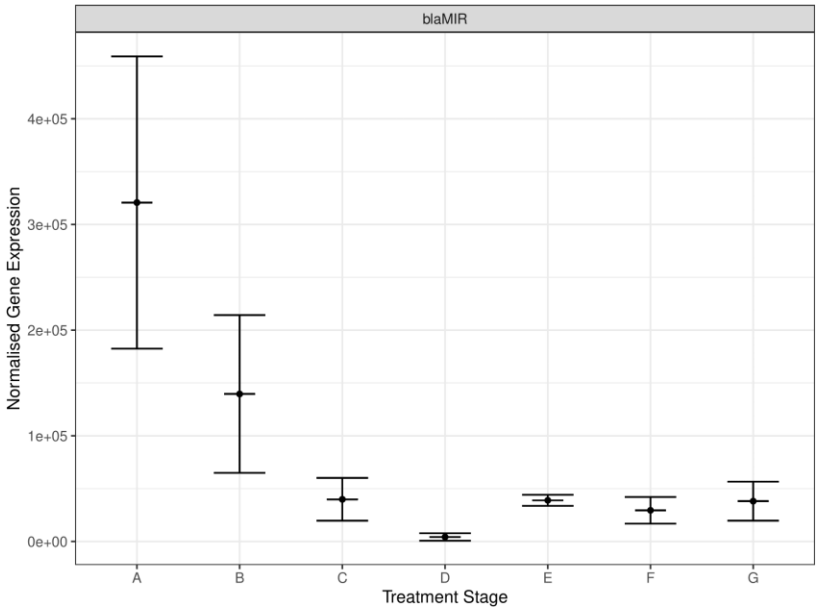

blaCARB
1.5e+10
1.0e+10
5.0e+09
Normalised Gene Expression
A
D
Treatment Stage

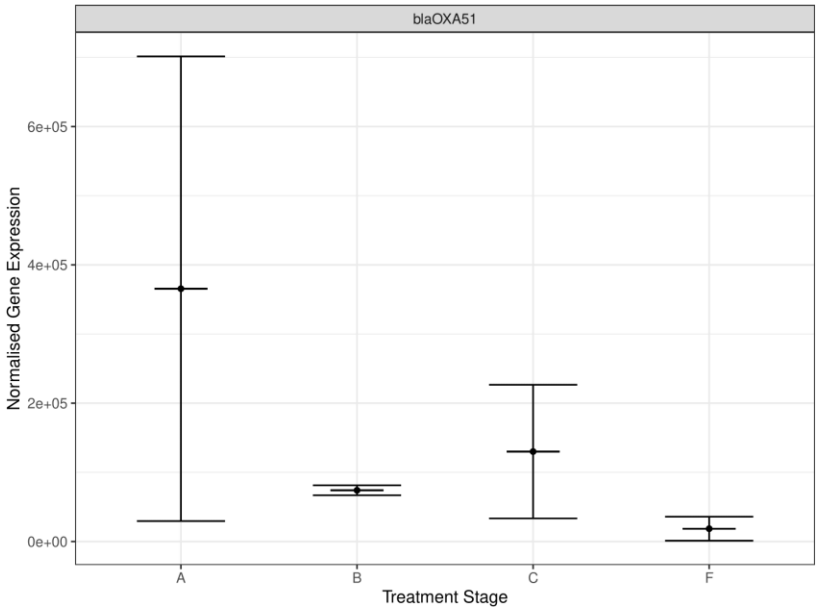

blaCTX−M
9e−05
6e−05
3e−05
0e+00
Normalised Gene Expression
A
F
G
Treatment Stage

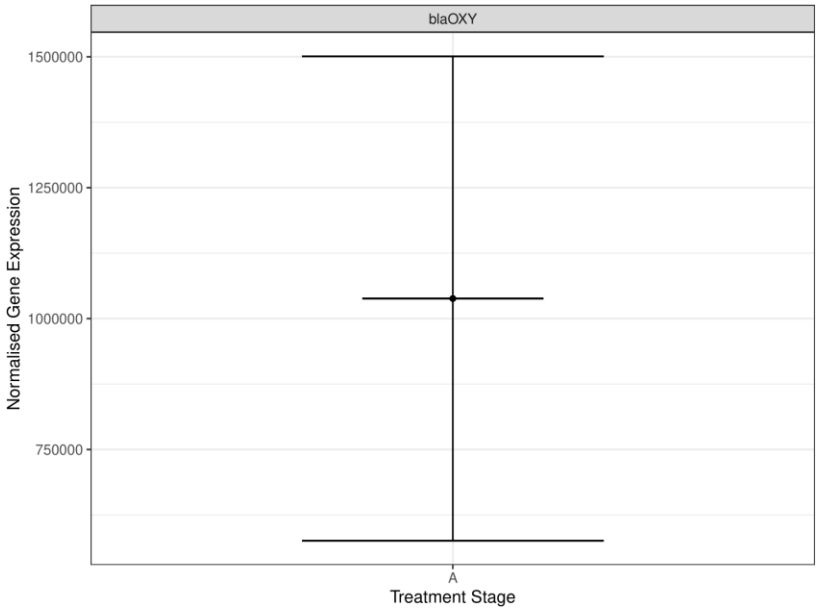

blaCTX−M_8
8e−05
6e−05
4e−05
Normalised Gene Expression
A
G
Treatment Stage

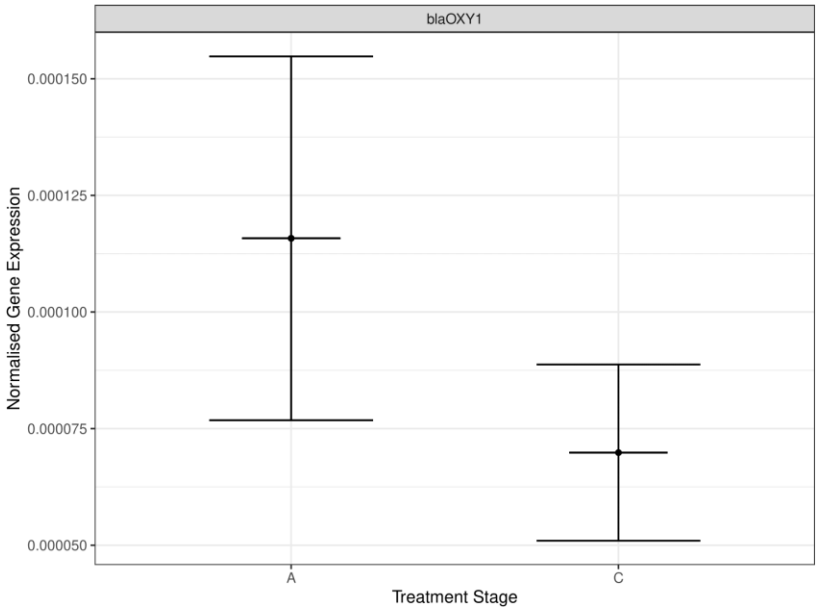

blaFOX
Normalised Gene Expression
2e−05
1e−05
0e+00
A
C
Treatment Stage

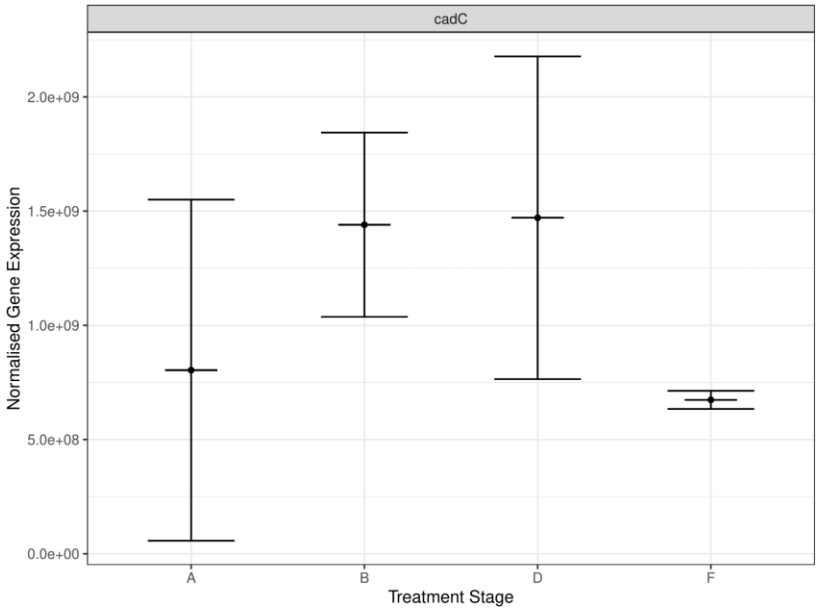

blaGES
Normalised Gene Expression
2.5e+09
2.0e+09
1.5e+09
1.0e+09
5.0e+08
A
B
D
F
G
Treatment Stage

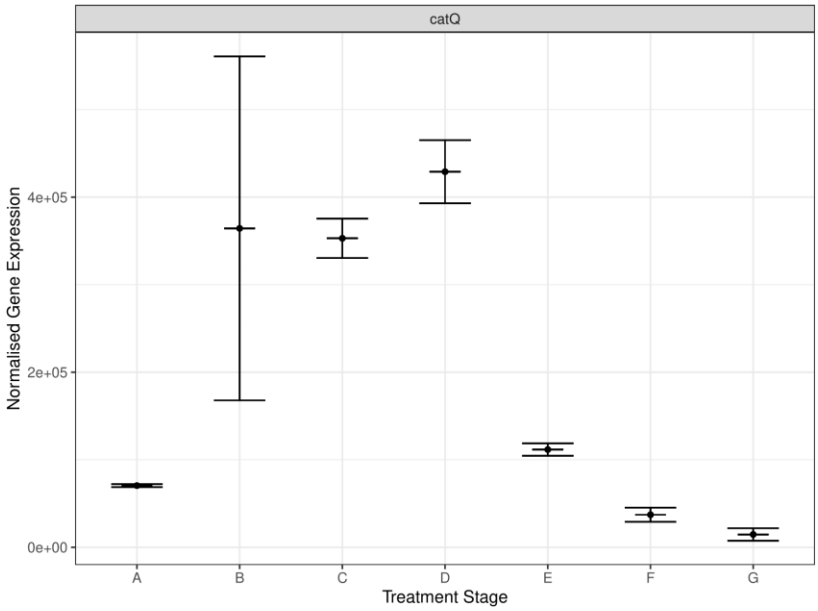

blaMIR
Normalised Gene Expression
4e+05
3e+05
2e+05
1e+05
0e+00
A
B
C
D
E
F
G
Treatment Stage

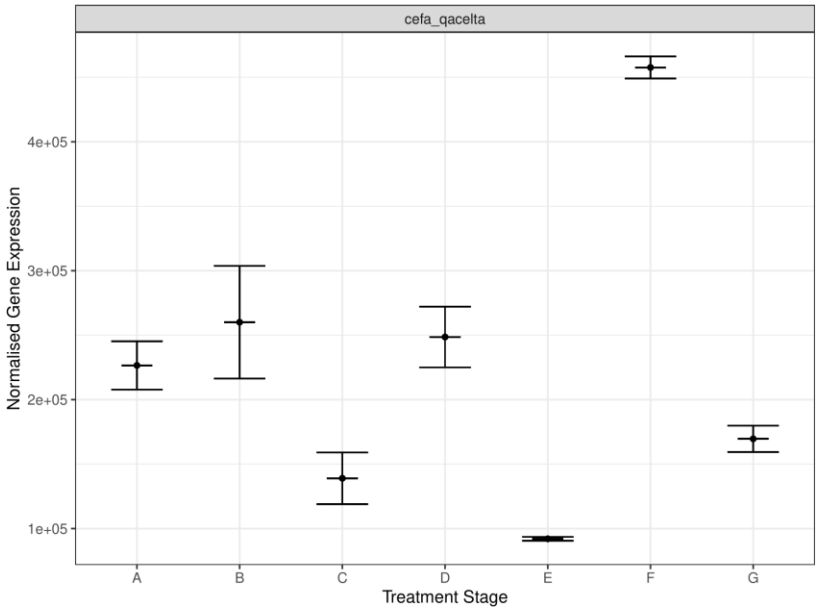

blaOXA51
Normalised Gene Expression
6e+05
4e+05
2e+05
0e+00
A
B
C
F
Treatment Stage

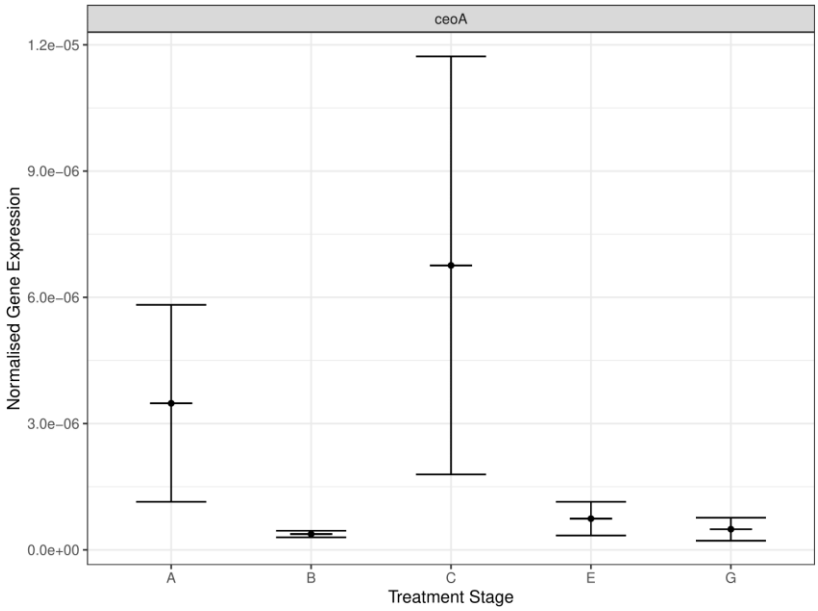

blaOXY
1500000
1250000
1000000
750000
Normalised Gene Expression
A
Treatment Stage

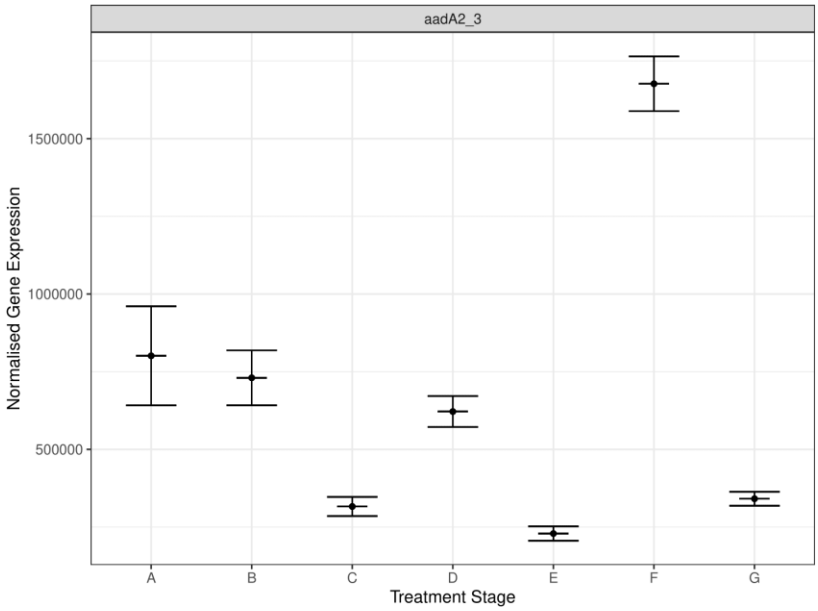

blaOXY1
0.000150
0.000125
0.000100
0.000075
0.000050
Normalised Gene Expression
A
C
Treatment Stage

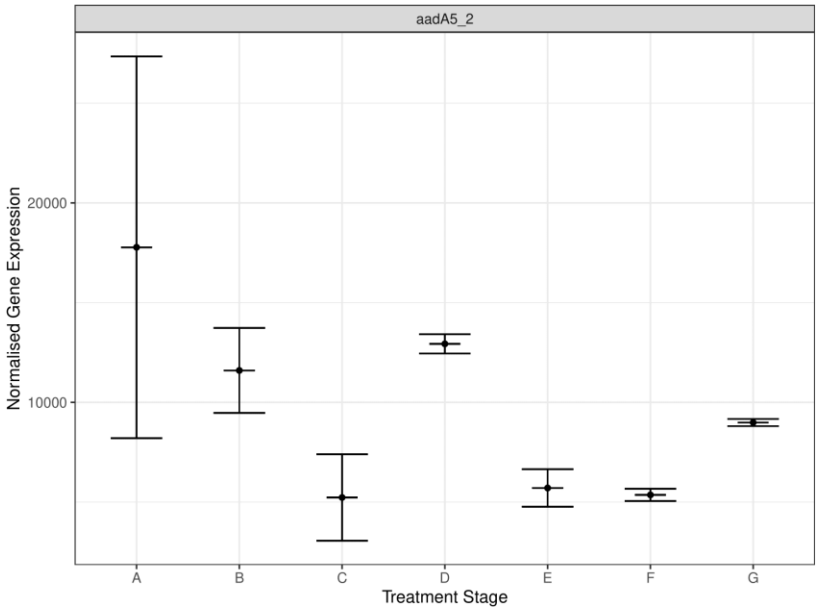

cadC
Normalised Gene Expression
2.0e+09
1.5e+09
1.0e+09
5.0e+08
0.0e+00
A
B
D
F
Treatment Stage

