Assessing an Algae-Based Treatment for Mitigation of Antibiotic Resistance

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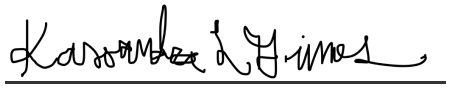
Author Signature: 

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Abstract

The widespread prevalence and growing dissemination of antibiotic resistant bacteria (ARB) via environmental pathways is becoming an issue of global proportions. Wastewater treatment plants are a source of constituents that may contribute to antibiotic resistance in the environment; namely, antibiotics, antibiotic resistance genes (ARGs), and ARB. The exposure of bacteria to antibiotics, ARGs, and ARB during bacteria-based wastewater treatment creates a selective pressure for the development of more ARB. Resistance may then be transmitted "vertically" to future offspring of those organisms or "horizontally" to other organisms in the same generation, resulting in proliferation of ARB within wastewater treatment plants and downstream receiving waters. Although ARB are theoretically deactivated during wastewater disinfection processes, many antibiotic drug compounds and their corresponding ARGs are present in the effluent and discharged to the environment. Thus, there is strong interest in developing wastewater treatment technologies that can deactivate these entities efficiently, inexpensively, and sustainably.

This dissertation assesses the capacity of a novel algae-mediated biological treatment using the freshwater alga, *Scenedesmus dimorphus*, to remove wastewater constituents that can stimulate antibiotic resistance in downstream environmental bacteria. Interdisciplinary techniques are brought together to measure the removal of the antibiotic ciprofloxacin (CIP), the residual potency of treated CIP effluents during short- and long-term exposures to model bacteria, and the deactivation of plasmid pEX18Tc, which carries the *tet* ARG. Results show significant CIP removal in light control samples without algae and algae treated samples: 53% and 93%, respectively, over 6 days. A residual antibiotic potency assay reveals that untreated CIP is significantly more growth-inhibiting to a model bacterium (*Escherichia coli)* than the algae-treated and light control samples during short exposures (6 hours). Adaptive laboratory evolution, again

using *E. coli*, reveals that treated samples exhibit reduced capacity to stimulate CIP resistance during sustained exposures compared to untreated CIP. Finally, observed CIP resistance in the CIP-exposed bacterial lineages is corroborated via genotype characterization, which reveals the presence of resistance-associated mutations in gyrase subunit A (*gyrA*) that are not present in bacterial lineages exposed to algae treated or light control samples.

Preliminary qPCR and transformation assay experiments reveal that the algae background matrix suppresses plasmid pEX18Tc amplification and transformation relative to controls in pure matrices. Final results for deactivation of the model plasmid pEX18Tc show 100% reduction in plasmid transformation efficiency in less than 3 days for both the algae treatment and light control. As such, algae-mediated tertiary treatment could be effective in deactivating wastewater constituents that stimulate antibiotic resistance in bacterial communities downstream from wastewater treatment plants. In addition, adaptive laboratory evolution and transformation assays are useful for assessing the potential of antibiotics and ARGs to stimulate antibiotic resistance downstream.

The results of this dissertation will not only inform decision-making about treatment technology options and facilitate meaningful risk analysis related to the dissemination of antibiotic resistance via environmental pathways, but also constitute methodological advancements in the environmental engineering field with a particular emphasis on effects-based treatment evaluations.

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Definition of Acronyms

ALE	Adaptive Laboratory Evolution
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Gene
CFU	Colony Forming Units
CIP	Ciprofloxacin
CS	Ciprofloxacin Standard
DC	Dark Control
EA	Experimental Algae
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gyrA	DNA gyrase subunit A
HPLC	High Pressure Liquid Chromatography
LB	Lysogeny Broth
LC	Light Control
MB3N	Modified Bold 3N Medium
MIC	Minimum Inhibitory Concentration
OD	Optical Density
parC	Topoisomerase IV
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
QRDR	Quinolone Resistance Determining Region
SPE	Solid Phase Extraction
tet	Plasmid pEX18Tc resistance gene encoding tetracycline resistance
WWTP	Wastewater Treatment Plant

Related Publications, Presentations, and Awards

Manuscripts

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- **Grimes, K.**, Dunphy, L., Loudermilk, E., Melara, A., Kolling, G., Papin, J., Colosi, L. "Evaluating the efficacy of an algae-based treatment to mitigate elicitation of antibiotic resistance," *Chemosphere.* 2019, 237. 10.1016/j.chemosphere.2019.124421

- Pruden, A., Alcalde, R., Alvarez, P., Ashbolt, N., Bischel, H., Capiro, N., Crossette, E., Frigon, D., **Grimes, K.,** Haas, C., Ikuma, K., Kappell, A., LaPara, T., Kimbell, L., Li, M., Li, X., McNamara, P., Seo, Y., Sobsey, M., Sozzi, E., Navab-Daneshmand, T., Nguyen, T., Raskin, L., Riquelme, M., Vikesland, P., Wigginton, K., Zhou, Z. An Environmental Science and Engineering Framework for Combating Antimicrobial Resistance. *Environ. Eng. Sci.* 2018, 35 (10), 1005-1011. 10.1089/ees.2017.0520

- Zhang, X., **Grimes, K.**, Colosi, L. M., Lung, W. Occurrence, Fate, Transport, and Management of Estrogens in the Environment: A Review. *Chemosphere.* 2019, 230, 462-478. 10.1016/j.chemosphere.2019.05.086

Presentations

Podium

- **Grimes, K.** (2020) "Assessing an Algae Treatment to Mitigate Elicitation of Antibiotic Resistance" University of Virginia Engineering Systems and Environment Department Seminar (Virtual), Charlottesville, VA.

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Awards and Fellowships

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Chapter 1: Introduction and Literature Review

1.1 Motivation, Background, and Literature Review

1.1.1 Overview of the global threat of antibiotic resistance

Antibiotic resistance and the ensuing evolution of antibiotic resistant bacteria (ARB) or "superbugs" is becoming an issue of global proportions. In the United States alone, over 249 million courses of antibiotics are prescribed each year and at least 47 million are prescribed unnecessarily (CDC, 2018). Many antibiotics are highly stable compounds that are not fully broken down by the body, so they remain active long after they are excreted or when incomplete doses are improperly disposed of. As a result of anthropogenic pressures, antibiotic compounds and ARB are detected in environmental hotspots around the world (Berendonk et al., 2015; Scott et al., 2016). This overabundance of antibiotics from medical, agricultural, and veterinary use, combined with the natural rapid evolutionary processes of bacteria, have sped up the rate at which bacteria develop resistance and increased the risk of contracting antibiotic resistant infections. As a result, more than 2.8 million antibiotic resistant infections resulting in more than 35,000 deaths occur each year in the United States (CDC, 2019). In order to save lives and preserve the efficacy of our valuable antibiotic fleet, we need a concerted interdisciplinary effort to curb the spread of antibiotic resistance not only in medical practice, but in environmental systems as well.

1.1.2 The role of wastewater treatment plants in antibiotic resistance

Antibiotics are currently unregulated in water and wastewater, but they are a particularly concerning class of environmental contaminants due to their biological potency and the risk of inadvertently promoting antibiotic resistance in downstream microbial communities. Significant fractions of each dose (up to 90%) remain unmetabolized and are excreted into the sewage collection system, thereby making their way to municipal wastewater treatment plants (WWTPs) (Tiwari et al., 2017). In addition to the lack of regulations, typical WWTP processes in the United States and Europe do not have the capacity to fully remove antibiotics before they are released into receiving waters. Activated sludge treatment is the most widely used wastewater treatment process around the world, but studies have proven the process to be ineffective at degrading most antibiotic compounds (Noguera-Oviedo and Aga, 2016). Further, some studies have found anaerobic/anoxic wastewater system conditions to be equally inefficient at reducing antibiotic concentrations. For example, Liu et al. 2013 analyzed the degradation of the antibiotic ciprofloxacin (CIP) under anaerobic/anoxic wastewater conditions and found little to no degradation of the antibiotic under either methanogenic or denitrifying conditions (Liu et al., 2013). Accordingly, many drugs of interest have been measured at appreciable concentrations (ng/L – mg/L) in WWTP effluents and in downstream receiving waters (Andreozzi et al., 2004; Ben et al., 2018; Golet et al., 2002; Hughes et al., 2012; Janecko et al., 2016; Li et al., 2010; Noguera-Oviedo and Aga, 2016; Tiwari et al., 2017; Xiong et al., 2017b). The presence of antibiotics in WWTP effluents is notable, as sustained exposure to low concentrations of antibiotics can elicit resistance in microbial organisms, including human pathogens (Gullberg et al., 2011; Li et al., 2010; Lupan et al., 2017). The environmental engineering community postulates that WWTPs "may represent a critical node for control of the global spread of antibiotic resistance" (Pruden et al., 2013) and seeks technologies

to remove constituents in the effluent that may contribute to antibiotic resistance stimulation in receiving waters.

In addition to the untreated parent drug and its principal metabolites, WWTP effluents contain other constituents that may stimulate antibiotic resistance in the environment; namely, ARB and their antibiotic resistance genes (ARGs) (Marti et al., 2013). From literature, ARB and ARGs flow into WWTPs via the influent (e.g., in hospital sewage) and can also be preferentially selected during the course of biological treatment as a result of ongoing selective pressure (i.e., the presence of antibiotics) (Costanzo et al., 2005; Dodd, 2012; Guo et al., 2017; Hultman et al., 2018; Karkman et al., 2017; Murray et al., 2018; Pruden, 2014; Quach-Cu et al., 2018; Rizzo et al., 2013). Within WWTPs and downstream receiving waters, ARGs can proliferate vertically through bacteria replication; i.e., one resistant organism passes down the resistance feature through multiple generations of the same lineage. ARGs can also be shared between bacteria of the same or different species horizontally via a process known as horizontal gene transfer. Antibiotic resistance originating in one bacteria species can spread to many other species, including nonpathogenic and pathogenic strains, via these horizontal gene transfer processes, which is a major contributor to widespread antibiotic resistance (von Wintersdorff et al., 2016).

Recent studies in Romania, the Netherlands, and China have detected antibiotics and ARGs in rivers up to 20 km downstream from WWTPs (Li et al., 2010; Lupan et al., 2017; Sabri et al., 2020). In designing our experiments for this dissertation, we presumed that ARB are more or less equally susceptible to traditional disinfection treatments as compared to non-resistant bacteria (Dodd, 2012). Accordingly, we expected that effluents do not typically contain appreciable quantities of viable ARB. However, it is uncertain to what extent traditional disinfection treatments deactivate genetic materials conferring antibiotic resistance. For this reason, it is still of concern

that ARG may be present in the effluent, either within dead ARB (i.e., in an intracellular form) or in a free-floating, extracellular form (Chang et al., 2017; Dodd, 2012; McKinney and Pruden, 2012; Yoon et al., 2017; Zhang et al., 2019).

1.1.3 Integrated algae-wastewater treatment systems: a potential solution?

WWTPs are increasingly expected to deliver effluents free from traditional regulated contaminants (e.g., biochemical oxygen demand (BOD), total suspended solids (TSS), etc.) and also unregulated "emerging contaminants" such as antibiotics, estrogens, and personal care products. Simultaneously, there is a strong desire to reduce the energy intensity and greenhouse gas footprint associated with conventional treatment (Batstone et al., 2015; McCarty et al., 2011; Melvin and Leusch, 2016). It has been suggested that integration of traditional wastewater treatment and algaculture could deliver enhanced effluent quality with reduced energy consumption compared to conventional tertiary treatments (Batstone et al., 2015; Colosi et al., 2015; McCarty et al., 2011; Melvin and Leusch, 2016; Salama et al., 2017). Bench-scale experiments have shown that freshwater microalgae deliver efficient removal of aqueous nutrients (i.e., nitrogen and phosphorus) that would otherwise contribute to downstream eutrophication as well as certain emerging contaminants (e.g., steroid hormones) (Ge et al., 2009; Shi et al., 2010; Zhang et al., 2014).

The idea of an integrated algae-wastewater treatment system has been conceptualized as a tertiary treatment method to improve wastewater treatment through enhanced removal of nitrogen and phosphorus while also generating energy through anaerobic digestion of biosolids to produce bio-electricity (Colosi et al., 2015; Menger-Krug et al., 2012). Colosi et al., 2015 performed a life cycle assessment of traditional tertiary WWTP systems compared with an algae treatment system

and compared them on the basis of energy efficiency using the energy return on investment (EROI) metric, which is the ratio of energy produced over energy consumed. EROI values less than 1 indicate systems that are net energy-consuming. Higher values are more energetically favorable than lower values. They computed EROI for the hypothetical algae treatment system to be 0.65, compared to values of 0.47, 0.24, and 0.35 for selected traditional tertiary treatments. A baseline WWTP configuration without tertiary treatment exhibited EROI of 0.50 (Colosi et al., 2015). Therefore, Colosi et al., 2015 showed the capability of an integrated algae-wastewater treatment system to benefit a WWTP from an energy perspective. Additional studies have taken this concept further and have investigated the potential for an integrated algae-wastewater treatment system to also remove unregulated contaminants. Zhang et al., 2014 performed an analysis on the ability of *Scenedesmus dimorphus* to degrade steroid estrogens and observed removals of 85-95% for the four estrogens studied. Matamoros et al., 2015 evaluated the degradation of 26 micro-contaminants in a pilot-scale microalgae-WWTP system. The pilot scale system achieved removal efficiencies of up to 90% for the compounds assessed (Matamoros et al., 2015). A schematic of the integrated algae-WWTP system, including potential emerging contaminant removal benefits, is presented in Figure 1-1.

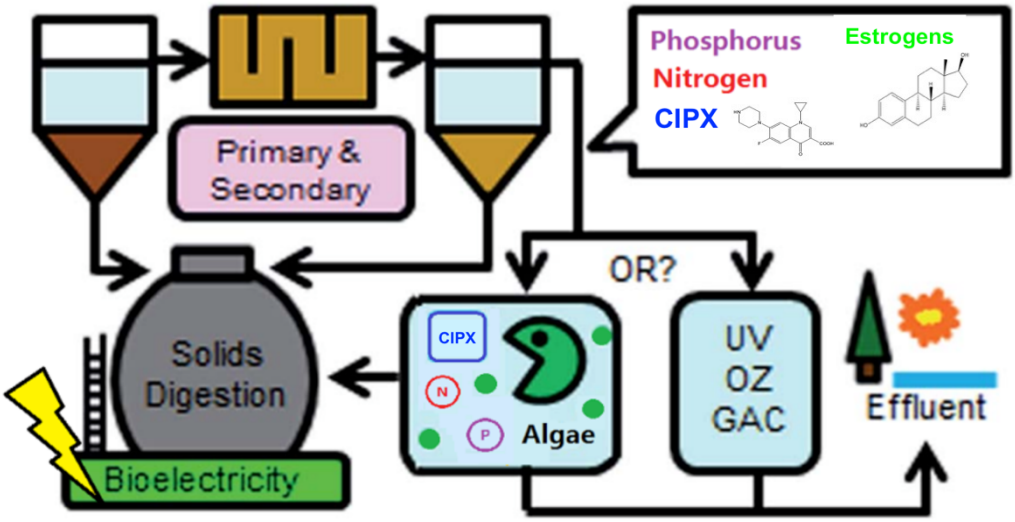

Figure 1-1. Diagram of an integrated algae-WWTP system including both nutrient and unregulated contaminant removals and energy generation from methane-derived electricity (Zhang et al., 2014).

Photodegradation, or photolysis, involves transformation of a molecule resulting from light exposure (most commonly solar or UV light). Many antibiotics and ARGs are considered susceptible to photodegradation, so understanding the reaction mechanisms and conditions for photo-transformation to occur is a major step in the process of developing technologies to successfully remove them (Chee-Sanford et al., 2009; Fatta-Kassinos et al., 2011; Homem and Santos, 2011; Paul et al., 2010; Zhang et al., 2019). In a traditional WWTP setting, the combination of high solids content and low light exposure relative to reactor volume makes substantial photodegradation unlikely to occur (Norvill et al., 2016). However, with the addition of an algae tertiary treatment system, WWTP effluents have the opportunity to gain significant exposure to light via circulation through a shallow, continuously mixed algae cultivation pond system.

The mechanism of photodegradation can be separated into two major reaction processes:

direct and indirect photolysis. In direct photolysis, photons are directly absorbed by the target molecule, which provides the energy to break down the chemical bonds and the compound's overall structure. The contribution of direct photolysis to overall photodegradation of a compound is dependent on many factors including light absorption of the compound, pH, temperature, and presence of other organic compounds in the system that may cause interference. Indirect photolysis occurs via oxidation of the target compound by radicals produced when photons excite other molecules in the system, known as photosensitizers (Norvill et al., 2016). Many common photosensitizers that can produce reactive radical species, such as organic acids, nitrate, and others are present in WWTP effluents. In an algae-WWTP system, both direct and indirect photolysis could play an important role in deactivating antibiotics and ARGs.

Incorporating an algae-based polishing step into a wastewater treatment process could deliver several environmental benefits: removal of nutrients, decreasing downstream eutrophication; production of algae biomass that can be converted into a carbon-neutral green energy source; and, potentially, mitigation of antibiotic resistance stimulating constituents.

1.1.4 Review of studies on treatment of antibiotic resistance constituents and research gaps

Recent reviews by Xiong, et al. 2017a and Wang, et al. 2017 have synthesized the existing literature pertaining to antibiotics removal by several microalgae (Bai and Acharya, 2017; Hom-Diaz et al., 2017; Wang et al., 2017; Xiong et al., 2017b, 2017a; Yu et al., 2017; Zhou et al., 2014). These studies focus on algae-mediated treatment of antibiotic compounds under bench- and pilot-scale conditions; however, an evaluation of how the treated effluents impact the stimulation of antibiotic resistance in the environment has not been previously performed. Evaluating the residual effects of treated effluents is especially important since studies have shown that reduction of a

parent drug compound can produce byproducts that are equally or more potent than the parent (Fatta-Kassinos et al., 2011; Noguera-Oviedo and Aga, 2016). Further, there is currently no information about algae-mediated deactivation of ARGs. Some advanced tertiary treatments, such as UV, UV-chlorination, and ozonation have been shown to deactivate ARGs to varying degrees (Chang et al., 2017; Czekalski et al., 2016; Destiani and Templeton, 2019; He et al., 2019; McKinney and Pruden, 2012; Quach-Cu et al., 2018; Yoon et al., 2017; Zhang et al., 2019). However, to the best of our knowledge, no one study has investigated the efficacy of a single treatment to remove an antibiotic parent compound, reduce its acute and chronic potency effects, and deactivate an ARG.

Figure 1-2 presents a schematic of the proposed algae treatment system and where it fits within the WWTP pathway and antibiotic resistance landscape. The goal of this dissertation is to evaluate the efficacy of the algae treatment in deactivating antibiotics and ARGs and examine its potential role in combatting the spread of antibiotic resistance from WWTPs.

