Evaluating Algae-mediated Removal of Ciprofloxacin

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Abstract

In this thesis, bench-scale laboratory experiments were used to evaluate the efficacy of an algae-based treatment process to remove ciprofloxacin (CIPX), a highly-prescribed human and veterinary antibiotic for treatment of bacterial infections. As a result of its widespread use, CIPX has been frequently detected in wastewater treatment plant (WWTP) influents and effluents, but it is unknown to what extent its presence affects organisms in receiving waters and natural systems downstream. Previous studies have shown that algae are able to effectively remove estrogens from artificially spiked growth medium. Based on this observation, it is hypothesized that algae may also remove other priority emerging contaminants; e.g., antibiotics, including CIPX. If so, the integrated algae-treatment system could improve WWTP effluent water quality while also providing energy to the WWTP through anaerobic digestion of the algae biomass into methane-derived electricity.

We examined the behavior and fate of CIPX under simulated algae cultivation conditions, using the model freshwater alga, *Scenedesmus dimorphus*. First, we analyzed biomass production of *S. dimorphus* during exposure to different concentrations of CIPX (0-5000 ng/L) over 192 hours. Interestingly, a dual effect was observed, whereby CIPX at an artificially high experimental concentration (5000 ng/L) was found to stimulate growth, while CIPX at lower environmentally relevant concentrations (0-600 ng/L) were found to inhibit cumulative algae growth. However, little to no impact on algae growth rate was observed within the first 24-48 hours of experimentation. Since it is anticipated that commercial-scale algae-treatment systems will use hydraulic residence times (HRT) on the order of 24 hours, the presence of CIPX in wastewater effluents is expected to have little to no observable impacts on algae productivity.

Second, we examined removal of CIPX as mediated by *S. dimorphus*. Appropriate controls were used to assist in allocating apparent removal among several possible removal mechanisms reported in literature: sorption, photolysis (i.e., light-mediated transformation), and algae-mediated biotransformation. Results show an average of 65% removal of CIPX over 8 days, mostly occurring within the first 24 hours. In contrast, negligible CIPX removal was observed in "dark reactors" and "light controls." These data rule out CIPX disappearance as an experimental artifact, and suggest that photolysis is not a significant contributor to CIPX removal under algae cultivation conditions. Negligible CIPX removal was also observed in foil-wrapped controls comprising autoclave-inactivated (i.e., dead) algae. This suggests that sorption is also not a significant contributor to CIPX removal in this system. As such, CIPX removal under the tested conditions is dominated by biotransformation reactions mediated by active algae biomass. This is consistent with previous investigations of algae-mediated removal of steroid estrogens (17α-estradiol, 17β-estradiol, estrone, and estriol); moreover, it brings to light many interesting questions about how algae-mediated biotransformation of CIPX and other antibiotics could impact the dissemination of antibiotic resistance. Overall, it was determined that the model alga does indeed provide rapid removal of the selected model antibiotic, which increases the appeal of algae-mediated WWTP effluent polishing.

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1. Introduction

The development of sewage treatment via wastewater treatment plants (WWTPs) is one of the most exemplary environmental engineering innovations in history. As global population continues to rise, proper treatment of human waste to protect both human and environmental health only grows in importance. However, current WWTP technologies are highly energy-intensive and costly to maintain and operate (Daw et al., 2012). With growing concerns for sustainability, research into improved wastewater treatment technologies that are more energy efficient has become a major priority.