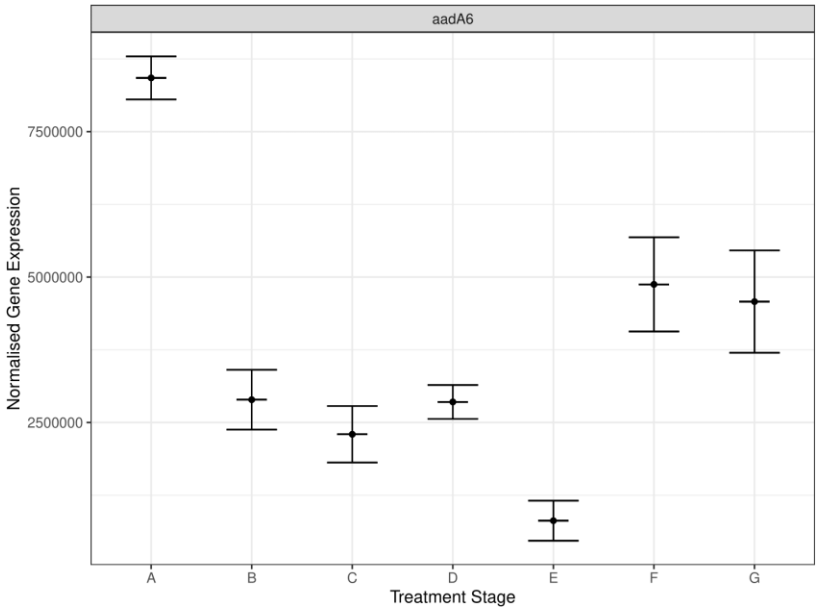

catQ
Normalised Gene Expression
4e+05
2e+05
0e+00
A
B
C
D
E
F
G
Treatment Stage

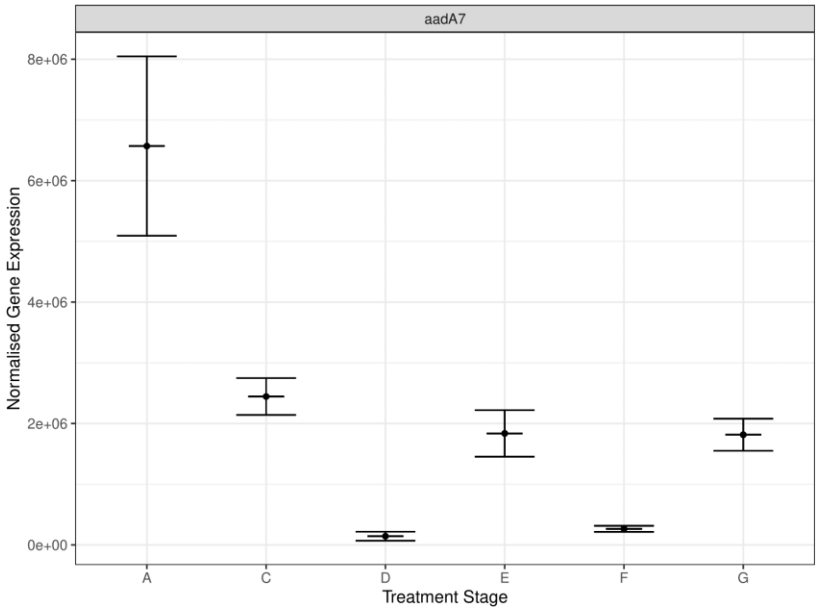

cefa_qacelta
Normalised Gene Expression
4e+05
3e+05
2e+05
1e+05
A
B
C
D
E
F
G
Treatment Stage

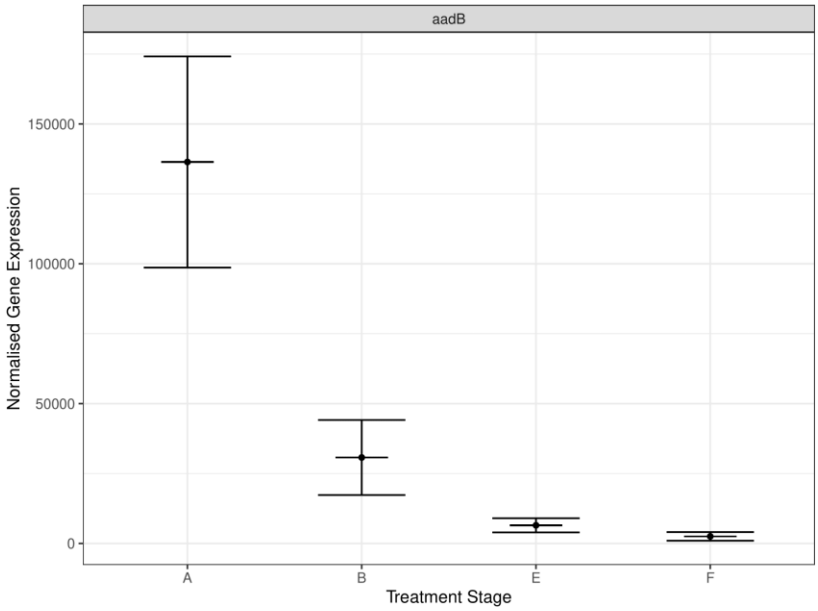

ceoA
Normalised Gene Expression
1.2e−05
9.0e−06
6.0e−06
3.0e−06
0.0e+00
A
B
C
E
G
Treatment Stage

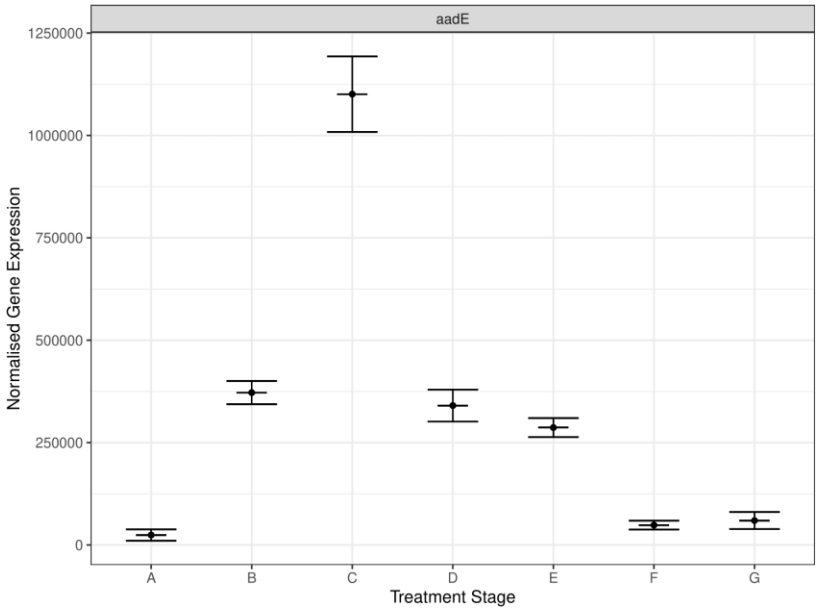

aadA2_3
Normalised Gene Expression
1500000
1000000
500000
A
B
C
D
E
F
G
Treatment Stage

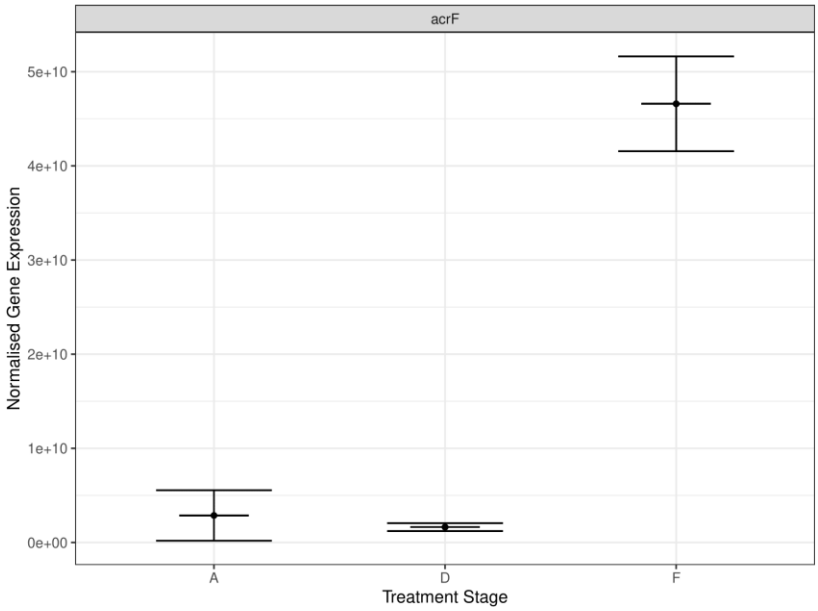

aadA5_2
Normalised Gene Expression
20000
10000
A
B
C
D
E
F
G
Treatment Stage

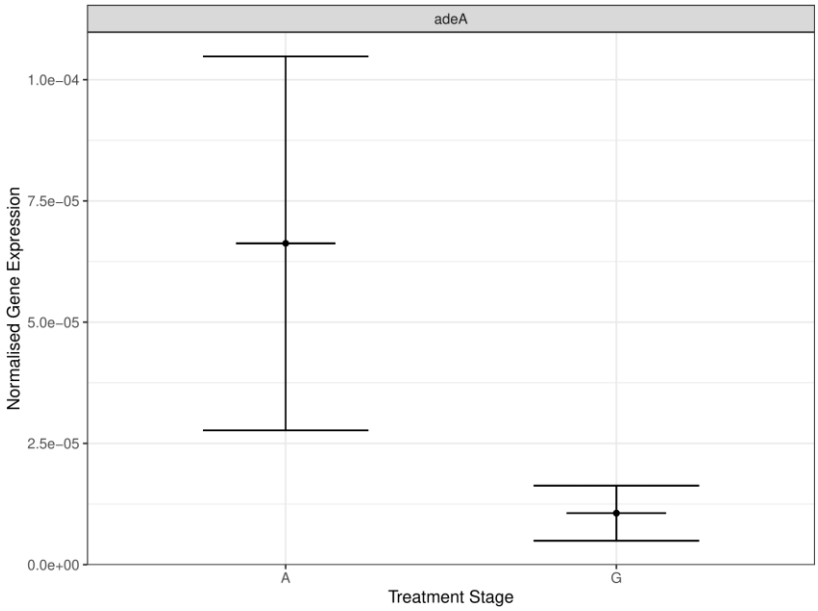

aadA6
Normalised Gene Expression
7500000
5000000
2500000
A
B
C
D
E
F
G
Treatment Stage

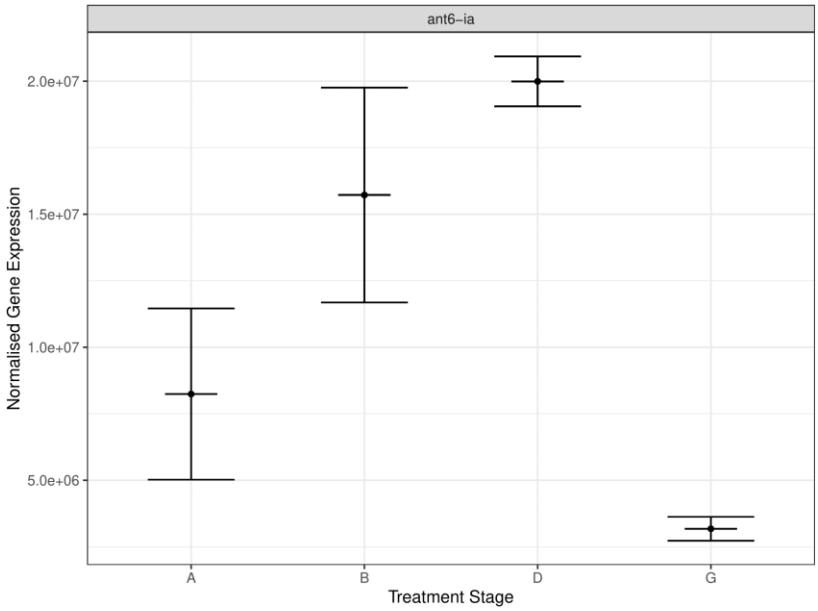

aadA7
Normalised Gene Expression
8e+06
6e+06
4e+06
2e+06
0e+00
A
C
D
E
F
G
Treatment Stage

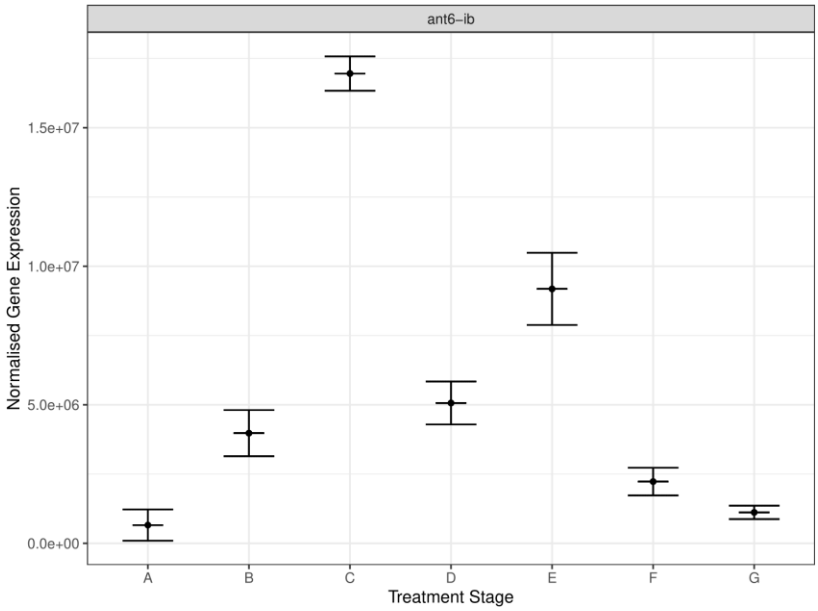

aadB
150000
100000
50000
0
Normalised Gene Expression
A
B
E
F
Treatment Stage

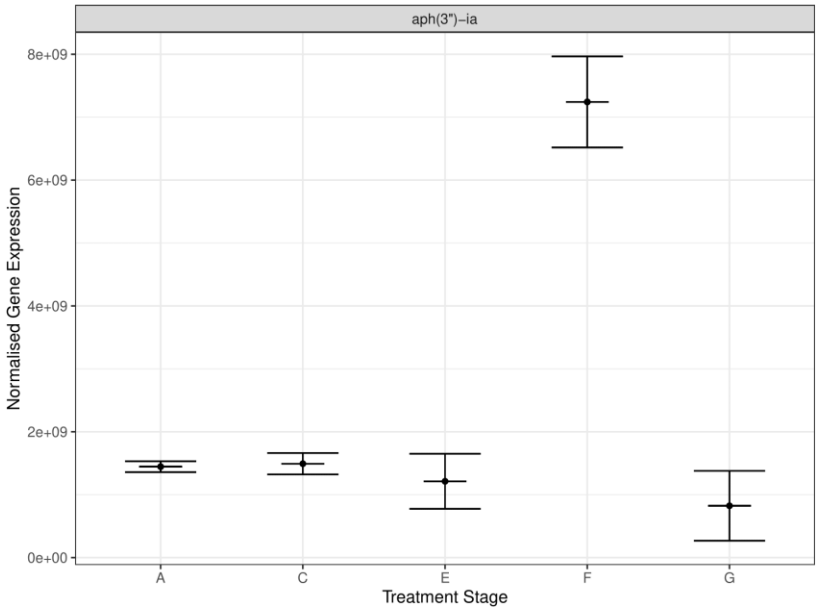

aadE
1250000
1000000
750000
500000
250000
0
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

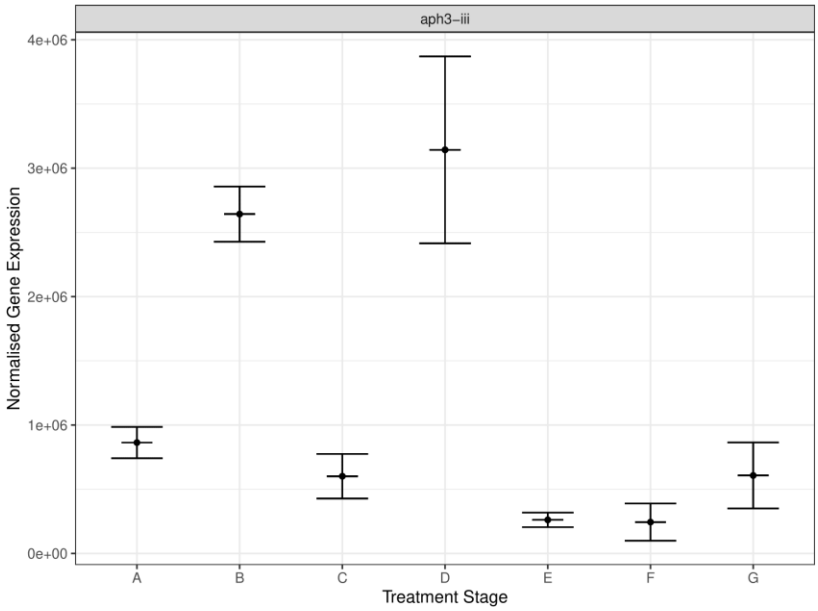

acrF
5e+10
4e+10
3e+10
2e+10
1e+10
0e+00
Normalised Gene Expression
A
D
F
Treatment Stage

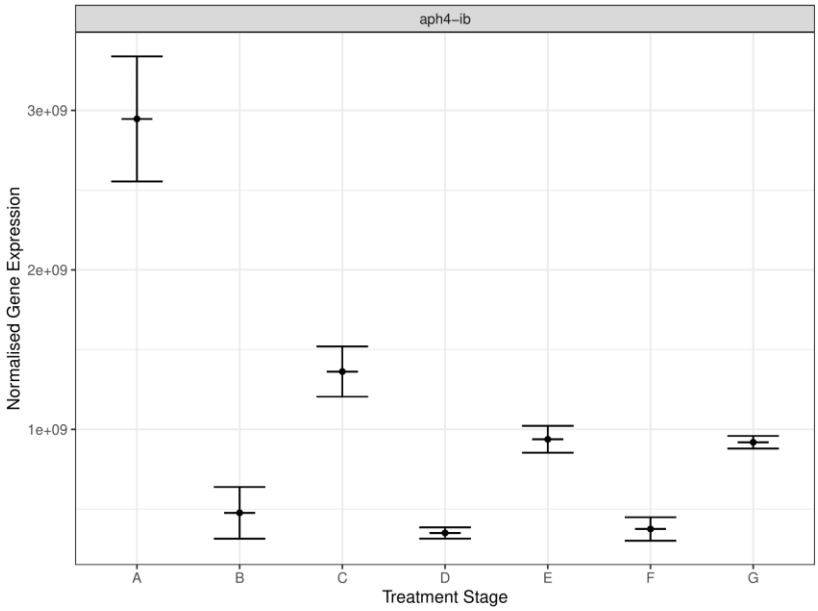

adeA
1.0e−04
7.5e−05
5.0e−05
2.5e−05
0.0e+00
Normalised Gene Expression
A
G
Treatment Stage