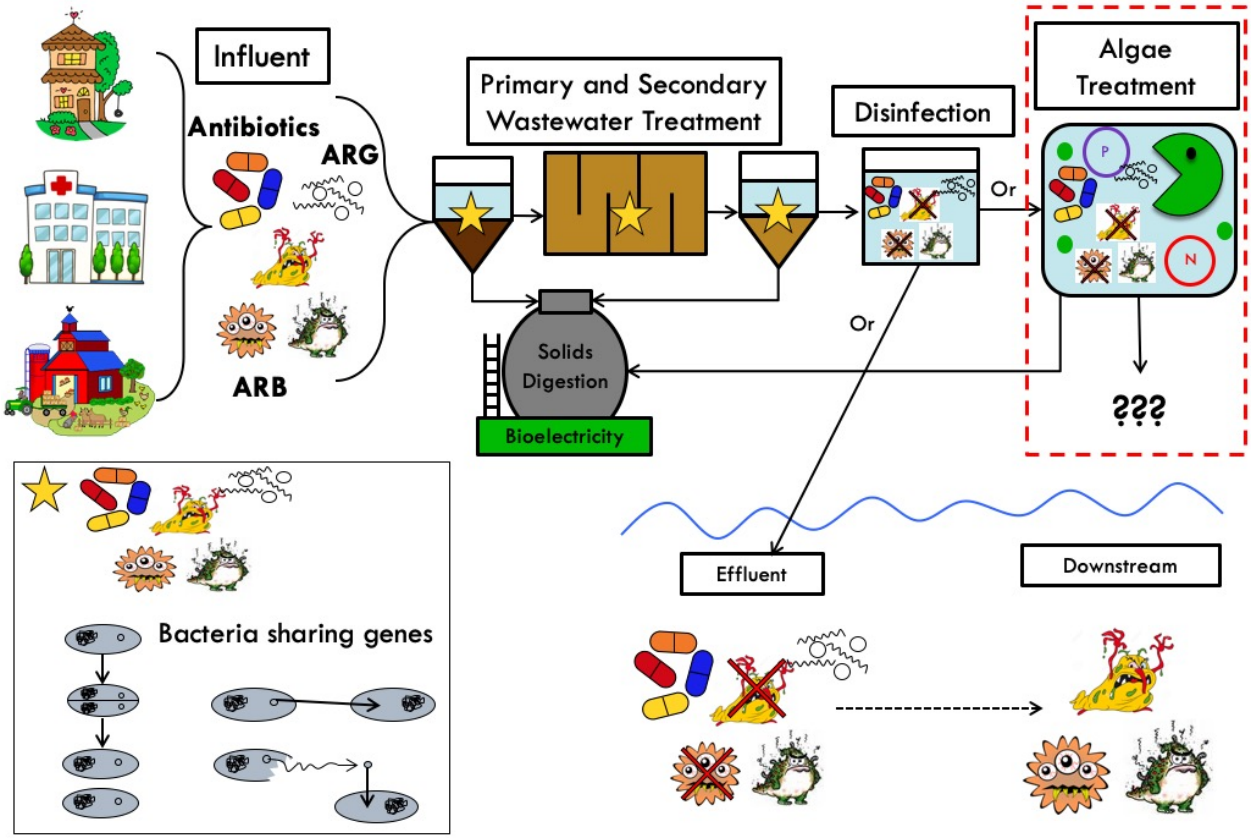

Figure 1-2. The pathway for antibiotic resistance-stimulating constituents to enter the wastewater treatment system and disseminate through effluents into the environment. This dissertation seeks to evaluate the potential role of an algae treatment system in mitigating antibiotics and ARGs. The star symbol represents areas of the wastewater treatment system where ARB are selectively amplified through both vertical and horizontal gene transfer processes.

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Chapter 2: Research Objectives

The overall goal of this dissertation is to evaluate the efficacy of a model algae-based tertiary wastewater treatment toward mitigation of wastewater constituents that can stimulate antibiotic resistance in downstream receiving waters. Our first step is to evaluate removal of a model antibiotic parent drug compound and assay corresponding potency/resistance effects of the resulting effluents. Our second step is to treat a model antibiotic resistance gene (ARG) and evaluate corresponding losses in ARG reactivity. For both steps, we perform bench-scale algae treatment experiments with subsequent effluent analysis and microbiological assays. We hypothesize that the algae treatment will reduce the capacity of these effluent constituents to stimulate antibiotic resistance in model downstream bacteria. Collectively, our analyses establish a comprehensive *effects-based assessment* of the algae treatment toward mitigating wastewater constituents that stimulate antibiotic resistance. This approach is useful for assessing the usefulness of other candidate treatment technologies.

Specifically, this dissertation has two key research objectives:

Objective 1. Evaluating the algae treatment for removal of a model antibiotic and its residual effect(s)

Objective 2. Evaluating the algae treatment for deactivation of a model ARG plasmid

In-depth analyses for Objectives 1 and 2 are presented in dissertation Chapters 3, 4, and 5. Contextualization and thematic conclusions are presented in Chapter 6. The overall project conclusions and future research recommendations are discussed in Chapter 7.

Figure 2-1illustrates how the methodology and findings for both objectives complement each other and together constitute a holistic effects-based framework for assessments of a wastewater treatment's capacity to remove not only target constituents, but also their effects in stimulating antibiotic resistance in the environment.

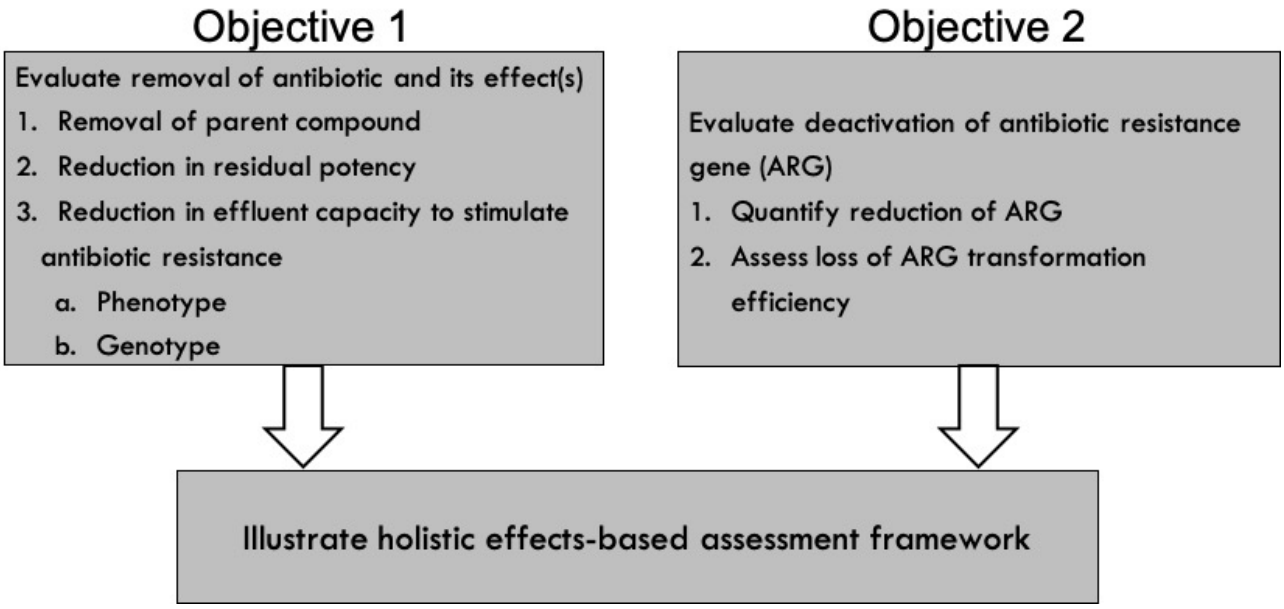
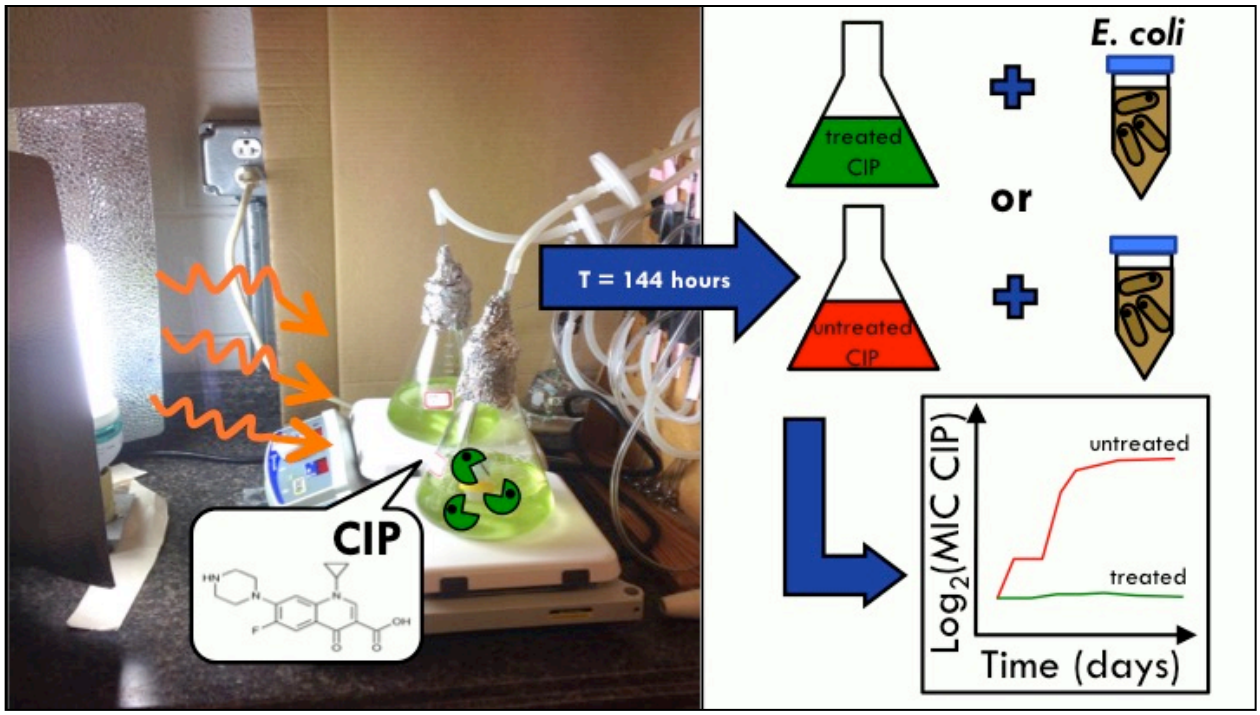


Figure 2-1. The research goals for objectives 1 and 2 illustrate a holistic effects-based assessment framework that can be adapted to evaluate other candidate wastewater treatments.

Chapter 3: Objective 1, Algae Treatment of Antibiotic

This chapter resulted in one publication in *Chemosphere*.

Grimes, K. L., Dunphy, L. J., Loudermilk, E. M., Melara, A. J., Kolling, G. L., Papin, J. A., Colosi, L. M. "Evaluating the efficacy of an algae-based treatment to mitigate elicitation of antibiotic resistance," *Chemosphere.* **2019, 237**. 10.1016/j.chemosphere.2019.124421



3.1 Introduction

The fluoroquinolone drug ciprofloxacin (CIP) is one of the most frequently prescribed broad-spectrum antibiotic drugs for human and veterinary use, accounting for 20 million prescriptions each year ("Drug Record Ciprofloxacin"). It has been measured at ng/L – μg/L concentrations in wastewater treatment plants (WWTPs) and downstream receiving waters (Janecko et al., 2016). The concentration of CIP in WWTP influents and effluents is highly dependent on location, wastewater source (e.g., hospital waste, livestock waste, domestic waste, etc.), and the type of treatment process. As a result of these and other processes, the global median concentration of CIP in freshwater ecosystems is 0.164 mg/L, with a maximum detected concentration of 6.5 mg/L (Hughes et al., 2012; Xiong et al., 2017b).

There is emerging interest in algae-mediated biological treatment of antibiotic drugs because unlike bacteria, eukaryotes are not the intended target of antibacterial drugs. Recent reviews by Xiong et al. (2017a) and Wang et al. (2017) offer good syntheses of experimental results pertaining to antibiotics removal by several microalgae species (Bai and Acharya, 2017; Hom-Diaz et al., 2017; Xiong et al., 2017b; Yu et al., 2017; Zhou et al., 2014). This body of work reveals that algae can remove certain antibiotic compounds under bench- and pilot-scale conditions; however, it is almost entirely unknown how the treatment affects the long-term antibiotic potency of the resulting treated effluents. This is a question of interest not only for the proposed algae treatment but also for conventional treatments (Fatta-Kassinos et al., 2011; Paul et al., 2010).

We investigated algae-mediated CIP removal with three key objectives: (1) evaluate CIP concentration over time under simulated algae treatment conditions; (2) evaluate the residual antibiotic potency of algae-treated samples during acute exposures to a model bacterium; and (3)

evaluate the residual capacity of algae-treated samples to elicit genetically transmissible CIP resistance, using adaptive laboratory evolution (ALE). ALE is a technique for assessing evolutionary changes in microbial communities in a controlled laboratory environment (Dragosits and Mattanovich, 2013; Yen and Papin, 2017). It is often used to understand the adaptive changes that develop from long-term exposure to selective pressures; however, it has not been widely used for environmental engineering applications. In this study, we use ALE to evaluate adaptation of *E. coli* lineages cultivated in treated or untreated CIP samples and assess development of antibiotic resistance over time, as quantified using minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antibiotic that prevents growth of bacteria. The three objectives of this study support a larger overarching goal of evaluating to what extent wastewater treatments can remove a parent drug compound with corresponding reductions in residual antibiotic potency and capacity to elicit transmissible antibiotic resistance.

3.2 Materials and Methods

3.2.1 Algae-mediated Removal of CIP

3.2.1.1 Algae Cultivation

We selected *Scenedesmus dimorphus* as our model alga for this study because it is an abundant species in temperate freshwater environments (Zhang et al., 2014) and has potential relevance for use in integrated algae-WWTP applications (González et al., 1997; Pushpakumari Kudahettige et al., 2018). We prepared pure cultures of *S. dimorphus* (UTEX 1237) based on a three-step procedure from previously published work (Zhang et al., 2014). See Chapter 4, Section 4.1 for more details.

3.2.1.2 CIP Removal Experiments

We prepared two sets of 500-mL reactors containing 25 µg/L of CIP in modified Bold 3N medium (MB3N), namely: 1) an experimental algae control (EA), which comprised CIP-spiked medium with 50 mg/L algae biomass, exposed to light; and, 2) a light control (LC), comprising CIP-spiked medium without algae and exposed to light. Reactors were cultivated under stirring, aeration, and 12 h of illumination per day (cool white full-spectrum fluorescent growth lamp with 125 W 6500 K, 30 µW/cm^2 UV penetration) for a period of 144 h. We also prepared dark control (DC) reactors, which contained CIP-spiked medium without algae under the same stirring and aeration conditions. The DC reactors were wrapped in foil to prevent light penetration. They comprised positive controls, to rule out apparent CIP removal in the absence of light and/or algae. All conditions (EA, LC, DC) were tested in triplicate. The 25 µg/L initial CIP concentration was selected because it is consistent with CIP measurements in WWTPs and the environment (Janecko et al., 2016). We observed that this CIP concentration did not have any appreciable effect on algae growth rate in preliminary growth experiments (Chapter 4, Section 4.1.1.2).

We collected 10-mL samples from the EA and LC reactors at 0, 6, 24, 48, 96, and 144 h. We assessed optical density at 662 nm (OD_{662}) to quantify algae biomass concentrations in the EA samples and then filtered them through 0.7-µm pore size glass microfiber filters (Millipore) to remove algae cells. We then applied a solid phase extraction (SPE) procedure modified from validated methods (Zhang et al., 2014; Zhou and Jiang, 2012) to concentrate the CIP in our samples and remove compounds in the media that interfere with CIP analysis (Chapter 4, Section 4.1.2).

We also applied two previously validated approaches to assess sorption of CIP to the algae biomass (Chapter 4, Section 4.1.3) (Wu et al., 2013; Zhang et al., 2014).

3.2.1.3 Analytical Methods

We measured CIP concentrations via high performance liquid chromatography (HPLC) based on previously validated methods using a Shimadzu 2010-AB HPLC with fluorescence and UV detectors (Idowu and Peggins, 2004; Lee et al., 2007; Muchohi et al., 2011; Piñero et al., 2013). Chapter 4, Section 4.1.4 provides details of the HPLC method as well as a representative chromatogram and calibration information.

3.2.2 Assessment of Residual Antibiotic Potency

3.2.2.1 Chemicals, Medium, and Inoculum Preparation

We analyzed the effects of treated CIP samples on *Escherichia coli* ATCC 25922™, a model Gram-negative bacterium. See Chapter 4, Section 4.2 for details about the *E. coli* preparation.

3.2.2.2 Assessment of Residual Acute Antibiotic Potency

We used a modified protocol from Paul et al. (2010) to assess residual antibiotic potency of the treated CIP solutions. We exposed *E. coli* to algae-treated (EA) and light control (LC) effluents collected at 144 hours (without subsequent SPE). We performed this assay in clear, flat-bottom 96-well plates. For reproducibility, we used three wells per experimental replicate. Each well received filter-sterilized (0.22-nm pore size) EA or LC post-treatment samples or a 25-µg/L untreated CIP standard (CS), each supplemented with concentrated LB media to support culture growth (Chapter 4, Table 4-1). We then added 25 µL of the 1:1000 diluted *E. coli* stock to each well. One row solely contained MB3N media and 25 µL of the 1:1000 diluted *E. coli* stock to

represent maximum growth of *E. coli* in the absence of CIP. Another row contained only MB3N without *E. coli* to represent no growth. All wells contained a total volume of 200 µL.

After loading the wells, we covered the plate with its lid to prevent evaporation and incubated it at 37 °C with 160-rpm shaking. We used a spectrophotometer (SpectraMax Plus 384) to measure OD_{625} to quantify *E. coli* growth over time until stationary phase (6 hours). We then used Equation 3-1, modified from Paul et al. (2010), to determine percent relative growth in the presence of EA samples, LC samples, or untreated CS relative to the media control.

Equation 3-1. $$\textbf{R.G. (\%)} = \frac{(A - A_{min})}{(A_{max} - A_{min})} * 100\%$$

R.G.(%) is percent relative growth; A is sample absorbance at OD_{625} after 6 hours of incubation; A_{min} = absorbance of an abiotic negative control comprising LB with no bacteria and no CIP (i.e., 0% growth); A_{max} = absorbance of a positive control corresponding to bacterial growth in LB with no CIP (i.e., 100% growth) (Paul et al., 2010). We used Microsoft Excel (2016) to compute *p*-values using two-sample student's *t*-tests for differences in mean assuming equal variances. Significance was established at $p < 0.05$.

3.2.3 Assessment of Residual Capacity to Elicit Antibiotic Resistance

3.2.3.1 Chemicals, Medium, and Inoculum

We purchased a fresh stock of *E. coli* ATCC 25922™ from Microbiologics™ and prepared experimental cultures according to the Microbiologics™ Kwik-Stik™ instructions. We filter-sterilized 4.5-mL aliquots of 144-hour treated EA samples, LC samples, and various control media

and froze them at -20 °C. EA and LC treated samples were not subject to SPE before freezing. We thawed and used individual aliquots once per day over the course of the ALE experiment.

3.2.3.2 ALE-based Assessment of Residual Capacity to Elicit Antibiotic Resistance

We exposed *E. coli* to EA and LC samples over many generations to assess whether or not the bacteria would exhibit changed CIP resistance compared to relevant controls. Resistance was quantified via changes in MIC (Yen and Papin, 2017). Our exposure conditions were as follows: 1) EA samples (collected at t = 144 hours); 2) LC samples (collected at t = 144 hours); 3) untreated 25-μg/L CIP standard (CS) in MB3N; 4) algae media control, comprising spent media collected at t = 144 hours from reactors containing algae in MB3N without CIP, with subsequent filter-sterilization to remove algae cells; and 5) LB media control, comprising fresh LB media without CIP. We simultaneously exposed *E. coli* to each of these five conditions in triplicate, such that fifteen parallel cultures were maintained concurrently. These fifteen cultures were subjected daily to three protocols: 1) propagation into fresh exposure media; 2) MIC plating to assess changes in CIP resistance; and 3) collection of samples for freezer storage. We performed all three procedures daily for ten days; except MIC plating was omitted on days seven and nine for convenience.