Emerging contaminants (ECs) constitute a subset of compounds that are unmonitored and uncontrolled, can be found in the environment, and are known or suspected to have adverse environmental and human health impacts. Some ECs of particular concern include environmental estrogens (e.g., oral birth control), pharmaceuticals (e.g., antibiotics, statins, etc.), and personal care products (e.g., shampoo, sunscreen). The majority of ECs make their way into the environment through anthropogenic outlets such as wastewater discharge and land surface runoff. WWTPs are especially recognized for discharging measurable concentrations of ECs into receiving waters (Kolpin et al., 2002; Ying et al., 2002; Ottmar et al., 2010, 2013; Jelić et al., 2012; Zhang et al., 2014; Matamoros et al., 2015). These contaminants are currently unregulated under the Safe Drinking Water Act at WWTPs, in part because they are so expensive to analyze and remove; but they are of significant interest to the water and wastewater treatment communities.

The water-energy nexus is a term referring to two interrelated challenges, whereby water is required to make energy and energy is required to make clean water. In this vein, there has been recent interest in the development of wastewater treatment technologies that are both net

energy-generating and have the capacity to remove ECs (Zhang et al., 2014; Ottmar, 2010; Ottmar et al., 2013; McCarty et al., 2011; Steele et al., 2014; Peng and Colosi, 2016). The synergy between algae cultivation and sewage treatment is especially interesting considering that algae utilize nitrogen and phosphorus, which are costly nutrients for algae farms to supply, to undergo photosynthesis and WWTPs have an abundance of these nutrients and are required to remove them. Therefore, incorporating an algae-based polishing step into a conventional WWTP could deliver several environmental benefits, including: removal of nutrients to decrease downstream eutrophication; production of algae biomass that can be converted into low-carbon bioelectricity; and, potentially, removal of priority emerging contaminants, thereby mitigating aquatic toxicity and human health impacts. A schematic of the proposed integrated algae-WWTP system is presented in Figure 1.1.

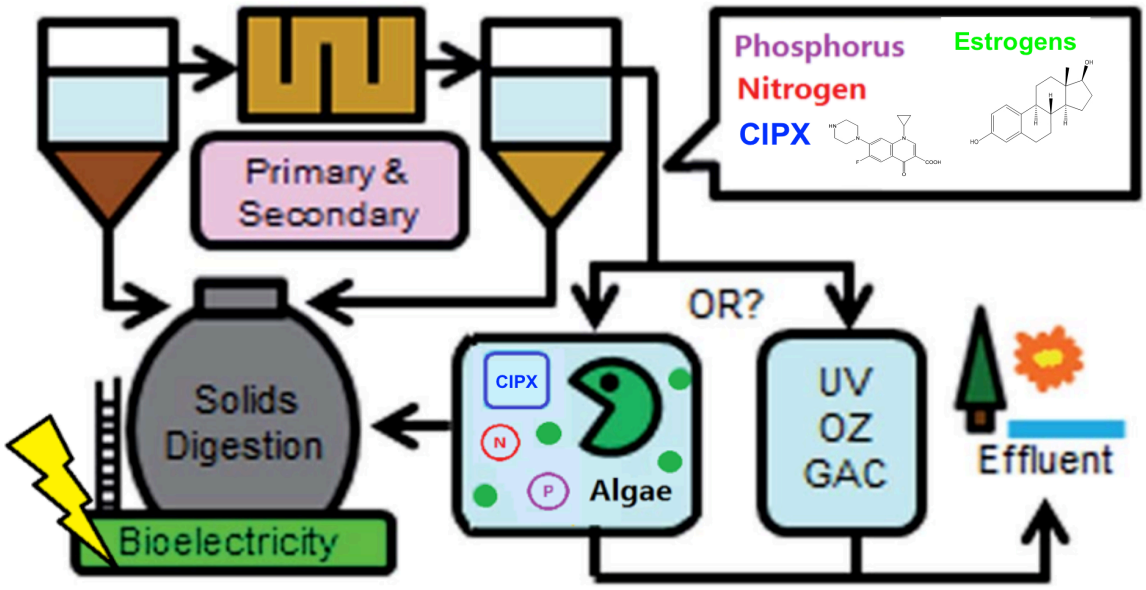


Figure 1.1. Diagram of the proposed integrated algae-WWTP system including both nutrient and EC removals and energy generation from methane-derived electricity. Modified from Zhang et al. (2014).