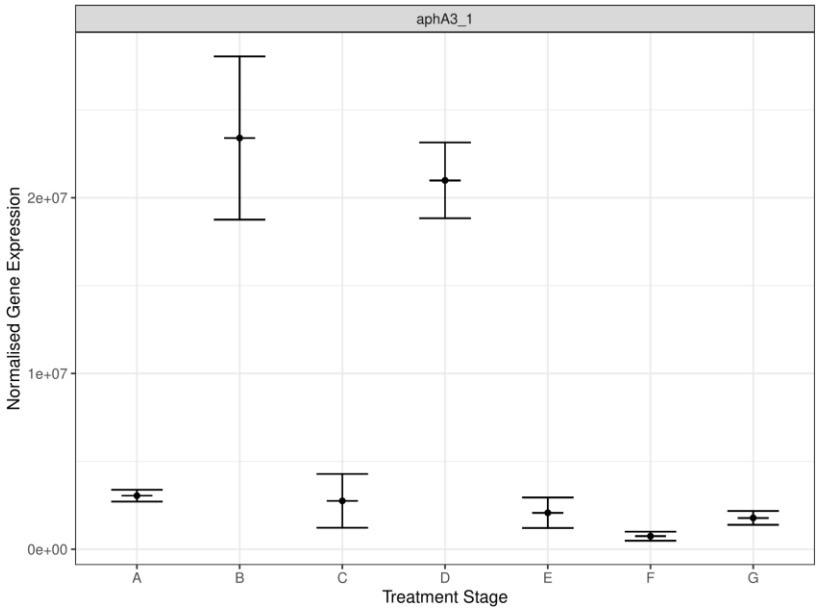

ant6−ia
Normalised Gene Expression
2.0e+07
1.5e+07
1.0e+07
5.0e+06
A
B
D
G
Treatment Stage

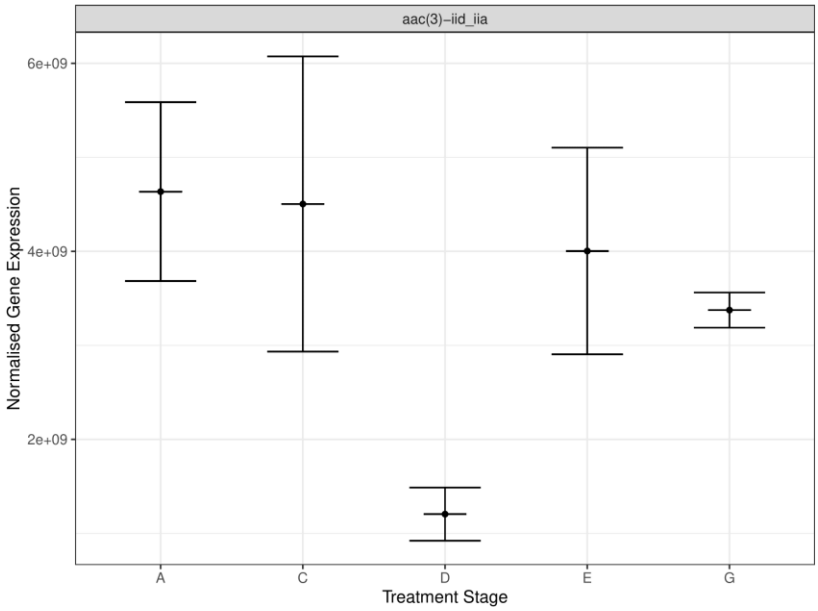

ant6−ib
Normalised Gene Expression
1.5e+07
1.0e+07
5.0e+06
0.0e+00
A
B
C
D
E
F
G
Treatment Stage

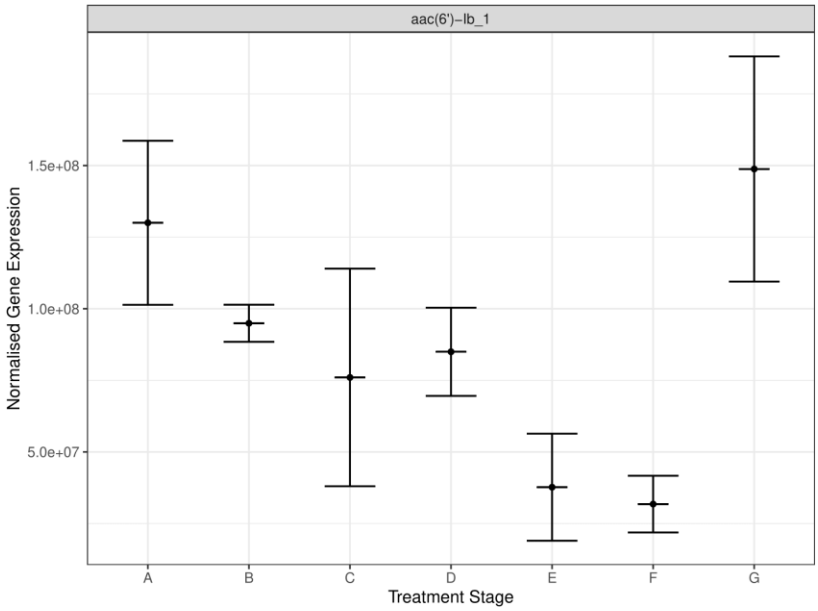

aph(3")–ia
Normalised Gene Expression
8e+09
6e+09
4e+09
2e+09
0e+00
A
C
E
F
G
Treatment Stage

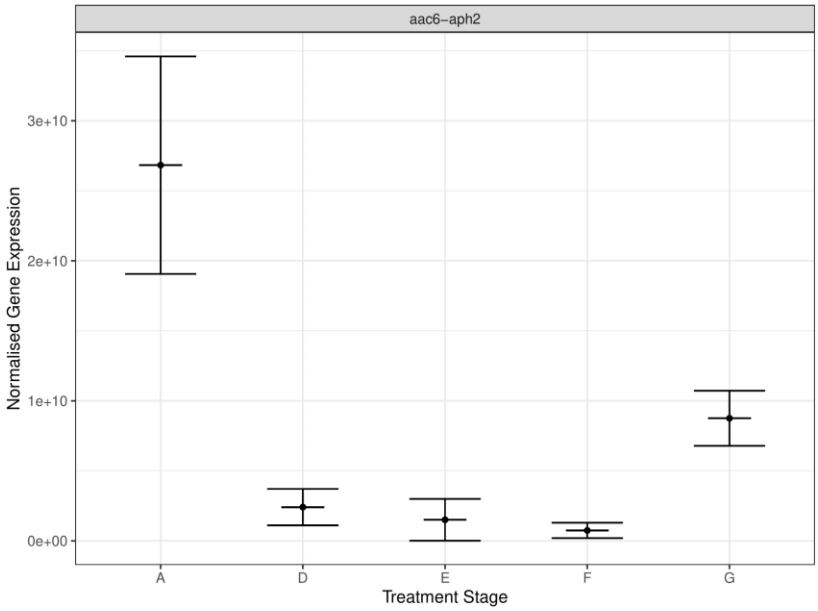

aph3–iii
Normalised Gene Expression
4e+06
3e+06
2e+06
1e+06
0e+00
A
B
C
D
E
F
G
Treatment Stage

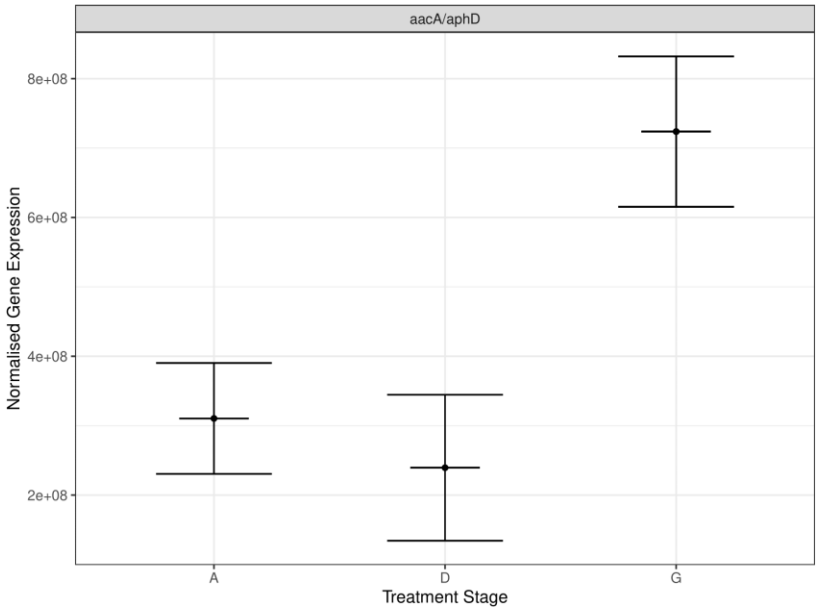

aph4−ib
Normalised Gene Expression
3e+09
2e+09
1e+09
A
B
C
D
E
F
G
Treatment Stage

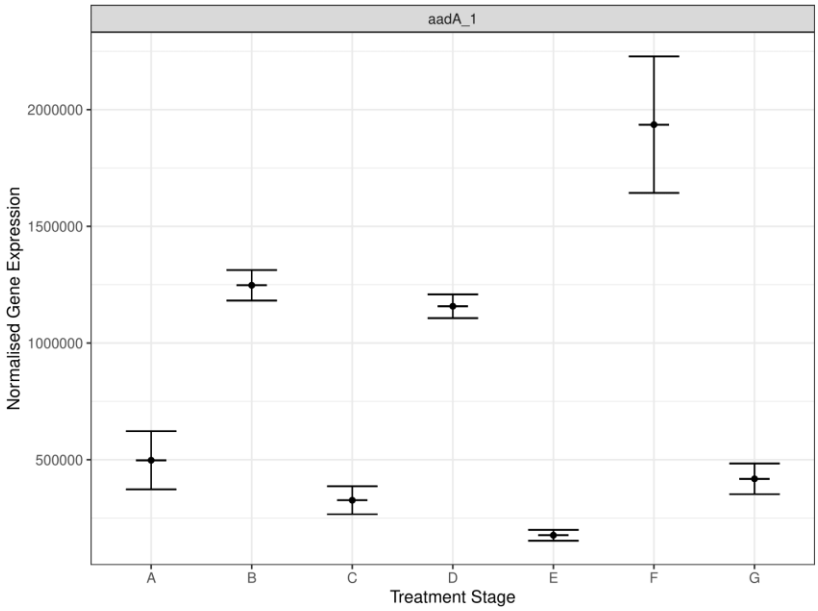

aphA3_1
Normalised Gene Expression
2e+07
1e+07
0e+00
A
B
C
D
E
F
G
Treatment Stage

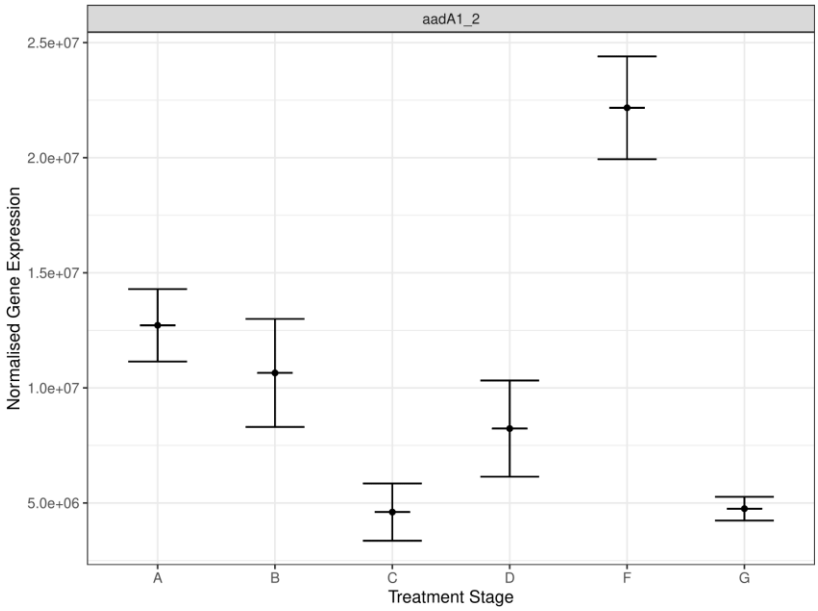

aac(3)−iid_iia
6e+09
4e+09
2e+09
Normalised Gene Expression
A
C
D
E
G
Treatment Stage

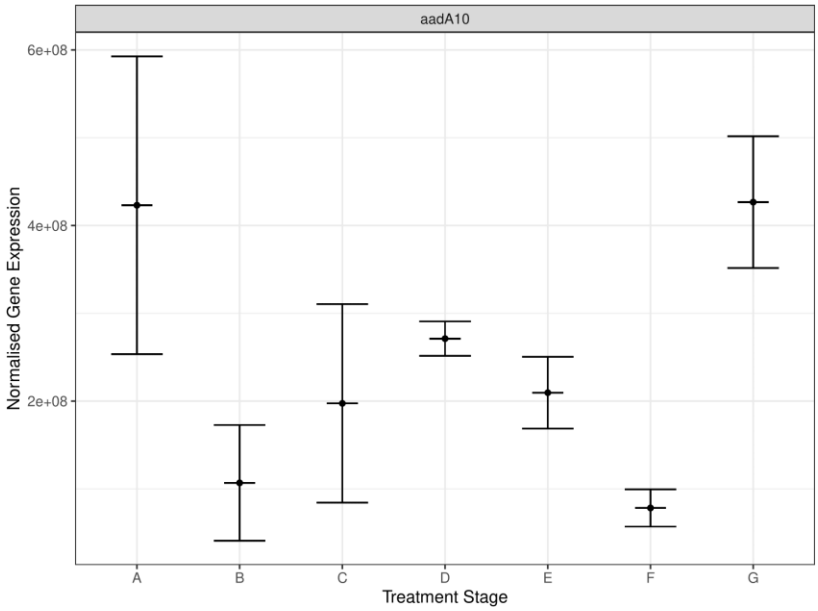

aac(6')−Ib_1
1.5e+08
1.0e+08
5.0e+07
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

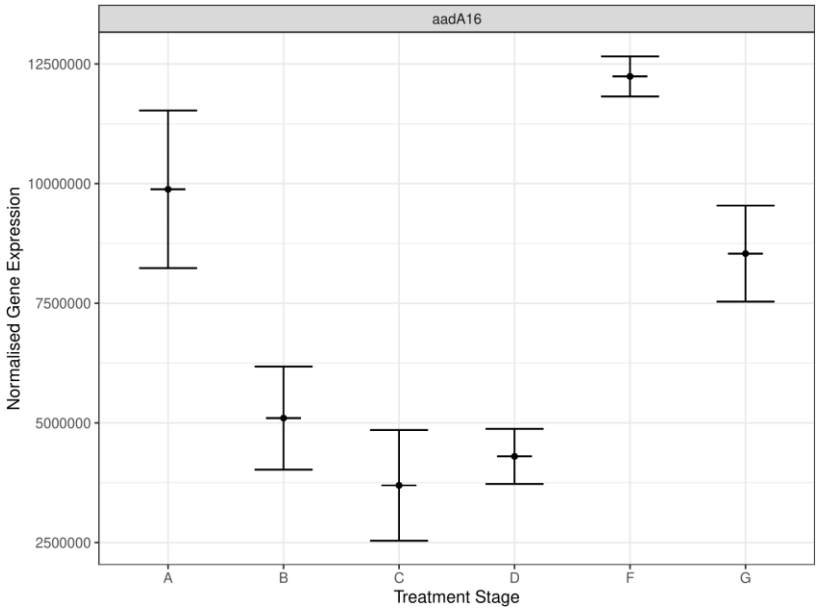

aac6−aph2
Normalised Gene Expression
3e+10
2e+10
1e+10
0e+00
A
D
E
F
G
Treatment Stage

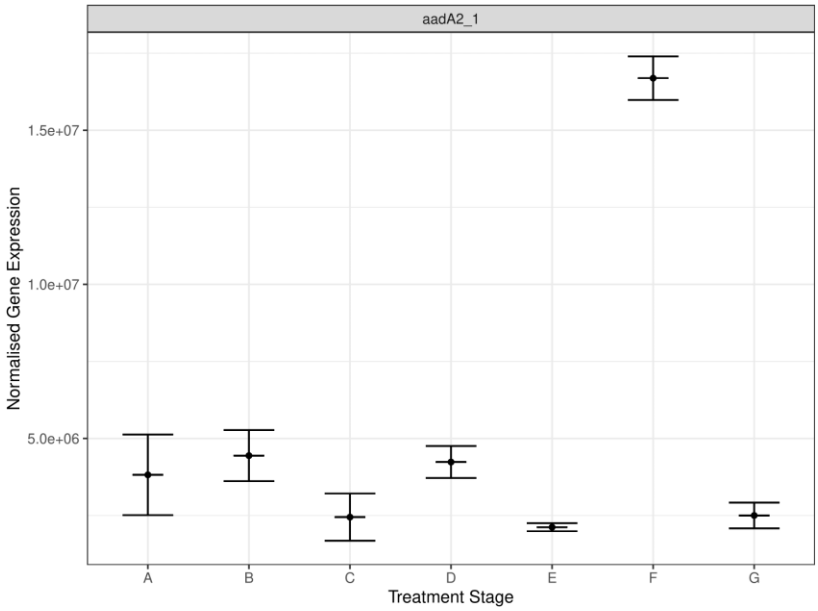

aacA/aphD
Normalised Gene Expression
8e+08
6e+08
4e+08
2e+08
A
D
G
Treatment Stage


aadA_1
Normalised Gene Expression
2000000
1500000
1000000
500000
A
B
C
D
E
F
G
Treatment Stage