We began by streaking a thawed stock culture of *E. coli* onto an LB agar plate and incubating it for 23 hours at 37 °C. We then inoculated three single colonies into 5 mL of LB and incubated these for 23 hours at 37 °C with 160-rpm shaking. We denoted these three cultures as Day-0 ancestors 1, 2, and 3. For culture propagation into fresh exposure media, we diluted the three Day-0 ancestor cultures by a factor of 111 to achieve OD_{600} values of approximately 0.01 in 5 mL of a fresh test solution: EA, LC, untreated CS, algae media control, or LB media control. Each solution was supplemented with concentrated LB nutrients to support *E. coli* growth. The

corresponding cell concentration was approximately 10^7 CFU/mL. Triplicate lineages were propagated for each exposure solution, each arising from a separate Day-0 ancestor. We incubated the resulting fifteen cultures for approximately 23 hours at 37 °C with 160-rpm shaking. The samples were collected at the same time each day and the 23 hours excludes approximately one hour of daily preparation time when samples were on the bench prior to incubation. We repeated this procedure daily for 10 days, or ~80 generations (Chapter 4, Section 4.3.1).

For the MIC assessments, we diluted each lineage by a factor of 111 to achieve an OD_{600} value of approximately 0.01 in 20 mL of fresh LB media. This value corresponded to approximately 10^7 CFU/mL. We then transferred the diluted lineages into a 96-well MIC plate containing a standard 2-fold dilution series spanning the range 0-4 μg/mL CIP (Andrews, 2001; Yen and Papin, 2017). From the literature, this range encompasses sub-inhibitory to much greater than inhibitory levels for wild-type *E. coli* (Chapter 4, Table 4-3) (Andrews, 2001; Becnel Boyd et al., 2009; Sharma et al., 2014). We also included wells for sterility control (LB media only) on all MIC plates. We enclosed the plates in rigid containers to prevent evaporation and incubated them at 37 °C with 140-rpm shaking. After 23 hours, we quantified MIC values based on visual observation. We confirmed these results using a plate reader, parameterizing "growth" as $OD_{600} > 0.2$ after background subtraction. The MIC value for each exposure condition was operationally defined as the lowest CIP concentration that did not show *E. coli* growth each day. We ended the experiment after ten days, when the apparent MIC values had stabilized across all fifteen lineages.

We froze samples of all fifteen lineages daily in 25% glycerol. We did this as a means to guard against accidental cross-culture or MIC plate contamination and enable future genotype evaluation of the samples (Chapter 4, Section 4.3.5).

3.2.3.3 Characterizing Genotype Basis for Observed Phenotype CIP Resistance

Frozen samples of the Day-0 ancestors and Day-10 *E. coli* lineages propagated in EA, LC, or untreated CS were streaked onto LB agar plates and incubated overnight at 37 °C. Two colonies were selected from each lineage, one for each gene of interest: DNA gyrase subunit A (*gyrA*), and topoisomerase IV (*parC*). These genes were selected based on existing literature that shows they are commonly associated with increased CIP resistance in *E. coli* (Chung et al., 2017; Hu et al., 2017; Johnning et al., 2015; Kim et al., 2012; Mavroidi et al., 2012; Morgan-Linnell et al., 2009). Polymerase chain reaction (PCR) was performed on each selected colony. Primers were designed using the NCBI primer BLAST tool for *E. coli* ATCC 25922™ (GenBank Accession Number: CP009072.1) (See Chapter 4, Table 4-4). DNA polymerase was One*Taq*® Hot Start 2X Master Mix with GC buffer, and PCR conditions were as follows: initial denaturation at 94 °C for 30 seconds; 35 cycles of second denaturation at 94 °C for 30 seconds; annealing at 57 °C for 30 seconds, and extension at 68°C for 3 minutes; final extension at 68 °C for 5 minutes; and hold at 4 °C. Amplified DNA products were purified from a 1% agarose gel (Qiagen), and Sanger sequencing was performed by a contract laboratory (Eurofins). Sequences were aligned to the reference genome corresponding to *E. coli* ATCC 25922 and mutations were identified using BLASTn (NCBI). Based on literature, the so-called quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* encode the amino acids that are most often the target of CIP (Hu et al., 2017; Johnning et al., 2015; Kim et al., 2012; Mavroidi et al., 2012; Morgan-Linnell et al., 2009). CIP resistance mutations can also occur in other gene regions (e.g., *gyrB*, *parE*, etc.) but these regions are less likely to develop mutations corresponding with a high MIC to CIP. The QRDR regions of *gyrA* and *parC* in *E. coli* typically encode codons 83-157 for *gyrA* and codons 56-84 for *parC*. Therefore, sequence data was assessed only if the QRDR were located in a high quality

region of the sequence (i.e., PHRED scores for each base pair > 50) and mutations were only reported if they resulted in a change of at least one amino acid.

3.3 Results and Discussion

3.3.1 Algae-mediated CIP Removal

The overall goal of this study was to determine whether algae-based tertiary treatment mediates effective removal of the CIP parent compound with corresponding reduction in acute antibiotic potency and capacity to elicit transmissible antibiotic resistance among bacteria downstream. Our first step was measuring reduction in CIP concentration during algae treatment. Results are presented in Figure 3-1. These data reveal that the algae treatment achieves effective removal of the CIP parent compound from an initial concentration of 25 μg/L. CIP removal was 53% for the light-exposed control (LC) reactors without algae biomass. CIP removal was even greater (93%) in illuminated reactors containing algae biomass (EA). Notably, most of the removal occurred within the first 48 hours for both sets of reactors. This duration corresponds with typical hydraulic retention times for some other WWTP processes.

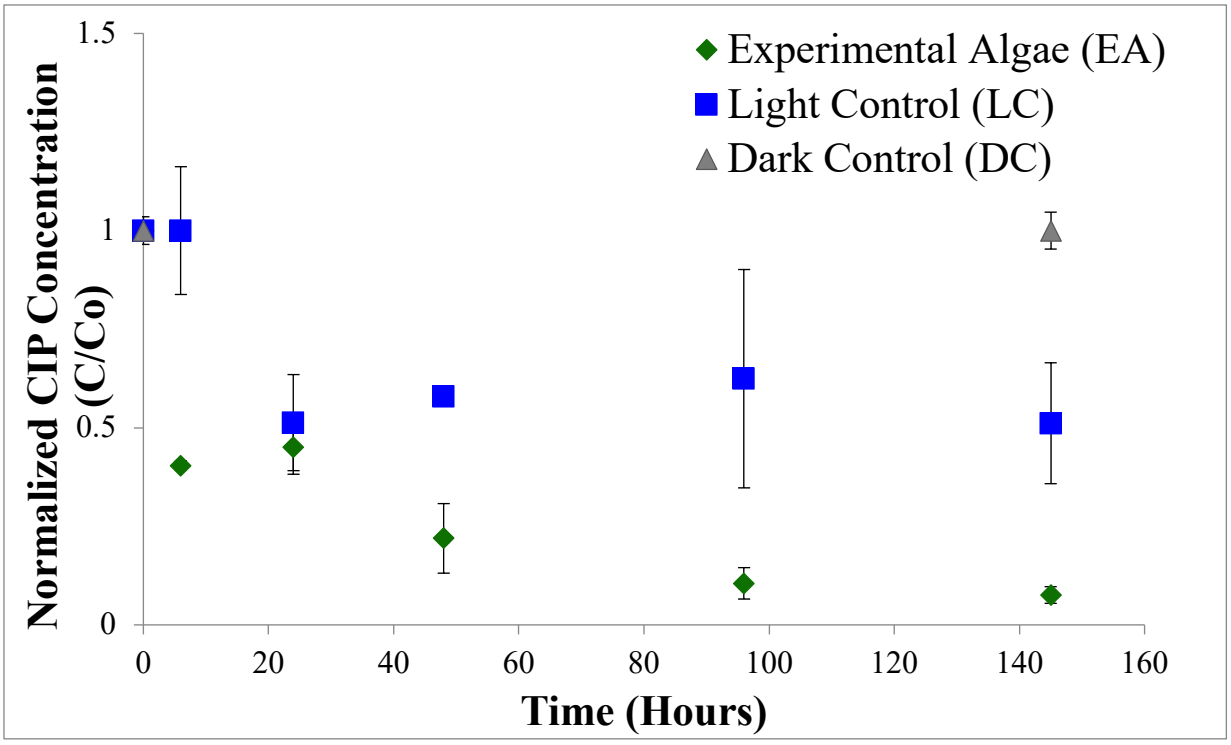


Figure 3-1. Normalized (C/C_0) concentrations of ciprofloxacin (CIP) over time for experimental algae (EA) and light control (LC) reactors over 144 hours. For dark control (DC) reactors, CIP concentration measurements were taken at hours 0 and 144. Normalized CIP concentrations refer to CIP concentrations at time = *t* divided by initial CIP concentration (C_0), where $C_0 = 25$ µg/L. Error bars are standard error for triplicate reactors of each condition.

The results in Figure 3-1 are consistent with emerging literature related to algae-mediated removal of CIP and other antibiotics. For example Bai and Acharya (2017) observed 100% CIP removal from spiked lake water by *Nannochloris sp*. over seven days. Zhou et al. (2014) investigated removal of some fifty organic compounds, including various antibiotics, by four separate axenic algae cultures: *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Chlorella vulgaris,* and *Scenedesmus obliquus*. They reported approximately 80% CIP reduction over seven days by all evaluated algae strains. They also observed comparable removals (50-80%) for several other fluoroquinolone drugs (e.g., enrofloxacin, lomefloxacin, norfloxacin, and ofloxacin).

Notably, they observed relatively uniform performances across all four evaluated algae cultures, which suggests that removal results may be somewhat generalizable across various algae strains for a specific drug of interest. Finally, Hom-Diaz et al. (2017) evaluated removal of diverse pharmaceuticals by an uncharacterized mixture of algae species inoculated from lake water. They observed CIP removal of approximately 50% over eight- or twelve-day durations.

We hypothesized that removal of CIP by different mechanisms could mediate different impacts on residual antibiotic potency and elicitation of transmissible microbial resistance. We therefore conducted a qualitative evaluation of CIP removal via several possible mechanisms. We evaluated CIP sorption to algae using two approaches (Chapter 4, Section 4.1.3). We observed negligible contribution of sorption using either of these protocols, which is consistent with the findings of Bai and Acharya (2017), who reported that CIP was not detectable in algae lipid extracts. Similarly, we used light-exposed control (LC) reactors to isolate abiotic photo-transformation in the absence of algae biomass. These analyses revealed that direct photo-transformation mediates significant reduction in CIP concentration but that overall CIP removal is appreciably greater in the presence of active algae biomass. This result indicates that the difference in CIP removal for reactors with and without active algae biomass must be attributable to some effect of the algae itself. For example, the algae cells may be actively metabolizing the CIP molecules in a manner that is consistent with "co-metabolism" reported by Xiong et al. (2017b). Concurrently, growth of the algae may create conditions that enhance CIP photo-transformation; e.g., by releasing algal organic matter (AOM) and/or increasing the dissolved oxygen concentration. These effects have been well documented in existing literature (Bai and Acharya, 2017; Fatta-Kassinos et al., 2011; Guo, Ruixin and Chen, Jianqu, 2015). We cannot conclusively identify the exact mechanisms by which the presence of the algae enhances overall reduction in

CIP concentration; therefore, this would be a valuable direction for future work. Finally, our results from the dark control reactors confirm that reduction in CIP concentration is not occurring via experimental artifacts (e.g., volatilization, sorption to reactor walls, etc). The measured CIP concentration in the dark control reactors at t = 144 hours was 100 $\pm$5% of the initial concentration.

With respect to how different removal mechanisms could affect residual sample potency, it is noteworthy that sorption plays an apparently negligible role in algae-mediated CIP removal. This process is undesirable for antibiotics because there is no change to the compound's intrinsic biological potency and its capacity to elicit antibiotic resistance. In contrast, both biotransformation and photo-transformation could convert a parent compound into products with different capacities to elicit antibiotic resistance (Fatta-Kassinos et al., 2011; Yu et al., 2017). It may be significant that most of our observed algae-mediated removal occurs via chemical transformations and that the treated samples likely contain a mixture of photo-products and biological metabolites. Existing literature confirms that CIP is susceptible to photo-transformation under conditions simulating "natural compartments" (Bai and Acharya, 2017; Vasconcelos et al., 2009) (e.g., sunlit fresh waters) or engineered UV treatment systems (Fatta-Kassinos et al., 2011; Paul et al., 2010). It has been shown that CIP undergoes defluorination, decarboxylation, and/or loss of the piperazine moiety to different degrees under different treatment conditions, but the core quinolone structure typically remains intact. It has also been widely observed that the corresponding photo-products retain some antibacterial activity but are generally less potent than the parent compound. Xiong et al. (2017b) suggest that algae may make use of cytochrome P450 (CYP450) enzymes to mediate CIP biodegradation reactions that are analogous to known bacterial transformations, again resulting in defluorination and loss of the piperazine ring. Thus, there is some similarity in key features between photo-products and biological metabolites. It is possible

that greater structural diversity of reaction products at lower residual concentrations could be valuable for mitigating the rise of antibiotic resistance among downstream organisms (Fatta-Kassinos et al., 2011). It would be worthwhile to identify CIP products in the LC and EA reactors in the future to help better understand how different removal mechanisms give rise to different products with potentially different residual antibiotic effects.

3.3.2 Assessment of Residual Acute Antibiotic Potency

Having observed that the algae treatment and light control mediate efficient transformation of the CIP parent compound, we also wished to evaluate residual antibiotic potency. That is, we wanted to know whether the effluents contained reaction by-products that were still capable of inhibiting bacterial growth. Results of the short-duration residual antibiotic potency analysis are presented in Figure 3-2.

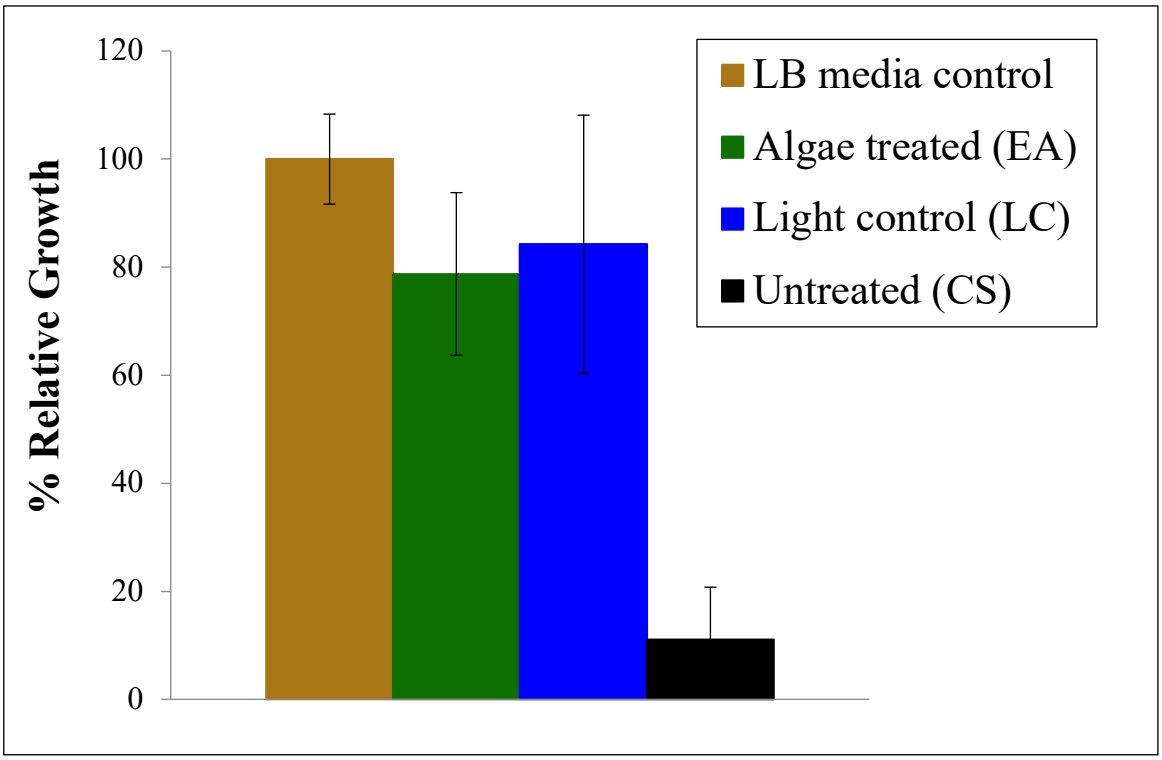


Figure 3-2. Growth of *E. coli* cultures exposed to (1) an LB media control without CIP (positive growth control), (2) algae treated (EA) samples, (3) light control (LC) samples, or (4) an untreated 25-µg/L CIP standard (CS). Error bars represent standard deviation for the mean of three replicates for each condition. *P*-values for comparisons of means are as follows: LB media control vs. EA ($p = 0.05$), LB media control vs. LC ($p = 0.32$), LB media control vs. untreated CS ($p = 0.002$), EA vs. LC ($p = 0.41$), LC vs. untreated CS ($p = 0.03$), and EA vs. untreated CS ($p = 0.04$).

From Figure 3-2, the algae treatment mediates appreciable reduction in CIP antibiotic potency during acute (short-duration) exposures to the model bacterium. The *E. coli* grew much more robustly in the presence of the treated samples from the experimental algae (EA) and light control (LC) than they did in the presence of the untreated CIP (CS). EA, LC, and LB media control samples exhibit a statistically significant difference in relative growth compared to the untreated CS samples at $\alpha = 0.05$.

One additional observation from Figure 3-2 is that the acute exposure assay did not reveal a clear difference in *E. coli* growth inhibition between the treated EA and LC samples. That is, relative growth rates for the EA and LC samples did not exhibit a statistically significant difference from one another or the LB media control. This result is perhaps unexpected, given the observed difference in overall reduction in CIP concentration under these two conditions: 53% for LC vs. 93% for EA (Figure 3-1). It is possible that direct photolysis of CIP in the LC samples generated transformation products that were sufficiently different from the parent compound so as not to impact *E. coli* growth. Although we expect that the EA samples contain more diverse transformation products than the LC samples due to multiple transformation reactions occurring simultaneously, it is possible that the extent of CIP transformation in the EA samples goes beyond the necessary threshold for mitigating the effect of CIP on *E. coli*. Either way, our results are consistent with scant previous literature related to assessment of post-treatment antibiotic potencies. Yu et al. (2017) evaluated the acute antibiotic potency of ceftazidime before and after algae treatment, using two model organisms: *E. coli* as a model for Gram-negative bacteria, and *Staphlycoccus aureus* as a model for Gram-positive bacteria. They observed minimal growth inhibition by the treated samples and concluded that the principal transformation by-products are not appreciably bacteriostatic. Similarly, Paul et al. (2010) reported rapid removal of CIP and its corresponding antibiotic "potency equivalent" (PEC) (as measured against *E. coli*) during photolytic and/or photocatalytic treatment using ultraviolet or visible light with or without a catalyst. They observed a nearly linear 1:1 correlation between residual CIP concentration and antibiotic potency for all treated samples, from which they concluded that the transformation products were negligibly bacteriostatic compared to the parent compound. This result was somewhat surprising given that all of their identified transformation products retained the core

quinolone moiety, which typically plays an essential role in antibacterial activity of CIP and other fluoroquinolones. Although algae-mediated CIP transformation products have not specifically been identified, additional literature on the photo-transformation products of CIP provides some insight into possible photo-products generated by our EA and LC samples. For the most part, photo-transformation of fluoroquinolones result in molecularly consistent byproducts, though the pathways, amount of formation, and extent of transformation vary with many factors (e.g., pH, irradiance type and duration, initial concentration of parent compound, and presence of organics). For example, Batchu et al., 2014 and Salma et al., 2016 both analyzed the photo-transformation products of CIP under different irradiation sources. Batchu et al., 2014 identified eight CIP metabolites and their potential degradation pathways in purified, fresh, and salt water matrices. Salma et al., 2016 identified eighteen transformation products and found that variation in solution pH resulted in different product generation. They concluded that the primary step in photo-transformation of CIP is defluorination followed by degradation of the piperazine ring (Salma et al., 2016); a process that has been shown to reduce the potency of fluoroquinolones (Calza et al., 2008; Paul et al., 2010). Neither Batchu et al. (2014) nor Salma et al. (2016) assessed the impacts of the transformation products on bacteria. Ultimately, it is not currently possible to understand why we observed no difference in acute potency for the algae-treated and light-control samples despite their difference in residual CIP concentrations, without knowing the identities of the LC and EA transformation products; therefore, it would be of interest to identify algae-mediated CIP transformation products in a future study.