Life Cycle Assessment (LCA) has been used to assess the energy efficiency of traditional WWTP processeses with and without conventional tertiary treatments or algae-medaited nutrient and EC removal. In particular, recent work by Colosi et al., 2015 focused on computed energy return on [energy] investment (EROI) for a WWTP-only system as compared to the same municipal WWTP system with implementation of one of four tertiary treatments; algae-medaiated tertiary treatment, ultraviolet irradiation (UV), ozonation (OZ), or adsoprtion onto granular activated carbon (GAC) . An EROI value greater than 1 indicates that a system is net-energy generating (i.e. energy in < energy out). The resulting EROI values for traditional tertiary wastewater treatment processes compared with the proposed algae tertiary treatment determined by Colosi et al. 2015 are presented in Table 1.1. The same basic WWTP configuration was assumed for each of the five WWTP systems listed in Table 1.1, which consisted of primary treatment, secondary treatment with biological nutrient removal, second-stage denitrification, solids handling and anaerobic digestion, and chlorination (Colosi et al., 2015). Therefore, the EROI values presented represent the net energy generated by the WWTP during anaerobic digestion divided by the net energy consumption for each tertiary treatment system and the primary and secondary treatment systems upstream.

From Table 1.1, the system that comprises WWTP + algae tertiary treatment has the greatest EROI value of 0.65 compared to traditional tertiary treatment processes and a WWTP without tertiary treatment. Both the additional input of algae biomass to the anaerobic digester as well as the offset of energy consumption due to algae-mediated nutrient removal account for the improved EROI of the algae tertiary treatment system. Although the EROI values are all less than one, meaning all of the systems consume more energy than they produce, it is promising that the algae treatment system is able to produce enough energy in the form of methane from

anaerobic digestion of biomass to offset some of the energy consumption for a WWTP. Additionally, the algae treatment system has the potential to remove emerging contaminants, which would add to its overall appeal.

Table 1.1. EROI values of conventional WWTP tertiary treatments compared with algae tertiary treatment (Colosi et al., 2015): 1) WWTP without tertiary treatment, 2) algae tertiary treatment, 3) UV radiation, 4) ozonation (OZ), and 5) granular activated carbon (GAC).

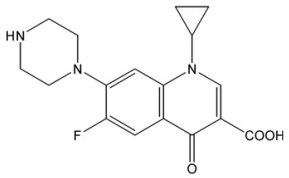
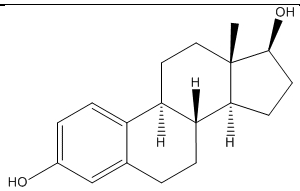
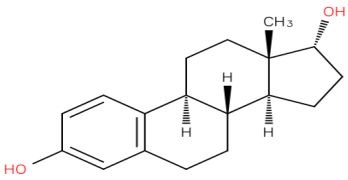
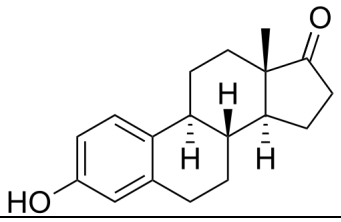
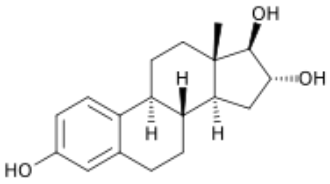
Treatment Systems:	**WWTP**	**Algae**	**UV**	**OZ**	**GAC**
EROI (MJ $energy_{OUT}$/MJ $energy_{IN}$)	0.50	0.65	0.47	0.24	0.35