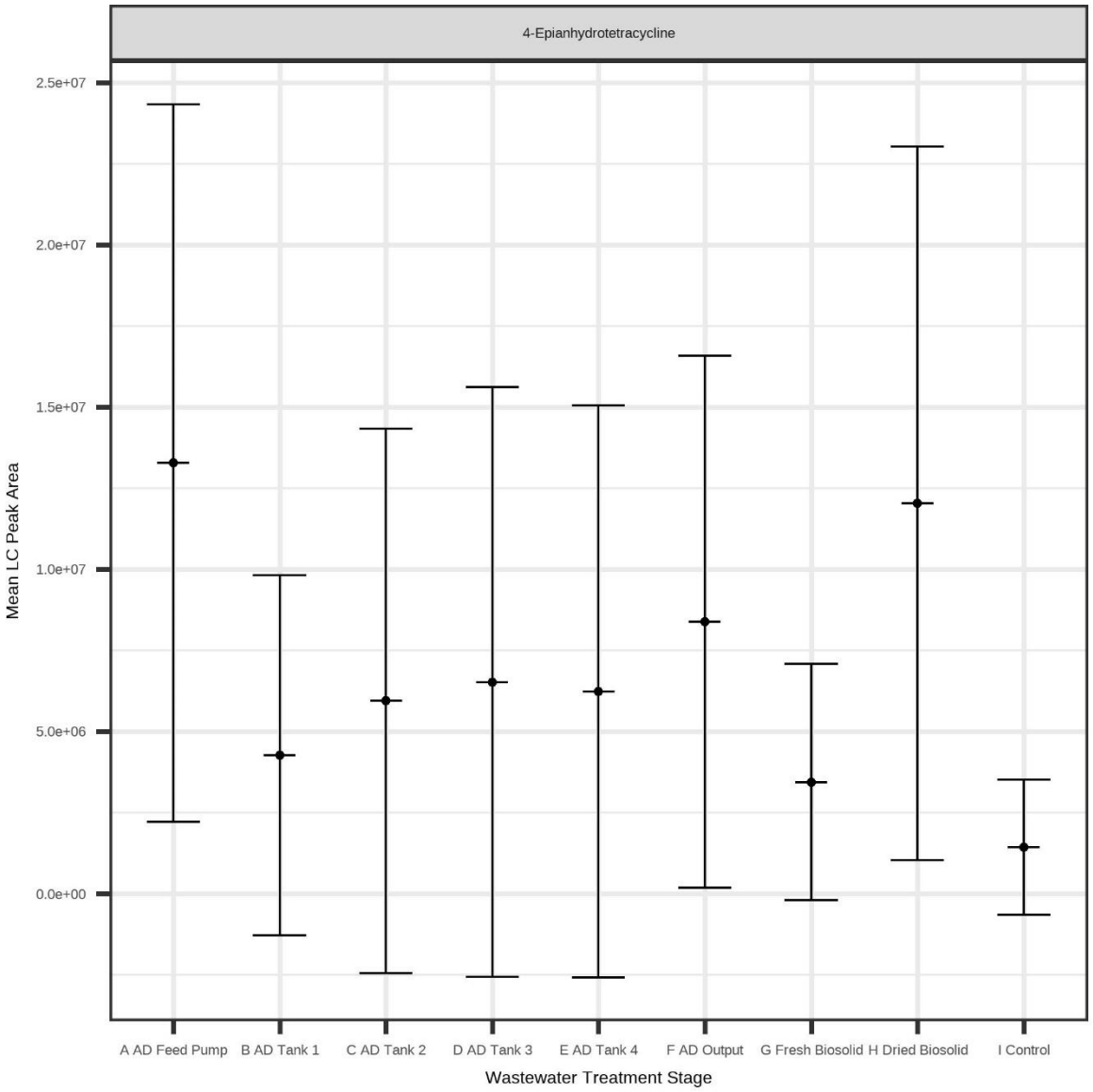

aadA1_2
Normalised Gene Expression
2.5e+07
2.0e+07
1.5e+07
1.0e+07
5.0e+06
A
B
C
D
F
G
Treatment Stage

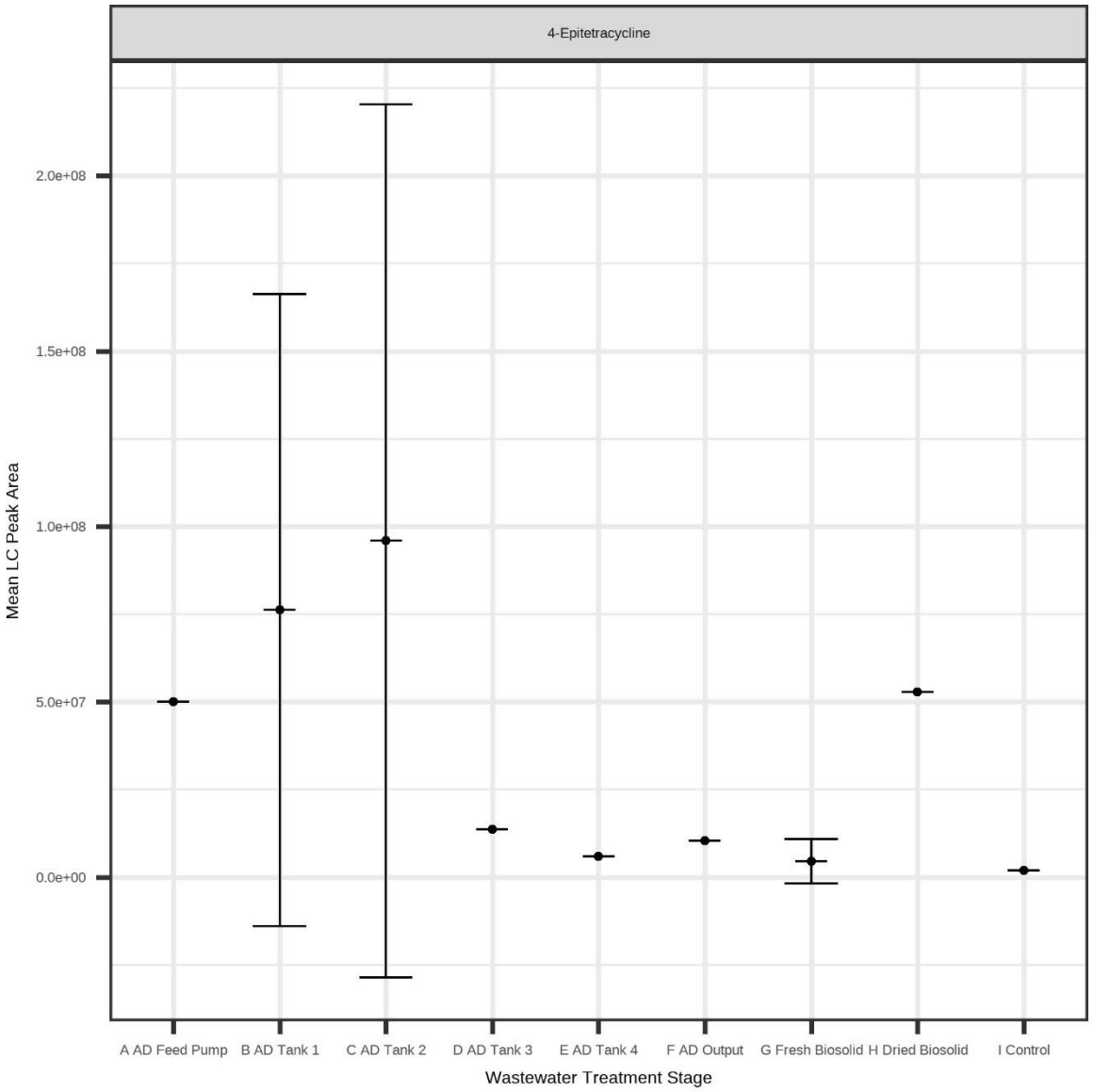

aadA10
6e+08
4e+08
2e+08
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

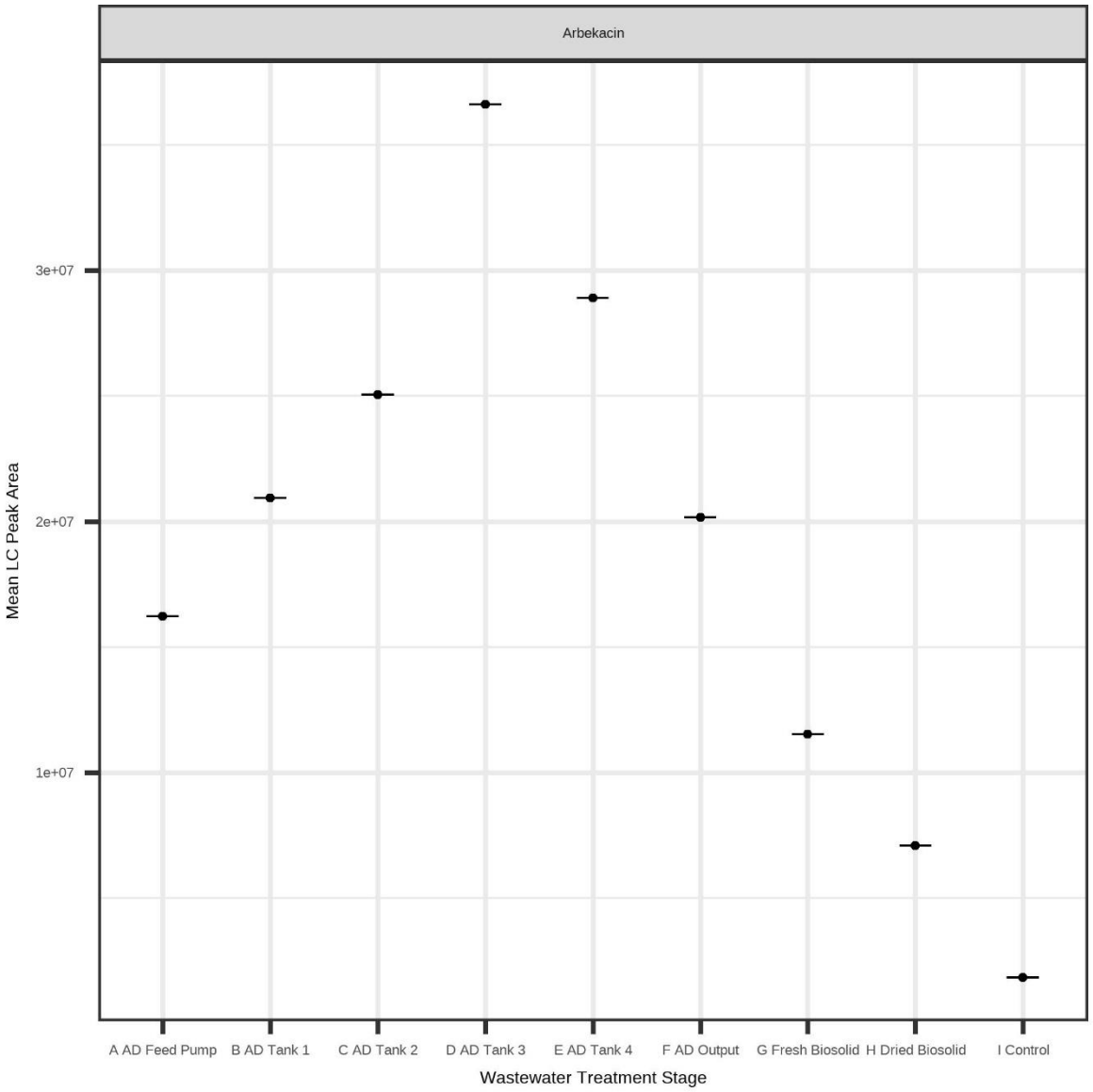

aadA16
12500000
10000000
7500000
5000000
2500000
Normalised Gene Expression
A
B
C
D
F
G
Treatment Stage

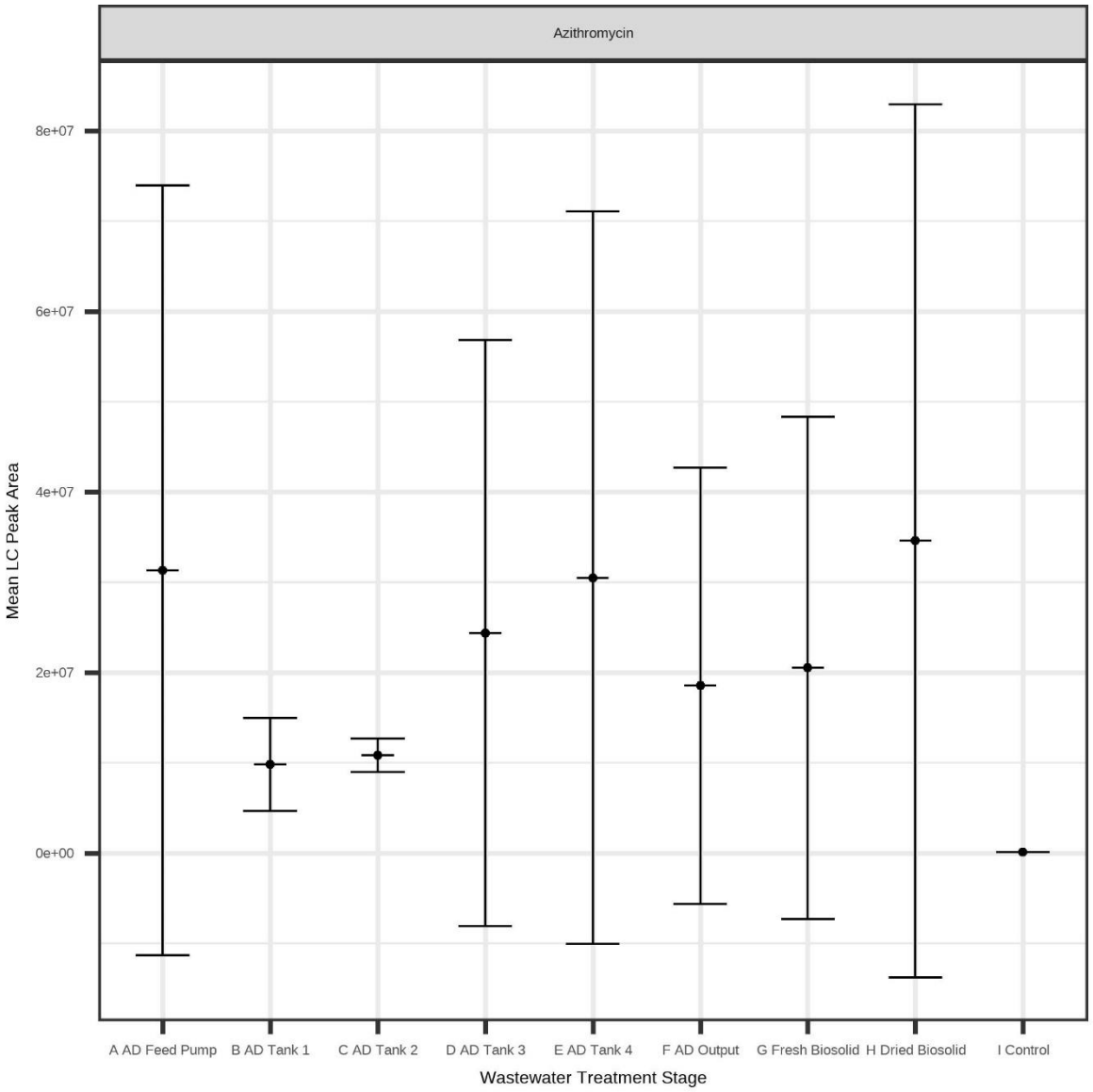

aadA2_1
1.5e+07
1.0e+07
5.0e+06
Normalised Gene Expression
A
B
C
D
E
F
G
Treatment Stage