3.3.3 Assessment of Residual Capacity to Elicit Antibiotic Resistance

Our results from the CIP removal experiments and the acute residual potency assay reveal that the algae treatment has the capacity to reduce CIP concentration in a manner that also reduces its acute antibiotic potency toward a model bacterium. However, neither experiment conclusively shows that the algae treatment delivers the ultimate desired effect of reducing elicitation of transmissible antibiotic resistance over sustained exposures. This question has not been well addressed in most existing studies evaluating the efficacy of novel or traditional wastewater treatments against various antibiotic drugs. One possible exception is a recent analysis of electron beam irradiation by Szabó et al. (2017) in which the authors co-cultivated a mixture of resistant and non-resistant *S. aureus* in effluents arising from different treatment extents and identified what minimum treatment was required to ensure that the resistant strain could no longer outcompete the non-resistant strain.

We performed an adaptive laboratory evolution (ALE) experiment to assess whether the algae treatment mitigates the development of antibiotic resistance during sustained exposures to a model bacterium. Results from this analysis are presented in Figure 3-3.

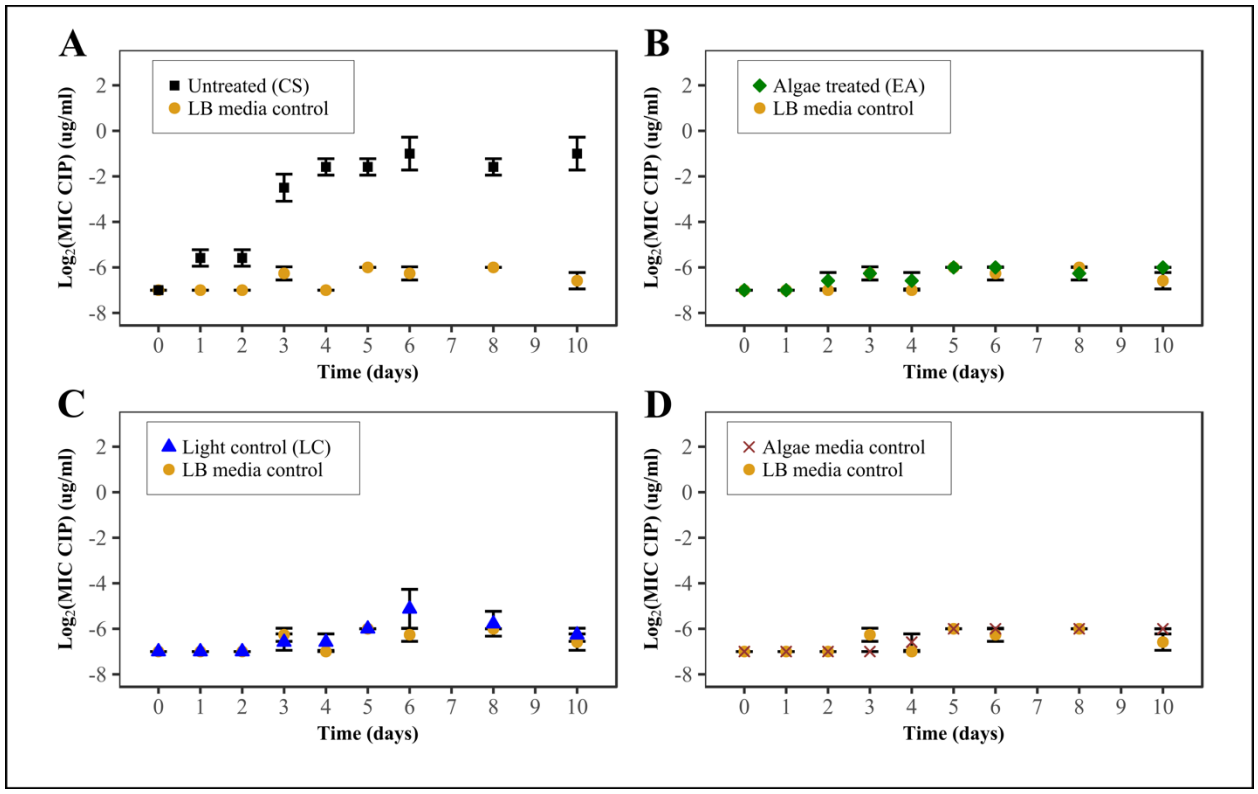


Figure 3-3. Observed minimum inhibitory concentrations (MICs) of CIP over time for *E. coli* lineages exposed to (A) 25-µg/L untreated CIP (CS), (B) algae treated (EA) samples, (C) light control (LC) samples, or (D) algae growth media (MB3N) without CIP ("algae media control"). Observed mean MIC values for *E. coli* lineages exposed to LB media without CIP ("LB media control") are included in all panels (A)-(D), to facilitate visual comparison with wild-type, CIP-sensitive *E. coli*. Error bars represent standard error for triplicate lineages within each exposure group.

From Figure 3-3, all lineages were initially sensitive to CIP with initial $\log_2$(MIC) ≈ -7. This value corresponds to a concentration of 0.01 µg/mL (10 µg/L), which is comparable with previously reported MIC values for wild-type, CIP-susceptible *E. coli* (Andrews, 2001; Becnel Boyd et al., 2009; Gullberg et al., 2011; Sharma et al., 2014). Within four days, lineages exposed

to the untreated CS exhibited markedly increased CIP resistance. The MIC exhibited by this lineage continued to rapidly increase before ultimately achieving a plateau of $\log_2$(MIC) = -1, which corresponds to 0.5 μg/mL (500 μg/L). The plateau concentration is 20-fold higher than the CIP concentration used to cultivate the CIP-exposed lineage. It is also 8-fold higher than the epidemiological cutoff (ECOFF) for CIP-resistant *E. coli* (0.064 μg/mL or 64 μg/L), as designated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) ("The European Committee on Antimicrobial Susceptibility Testing," 2018). In contrast, we did not observe a change in measured MIC for lineages exposed to EA, LC, or the various media controls. After ten days (~80 generations), MIC values for these lineages were still within the range 0.0078-0.016 μg/mL (7.8-16 μg/L), consistent with EUCAST's characterization of CIP-susceptible, wild-type *E. coli* ("The European Committee on Antimicrobial Susceptibility Testing," 2018). Thus, the EA samples and LC samples did not induce CIP resistance in *E. coli* during long-term exposures. This result offers definitive indication that the algae treatment reduces not only CIP parent compound concentration but also its acute antibiotic potency and its capacity to elicit antibiotic resistance over time.

As in the acute exposure assay, we did not observe a difference in residual effect between the EA samples (Figure 3-3B) and LC samples (Figure 3-3C) during the longer-term exposure assay. That is, *E. coli* exposed to EA and LC samples exhibited no statistically significant difference in MIC value. This result is a subtle but potentially important observation as it provides insight about how the proposed treatment delivers its desired outcomes. For example, the LC results reveal that light exposure alone enables rapid transformation of CIP into photo-products with marked reductions in both acute antibiotic potency and capacity to elicit antibiotic resistance over time. This result is intriguing, since most conventional treatments do not incorporate

substantial sunlight exposure. However, it may be premature to conclude that the principal benefit of the proposed algae treatment is its ability to leverage "free" sunlight-powered photo-transformations. As we have seen, the addition of active algae biomass markedly increases overall reduction in CIP concentration. Further, it has been previously shown that algae treatment is less energy consuming than conventional tertiary treatments such as UV irradiation (Colosi et al., 2015).

3.3.4 Characterizing the Genetic Basis for Observed Phenotype CIP Resistance

Having observed that the algae treatment reduces elicitation of phenotype-level CIP resistance, as revealed by no increase in MIC over time, it was also of interest to investigate the genetic basis for this effect. We hypothesized that there may be relevant mutations present in the genomes of the CIP-resistant *E. coli* lineages that were absent from the non-resistant lineages cultivated in the treated effluents or media controls. Previous literature indicated that CIP resistance in *E. coli* and other bacteria corresponding with a high MIC to CIP is most frequently correlated with mutations in the QRDR of two genes: *gyrA* and/or *parC* (Chung et al., 2017; Hu et al., 2017; Johnning et al., 2015; Kim et al., 2012; Mavroidi et al., 2012; Morgan-Linnell et al., 2009). The presence or absence of mutations in the QRDR of these two genes was determined with Sanger sequencing and compared to mutations found in literature (Table 3-1).

Table 3-1. Mutations in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC* genes for *E. coli* lineages arising from adaptive laboratory evolution (ALE). Different lineages correspond to propagation in algae treated (EA) samples, light-exposed (LC) samples, or an untreated CIP standard (CS). Genomes from these lineages were compared to the Day-0 ancestors that had never been exposed to CIP.

Exposure Condition	**Replicate #**	**Observed Mutations in QRDR**	
		gyrA	***parC***
Ancestors *(Pre-ALE)*	1	None	None
	2	None	None
	3	None	None
Untreated CIP standard (CS) *(Post-ALE)*	1	Ser83Leu	None
	2	Asp87Gly	None
	3	None	None
Algae treated (EA) samples *(Post-ALE)*	1	None	None
	2	None	None
	3	None	None
Light control (LC) samples *(Post-ALE)*	1	None	None
	2	None	None
	3	None	None

The results in Table 3-1 confirm our observations in Figure 3-3. Two of the three *E. coli* lineages cultivated in untreated CS developed mutations in the QRDR of *gyrA*. Both mutations resulted in amino acid substitutions that have been associated with increased resistance to CIP in

literature (Chung et al., 2017; Hu et al., 2017; Johnning et al., 2015; Morgan-Linnell et al., 2009). In contrast, the *E. coli* lineages cultivated in treated samples did not develop mutations in the QRDR of *gyrA*, which is consistent with our observations from Figure 3-3. Across all lineages, no mutations in the QRDR of *parC* were detected; however, existing literature indicates that mutations in the QRDR of *gyrA* have been observed at a higher frequency in CIP resistant *E. coli* than *parC* mutations (Chung et al., 2017; Hu et al., 2017; Johnning et al., 2015; Morgan-Linnell et al., 2009). The third replicate of *E. coli* exposed to untreated CS did not develop mutations in the QRDR of *gyrA*. This result is unexpected given that this replicate, as well as replicates 1 and 2, developed a high MIC to CIP during ALE (Figure 3-3A). However, it is possible that this replicate contains a mutation within a gene region that we did not assess for this study such as *gyrB* or *parE*. These gene regions are less frequently associated with CIP resistance.

3.4 Conclusions

The results of this study demonstrate the efficacy of the algae treatment in reducing not only the CIP parent drug concentration, but also its corresponding acute potency and capacity to stimulate antibiotic resistance during chronic exposure to a model bacterium. It is pertinent to conduct comprehensive effects-based evaluations of all candidate treatment technologies to assess whether removal of a parent drug compound delivers reduced capacity of the effluent to elicit antibiotic resistance downstream. In our case, algae-mediated CIP transformation did correspond with a reduced likelihood of stimulating antibiotic resistance, but some studies have shown no correlation between reduction of the parent drug compound and reduction in stimulating antibiotic resistance. Therefore, previous studies of other candidate treatments should incorporate effects-based methods to validate their parent drug compound removal results. ALE is a valuable tool to achieve this goal. Beyond this, it remains to be seen whether the treatment is also effective in

deactivating another WWTP effluent constituent that is known to stimulate antibiotic resistance in the downstream environment; namely, antibiotic resistance genes (ARG). This assessment forms the basis for work presented in Chapter 5, with a goal of providing a more robust evaluation of the performance of the proposed algae treatment.

Supplementary Information: Chapter 4

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Chapter 4: Objective 1, Supplementary Information

Evaluating the efficacy of an algae-based treatment to remove an antibiotic compound and its corresponding antibiotic resistance effects

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4.1 Algae-mediated removal of CIP

4.1.1 Algae Cultivation

4.1.1.1 Chemicals and Medium

We purchased reagent-grade ingredients for algae culture media and other chemicals from Fisher Scientific Inc. (Pittsburgh, PA). HPLC-grade analytical solvents (acetonitrile and methanol) were from Sigma-Aldrich (St. Louis, MO). We prepared CIP standards over the range of 0-250 μg/L via serial dilution in an HPLC-grade solvent mixture of formic acid, methanol, and acetonitrile (2/49/49 v/v) from a 0.25-g/L stock solution of CIP in the same solvent mixture.

4.1.1.2 Growth Curves

In order to assess the potential potency of CIP to our model alga, *S. dimorphus*, We assessed and compared its growth over time with or without 25 μg/L of CIP. We sampled from triplicate reactors for each condition at predesignated time intervals over 144 hours and measured growth via optical density at a wavelength of 662nm. The results of the analysis are presented in Figure 4-1. Based on these data, we observed no statistically significant difference in growth with and without 25 μg/L CIP. This confirms that CIP does not inhibit algae growth at this concentration.

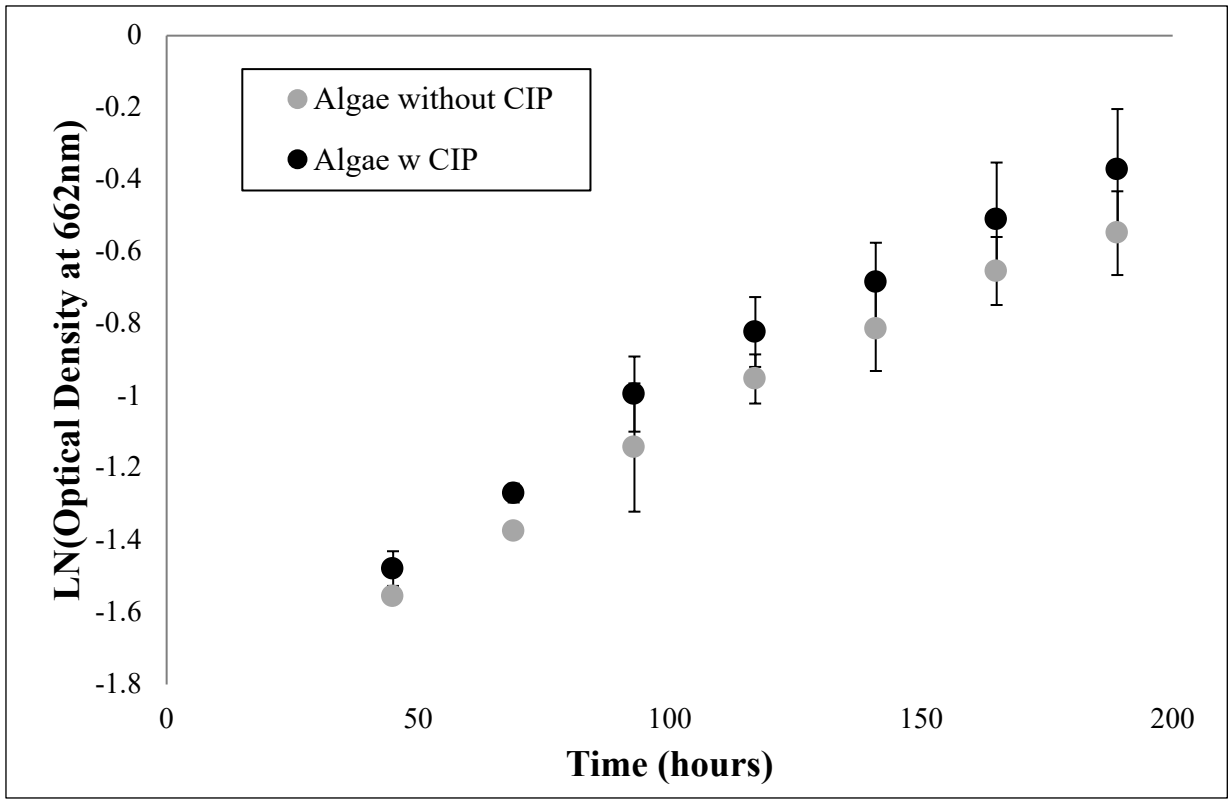

Figure 4-1. Algae growth over time during exponential phase, in the presence and absence of 25 µg/L CIP. Error bars represent 90% confidence for triplicate reactors. P-value for the t-test to compare two regression slopes (assuming non-pooled standard error) is 0.30. This analysis was conducted using the Real Statistics Resource Pack software (Release 5.4). Copyright (2013 – 2018) Charles Zaiontz (www.real-statistics.com).

4.1.2 SPE Protocol

In order to concentrate and purify our experimental samples, we applied a solid phase extraction (SPE) method based on previously validated methods from Zhang et al. 2014 and Zhou et al. 2012 (Zhang et al., 2014; Zhou and Jiang, 2012). We pretreated Oasis® HLB SPE 3cc (160 mg) cartridges with 1 mL of methanol and equilibrated with 1 mL of HPLC-grade water. We then loaded 10-mL samples at a rate of 5 mL/min. Next, we washed the loaded cartridges with 2 mL of

a 50:50 (v/v) HPLC-grade methanol and water solution, vacuum dried them for 15 minutes, and then eluted them in 4 mL of a 2/49/49 formic acid-methanol-acetonitrile (v/v/v) solvent mixture. We then sealed the eluted samples in amber, crimp-top HPLC vials.

4.1.3 Approaches to Account for CIP Sorption

The unique characteristics of both the sorbate (CIP) and sorbent (algae) made it challenging to develop a method for allocating the contribution of sorption to the total apparent CIP removal observed in our EA reactors. We therefore applied two literature protocols to assess the contribution of sorption to CIP apparent removal. One protocol was from Wu et al. 2013, and the other was from Zhang et al. 2014 (Wu et al., 2013; Zhang et al., 2014).

The protocol from Wu et al. 2013 is optimized for assessing the desorption of CIP from clay minerals. Although the sorbate is the same between our experiment and the one conducted by Wu et al. 2013, our sorbents have very different properties. Following a modification of the method from Wu et al. 2013, we measured the sorption of CIP to live algae cells in the experimental algae (EA) reactors at t = 24 hours (to capture possible instantaneous sorption) and 144 hours (to capture overall net sorption) using the following steps:

1. Centrifuge 45-mL samples from each EA reactor at 3000 rpm for 20 minutes to separate algae solids from medium.
2. Decant supernatant (algae medium) and add 20 mL of 50-mM $AlCl_3$ solution to the algae biomass in the tube. Shake to combine and dissolve algae pellet.
3. Attach the centrifuge tubes to a crab shaker at 25 °C (77 °F) in the dark to prevent further photolysis of CIP; shake for 24 hours.

4. Centrifuge samples at 3000 rpm for 20 minutes and remove 20 mL of liquid into a fresh centrifuge tube for SPE and HPLC analysis.
5. Repeat steps 3-5 until one or more replicates exhibit non-detectable (ND) CIP. ND was operationally defined as < 10% of the minimum detection level (see Chapter 3, Section 3.2.1).

Using this approach, and extrapolating from our calibration data (see Figure 4-4) we estimated that sorption may have accounted for < 1% of CIP apparent removal at t = 24 hours and that this was unchanged after t = 144 hours.

The protocol developed by Zhang et al. 2014 assesses the sorption of estrogens to *S. dimorphus* with a separate set of experimental control reactors. They used an autoclaved algae sorption control (AASC), comprising autoclave-deactivated algae biomass spiked with 5 μg/L of estrogens and completely covered (to prevent light penetration) to account for sorption of the estrogens to the algae. Samples from the AASC reactors were filtered to remove the algae cells and the filtrate was collected to assess estrogen concentrations at the start and end of the experiment using HPLC. The difference between the concentration of estrogens spiked into the reactor at the start of the experiment and the concentration of estrogens measured in the filtrate is the concentration of estrogens that sorbed to the algae. However, it is uncertain whether or not autoclaving the algae changes its sorptive properties, so it is possible that this method may not be entirely representative of the sorption that may occur in the EA reactors with live algae.