Previous bench-scale studies of the theoretical algae cultivation and wastewater treatment system for removal of ECs have showed promise. Zhang et al. (2014) performed bench-scale experiments using pure cultures of the model alga *Scenedesmus dimorphus* and observed its ability to remove four of the most commonly found steroid estrogens in municipal and livestock wastewaters: 17α-estradiol, 17β-estradiol, estrone, and estriol. Observed removal efficiencies were 95% for the 17β-estradiol and estriol and 85% for the 17α-estradiol and estrone, over 8 days. Zhang et al. (2014) also studied the major pathways of removal of the steroid estrogens. They found that approximately 10% of the observed removal occurred via sorption while 90% of the removal was attributed to algae-mediated biotransformation. No removal via photolysis was observed for the estrogens. These results were extremely promising, and exploration of algae-mediated removal of other ECs is a logical next step for bench-scale experimental research related to the proposed algae treatment system.

In order to further prove the effectiveness of algae mediated removal of a variety of ECs, it was important to consider studying a subset of ECs that are both of environmental interest and

chemically different from the estrogens analyzed by Zhang et al. (2014). In the US alone, over 262.5 million courses of antibiotics are prescribed each year (CDC, 2016). Many of these antibiotics are highly stable compounds that are not fully broken down by the body, so they remain active long after they are excreted (eMedExpert, 2016). Antibiotics are of particular concern due to their biological potency and the risk of inadvertently promoting antibiotic resistance via chronic exposure of pathogenic agents to very low concentrations in partially treated effluents. Ciprofloxacin (CIPX) is a highly prescribed, potent antibiotic used to treat bacterial infections in both humans and animals. More than 20 million CIPX prescriptions were written in the US in 2010, making it the 5th most-prescribed antibiotic in the country (Lanphier, 2013). Of particular interest are the differences in chemical structure and properties between CIPX and steroid estrogens, presented in Table 1.2. Analysis of algae's ability to remove CIPX in comparison to its removal of estrogens can potentially provide a better understanding of how chemical parameters of EC compounds affect the removal capabilities of the proposed system. Ideally, a better understanding of how compound structure and parameters affect algae mediated removal of some ECs could allow for future evaluations of other ECs without the need for physical experimentation.

Table 1.2. Comparison between the chemical parameters of interest for CIPX (Batchu et al., 2014) and the steroid estrogens analyzed by Zhang et al., (2014) (Ying et al., 2002; Ren et al., 2016).

	Chemical Structure	Log K_{ow}	pKa	Water Solubility (mg/L)	Quantum Yield, Φ (mol/Einstein)
Ciprofloxacin		0.28	6.09	30,000	0.158
17 β-estradiol		3.94	10.33	3.6	0.07
17 α-estradiol		4.15	10.33	6.67	0.0102
Estrone		3.13	10.33	3.94	0.0246
Estriol		2.81	10.33	11.9	NA

The overall goal of this study was to explore whether or not integrated algae and WWTP systems can contribute to removal of other chemicals in addition to estrogens through bench scale experiments with the model alga, *S. dimorphus*. Antibiotics were targeted as a subset of ECs of interest and CIPX was chosen as a model antibiotic to perform the study. The following

three objectives were addressed and evaluated to assess the capability of the proposed algae treatment system to remove CIPX:

1. How does exposure to CIPX affect cumulative algae growth?
2. Can algae be used to effectively reduce EC concentrations in WWTP effluent?
3. What removal pathways occur during algae-mediated EC removal?

Comparisons between the CIPX removal results were then compared with the results of the estrogen studies performed by Zhang et al. (2014) to achieve an overall assessment of the known removal interactions of the theoretical algae treatment system.

2. Materials and Methods

2.0 Materials

Reagent-grade ingredients for culture media and other chemicals were purchased from Fisher Scientific Inc. (Pittsburgh, PA). HPLC-grade analytical solvents (acetonitrile and methanol) were from Sigma-Aldrich (St.Louis, MO).