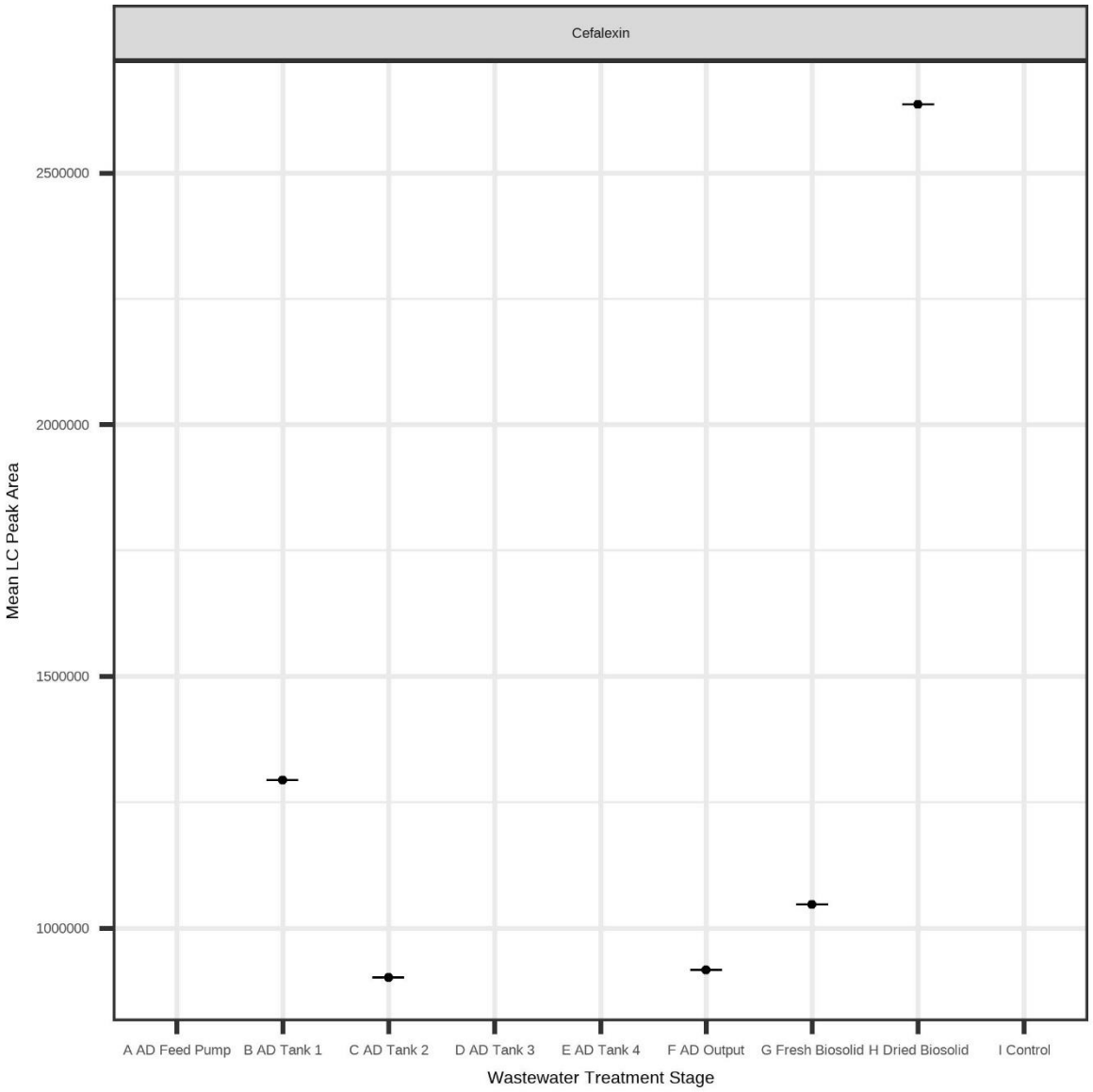
5.3. HPLC-MS/MS Peak Area Data

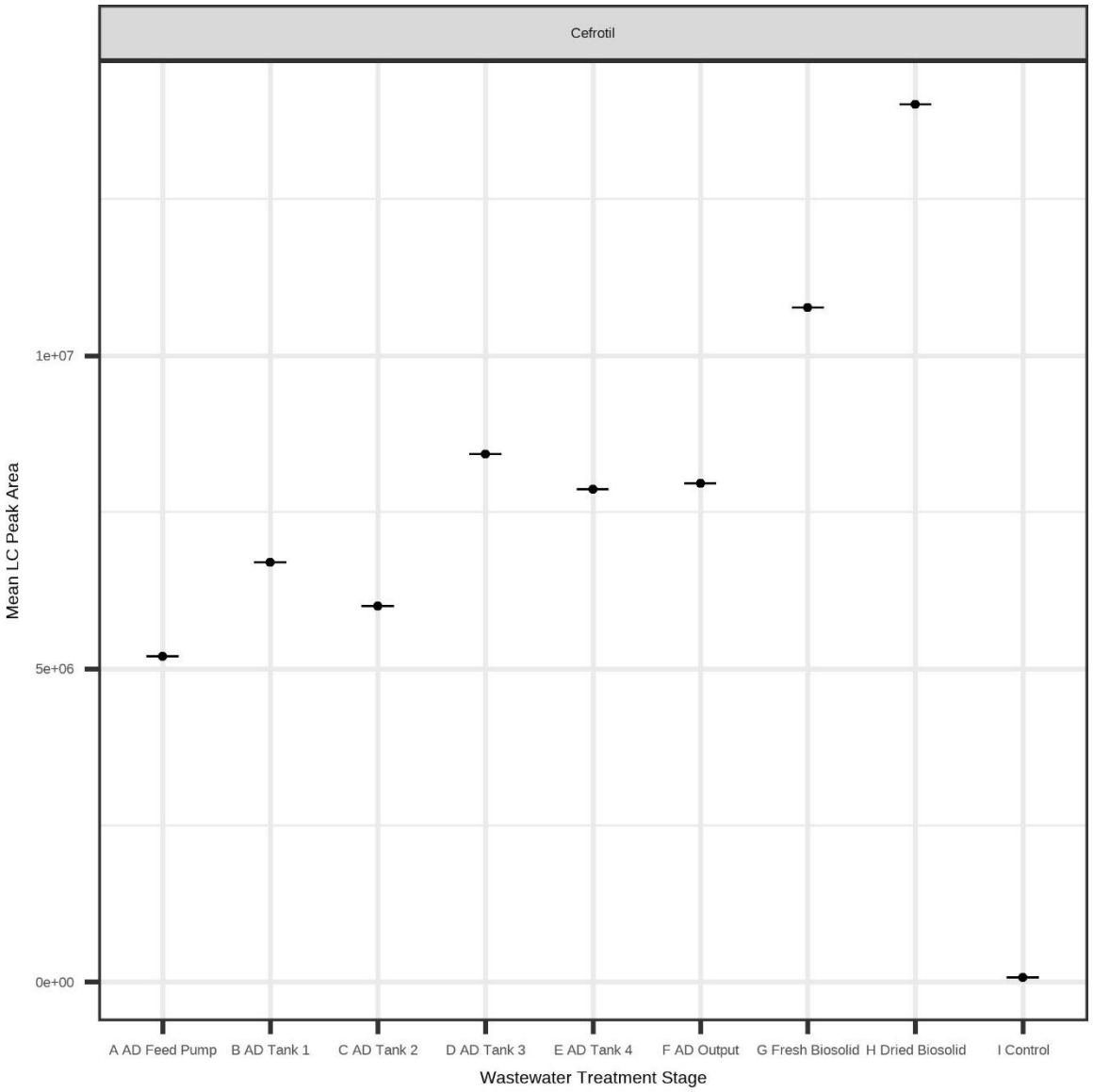

4-Epianhydrotetracycline
Mean LC Peak Area
2.5e+07
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

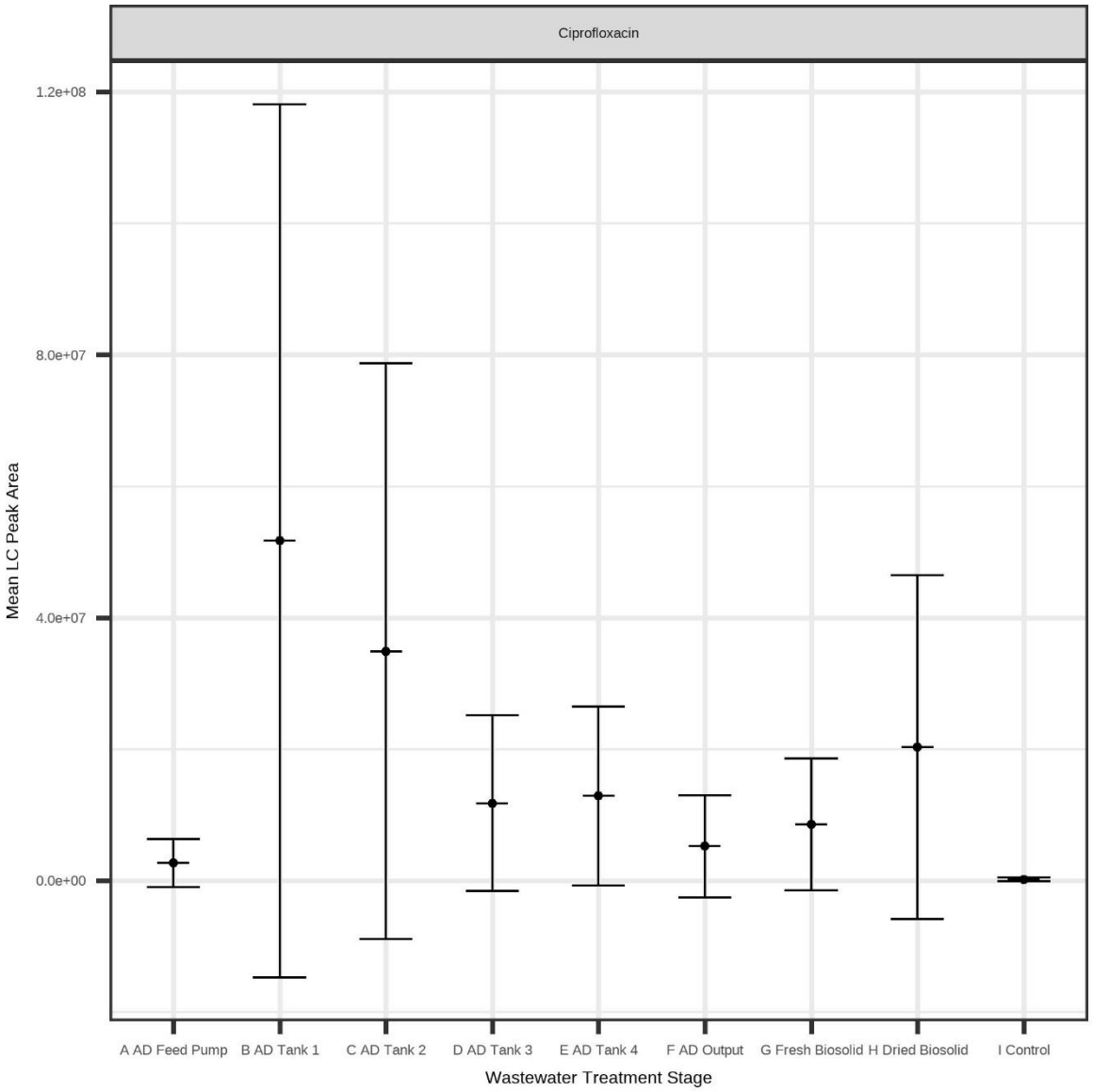

4-Epitetracycline
Mean LC Peak Area
2.0e+08
1.5e+08
1.0e+08
5.0e+07
0.0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

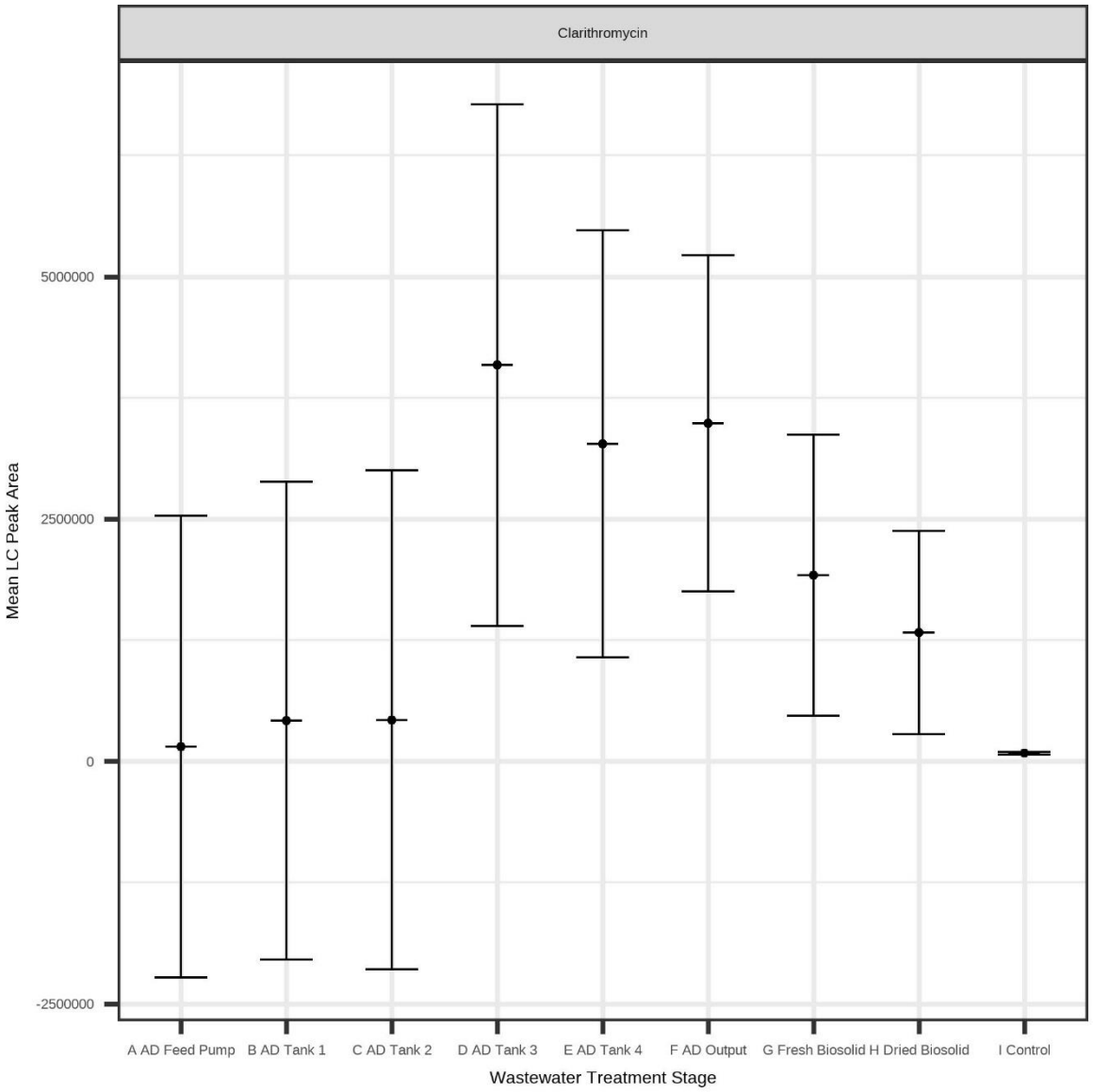

Arbekacin
Mean LC Peak Area
3e+07
2e+07
1e+07
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

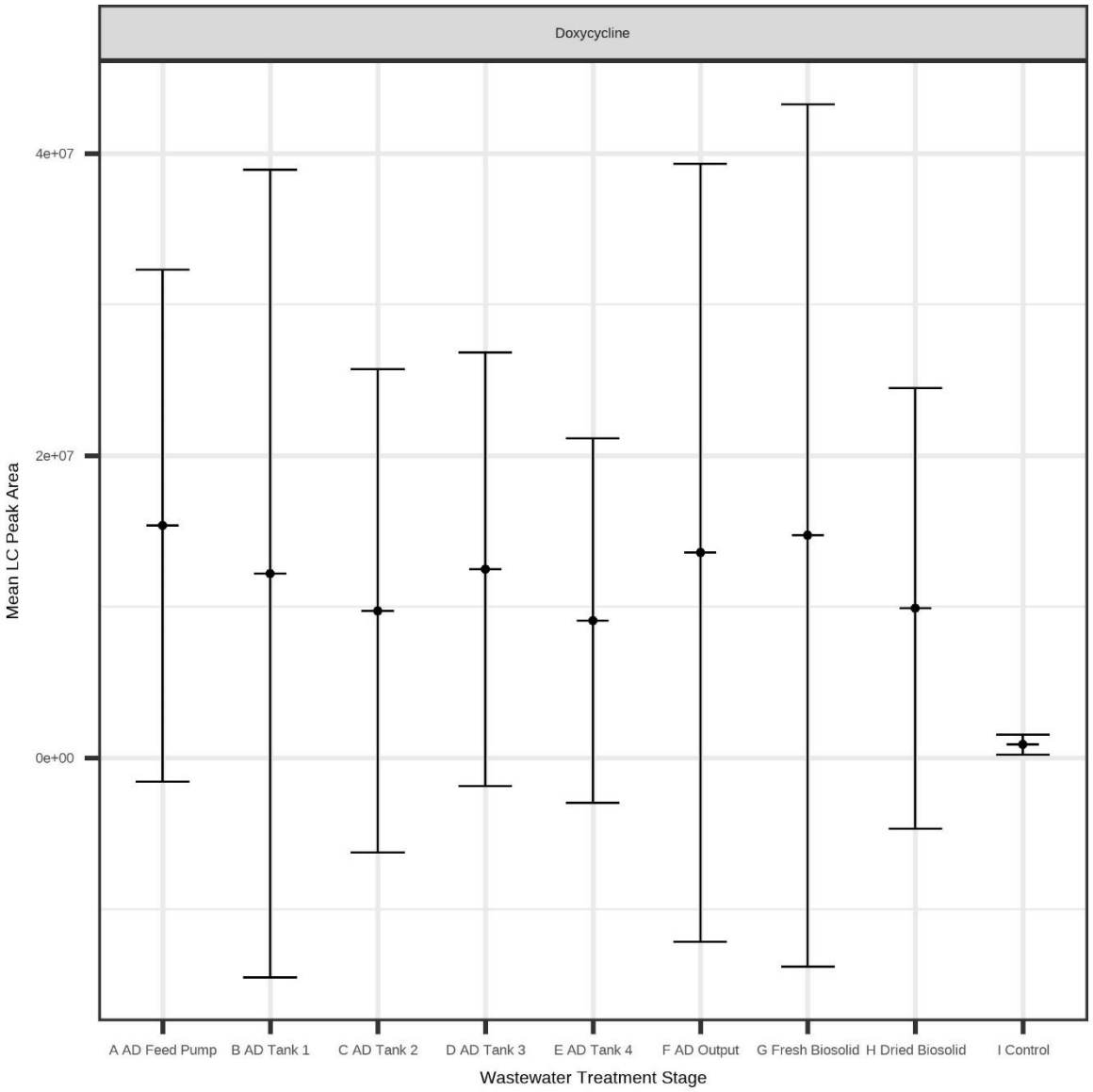

Azithromycin
8e+07
6e+07
4e+07
2e+07
0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

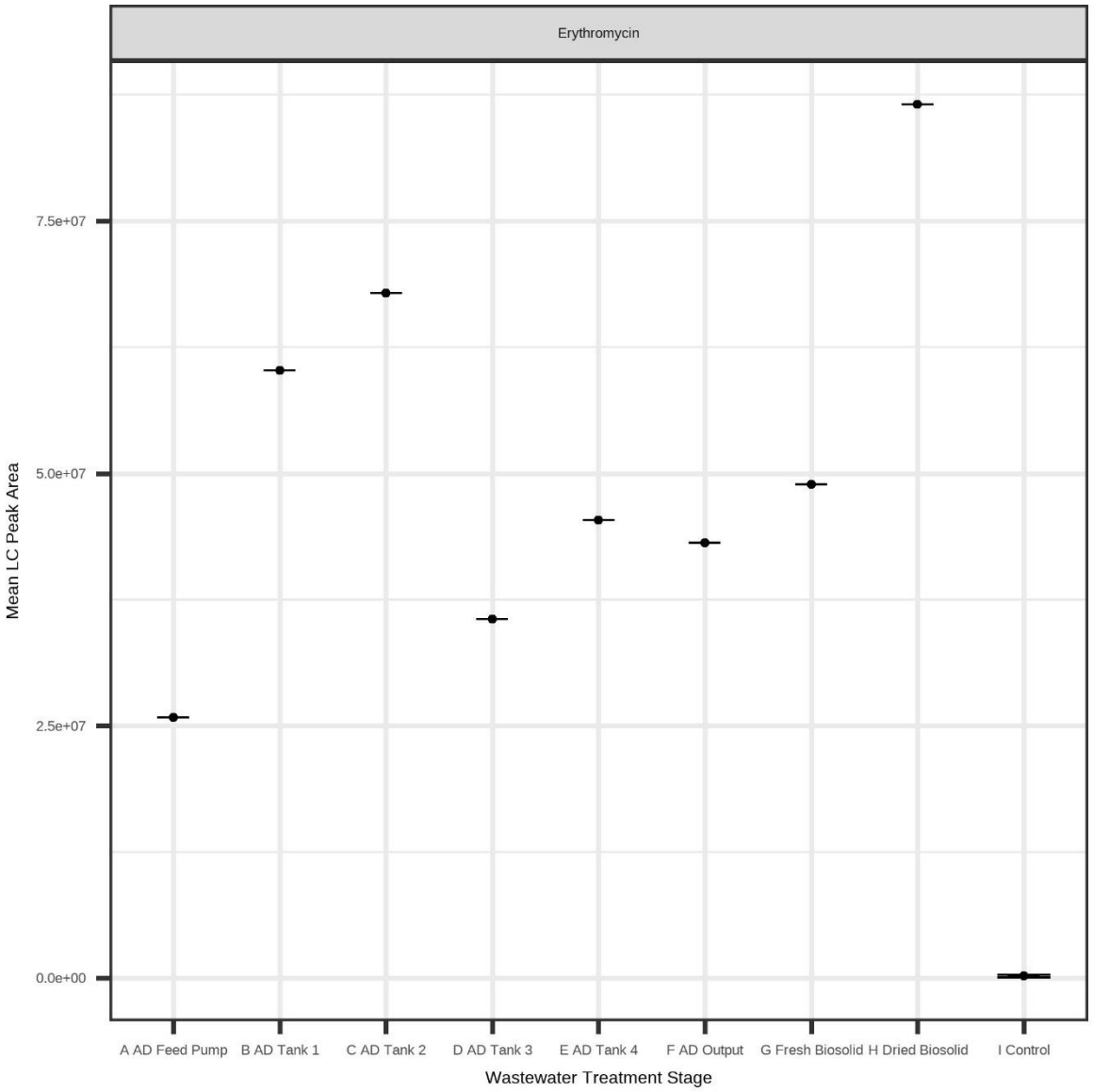

Cefalexin
2500000
2000000
1500000
1000000
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