Based on the method from Zhang et al. 2014, we also used a set of two AASC reactors, which comprised of 50 mg/L of previously autoclave-deactivated (60 minutes at 121 °C and 260 psi/°F) algae biomass and media spiked with 25 μg/L CIP and wrapped in foil to prevent light

penetration. These reactors were cultivated under stirring, aeration, and illumination conditions for 144 hours as described in Chapter 3, Section 3.2.1. Samples from the AASC reactors were collected at the start and end of the experiment, filtered through 0.7 μm pore size glass microfiber filters to remove the algae cells, and residual CIP concentration in the filtrate assessed using SPE and HPLC.

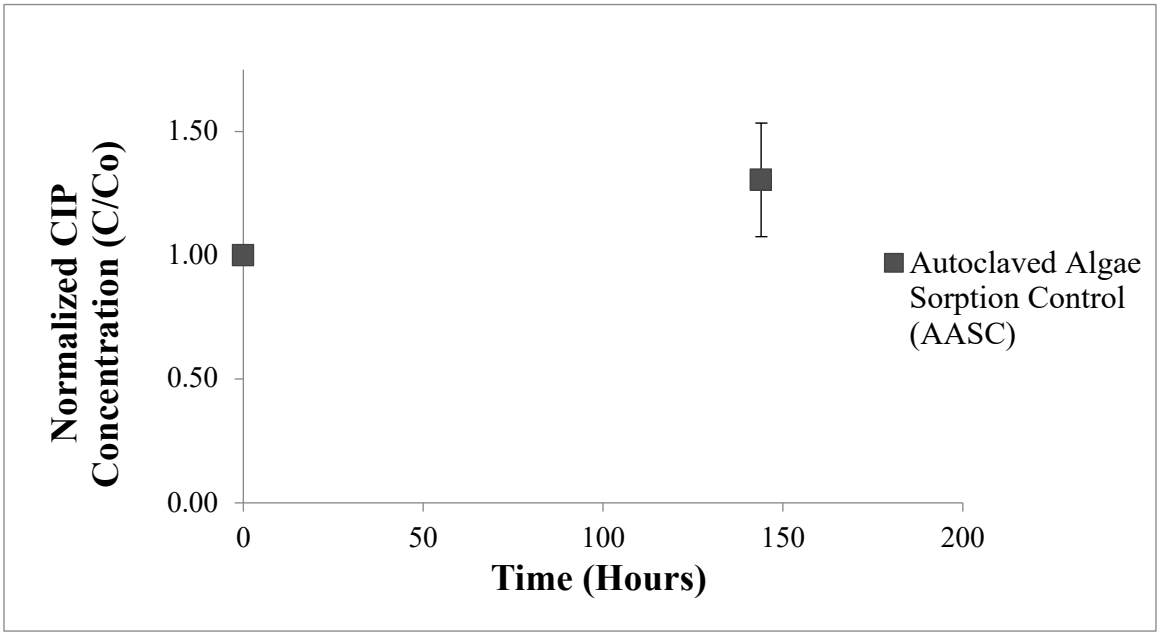

Figure 4-2. Normalized (C/C_0) concentrations of ciprofloxacin (CIP) over time for autoclaved algae sorption control (AASC) reactors over 144 hours. Error bars are standard error for the duplicate AASC reactors.

The CIP concentrations in the filtrate of our AASC reactors at the start and end of the experiment do not decrease over time as shown in Figure 4-2. This result indicates that the CIP did not sorb to the autoclaved algae in the reactor. This result suggests that sorption was not a significant contributor to CIP apparent removal observed in the EA system.

Based on our observations with both of these methods, we conclude that sorption of CIP to algae does not play a significant role in the overall apparent CIP removal.

4.1.4 HPLC Method, Chromatogram, and CIP calibration curve

We used a Shimadzu 2010-AB high performance liquid chromatography (HPLC) with fluorescence and UV detectors to measure CIP concentrations based on previously validated methods (Idowu and Peggins, 2004; Lee et al., 2007; Muchohi et al., 2011; Piñero et al., 2013). We used the fluorescence detector as the primary detector. Excitation and emission wavelengths were 280 and 445 nm, respectively. The mobile phase comprised a mixture of: A) 1% formic acid in DI water (74% of total flow); and B) 50:50 methanol and acetonitrile (16% of total flow). This was pumped isocratically at 0.5 mL/min. We used a 125 mm x 3.2 mm C18 column (Phenomenex) and a sample injection volume of 20 µL. Under these conditions, CIP retention time was 1.9 minutes and limit of detection was 2 µg/L.

HPLC Chromatogram

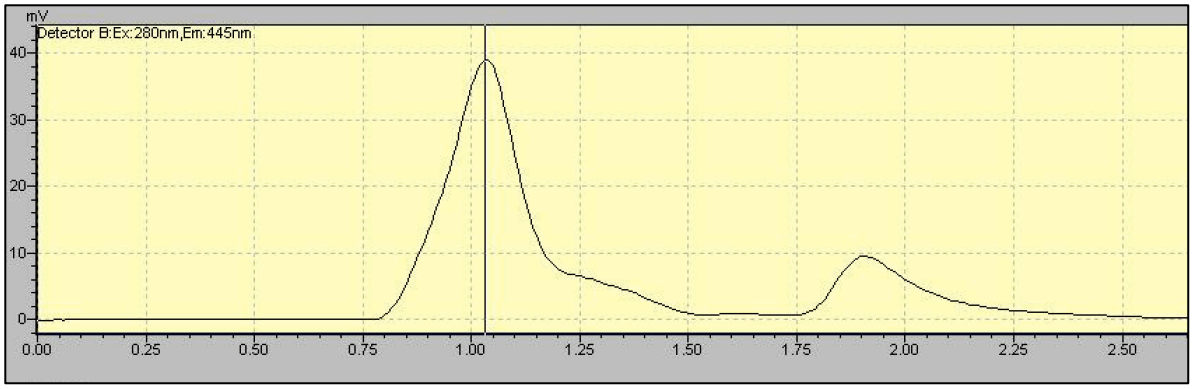

Figure 4-3. Example chromatogram for 80 µg/L CIP standard in a mixed solution of formic acid/acetonitrile/methanol (2/49/49) under HPLC (Shimadzu) fluorescence detection. CIP elutes at roughly 1.9 minutes. Formic acid in the mobile phase generates a peak at roughly 1.0 minute.

CIP Calibration Curve

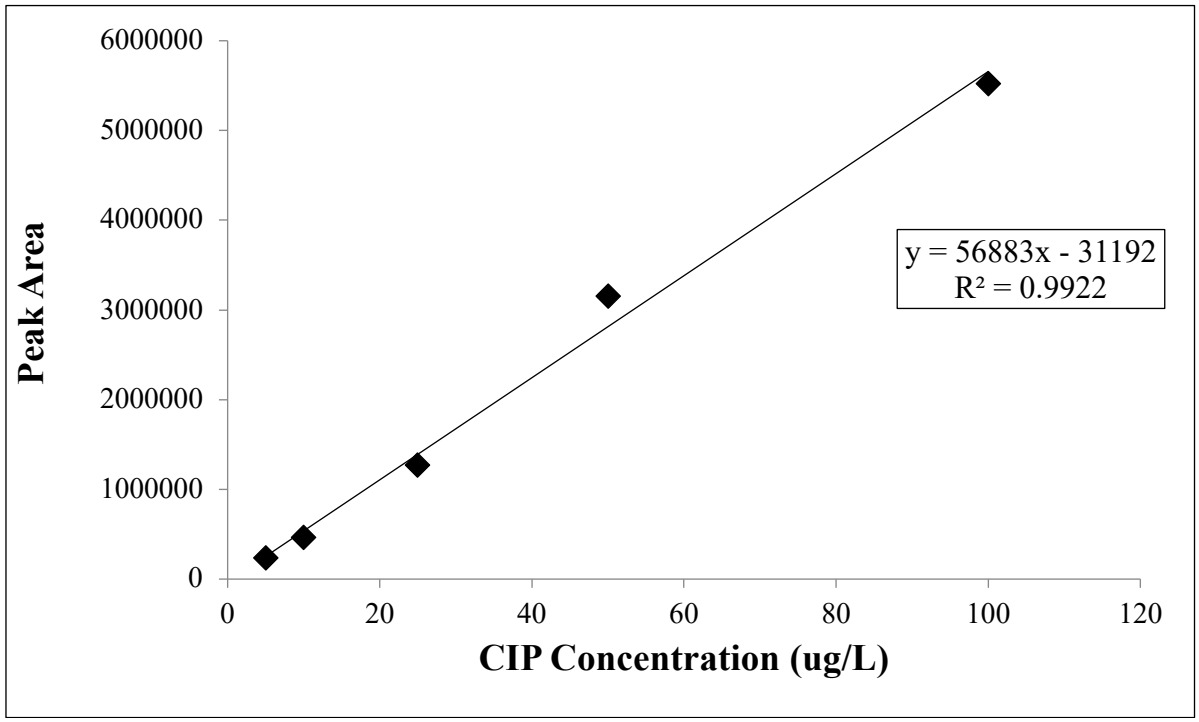

Figure 4-4. Calibration curve for CIP concentrations over the range 2-100 µg/L. The minimum detection level of CIP for this method was 2 µg/L.

The minimum detection level of CIP for this method is 2 µg/L. This was the lowest standard for which peak area could be reproducibly quantified (< 1% variation over 5-10 injections). Our concentration factor for SPE was 4X. Our SPE recovery was approximately 100%.

4.2 Assessment of Residual Acute Antibiotic Potency

4.2.1 E. coli Preparation

Our *E. coli* stock culture was made in lysogeny broth (LB) and stored at -20 °C in a 25% glycerol solution. We prepared fresh *E. coli* cultures from a frozen stock at the start of each exposure experiment, adding 100 µL of the concentrate pellet to 50 mL of autoclaved LB in a 250-mL flask. We incubated this culture with 200-rpm shaking for 12 hours at 37 °C, then centrifuged and poured off the supernatant. We determined culture CFUs using the IDEXX protocol and compared the results with OD_{625}. The concentration of the stock was 2.6 x 10^{12} CFU/100 mL, which we then diluted 1:1000 with fresh LB to use in experiments.

4.2.2 Lysogeny Broth (LB) Recipe

To make lysogeny broth (LB) culture media, we added 0.5 g yeast extract, 0.5 g sodium chloride, and 0.25 g tryptone to 50 mL of DI water in a 250-mL flask, mixed until completely dissolved, and autoclaved.

4.2.3 Controls used for CIP Acute Potency Bioassay

Table 4-1. Micro-well contents for the CIP acute potency bioassay

Control	**Supplemented with concentrated LB nutrients?**	**CIP, concentration (µg/L)**	***E. coli*, stock concentration (CFU/100mL)**
A_{max}[1]	Yes	No, 0	Yes, $2.6x10^9$
A_{min}[2]	Yes	No, 0	No, 0
Untreated CIP Standard (CS)	Yes	Yes, 25	Yes, $2.6x10^9$
Treated samples from algae (EA) and light-exposed (LC) experimental reactors	Yes	Yes, residual CIP concentration and by-products measured at 144 hours	Yes, $2.6x10^9$

[1]Absorbance of a positive control corresponding to bacterial growth in LB with no CIP (i.e., 100% growth)

[2]Absorbance of an abiotic negative control comprising LB with no bacteria and no CIP (i.e., 0% growth)

4.3 Assessment of Residual Capacity to Elicit Antibiotic Resistance

4.3.1 Calculation of Number of Generations

The doubling time and total number of generations during our 10-day ALE experiment were estimated as follows. Each day, bacterial cultures were propagated to fresh media with a dilution factor of 1/250. Prior to dilution, the optical density at 600nm (OD_{600}) of each culture was measured. The OD_{600} values of the daily cultures remained relatively stable throughout the evolution experiment. The number of generations per day was therefore calculated as

log(250)/log(2), or approximately 8 generations per day. The 10-day adaptation experiment therefore accounted for about 80 generations.

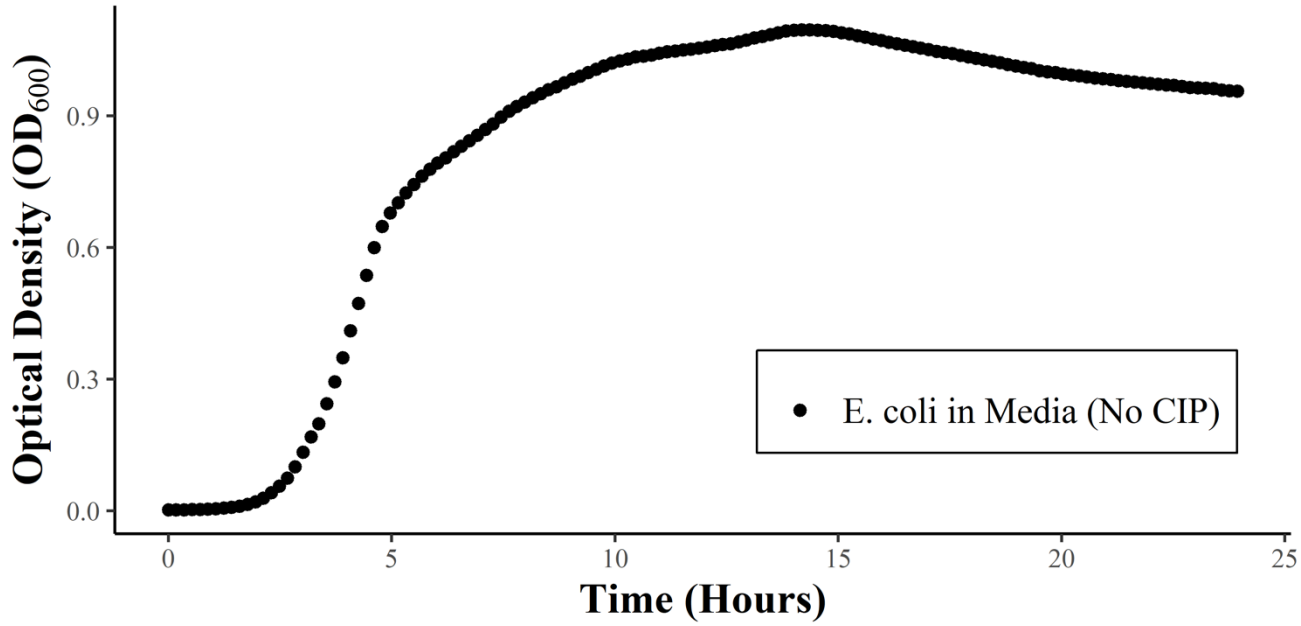

Figure 4-5. Background subtracted growth curve for *E. coli* grown in LB media without CIP. Optical density readings were taken every 10 minutes and the background blank media was subtracted (black). Exponential growth ends after roughly 10 hours and stationary phase continues until roughly 23 hours. We chose to incubate all lineages for the full 23-hour growth phase to allow ample time for each lineage to reach stationarity.

4.3.2 MIC Plate Dilution Calculations for E. coli

Table 4-2. CIP concentration gradient in each well to assess MIC of *E. coli* to CIP during ALE

	1	2	3	4	5	6	7	8	9	10	11	12
A	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
B	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
C	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
D	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
E	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
F	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
G	[0 ug/mL]	[0.0039 ug/mL]	[0.0078 ug/mL]	[0.0156 ug/mL]	[0.03125 ug/mL]	[0.0625 ug/mL]	[0.125 ug/mL]	[0.25 ug/mL]	[0.5 ug/mL]	[1 ug/mL]	[2 ug/mL]	[4 ug/mL]
H	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank

4.3.3 Reference MICs for CIP-susceptible E. coli and CIP

Table 4-3. Reference MICs of CIP for "wild-type" (i.e. CIP-susceptible) *E. coli*

MIC (µg /mL)	Source
0.023	Gullberg, E., et al. 2011
0.015	Andrews, J.M., 2001
0.017	Boyd, L.B., et al. 2009
0.015 - 0.03	Sharma, R. et al. 2014

4.3.4 Sanger Sequencing – Primer Design

Table 4-4. Primers used in this study.

Gene	Primer (5'-3')	Gene Size (base pairs)	PCR Annealing Temperature (°C)	Melting Temperature (°C)
gyrA Forward	GCTCCCTTTTGGCATGAAGC	2662	57	62.4
gyrA Reverse	GCGGTTAGATGAGCGACCTT		57	62.4
parC Forward	GATCTCCTGTGACTCGACGC	2433	57	64.5
parC Reverse	GTTGAATACGCTGCCGGATG		57	62.4

4.3.5 Retrospective MIC Analyses

We retrospectively re-measured the 10-day MIC values for the third algae treated (EA) replicate due to suspected contamination during the Day 1 lineage propagation. Following protocol used by Yen and Papin, 2017, we revived the Day-0 frozen stock of ancestor 3 and plated it on an LB agar plate incubated for 23 hours at 37 °C (Yen and Papin, 2017). After incubation of the Day-0 ancestor 3, we performed the same process of single colony inoculation, daily propagation into algae treated exposure media, sample storage, and MIC plating for ten days as described in Section 3.2.3 of the manuscript methodology. Our retrospective analysis of the EA replicate 3 lineage resulted in MIC data that was consistent with that of EA replicates 1 and 2.

Each MIC assay contained seven rows of identical CIP concentration gradient (technical replicates) and 1 row of blank media as a sterility control. When assessing daily MICs for each lineage, we used the criteria that there had to be a 4-row MIC consensus in order to conclusively designate a MIC for each lineage each day. For assays that did not meet the 4-row MIC consensus on specific days, we revived frozen stock cultures of the corresponding lineage from the day prior to the MIC error and inoculated directly into fresh exposure media. We then incubated the revived cultures for 23 hours at 37 °C with 160-rpm shaking and diluted cultures to an OD_{600} of 0.01 for MIC plate analysis as described in Section 3.2.3 of the manuscript methodology. Our retrospective analysis of individual lineage MIC plates resulted in better 4-row MIC consensus and data that was consistent with the other biological replicates for each condition.

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Chapter 5: Objective 2, Algae Treatment of a Model ARG Plasmid

This chapter will result in one publication.

Grimes, K. L., Dunphy, L. J., Kolling, G. L., Papin, J. A., Colosi, L. M. (2020) "Assessing an algae-based treatment to deactivate a plasmid carrying tetracycline resistance." *(In preparation)*

5.1 Introduction

In addition to the untreated antibiotic drug compounds and their principal metabolites, wastewater treatment plant (WWTP) effluents are also a source of antibiotic resistance genes (ARG) into the environment, which can stimulate antibiotic resistance in environmental bacteria (Marti et al., 2013). Objective 2 of this dissertation focuses on methodological development to assess of the efficacy of the algae treatment in degrading or deactivating ARG; as measured using two different approaches: qualitative polymerase chain reaction (qPCR) and transformation assay.

ARG can exist as two general classes: (1) as part of the organism's genome (i.e., "genomic ARG"); or (2) on a separate genetic structure that can replicate independently of its genome (i.e., "plasmid-mediated ARG"). Genomic ARGs are transmitted to other organisms primarily via vertical gene transfer. This refers to the process by which a viable bacterium replicates its genome and divides into two identical cells. In contrast, plasmid ARG may be transmitted to other organisms either vertically or horizontally. Horizontal gene transfer comprises the process by which viable bacteria share genes with other viable bacteria within the same generation, or viable bacteria take up free-floating genes from the surrounding environment (von Wintersdorff et al., 2016). This dissertation will focus on algae treatment of a plasmid-mediated ARG, since it is presumed that bacteria carrying genomic ARG will not be able to transmit resistance to other organisms (i.e., their offspring) once they have been killed via disinfection. Also, horizontal gene transfer is of much greater concern for public health than vertical gene transfer because of the potential for genes to be shared among bacteria species and in particular, potential for non-pathogenic bacteria to share ARG with pathogenic bacteria and then rapidly proliferate (Guo et al., 2017; Li et al., 2010; von Wintersdorff et al., 2016).