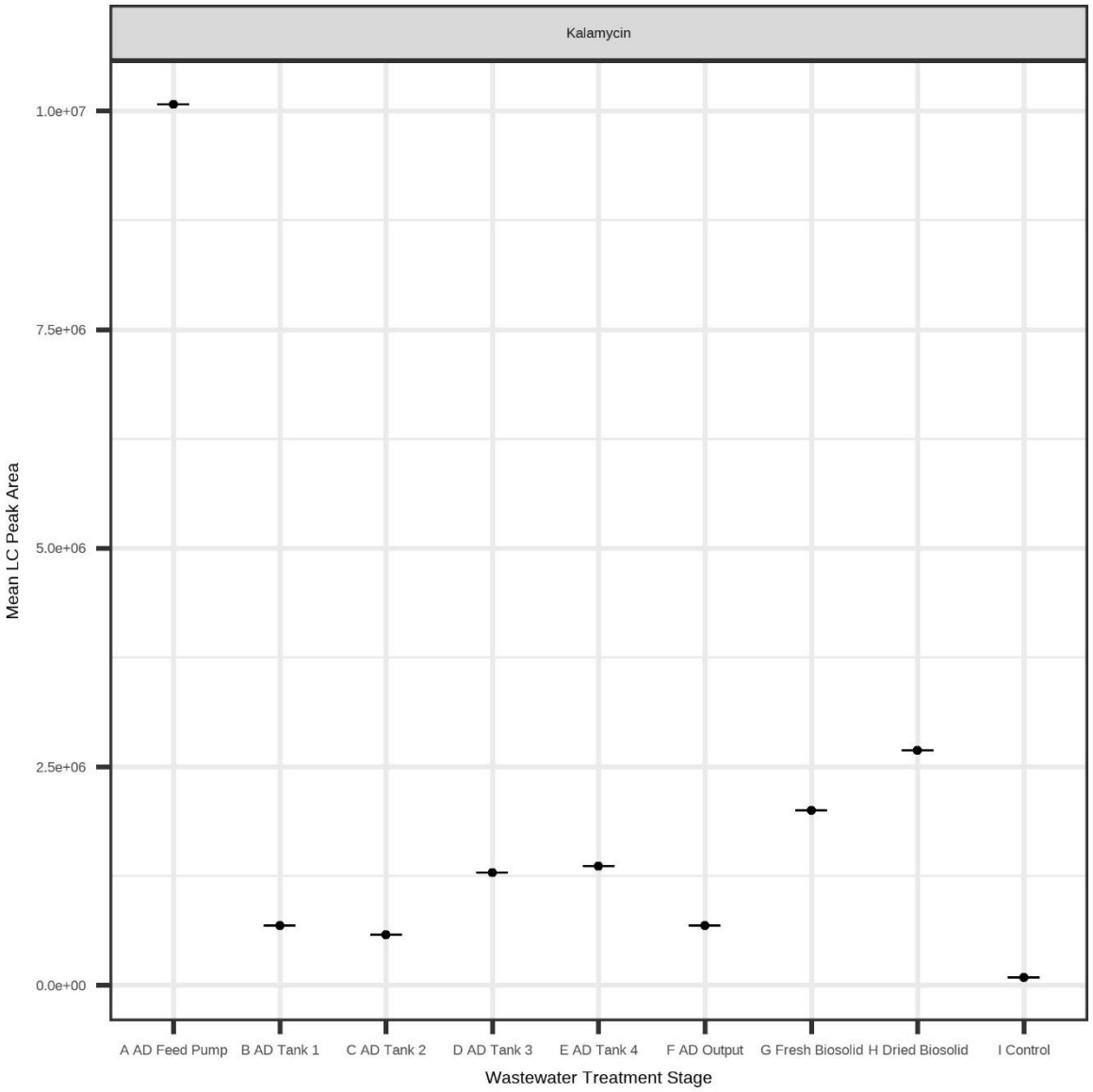

Cefrotil
Mean LC Peak Area
1e+07
5e+06
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

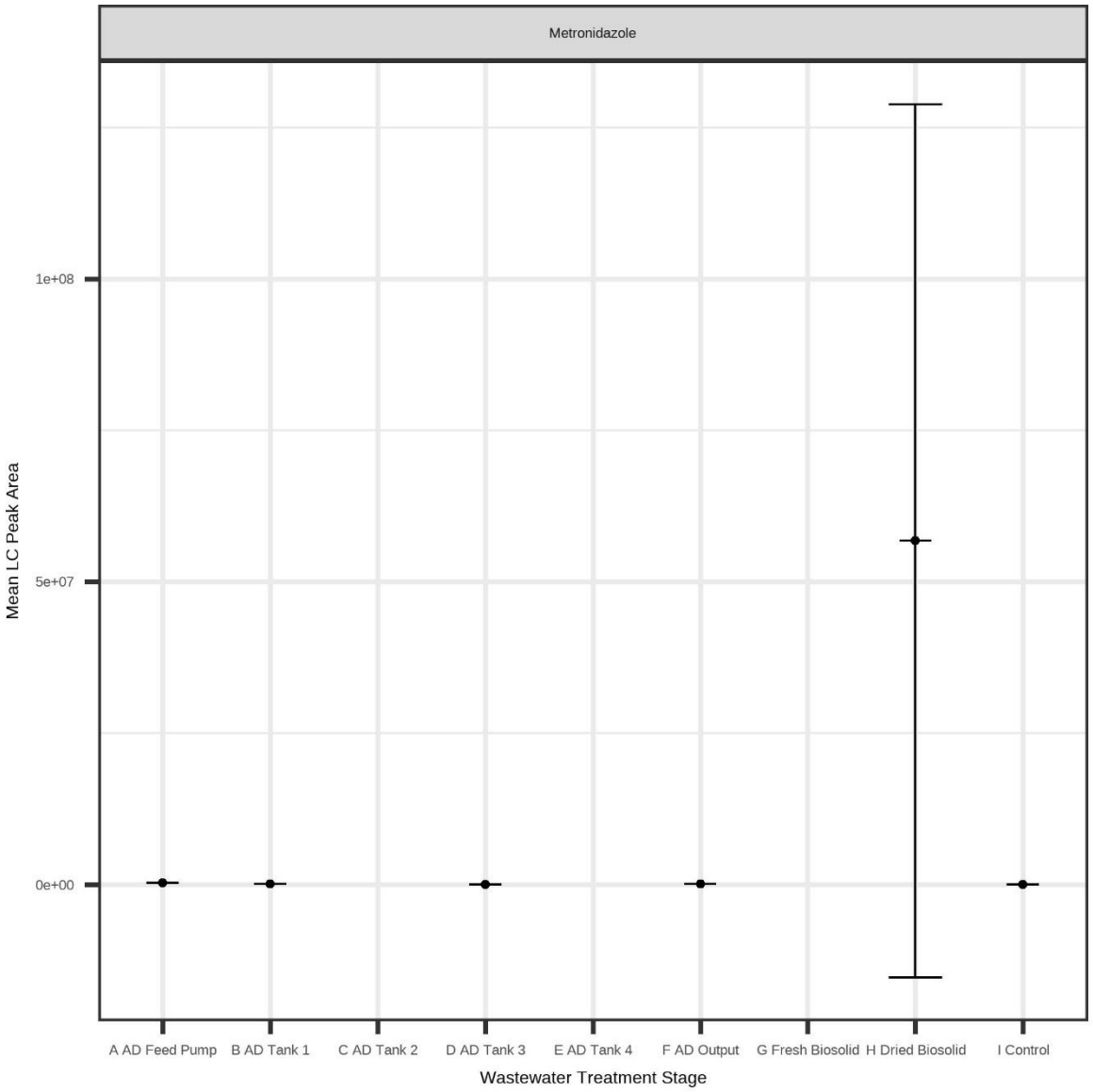

Ciprofloxacin
1.2e+08
8.0e+07
4.0e+07
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

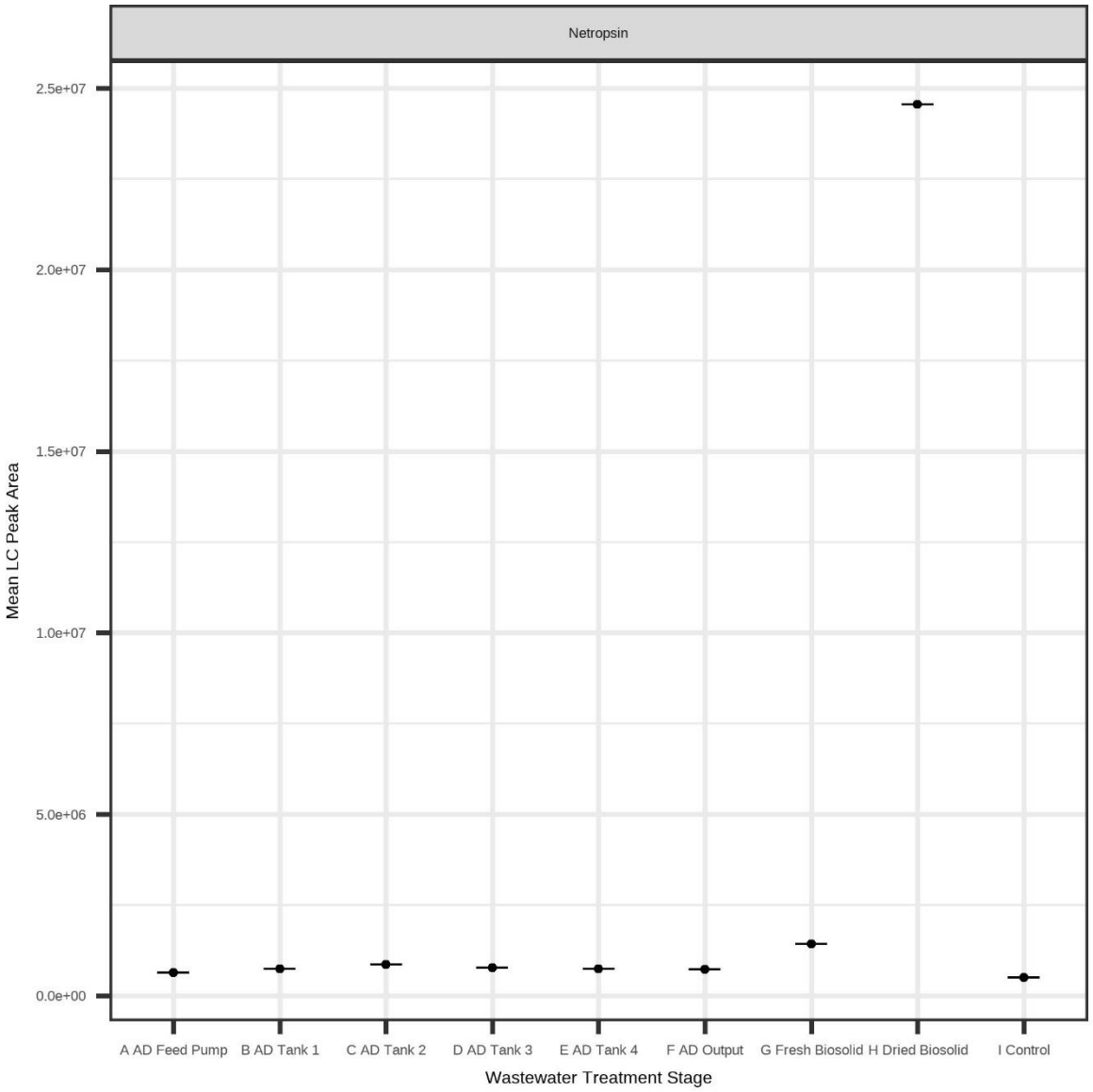

Clarithromycin
Mean LC Peak Area
5000000
2500000
0
-2500000
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

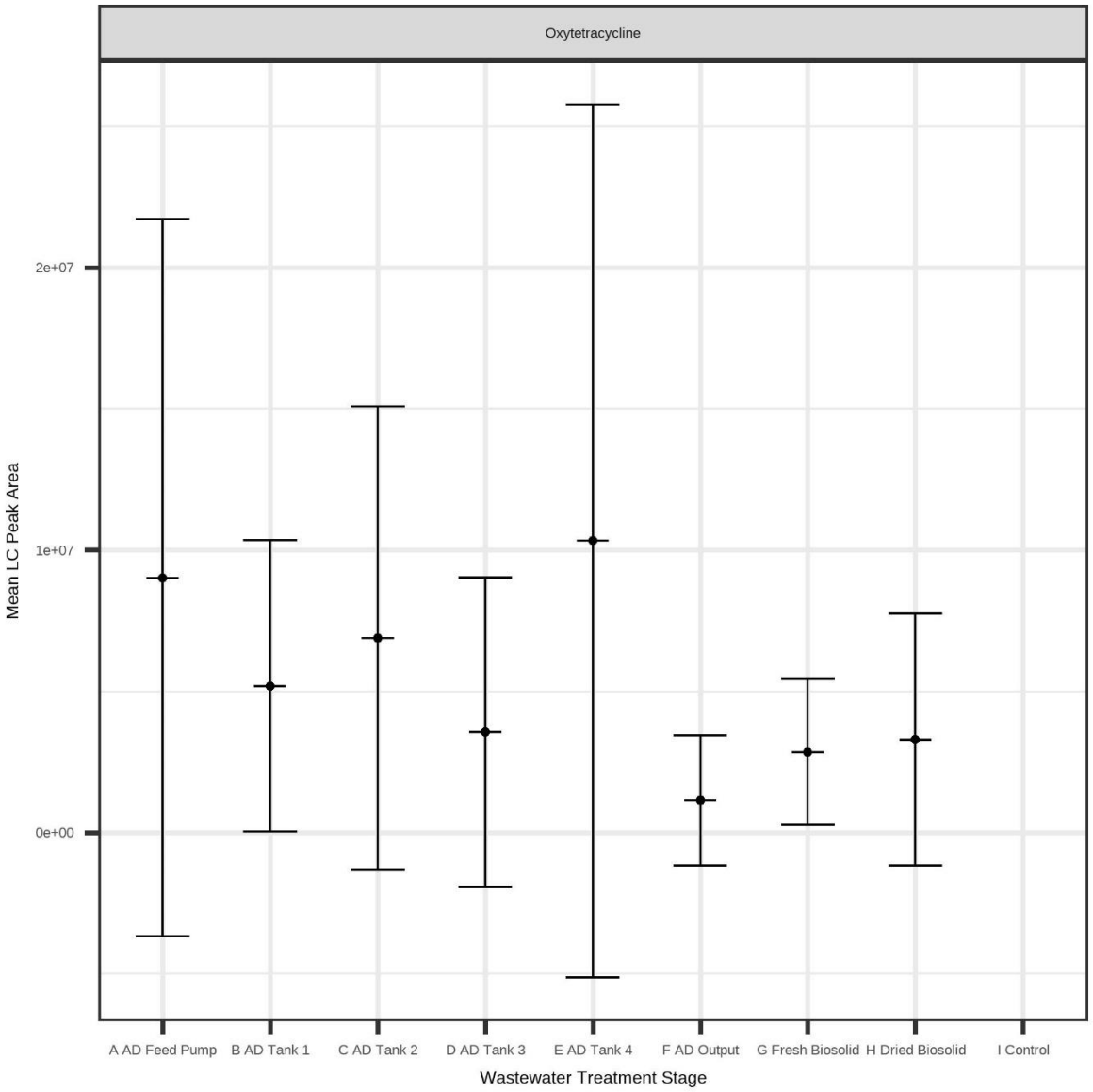

Doxycycline
4e+07
2e+07
0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

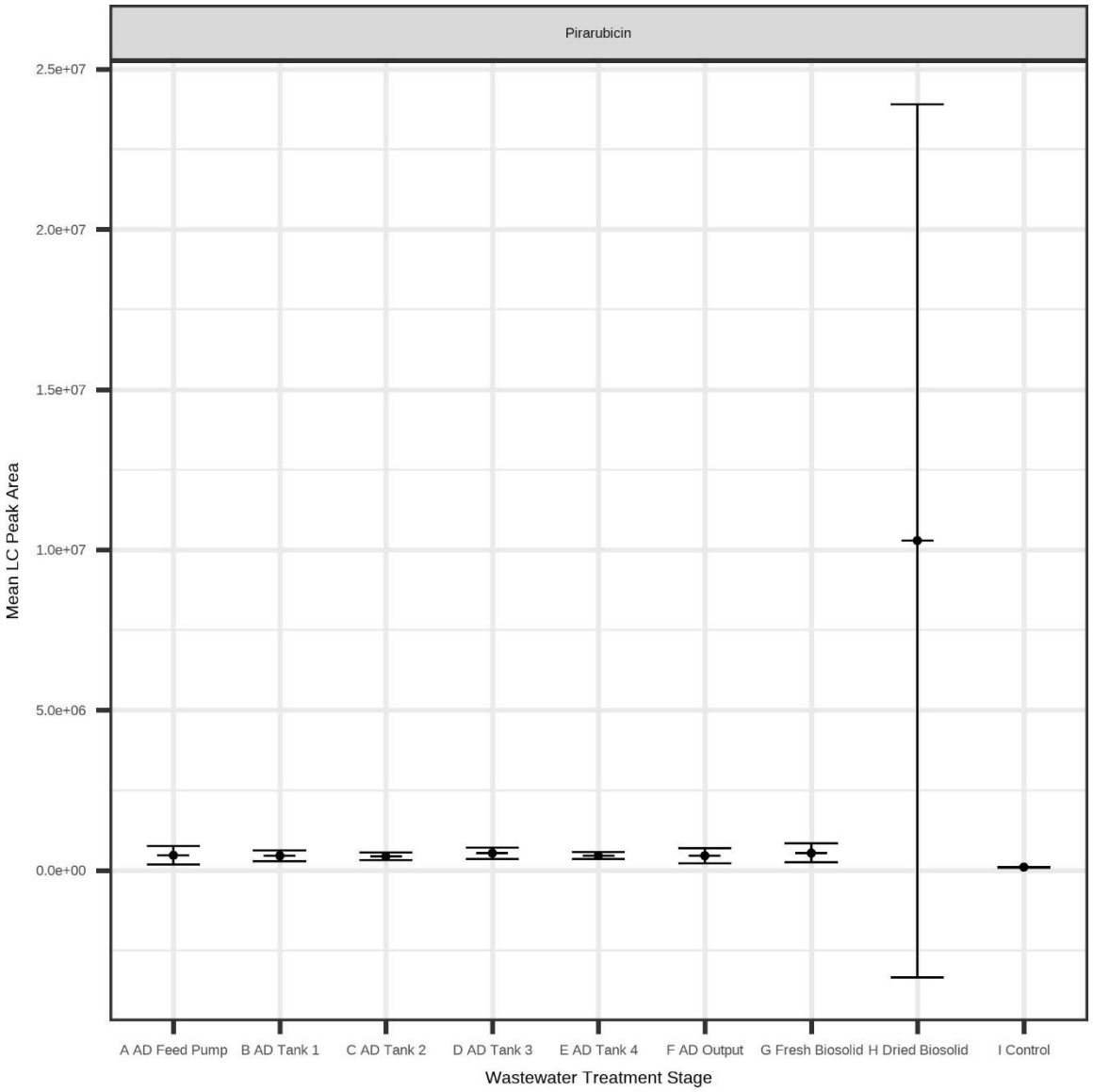

Erythromycin
7.5e+07
5.0e+07
2.5e+07
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