Existing literature indicates that ciprofloxacin (CIP) resistance is predominantly encoded via genomic ARG in environmentally relevant bacteria; e.g., *Escherichia coli* (Chung et al., 2017; Hu et al., 2017; Johnning et al., 2015; Kim et al., 2012; Mavroidi et al., 2012; Morgan-Linnell et al., 2009). This makes CIP-relevant ARG somewhat uninteresting in the context of evaluating ARG deactivation. Therefore, Objective 2 experiments focus on a model plasmid, pEX18Tc, that contains the *tet* gene encoding resistance to tetracycline. Like CIP, tetracycline is a widely prescribed, therapeutically important antibiotic drug. Plasmids encoding tetracycline resistance have been measured in WWTP influent and effluent (Dodd, 2012; Guo et al., 2017; Hultman et al., 2018; Karkman et al., 2017; May et al., 2009). Also, the genes that encode tetracycline resistance may also confer resistance to certain other antibiotics (i.e., multi-drug resistance) such that our findings will have relevance to not only tetracycline resistance mitigation but also other kinds of resistance mitigation (May et al., 2009).

Currently, there is limited information available on the fate of ARG in and downstream of WWTPs. Also, thousands of ARG have been identified, which makes them difficult and expensive to assess on an individual basis. As discussed in Chapter 1, preliminary reports reveal that different wastewater treatments are effective against various ARG to different extents. For example, in a study at a WWTP in Cluj-Napoca, Romania, elevated levels of ARG and ARB were measured 10 km downstream of the plant indicating that the primary, secondary, and tertiary (chlorination) treatments of the plant were ineffective at deactivating ARG (Lupan et al., 2017). Likewise, ARGs encoding tetracycline resistance were detected within and downstream from an oxytetracycline production facility WWTP in China. They were not detected upstream of the plant, which indicates that the treatment processes directly contributed ARG to the receiving water (Li et al., 2010).

As discussed in Chapter 1, lab-scale studies of different types of tertiary wastewater treatments have demonstrated varying levels of effectiveness toward deactivating ARGs. Additionally, some studies have confirmed instances of ARG reactivation after application of traditional wastewater treatments such as UV and ozone. Previous studies have not assessed the effectiveness of an algae treatment system toward ARG deactivation. The energy benefits of integrated algae wastewater systems have been previously established (Colosi et al., 2015; Ge et al., 2009; Menger-Krug et al., 2012; Shi et al., 2010; Zhang et al., 2014).

We investigated algae treatment of the pEX18Tc tetracycline resistance conferring plasmid with two key objectives: (1) quantify plasmid *degradation* via qPCR; and (2) assess plasmid *deactivation* via transformation assays. This chapter also emphasizes the method development for assessing algae treatment of ARGs. In the literature, qPCR is often used to quickly quantify ARG in environmental systems. To align our results with those of other ARG studies, we worked to develop a comparable qPCR method for the algae treatment system, but were ultimately unsuccessful. A few studies have also used transformation assays, which are culture-based assessments, to evaluate ARG deactivation. Transformation assays provide a better interpretation of a treatment's ability to reduce the capacity of the ARG to transform in other host cells via horizontal gene transfer. Therefore, we prioritized the development of a transformation assay over qPCR for our assessment of the algae treatment of plasmid pEX18Tc. The objectives of this study build on our previous work to assess the efficacy of a single treatment to remove an antibiotic parent compound, reduce its acute and chronic potency effects, and deactivate ARG.

5.2 Materials and Methods

5.2.1 Algae Treatment

5.2.1.1 General Experimental Approach

We exposed an ARG plasmid stock solution to a model algae treatment to assess the treatment efficacy toward deactivating the ARG. We also exposed the plasmid stock solution to relevant controls to ascertain potential effects of concurrent deactivation processes in the algae treatment. We used qPCR, an assay commonly used for ARG detection, to assess changes in plasmid quantity. We designed a short-amplicon qPCR method (< 200 bps) similar to those commonly used for ARG detection as well as a long-amplicon qPCR method (~1200 bps) to assess impacts over the entire ARG (1190 bps). In a separate analysis, we evaluated reduction in ARG transformation efficiency for untreated versus treated plasmid stock solutions, to qualitatively characterize the extent to which algae treatment mediates the risk of ARG dissemination via discharged wastewater effluents. Our use of the transformation assay as an effects-based assessment offers some insight into the effects of treated ARG on downstream organisms in environmental settings.

5.2.1.2 Algae Cultivation

Our research group maintains pure cultures of the freshwater algal species *Scenedesmus dimorphus* (UTEX 1237) for use in the treatment experiments as previously published. (Grimes et al., 2019; Zhang et al., 2014)

5.2.1.3 ARG Plasmid Selection, Preparation, and Extraction

We assessed plasmid pEX18Tc (originally provided by Dr. Joe J. Harrison, University of Calgary, Calgary, Canada), which is a multi-host cloning vector that carries the *tet* resistance gene (Hmelo et al., 2015). The pEX18Tc was propagated in *E. coli* strain DH5α and extracted for use in the plasmid treatment experiments.

We performed a two-step *E. coli* cultivation procedure to produce enough plasmid copies to prepare stocks for various experiments. First, we inoculated host *E. coli* carrying the plasmid in 5 mL of lysogeny broth (LB) with 10 μg/mL tetracycline and incubated at 37 °C with 200-rpm shaking for 24 hours (See Chapter 4, Section 4.2.2 for LB recipe). Next, we propagated the 5-mL cultures into 150 mL of LB with 10 μg/mL tetracycline and incubated at 37 °C with 200-rpm shaking for an additional 24 hours before subsequent plasmid extraction.

Initially, we used the QIAPrep Spin MiniPrep Kit (Qiagen, Valencia, CA) according to kit instructions to extract the plasmid from the host *E. coli*. Plasmid yields were consistently around 50 ng/μL, as measured with a DeNovix DS-C spectrophotometer using the DeNovix dsDNA broad range assay (DeNovix, Wilmington, DE). When added to the 150-mL reactor volumes used in our treatment experiments, the initial plasmid concentration was reduced to 0.016 ng/μL. During preliminary qPCR analyses, we determined that the 0.016 ng/μL initial plasmid concentration was too low for subsequent detection during our treatment experiments.

In order to increase the plasmid yield from the host *E. coli*, we used the ZymoPURE II Plasmid MaxiPrep Kit (Zymo Research, Irvine, CA) according to kit instructions. Plasmid yields were approximately 2000 ng/μL, which, when added to the 150-mL reactor volumes used in our treatment experiments, was reduced to 2 ng/μL. The higher plasmid yield with the ZymoPURE II Plasmid MaxiPrep Kit improved plasmid detection for our analyses.

5.2.1.4 Algae Treatment Setup

We prepared experimental and control solutions in modified Bold 3N medium (MB3N) using 250-mL reactors containing 150-mL liquid volume for each condition. The experimental and control conditions included: 1) the algae treatment, which comprised 50 mg/L algae biomass in MB3N, exposed to light, and contained 5.5 ng/μL plasmid; 2) an autoclaved algae control, comprising 50 mg/L autoclaved algae biomass in MB3N with 5.5 ng/μL plasmid and covered with aluminum foil to prevent light exposure; 3) a light control, comprising 5.5 ng/μL plasmid in MB3N without algae and exposed to light; and, 4) a dark control, comprising 5.5 ng/μL plasmid in MB3N without algae and covered with aluminum foil to prevent light exposure. Blank controls containing MB3N and no plasmid were used as negative controls for plasmid analysis via qPCR and transformation assays. All reactors were cultivated under stirring, aeration, and 12 hours of illumination per day (cool white full-spectrum fluorescent growth lamp with 125 W 6500 K, 30 $\mu W/cm^2$ UV penetration) for the treatment duration.

We collected 5-mL samples from all reactors at time 0 and every 24 hours thereafter. We measured optical density at 662 nm (OD_{662}) to quantify algae biomass concentrations in the algae treatment series and autoclaved algae series at time 0 and every 24 hours thereafter. We syringe-filtered the collected samples from all reactors through 0.7-μm pore size glass microfiber filters (Millipore) prior to qPCR and transformation assays. Filtered samples were wrapped in aluminum foil to prevent light exposure and stored at -20 ºC until analysis.

5.2.2 Quantification of ARG Plasmid

5.2.2.1 Primer Design

We selected primers using the NCBI primer BLAST tool for plasmid pEX18Tc (GenBank Accession Number: AF047519.1) (Hoang et al., 1998). We designed both short and long amplicon primers for the *tet* gene (1190 bps) of plasmid pEX18Tc and optimized one set of primers for each amplicon size (Table 5-1). The short amplicon primers covered a short section of the gene (< 200 bps) and the long amplicon primers covered the entire gene (~1200 bps).

Table 5-1. Long amplicon and short amplicon primers used for the *tet* gene of pEX18Tc qPCR.

Gene	Primer (5'-3')	Gene Size (base pairs)	PCR Annealing Temperature (°C)	Melting Temperature (°C)
Long Forward	CATTCAGGTCGAGGTGGC	1203	61	62.2
Long Reverse	CCTGGATGCTGTAGGCATAGG		61	64.5
Short Forward	GCTCTCCCTTATGCGACTCC	163	61	64.5
Short Reverse	CTCATGAGCGTTGTTTCGG		61	62.4

5.2.2.2 qPCR Protocol

We performed qPCR using a Bio-Rad CFX autocycler (Bio Rad, Hercules, CA). We tested multiple enzyme kits, but found the KAPA SYBR FAST qPCR Master Mix (2X) Kit (Kapa Biosystems, Inc, Wilmington, MA) to be the most effective for analyzing plasmid pEX18Tc. We performed qPCR in 96-well microplates. Each 25-µL qPCR reaction contained 0.4 µL of forward

and reverse primers at 100 µM, 10 µL of master mix, 1 µL of DNA template, and 8.2 µL of sterile nuclease free water. The qPCR conditions were as follows: one cycle at 95 °C for 3 minutes, 40 cycles of 95 °C for 3 seconds followed by 20 seconds annealing at 61 °C, and a melt curve to assess specificity. Our qPCR method development was inconclusive due to challenges related to primer specificity, background matrix interference, and low plasmid concentration. Our approach to resolving the qPCR challenges is detailed in Section 3.1 of the results and discussion.

5.2.3 Assessment of ARG Plasmid Transformation Efficiency

5.2.3.1 Transformation Assay Protocol

For our transformation assays, we used *Zymo Mix & Go! Competent Cells E. coli* strain DH5α in the 50 µL 96-well plate format (Zymo Research, Irvine, CA). Initially, we performed the assay according to the vendor instructions for tetracycline resistance markers, but we were met with challenges of transformation reproducibility. In order to improve reproducibility, we increased the number of technical replicates for each sample to no less than three and added additional steps to the protocol. We performed the transformation assays as follows: 1) thaw *Mix & Go! Competent Cells* tubes on ice; 2) once thawed, add 2.5 µL filtered sample DNA to each tube; 3) incubate tubes on ice for 30 minutes; 4) "heat shock" tubes at 42 °C for 45 seconds; 5) place tubes immediately back on ice for 2 minutes; 6) add 200 µL of SOC medium to each tube; 7) incubate at 37 °C for 1 hour; and 8) plate 40 µL of sample onto pre-warmed LB plates.

We evaluated plasmid transformation efficiency based on a previously developed method (Chang et al., 2017). Specifically, we quantified the plasmid transformation efficiency by comparing the number of *E. coli* colonies on the nonselective plate to the number of *E. coli* colonies on the tetracycline selective plate, as expressed by Equation 5-1:

Equation 5-1. $$\text{Transformation Efficiency} = \frac{transformant\ CFU\ on\ selective\ plate}{total\ CFU\ on\ nonselective\ plate}$$

where CFU stands for colony forming units.

We evaluated reduction of transformation efficiency by comparing the pre-treated and post-treated samples for each condition. Our negative controls revealed no artifactual growth of DH5α without plasmid exposure on tetracycline selective plates, indicating that the DH5α strain contained no inherent resistance to tetracycline.

5.3 Results and Discussion

5.3.1 Quantification of ARG Plasmid

The overall goal of this study was to determine whether algae-mediated tertiary treatment mediates effective deactivation of the pEX18Tc plasmid. Deactivation effectiveness was assessed in two ways: first by quantifying plasmid reduction, and then by measuring loss of transformation efficiency pre- and post-treatment.

Our first step was to develop a method for quantifying pre- and post-treatment plasmid concentrations using qPCR. qPCR has been widely used in previous studies to monitor the presence and quantity of ARGs in environmental samples; much more so than transformation assays. qPCR has been widely used mostly due to convenience. Method development is typically fairly straightforward, and data collection is fairly straightforward once a robust method is in place. In order to optimize qPCR efficiency, it is recommended to amplify DNA regions within the range of ~75-200 base pairs (bps), since larger amplicons can inhibit amplification (Yuan et al., 2015;

Zhang et al., 2015; Zhuang et al., 2015). However, ARG amplicons are typically much larger than the recommended amplicon size for optimal qPCR, so methods to assess sections greater than 1000 bps in phosphate buffer solution or filtered wastewater matrices have been previously developed (Chang et al., 2017; He et al., 2019; McKinney and Pruden, 2012; Yoon et al., 2017; Zhang et al., 2019). We were unsuccessful in our attempts to develop a qPCR method for the pEX18Tc plasmid as a result of inhibitory effects of the algae matrix and also plasmid concentrations that were below qPCR's reliable detection limit.

The first major challenge we faced with qPCR method development was the fact that our algae treatment needs to operate at a volume of at least 150 mL to maintain normal algal growth. This volume is much larger than what has been used in previously published bench-scale plasmid treatment experiments (Chang et al., 2017; McKinney and Pruden, 2012; Yoon et al., 2017; Zhang et al., 2019). In our case, the plasmid stock (50 ng/µL) was diluted by roughly three orders of magnitude (1,000x) upon introduction to the treatment system. For this reason, our calibration standards for qPCR had to span this entire range plus additional dilute concentrations to reach the desired post-treatment concentration. All told, our plasmid standard serial dilutions spanned 10 orders of magnitude, ranging from a maximum of 6.132 ng/µL to a minimum of 6.132×10^{-10} ng/µL, which would have corresponded with greater than 4 log reduction of the initial in-reactor concentration of 0.016 ng/µL (Table 5-2).

Table 5-2. Concentrations of plasmid pEX18Tc standard series with QIAPrep Spin MiniPrep Kit and anticipated experimental log reduction concentrations of pEX18Tc.

Standard Concentration (ng/μL)	Anticipated Experimental Concentrations (ng/μL)	Percent Removal	log Reduction
6.132	0.016352	Initial (C_0)	
6.13E-01	0.0147168	10% Removal	
6.13E-02	0.012264	25% Removal	
6.13E-03	0.008176	50% Removal	
6.13E-04	0.004088	75% Removal	
6.13E-05	0.0016352	90% Removal	1 log
6.13E-06	0.0008176	95% Removal	
6.13E-07	0.00016352	99% Removal	2 log
6.13E-08	1.6352E-05	99.9% Removal	3 log
6.13E-09	1.6352E-06	99.99% Removal	4 log
6.13 E-10			
Blank			

Figure 5-1 shows one example of a preliminary qPCR standard curve for plasmid pEX18Tc. Typically, samples with a cycle threshold (Ct) value greater than 30 or 35 are considered non-specific amplifications and are therefore deemed below the method's minimum detection level. Only the four plasmid standards with the highest concentrations met the criteria of having Ct values below 30 and generated a calibratable standard curve. Plasmid standard concentrations below 0.00613 ng/μL were not reliably quantifiable, which indicated that we would only be able to capture a maximum of 1 log reduction in our experiments. In order to quantify greater removal extents for plasmid pEX18Tc, we needed a higher initial plasmid concentration.

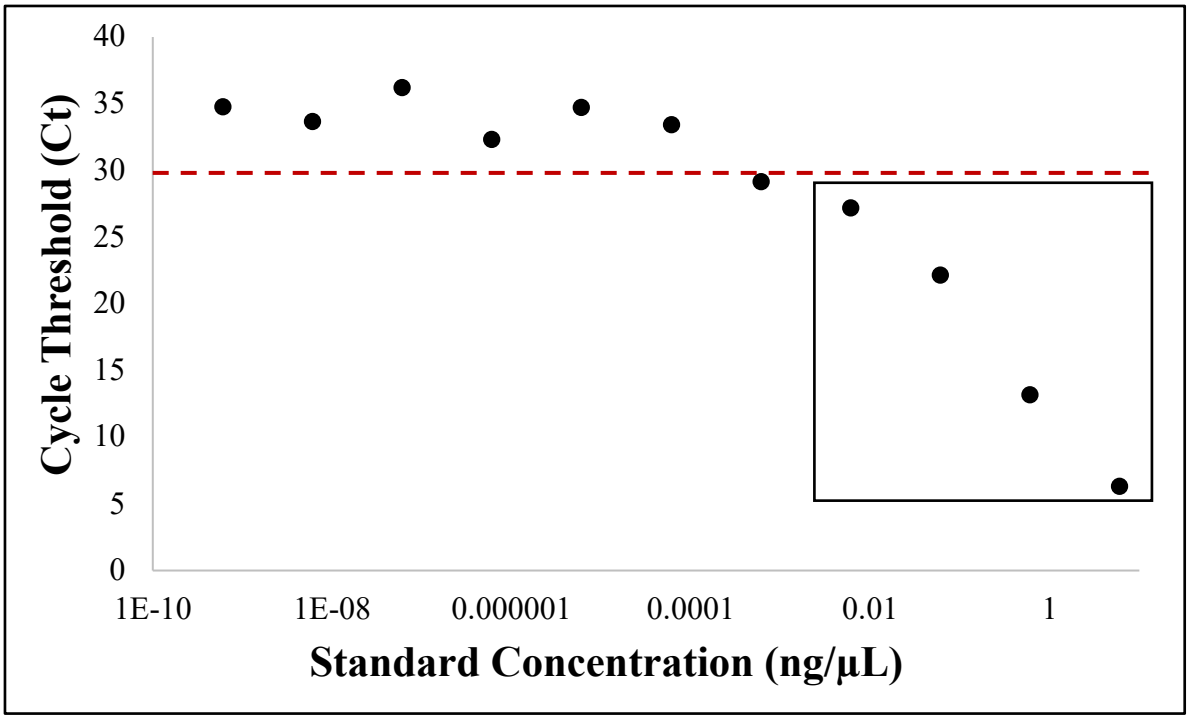

Figure 5-1. Preliminary qPCR standard curve using short amplicon primers for plasmid pEX18Tc. The black box frames the calibratable range of the standards and the red dashed line denotes the qPCR detection limit (Ct = 30 cycles). Standard concentrations are presented in Table 5-2.

We used the high concentration plasmid extraction kit from Zymo to improve plasmid yield and increase our initial experimental concentration. Despite the higher plasmid concentrations (Table 5-3), we continued to observe non-specific amplification at low plasmid concentrations as well as a reduction in standard curve linearity (Figure 5-2). In order to address the standard curve linearity, we generated new plasmid standard serial dilutions and reconstituted the primers, but the inconsistencies in the standard curves persisted.

Table 5-3. Concentrations of plasmid pEX18Tc standard series with ZymoPURE II Plasmid MaxiPrep Kit and anticipated experimental log reduction concentrations of pEX18Tc.

Standard Concentration (ng/μL)	Anticipated Experimental Concentrations (ng/μL)	Percent Removal	log Reduction
1326	3.476666667	Initial (C_0)	
1.33E+02	3.129	10% Removal	
1.33E+01	2.6075	25% Removal	
1.33E+00	1.738333333	50% Removal	
1.33E-01	0.869166667	75% Removal	
1.33E-02	0.347666667	90% Removal	1 log
1.33E-03	0.173833333	95% Removal	
1.33E-04	0.034766667	99% Removal	2 log
1.33E-05	0.003476667	99.9% Removal	3 log
1.33E-06	0.000347667	99.99% Removal	4 log
1.33E-07			
Blank			

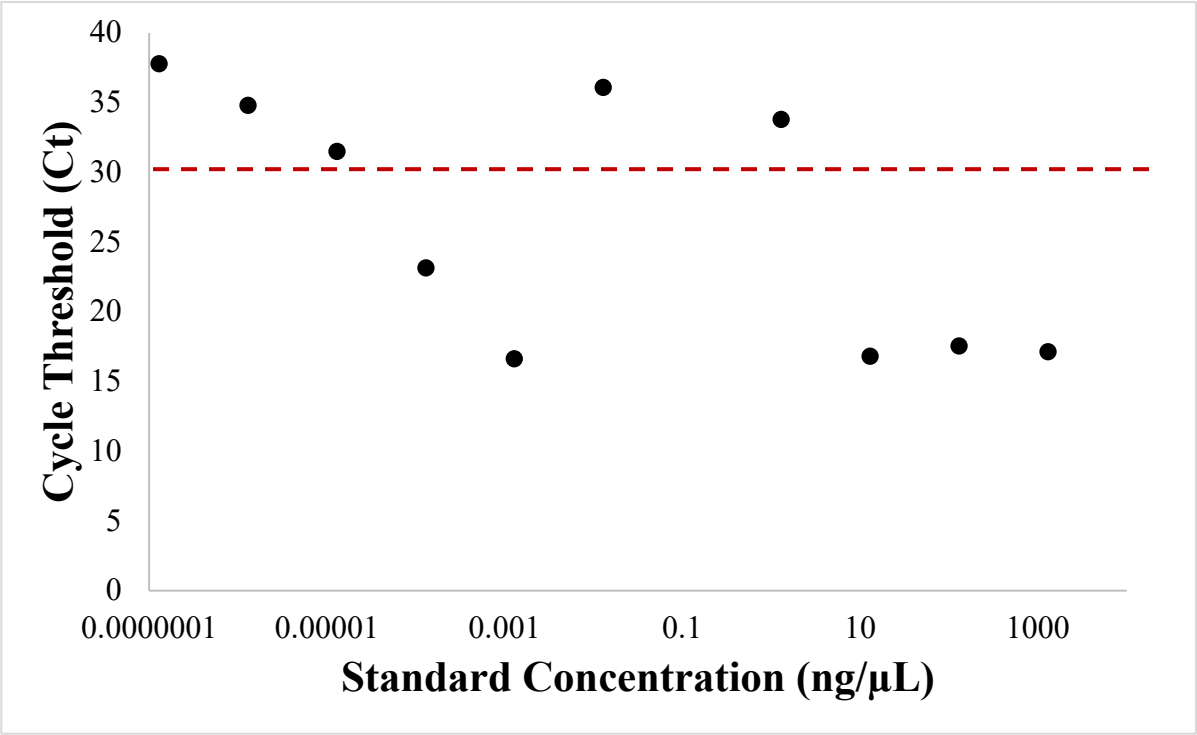

Figure 5-2. Preliminary qPCR standard curve using short amplicon primers for plasmid pEX18Tc. There was no calibratable region for this qPCR run and the red dashed line denotes the qPCR detection limit (Ct = 30 cycles). Standard concentrations are presented in Table 5-3.

The second major challenge we faced with qPCR method development was detecting the plasmid in the algae background matrix. Even with sample filtration to remove algae cells prior to qPCR, we observed inhibitory effects of the filtered algae background matrix on plasmid amplification. This inhibition effect was specific to both the viable (i.e., non-autoclaved) and autoclaved algae matrices since the effect was not evident in the other experimental matrices. To address this phenomenon, we tested sample preparation and cleanup kits such as the DNeasy PowerClean Pro Cleanup Kit (Qiagen, Valencia, CA) to remove potential PCR inhibitors such as humic acids, but we still could not detect the plasmid in the algae matrix. This indicates that the inhibitor in the algae background matrix is likely an uncommon and difficult to remove compound.

In both the algae treatment samples and the autoclaved algae control samples, it was likely that there would be algae DNA in the background matrix. We wanted to be sure that the primers we had optimized for our plasmid would not inadvertently amplify the algae DNA. To address this, we used a ZymoBIOMICS DNA Microprep Kit (Zymo Research, Irvine, CA) on our algae samples to extract the algae DNA and performed qPCR on the samples using our long and short amplicon primers. We ran qPCR on our algae DNA samples both with and without plasmid and observed that both sets of primers were indeed cross-amplifying sections of the algae DNA in samples in the absence of the target plasmid. This indicates that the primers for pEX18Tc will need to be redesigned to be more specific to the plasmid and reduce co-amplification of algae DNA. This step has not been taken to date. Re-optimizing the primers for plasmid pEX18Tc will prove challenging since the genome of our algae species, *S. dimorphus*, has not been sequenced; which means there is no way to confirm that new primers won't co-amplify the algae genome without testing it experimentally.

An example of our melt temperature results from a qPCR run with samples of our algae background matrices using both long and short amplicon primers is presented in Table 5-4. Specifically, melt temperatures and Ct values for the background matrices with and without a plasmid concentration of 6.132 ng/μL are shown. The melt temperature results are used to identify specific qPCR products. Ideally, different products should have melt temperatures that are at least 2 °C different from one another. From Table 5-4, the samples containing extracted algae DNA and those with plasmid had melt temperatures within 2 °C of each other for both primer sets (i.e. 86.5 and 85 for the plasmid and algae DNA, respectively for the short amplicon primers and 88.5 and 89 for the plasmid and algae DNA, respectively for the long amplicon primers). The close proximity of the two melt temperatures makes it difficult to distinguish between the amplification

products for the algae DNA versus the plasmid. This means that in our experiment, the plasmid quantities could be artificially inflated in samples containing algae DNA. In addition, the live and autoclaved algae matrix contained inhibitors that suppressed plasmid amplification as demonstrated by comparing the plasmid standard melt temperature and Ct values with those of the algae matrix and autoclaved algae with plasmid (Table 5-4).

Table 5-4. Melt temperature and cycle threshold (Ct) results for the live and autoclaved algae background matrices for both the short and long amplicon primers. Samples that contained plasmid had a plasmid concentration of 6.132 ng/μL.

Matrix	Short Amplicon Primers		Long Amplicon Primers	
	Melt Temperature (°C)	Ct	Melt Temperature (°C)	Ct
Plasmid Standard	86.5	27.12	88.5	4.46
Algae DNA with Plasmid	86.5	19.12	88.5	18.37
Algae Matrix with Plasmid	None	20.58	88.5	20.55
Autoclaved Algae with Plasmid	None	18.19	None	20.29
Algae DNA	85	27.76	89.0	30.72
Algae matrix	None	36.16	None	31.77
Autoclaved Algae	None	32.53	None	None
Negative control	75	31.27	None	None

In the end, we decided not to move forward with qPCR-based analysis of the extent to which algae treatment mediates a reduction in ARG concentration over time, as a result of challenges described above: the need for very large plasmid copy numbers in the initial stock,

based on reactor volume; the need for an unworkably large calibrated range; significant matrix inhibition on plasmid amplification; and significant cross-amplification of background algae DNA. These difficulties dramatically undermine the key benefit of qPCR for analyzing environmental samples, which is its speed and convenience. Emerging literature corroborates our findings by documenting disadvantages of using qPCR for assessment of ARGs in environmental samples. For example, it has been shown that qPCR provides an overly conservative estimate of the effectiveness of a treatment in deactivating ARGs (Chang et al., 2017; McKinney and Pruden, 2012; Yoon et al., 2017). Overall plasmid damage is often underestimated by qPCR because it can only effectively assess short sections of DNA at a time (Chang et al., 2017; McKinney and Pruden, 2012; Zhang et al., 2019). A plasmid can be deactivated as a result of damage to gene regions outside of the ARG portion captured by qPCR. This phenomenon is illustrated by recent work from two different groups: Chang et al. 2017 and Zhang et al. 2019 (Chang et al., 2017; Zhang et al., 2019). In their comparison of short and long amplicon qPCR methods to assess deactivation of a plasmid carrying *tet A*- and bla_{TEM-1}-resistance genes during UV treatment, Chang et al. 2017 observed that the first-order deactivation rate constants measured using a long amplicon protocol were larger than those measured using a short amplicon protocol. They concluded this was likely due to the presence of more target sites in longer DNA sequences, such that the longer amplicon protocol had higher likelihood of capturing UV-induced plasmid damage (Chang et al., 2017). Zhang et al. 2019 made the same observation in their comparison of long and short amplicon protocols for evaluating dissolved organic matter photosensitization of a plasmid carrying *tet A*- and bla_{TEM-1}-resistance genes.

Additionally, Chang et al. 2017 and Zhang et. al. 2019 observed that qPCR provided a more conservative assessment of plasmid deactivation compared to relevant transformation assays

(Chang et al., 2017; Zhang et al., 2019). They posited that this result was likely due to the treatment's effects on gene regions that weren't captured in qPCR of the ARG alone. In their view, deactivation of plasmid gene regions outside of the ARG affected the plasmid's ability to be transformed by bacteria, which means that despite the lower degradation of the resistance genes themselves, the genes cannot spread to other hosts because the plasmid they reside on is unable to transform. This means that even if the ARG region of the plasmid remains intact, the plasmid's capacity to transmit antibiotic resistance in downstream organisms is likely reduced as a result of the applied treatment.

Overall, recent plasmid treatment studies provide evidence for the advantages of using an effects-based assessment approach, such as transformation assays, when evaluating a treatment's effectiveness in mitigating the spread of antibiotic resistance. Considering both our difficulties with qPCR method development and the literature evidence pointing toward the benefit of alternative assessments, we decided to stop qPCR troubleshooting and focus on our transformation assays. However, for sample matrices where qPCR protocols are readily available, qPCR is still a valuable tool for obtaining rapid and conservative estimates of ARG quantity.

5.3.2 Assessment of ARG Plasmid Transformation Efficiency

Our next goal was to assess the algae treatment's effectiveness in reducing the transformation efficiency of the pEX18Tc plasmid. The primary advantage of a transformation assay for this purpose is that it provides a more direct evaluation of the plasmid's ability to transform and confer resistance in downstream organisms before and after application of a water treatment.

Prior to performing transformation assays with our experimental samples, we assessed the transformation efficiencies of the plasmid in two control solutions (Figure 5-3). The first control solution contained 3.48 ng/μL of plasmid in nuclease-free water and the second contained 3.48 ng/μL of plasmid in filtered algae matrix. We observed that the plasmid transformation was suppressed in the presence of the filtered algae background matrix, as compared to plasmid transformation efficiency measured in the pure nuclease free water matrix. This preliminary result provided a promising indication of the potential effectiveness of the algae treatment over a longer treatment duration.

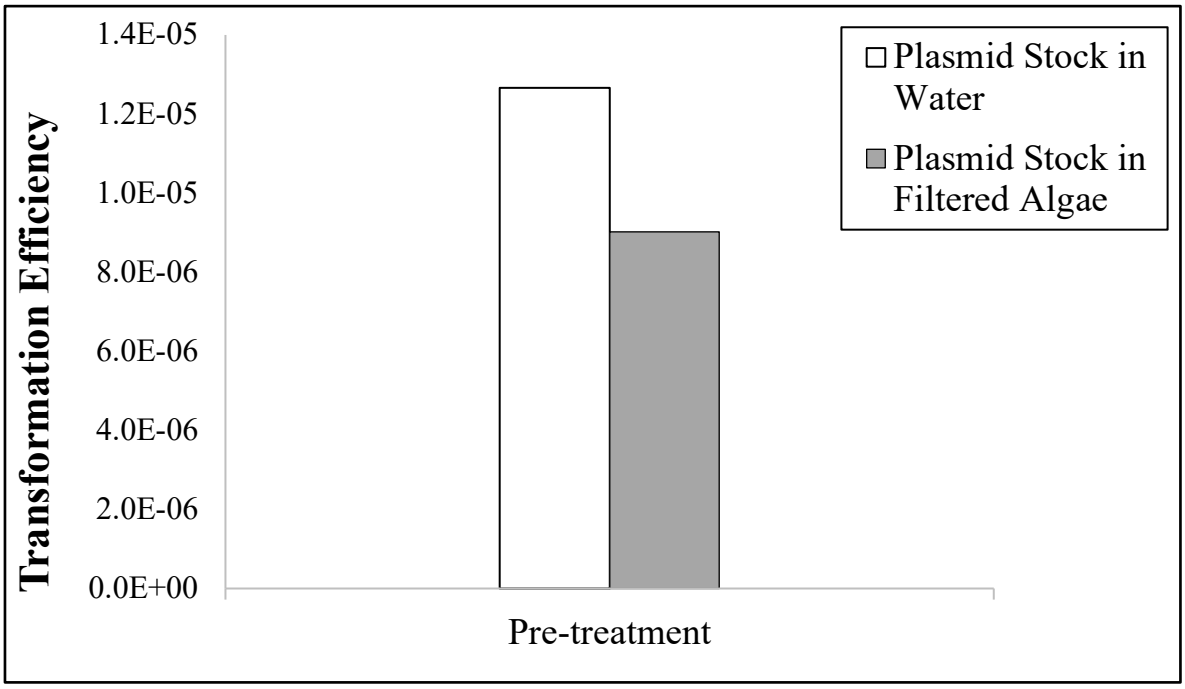

Figure 5-3. Preliminary transformation efficiencies of 3.48 ng/μL pEX18Tc plasmid standards in nuclease-free water and filtered algae background matrix. See Equation 5-1 for the transformation efficiency equation.

After optimization of our transformation assay protocol, our transformation efficiencies for tetracycline resistance were consistently between 10^{-5} and 10^{-6} for competent cells exposed to pEX18Tc in either a nuclease-free water solution (control), MB3N, or the experimental algae matrix. This means that one out of every 100,000 or 1,000,000 competent *E. coli* cells developed tetracycline resistance when exposed to our plasmid.

We then applied the optimized transformation assay protocol to our experimental pre- and post-treatment samples and calculated the corresponding transformation efficiencies. These results are presented in Figure 5-4. Consistent with our observations in the preliminary transformation assays, the transformation efficiencies for pre-treated plasmid in the autoclaved and live algae matrices are lower than those for the dark and light controls. Given the differences in pre-treated plasmid transformation efficiencies between the algae matrices and control samples, and in order to quantify transformation efficiency reduction, we normalized the data from Figure 5-4 using Equation 5-2. The resulting percent reductions in transformation efficiency are presented in Figure 5-5.

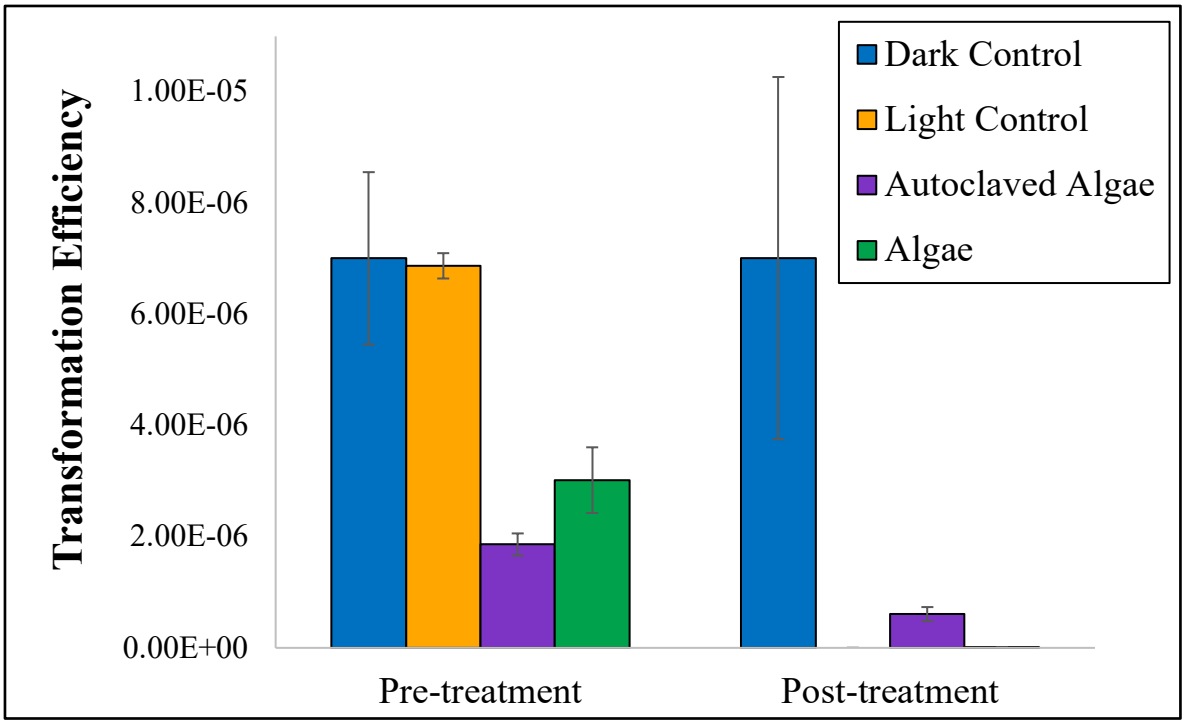

Figure 5-4. Pre- and Post-treatment transformation efficiencies of the pEX18Tc plasmid exposed to (1) dark control, (2) light control, (3) autoclaved algae control, or (4) algae treatment. Treatment duration was 3 days. Error bars represent standard deviation for the mean of three experimental replicates for each condition. See Equation 5-1 for the transformation efficiency equation.

Equation 5-2.

$$\text{Percent Reduction in Transformation Efficiency} = \frac{Pre{-}Treatment - Post{-}Treatment}{Pre{-}Treatment} * 100\%$$

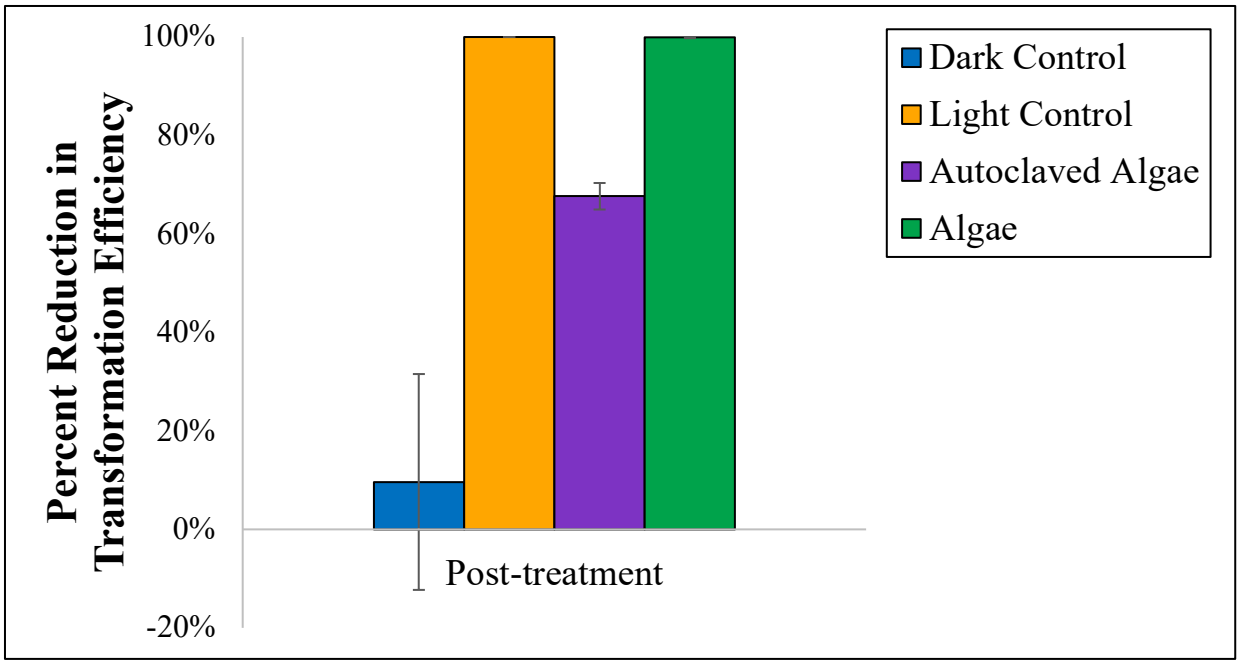

Figure 5-5. Percent reduction in transformation efficiency of the pEX18Tc plasmid exposed to (1) dark control, (2) light control, (3) autoclaved algae control, or (4) algae treatment. Post-treatment corresponds with a treatment duration of 3 days. Error bars represent standard deviation for the mean of three experimental replicates for each condition. See Equation 5-2 for the percent reduction in transformation efficiency equation.