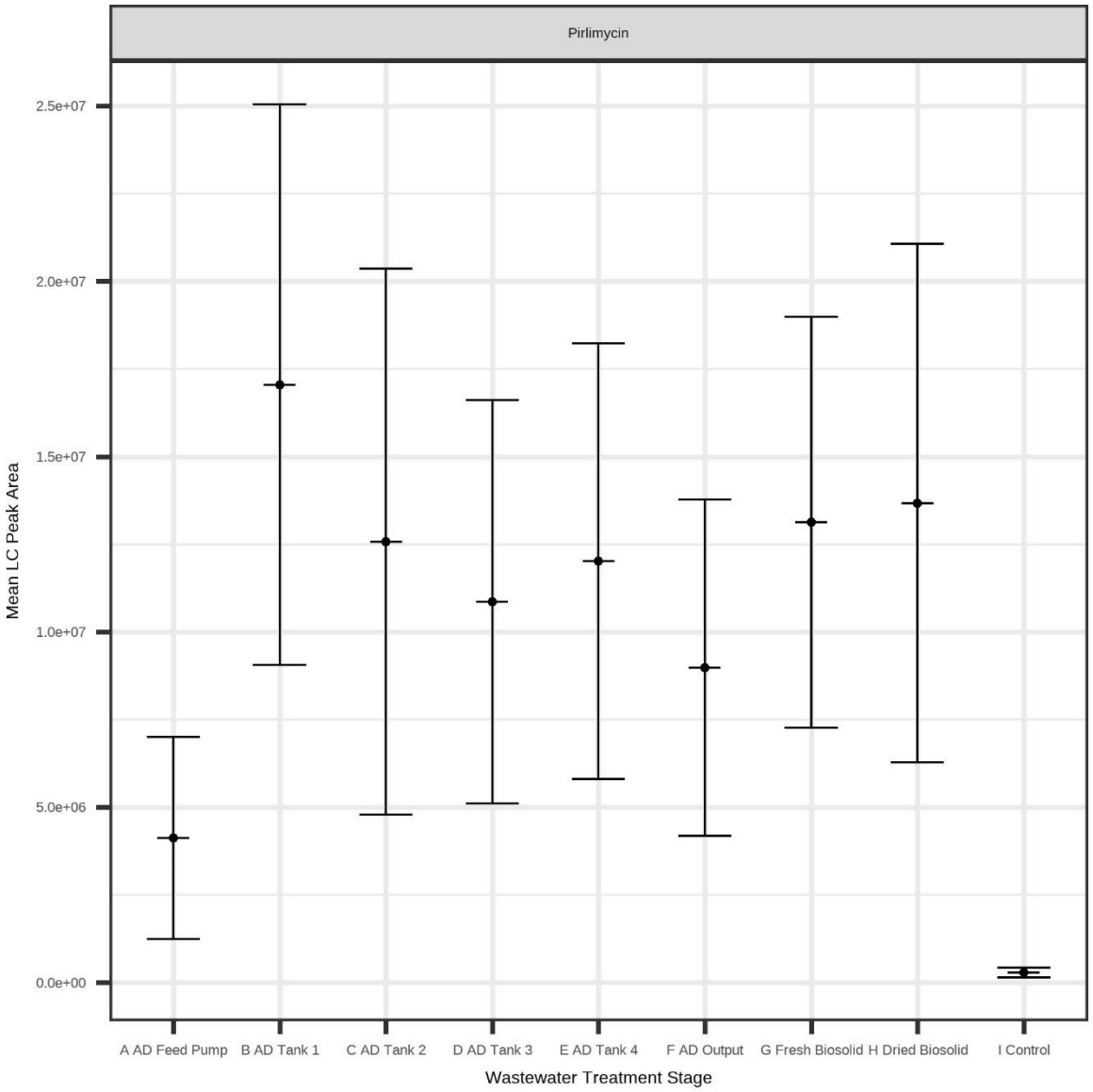

Kalamycin
1.0e+07
7.5e+06
5.0e+06
2.5e+06
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

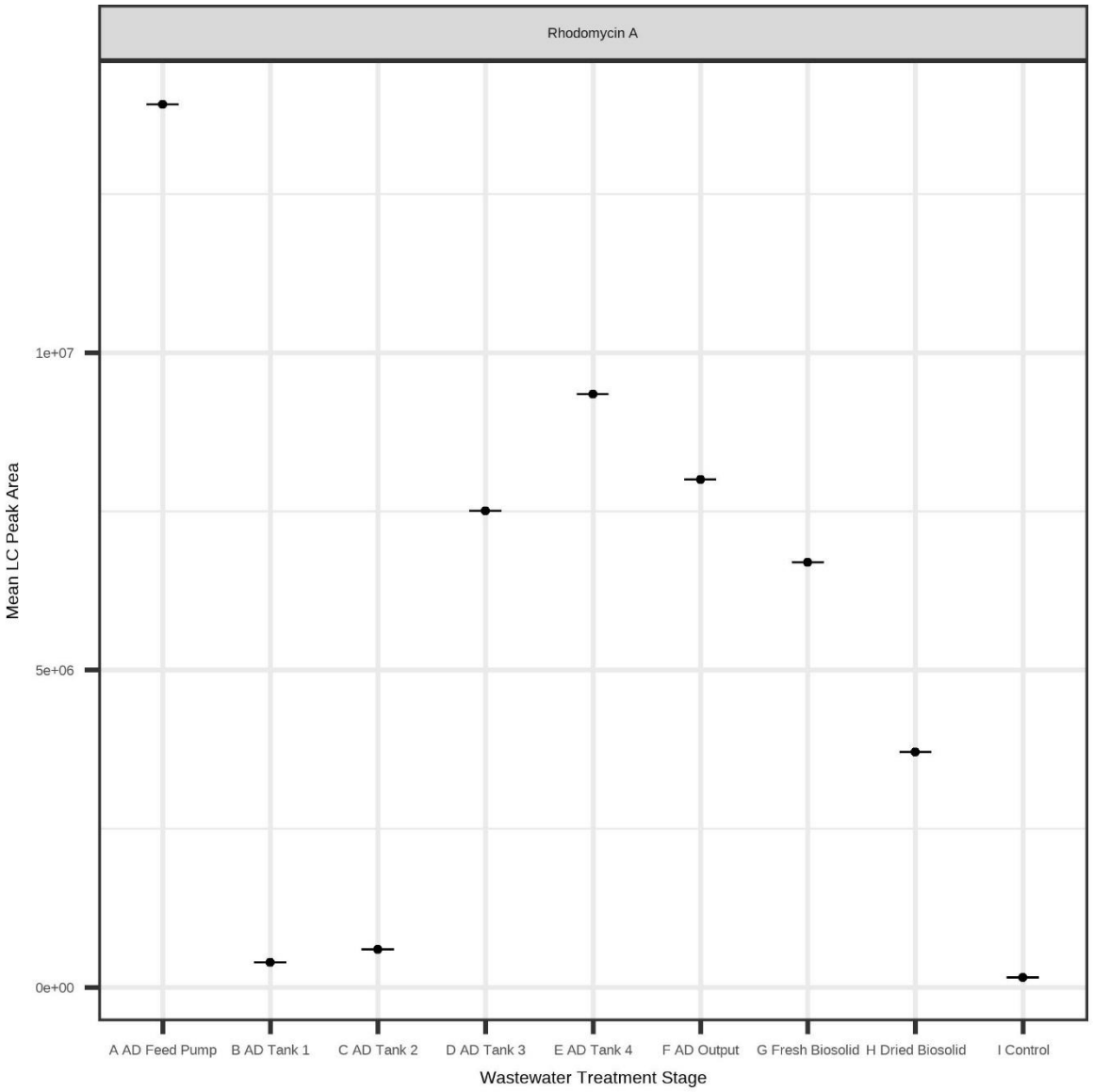

Metronidazole
Mean LC Peak Area
1e+08
5e+07
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

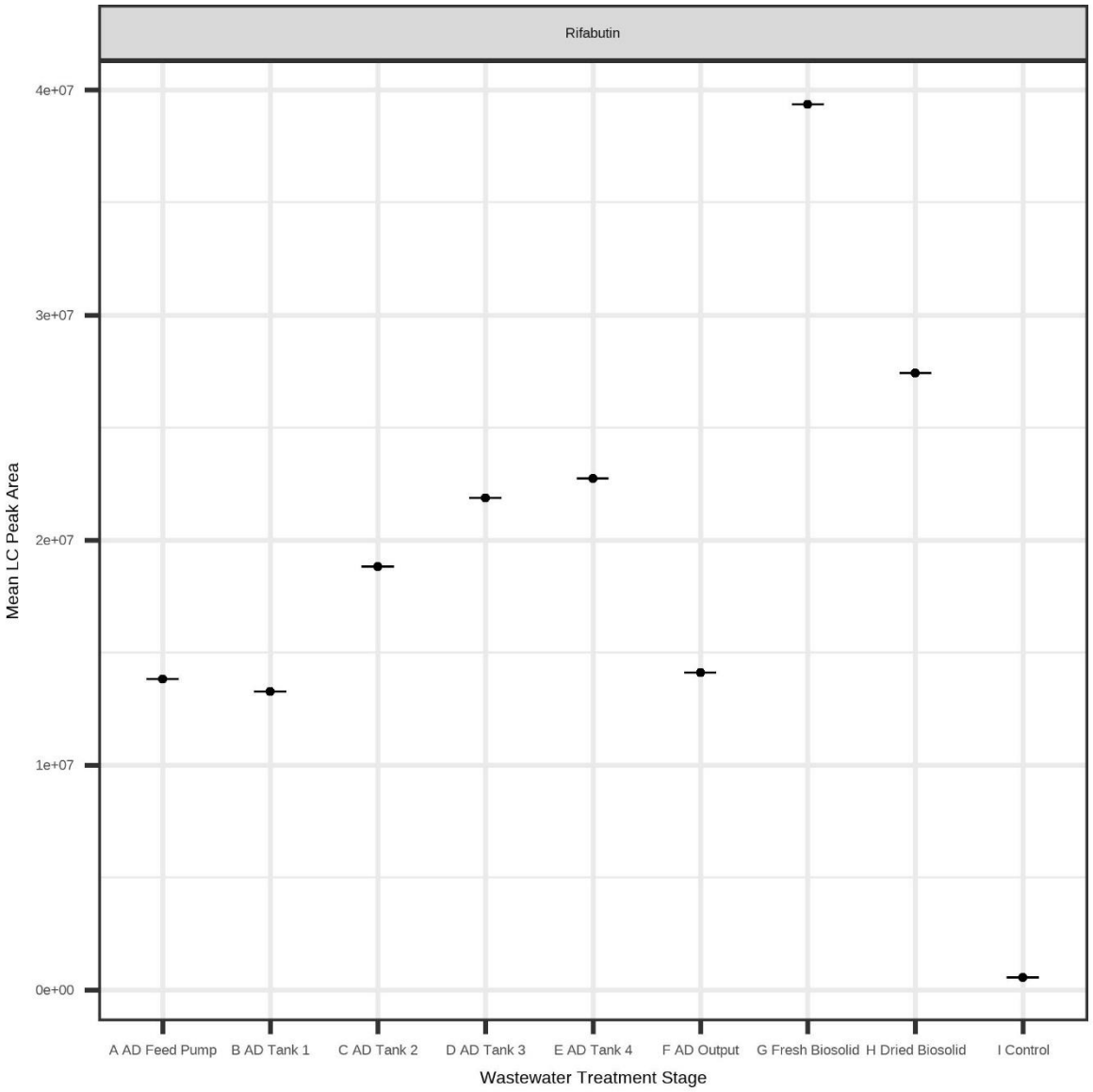

Netropsin
2.5e+07
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

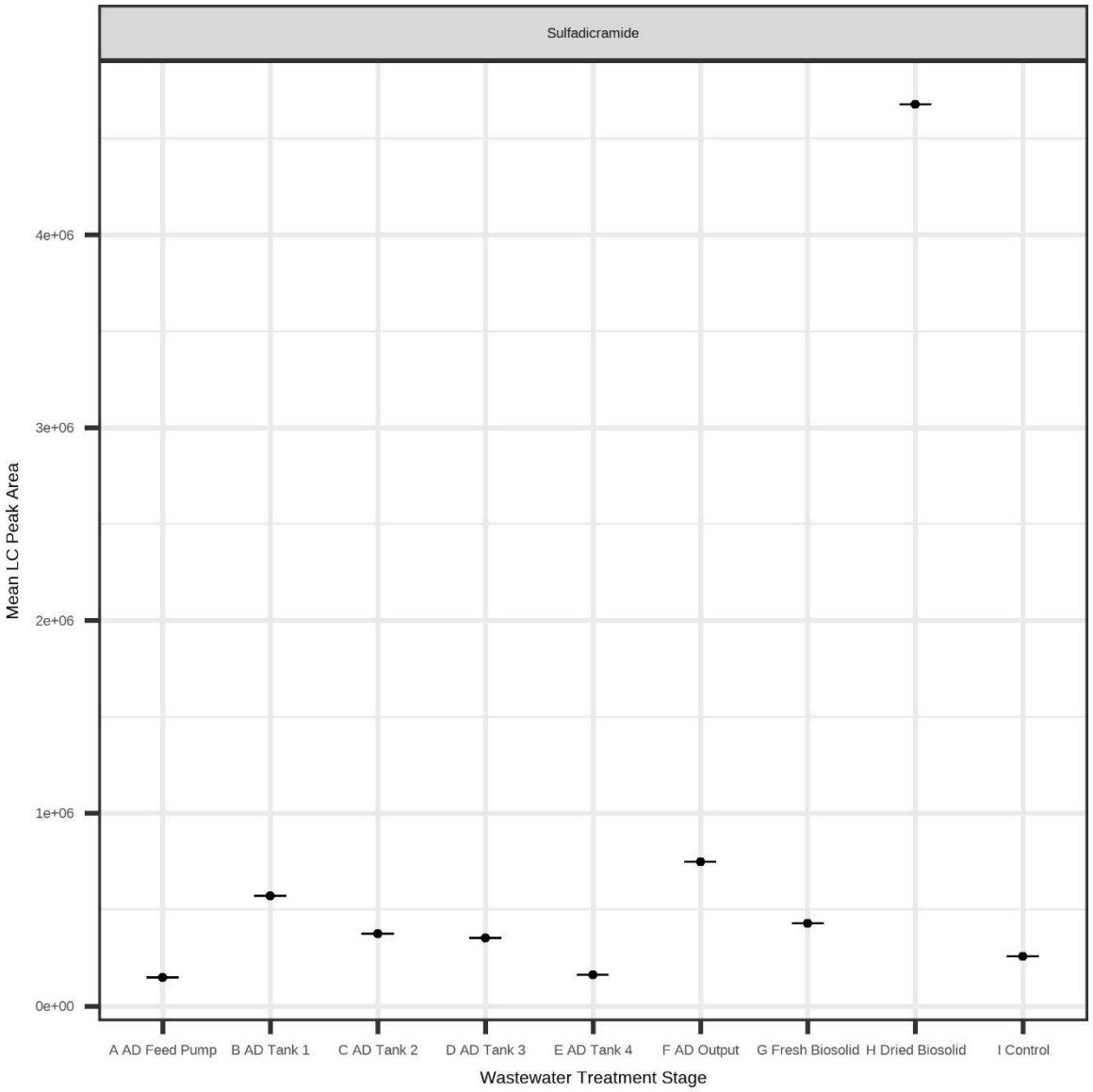

Oxytetracycline
Mean LC Peak Area
2e+07
1e+07
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

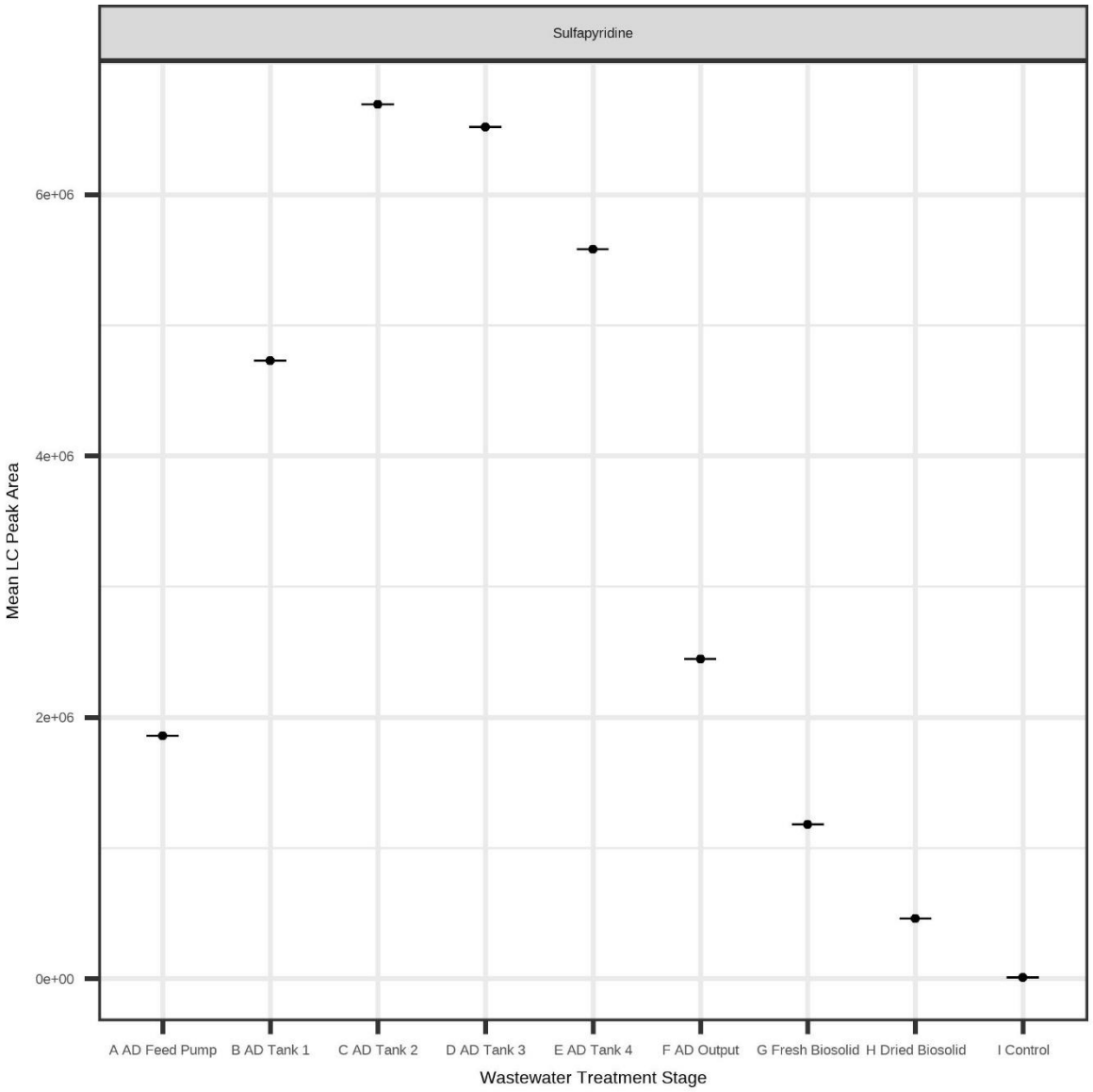

Pirarubicin
2.5e+07
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

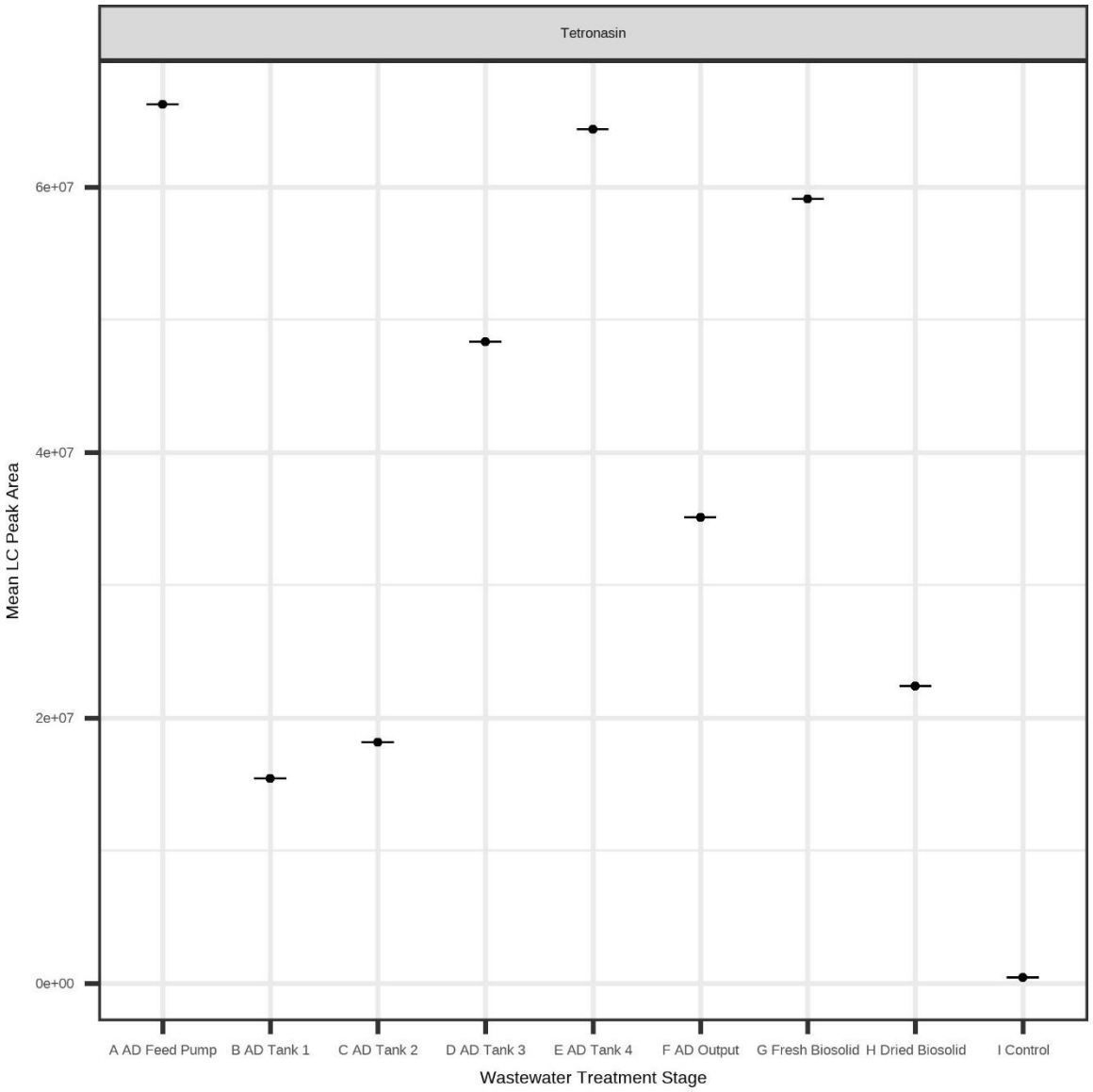

Pirlimycin
2.5e+07
2.0e+07
1.5e+07
1.0e+07
5.0e+06
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

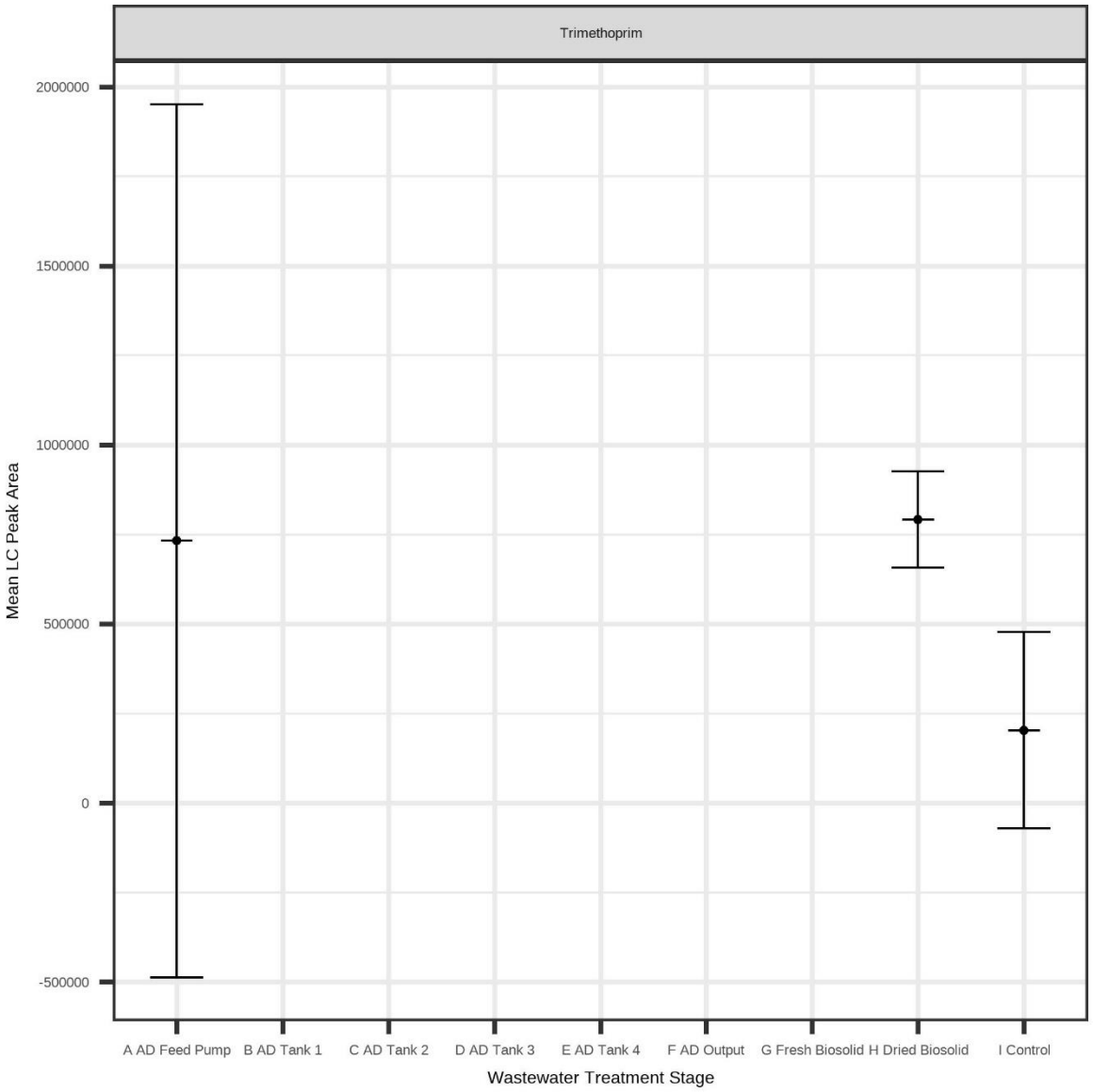

Rhodomycin A
Mean LC Peak Area
1e+07
5e+06
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

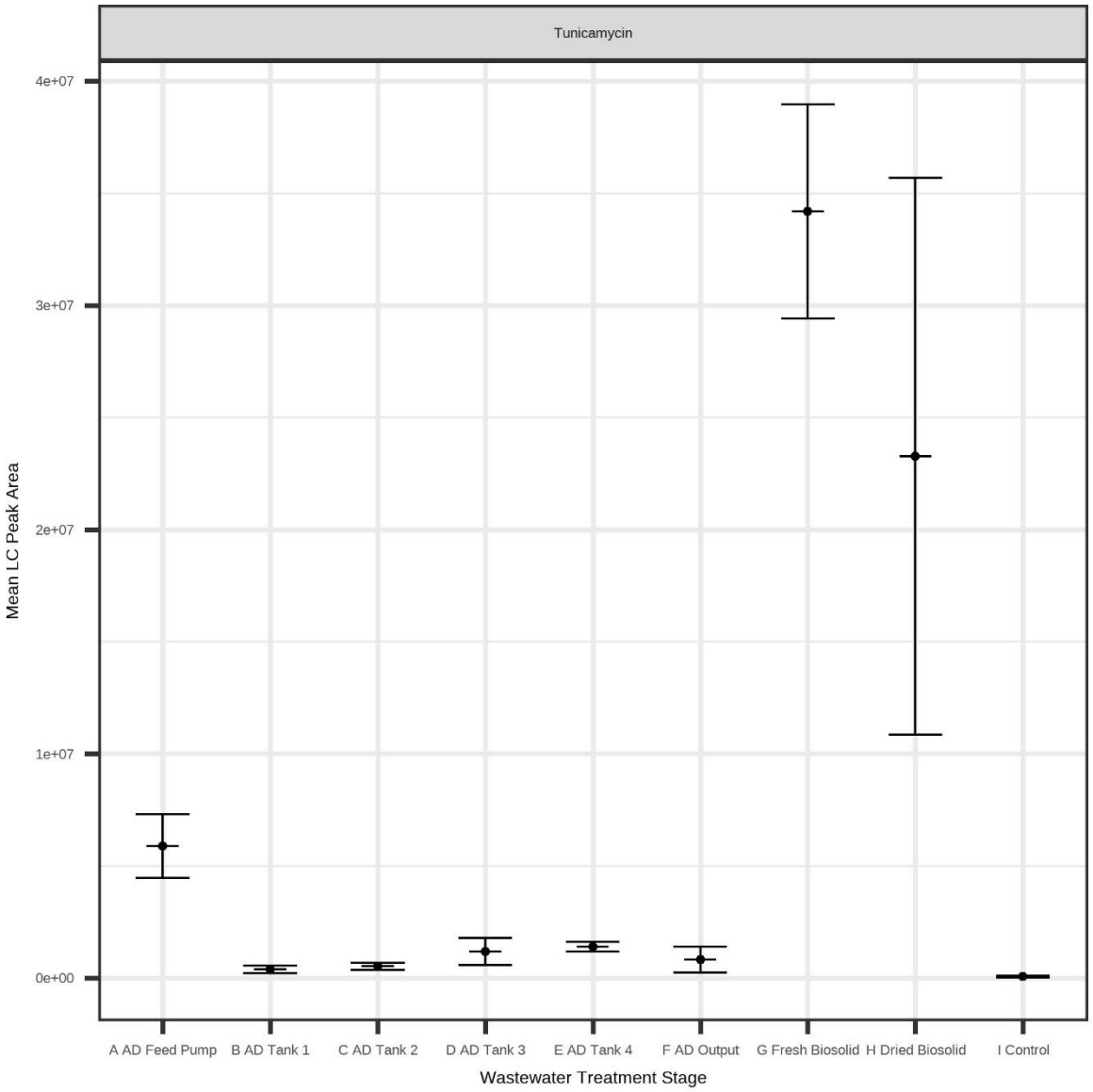

Rifabutin
4e+07
3e+07
2e+07
1e+07
0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Sulfadicramide
Mean LC Peak Area
4e+06
3e+06
2e+06
1e+06
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Sulfapyridine
Mean LC Peak Area
6e+06
4e+06
2e+06
0e+00
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Tetronasin
6e+07
4e+07
2e+07
0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Trimethoprim
2000000
1500000
1000000
500000
0
-500000
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Tunicamycin
4e+07
3e+07
2e+07
1e+07
0e+00
Mean LC Peak Area
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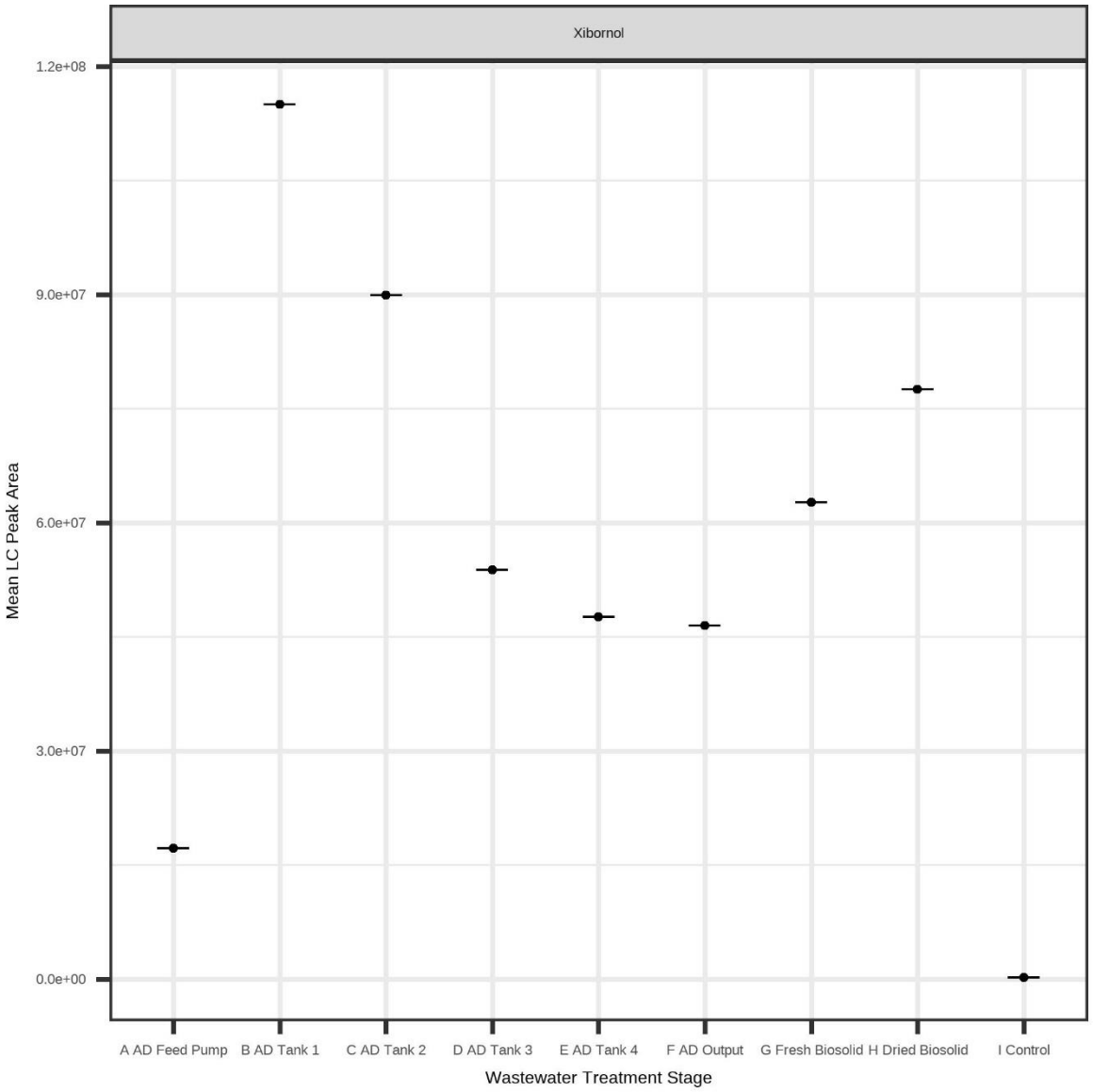
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage


Xibornol
1.2e+08
9.0e+07
6.0e+07
3.0e+07
0.0e+00
Mean LC Peak Area
A AD Feed Pump
B AD Tank 1
C AD Tank 2
D AD Tank 3
E AD Tank 4
F AD Output
G Fresh Biosolid
H Dried Biosolid
I Control
Wastewater Treatment Stage

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