Based on our previous analysis of algae treatment of ciprofloxacin (CIP), we hypothesized that reduction in plasmid transformation efficiency could happen to varying degrees by different mechanisms occurring in the algae treatment system. In our autoclaved algae control, we observed an immediate reduction in pre-treated transformation efficiency (at time t = 0) compared with the algae-free dark controls. We also observed an additional reduction in transformation efficiency of 67% following 3 days of exposure to the autoclaved algae samples. It is not clear by what mechanism the conditions of the autoclaved algae series reduced the transformation efficiency for the pEX18Tc plasmid. We included the light control to isolate the effects of photolytic processes

on plasmid transformation efficiency. We observed a 100% loss of transformation efficiency over 3 days in the light control. We also observed 100% reduction in plasmid transformation efficiency over 3 days in the algae treated samples. Based on our observations for the controls, it is not possible to apportion the reduction in transformation efficiency among various mechanisms including photolytic processes, sorption, aggregation, etc. However, our dark control data confirms that loss of plasmid transformation efficiency is not occurring as a result of experimental artifacts.

With regards to the treatment duration, it is noteworthy that complete loss of transformation efficiency was observed in only 3 days; a duration that corresponds with typical hydraulic retention times for algal ponds (Craggs et al., 2014; García et al., 2000). In our previous assessment of algae treatment of CIP, we also observed rapid initial removal of the parent compound (~80% within 2 days), but only 93% of the initial parent compound concentration was removed after 6 days (Grimes et al., 2019). The experimental conditions were essentially the same in both studies. Future transformation analyses of samples collected at earlier time points would help elucidate the exact treatment duration needed to mediate 100% loss of plasmid transformation efficiency.

Our observations of rapid and complete loss of transformation efficiency via the algae treatment and light control align with the limited existing literature on changes in transformation efficiency for ARGs exposed to different water/wastewater treatments. Chang et al. 2017 performed transformation assays on a UV_{254}-treated plasmid carrying ARGs with *Acinetobacter baylyi* as the competent host bacteria. They observed increasingly rapid reduction in transformation efficiency with increasing UV doses. They concluded that their plasmid required a UV_{254} dose of 20-25 mJ/cm^2 per $\log_{10}$ of transformation efficiency (Chang et al., 2017). Similarly, Zhang et al. 2019 performed an analysis using transformation assays to evaluate deactivation of a plasmid via dissolved organic matter (DOM) photosensitization using a mercury lamp

photochemical reactor. The presence of DOM in the system increased the rate of transformation efficiency reduction compared with reactors that did not contain DOM (Zhang et al., 2019). Our algae system, which also contains DOM, is similar to that of Zhang et al. 2019, although our data showed no difference in plasmid transformation efficiency reduction between irradiated reactors with or without live algae. Future assessments of the algae treatment including earlier time point measurements could elucidate whether the presence of algae increases the rate of transformation efficiency reduction compared to the light control (e.g., by acting as photosensitizer) or decreases it (e.g., by reducing light penetration). Similarly, He et. al. 2019 observed changes in transformation efficiency of ARGs after treating with free chlorine, monochloramine, chlorine dioxide, ozone, UV, or hydroxyl radical. They observed > 90% reduction in transformation efficiency at typical WWTP disinfection exposures and reported higher rates of ARG deactivation for higher treatment doses (He et al., 2019).

It is clear from our results, and those of Chang et. al. 2017 and Zhang et al. 2019 that illumination has a major impact on plasmid deactivation. However, some studies have demonstrated ARG repair and reactivation after application of UV and other traditional wastewater treatments (Chang et al., 2017; Czekalski et al., 2016; Destiani and Templeton, 2019; Dodd, 2012; Lamba and Ahammad, 2017; McKinney and Pruden, 2012). Given the significant role of the light in deactivating the pEX18Tc plasmid (as made evident in the light control), future work should examine potential plasmid reactivation after an extended post-treatment duration. It would be of interest to examine what effect, if any, the algae background matrix may have on rate and extent of photorepair. Several studies have also shown that different treatments have varying degrees of effectiveness in degrading or deactivating intracellular ARGs compared with extracellular ARGs (Czekalski et al., 2016; Destiani and Templeton, 2019; Dodd, 2012; He et al., 2019; McKinney

and Pruden, 2012; Yoon et al., 2017). In this study, we focused solely on assessing algae treatment of an extracellular plasmid. Future work to assess deactivation of intracellular ARGs would provide a more holistic interpretation of the algae treatment's effectiveness in reducing the risk of disseminating antibiotic resistance.

In terms of environmental relevance, it is noteworthy that the algae background matrix mediated an immediate suppression of plasmid transformation compared with control stocks. The algae system has some similarities with natural receiving waters, such as the presence of DOM and solar light exposure. While receiving water conditions and light penetration can vary widely, our data suggest that freshwater systems containing algae may be conducive for suppressing some or all of a plasmid's ability to be transformed. However, the algae system has only been evaluated for one plasmid with one host bacterium, and the implications for the treatment's effectiveness against intracellular ARGs is not yet known.

Finally, it is imperative that we reiterate the importance of using transformation assays or other culture-based methods to evaluate the effectiveness of wastewater treatment technologies in deactivating ARGs. As studies have shown, quantitative methods (e.g. qPCR) can provide an estimate of a treatment's effectiveness, but quantitative reduction does not always correspond with a reduction in the likelihood of stimulating antibiotic resistance. Therefore, quantitative methods alone cannot provide the holistic picture of a treatment's effectiveness. Although it requires more time to obtain results for the transformation assay than for qPCR, culture-based methods are the "gold standard" to gain insight into a treatment's effectiveness toward reducing the risk of dissemination of antibiotic resistance in downstream waters. However, there are also caveats to the reliability of typical transformation assay protocols as a holistic effects-based assessment of a treatment. For example, they may provide an underestimation of ARG deactivation because the

competence of only one pure-culture host bacterium is assessed. This challenge can be mitigated by assessing transformation efficiency for multiple host bacteria strains. On the other hand, transformation assays may overestimate ARG deactivation because most laboratory protocols involve steps to increase the competence of the host cells. To better simulate environmental transformation conditions, some studies have developed natural transformation protocols (He et al., 2019) or microcosm reactors (Burch et al., 2014; Fahrenfeld et al., 2013; Luo et al., 2014). Future studies that use natural transformation methods to assess treatment of ARGs should be performed.

5.4 Conclusions

The results of this study demonstrate the efficacy of the algae treatment in rapidly reducing the extent to which the pEX18Tc plasmid is transformed by bacteria. In both the algae treatment and light control, 100% reduction in plasmid transformation efficiency was achieved in less than 3 days. A 67% loss of plasmid transformation efficiency was observed in the autoclaved algae control. Notably, the live and autoclaved algae background matrices suppressed plasmid transformation in pre-treatment samples relative to pure water and control background matrices. The similarities between the algae matrix and environmental matrices may indicate that environmental conditions are not conducive to maintaining extracellular plasmid in a state that is readily transformable, although future study is needed to confirm this hypothesis. Future work could focus on developing a qPCR methodology to address the algae background matrix interference and performing an evaluation of the algae treatment's efficacy in deactivating intracellular ARGs. While plasmid quantification with qPCR can be a useful method to estimate

plasmid deactivation, the results of this study emphasize the importance of prioritizing transformation assays as an effects-based evaluation tool.

5.5 References

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Chapter 6: Contextualization and Thematic Conclusions

Throughout the course of this research, we explored and unearthed several noteworthy themes. The overarching theme of this work is that it is more valuable to assess treatment effects than it is to quantify removal. In the case of algae treatment of ciprofloxacin (CIP), we found that 93% removal of the parent compound resulted in effluents that did not stimulate CIP resistance. However, this observation is not always true for other antibiotics and treatments, as studies have shown that transformation reactions can generate byproducts that are equally or more potent than the parent compound (Fatta-Kassinos et al., 2011; Noguera-Oviedo and Aga, 2016). The development of antibiotic resistance is a complex process with many factors, so the quantity of antibiotics, antibiotic resistant bacteria (ARB), and antibiotic resistant genes (ARGs) in effluents may not directly correspond with their level of risk for disseminating antibiotic resistance. Traditional focus on antibiotic and ARG quantification methods should be shifted in favor of culture-based methods like adaptive laboratory evolution for antibiotics treatment and transformation or microcosm assays for ARG treatment to move the field forward. In instances where treatments have only been assessed with quantification methods, culture-based methods should be used to verify previous observations.

A second theme that has emerged from this work is insight related to which wastewater constituents pose the greatest risk for antibiotic resistance dissemination in the environment and should subsequently be highest priorities at wastewater treatment plants (WWTPs). Generally, ARB and antibiotics are considered a major threat in environmental systems, and previous studies

have also stressed the importance of mitigating extracellular ARGs in WWTPs (Amarasiri et al., 2019; Chang et al., 2017; Dodd, 2012; McKinney and Pruden, 2012; Zhang et al., 2019). However, the significant loss of plasmid transformation efficiency observed upon initial exposure to the algae matrix, together with similar observations in other studies, could suggest that extracellular ARG pose less risk than other relevant wastewater constituents (Lorenz and Wackernagel, 1994; Munck et al., 2015). Based on our results, we would rank the priority for mitigation of these constituents as ARB > antibiotics > extracellular ARGs. However, it would be unwise to completely disregard the impact of extracellular ARGs in the environment based on the limited data available, since there is evidence that natural transformation can occur under certain circumstances, particularly when naturally competent environmental bacteria are exposed to plasmids that remain viable despite harsh environmental conditions (Domingues et al., 2019, 2018; Hasegawa et al., 2018; Lorenz and Wackernagel, 1994; Mantilla-Calderon et al., 2019; von Wintersdorff et al., 2016). Additional studies on treatment of different ARGs and transformation assays with different strains of competent bacteria are needed before a definitive conclusion about their risk toward stimulating antibiotic resistance relative to other constituents can be made. Ultimately, treatments that can deactivate antibiotics, ARB, and ARGs collectively will be the most effective in reducing dissemination of antibiotic resistance in the environment.

Finally, it is necessary to address the role of algae in this research and contextualize its implications for full-scale wastewater treatment and impacts on the natural environment via receiving waters. It is clear from our results in Chapters 3 and 5 that the algae cultivation conditions contribute to both CIP removal and plasmid pEX18Tc deactivation. Although our results suggest algae treatment could be a promising technology for the wastewater antibiotic resistance mitigation fleet, many challenges still exist with scaling up the technology beyond current bench and pilot-

scale systems; e.g., its large land footprint and significant consumption of carbon dioxide and other growth nutrients, variations in its effectiveness toward pollutant removal in mixed algae cultures with wastewater bacteria, and the energy consumption associated with harvesting the algae for bioenergy (Colosi et al., 2015; Lavrinovičs and Juhna, 2018).

Interestingly, some aspects of the algae cultivation system are similar to environmental receiving waters; namely, the presence of dissolved organic matter, dissolved oxygen, and diurnal solar irradiation. The typical reactive conditions of environmental receiving waters have been shown to mitigate some antibiotics, ARB, and ARGs. However, receiving water conditions are highly variable, meaning that environmental deactivation of these constituents is inconsistent. Despite the potential for photolytic, biochemical, and other reactions to occur, some antibiotics, ARB, and ARGs are persistent and have been measured in environmental systems. This observation may be a result of variable factors in environmental systems such as variations in light penetration, seasonal changes, variations in upstream wastewater treatment, location, etc. Therefore, our observations of the algae system are not entirely analogous to environmental systems. Another system that may provide similar conditions conducive to antibiotic and ARG removal without the operational burden and maintenance costs associated with large-scale microalgae cultivation systems are constructed wetlands. Recent studies of constructed wetlands have demonstrated effective removal of antibiotics, although more studies of constructed wetland deactivation of ARB and ARGs is needed (Berglund et al., 2014; Bôto et al., 2016; Chen et al., 2016; Hijosa-Valsero et al., 2011; Santos et al., 2019; Shan et al., 2020). Future work in this field should investigate constructed wetland treatment of ARB and ARGs. It is possible that constructed wetlands may be a more realistic and sustainable alternative to an algae system to effectively treat antibiotics, ARGs, and ARB at a lower cost.

Overall, antibiotic resistance presents a leading threat to global public health, and WWTPs are widely considered to be a critical node for controlling its spread (Manaia et al., 2018; Pruden et al., 2013). Traditional treatments such as UV have demonstrated effective deactivation of some antibiotics, ARB, and ARGs, but they come with high energy costs to deliver the high doses required. Moreover, there is evidence that ARGs may reactivate via photo-repair following UV-based deactivation. These are serious shortcomings of widely used conventional water/wastewater treatments currently available on the market, especially given that the constituents of interest are currently unregulated. Although the algae treatment has shown promising effectiveness for antibiotic removal and ARG deactivation, the looming threat of antibiotic resistance requires immediate action and likely cannot wait for the additional treatment assessments needed or the development of full-scale algae treatment. Therefore, I believe the path forward for the environmental engineering community is to 1) explore methods to reduce energy consumption of traditional treatments such as implementing solar-powered UV; 2) evaluate and identify levels of treatment that prevent ARG reactivation; and 3) explore constructed wetlands as a potential low-cost treatment. Notably, these options deliver valuable removal of various regulated wastewater constituents, such that removal of unregulated, antibiotic resistance-relevant constituents is an additional, externalized benefit of their application.

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Chapter 7: Conclusions and Future Study

7.1 Research Summary and Conclusions

This dissertation explored the efficacy of a novel bench-scale algae treatment system toward deactivation of wastewater constituents that can stimulate antibiotic resistance. Specifically, we studied the treatment of two major constituents: a model antibiotic, ciprofloxacin (CIP), and a model antibiotic resistance gene (ARG), plasmid pEX18Tc, which carries the tetracycline resistance gene, *tet*. The algae treatment system consists of a monoculture of *S. dimorphus* exposed to twelve hours of simulated solar irradiation daily. Previous studies have demonstrated the effectiveness of UV and dissolved organic matter photosensitization systems toward ARG deactivation, so we hypothesized that the algae system would be similarly effective with the added wastewater treatment plant benefit of being a net-energy generating treatment. First, we evaluated the algae treatment's efficacy toward reducing CIP and its residual effects through the use of liquid chromatography, microbiological assays, and adaptive laboratory evolution. Next, we developed qPCR and transformation assay methods to evaluate the treatment's efficacy toward deactivating plasmid pEX18Tc. Our qPCR method development was inconclusive due to low plasmid concentrations and background matrix interference, but we were successful in developing a transformation assay to measure reduction in transformation efficiency. Our results indicate that the algae treatment is effective at removing CIP and its residual effects and deactivating plasmid pEX18Tc. Together, our analyses provide a comprehensive understanding of the algae treatment's

effectiveness toward reducing the impacts of both an antibiotic and an ARG on downstream bacteria through the emphasis and development of effects-based assessment methods.

With regards to algae treatment of CIP, we observed rapid removal of the parent compound that was primarily driven by algae and light mediated transformation reactions. Maximum removals of 93% for algae treatment and 53% for the light control occurred after six days, but with the bulk of the removal occurring within a typical residence time of two days. Removal of the parent CIP compound corresponded with a reduction in acute potency of the treated effluents toward *E. coli*. Our adaptive laboratory evolution experiment revealed that treated effluents did not stimulate phenotype or genotype CIP resistance.

With regards to algae treatment of plasmid pEX18Tc, we observed that the algae background matrix not only suppressed plasmid amplification during qPCR, but also reduced plasmid transformation efficiency in pre-treatment controls. In less than three days of treatment, the algae treated and light control samples mediated a 100% loss of plasmid transformation efficiency.

Overall, this research has provided a framework for evaluating the efficacy of other candidate treatments for mitigating antibiotic resistance disseminated from wastewater effluents. In particular, it emphasizes the need to assess effluent characteristics *beyond* residual concentrations of the parent drug compound or quantities of ARGs, most notably: residual capacity to stimulate antibiotic resistance via chronic exposure; and loss of transformable ARGs.

7.2 Recommendations for Future Study

Although we ultimately discovered that it was not worthwhile to continue pursuing qPCR method development for the algae treatment system for the purposes of this dissertation, we can provide recommendations for future study based on our findings. Inconclusive results from this dissertation reveal that there are methodological challenges to applying qPCR to the algae treatment system including low plasmid yields, background matrix interference with plasmid amplification, and challenges with primer specificity. Additionally, previous literature suggests that qPCR is a less useful tool than transformation assays for determining a treatment's effectiveness. However, a developed qPCR method could be useful for quickly estimating ARG deactivation and for comparing the algae treatment with other treatments. In order to continue qPCR method development, I would recommend the following:

1) Scale up plasmid extraction. Methods for scaling up could include growing plasmid host cultures in waves and freezing large quantities of extracted plasmid to combine and use in later experiments, or increasing laboratory capacity to grow more host cultures simultaneously.

2) Re-optimize the primers or select a different ARG. Our challenge with this research was the co-amplification of the *tet* gene and the extracted algae DNA. To address this issue, different primers could be selected. However, since the genome for *S. dimorphus* has not been sequenced, it would not be possible to optimize primer specificity without testing new primers experimentally each time. Optimizing primers to a genome of an algae species with similar characteristics to *S. dimorphus* may help to some degree, but genomic variations between different strains make this not an ideal solution. Processes for sequencing the *S.*

dimorphus genome or switching to a different algal strain for the treatment experiments would likely be laborious and present their own complex challenges. Most likely the simplest solution would be to analyze a different ARG that has primer binding sites that are unique from the algae genome.

3) Identify constituents in the algae background matrix that interfere with ARG amplification and develop a filtration method to remove those compounds. It may be possible that other commercially available kits for purifying plasmid DNA may be more effective than those we tried. Solid phase extraction could also be a useful tool for removing algae matrix inhibitors and eluting only the plasmid. Most likely the simplest option would be to scale up the plasmid yields so that concentrations are high enough to be detected beyond the background baseline (See recommendation 1).

Additionally, Chapter 4 of this dissertation focused only on deactivation of extracellular plasmids. While we observed the algae treatment to be especially effective at plasmid deactivation, our observations of a rapid loss of transformation efficiency may indicate that extracellular ARGs are less likely to transform in the environment than intracellular ARGs. Although most studies to date postulate that typical wastewater disinfection processes likely disinfect antibiotic resistant bacteria (ARB) to an insignificant level, it is possible that the ~0.01% of ARB that survive wastewater disinfection are driving antibiotic resistance stimulation in downstream environments. Similarly, bacteriophage transmission of antibiotic resistance may also play a major role in downstream antibiotic resistance stimulation and a small percentage of these viruses can survive disinfection. Therefore, it is of interest for future research to investigate treatment of intracellular ARGs, ARB, and bacteriophage.

The central theme of this dissertation is the emphasis on the broader practicality of using effects-based evaluation methods. Other researchers in the wastewater treatment of ARGs field have also begun to take note of the need for more practical assessments and are developing assays that can better replicate some of the complexities of natural microbial interactions. Specifically, a few recent studies have introduced the concept of microcosm studies. The main idea behind microcosms is to simulate the interactions of treated effluents with natural bacteria populations. Generally, researchers collect an environmental water or sediment sample and expose the native bacteria to lab-scale or wastewater treated effluents and observe whether or not natural transformation of ARGs occurs via selective plating. The findings from this dissertation have influenced the next generation of researchers in our team to continue this line of work and adapt a microcosm study to assess a local WWTP for its effectiveness toward ARG and ARB deactivation. We believe microcosm assessments are the next evolution in effects-based assaying of water and wastewater treatment technologies.