2.1 Algae Cultivation

Pure cultures of the freshwater alga, *Scenedesmus dimorphus*, were prepared based on a three-step procedure from Zhang, et. al. (2014). First, scrapings were transferred from an agar slant tube stock purchased from the UTEX culture collection and inoculated into 50 mL of protease-peptone medium in a capped 125-mL flask. These flasks were then incubated aseptically and agitated using a crab shaker (Lab-Line Instruments, Inc.) and exposed to 12 hours of illumination (cool white fluorescent growth lamp with 125-W 6500-K) and 12 hours of darkness per day to simulate natural light conditions for 5 days. For step 2, aliquots of algae suspension were then transferred to 1-L flasks containing 500 mL of modified Bold 3N medium (MB3N) and subjected to the same light-dark sequencing. The flasks were stirred on a magnetic stir plate (200-300 rpm) and continuously aerated with filtered air flowing at 0.7 scfh for 8 days (i.e. when stationary phase was reached). Step 3 of the cultivation procedure comprised the various experimental reactions of interest, as described in the paragraphs below and summarized schematically in Figure 2.1.

2.2 Assessing the Impact of CIPX on Algae Growth Rate

A 0.25-g/L CIPX stock solution was prepared in DI water containing 10% formic acid (Fisher Scientific Inc.) and serially diluted with pure DI water. 1-mL aliquots of each dilution were then added to 499-mL volumes of MB3N medium to generate 7 concentrations of CIPX in

medium: 0, 25, 50, 100, 300, 600, and 5000 ng/L. Final formic acid concentration in each reactor was <0.0004% and pH in all reactors was roughly 7.0. These secondary stocks of CIPX in MB3N were refrigerated in sealed, foil-wrapped containers for up to one month, as needed to complete the experiments summarized in subsequent sections.

In order to characterize the affects of CIPX on algae biomass growth, 500-mL reactors containing each of the CIPX in MB3N solutions referenced above were prepared in duplicate. These reactors were cultivated under stirring, aeration, and illumination conditions corresponding to Step 3 of the protocol described in Section 2.1. 5-mL samples of algae suspension were collected from each reactor over the course of eight days at 0, 6, 24, 48, 96, 144, and 192 hours. These were analyzed for optical density (OD) using a spectrophotometer at a wavelength of 662 nm. It had been previously determined in preliminary tests that there was negligible interference from MB3N medium or CIPX at this wavelength. Measured ODs were then converted to biomass concentrations using a previously validated calibration equation for this alga under identical cultivation conditions (Zhang, 2013) (See Appendix A, Figure A1).

2.3 Assessing the Impact of Algae on CIPX Concentrations

CIPX standards over the range of 0-100 μg/L were prepared via serial dilution in an HPLC-grade solvent mixture of formic acid, methanol, and acetonitrile (2/49/49 on a volume basis) from a 0.25-g/L stock solution of CIPX made with the same solvent composition.

Four sets of duplicate 500-mL reactors containing 5000 ng/L CIPX in MB3N were prepared according to the procedure described in Section 2.2, including: 1) a dark control (DC), which comprised CIPX-spiked medium without algae, wrapped in foil to prevent light penetration; 2) a light control (LC), comprising CIPX-spiked medium without algae 3) an autoclaved algae sorption control (AASC), which comprised 40 mg/L of previously autoclave-

deactivated (60 min at 121 °C and 260 psi/°F) algae biomass in CIPX-spiked medium, wrapped in foil to prevent light penetration; and, 4) an experimental algae (EA) reactor, which consisted of 20 mg/L algae biomass cultivated in CIPX-spiked growth medium. These reactors were cultivated under stirring, aeration, and illumination conditions corresponding to Step 3 of the protocol described in Section 2.1, for 8 days. An additional negative control (NC), comprising 40 mg/L of algae biomass in medium without CIPX and exposed to light, was also created in duplicate. These were subjected to the same cultivation conditions as the DC, LC, AASC, and EA reactors. Figure 2.1 presents the setup for each of the experimental and control reactors.

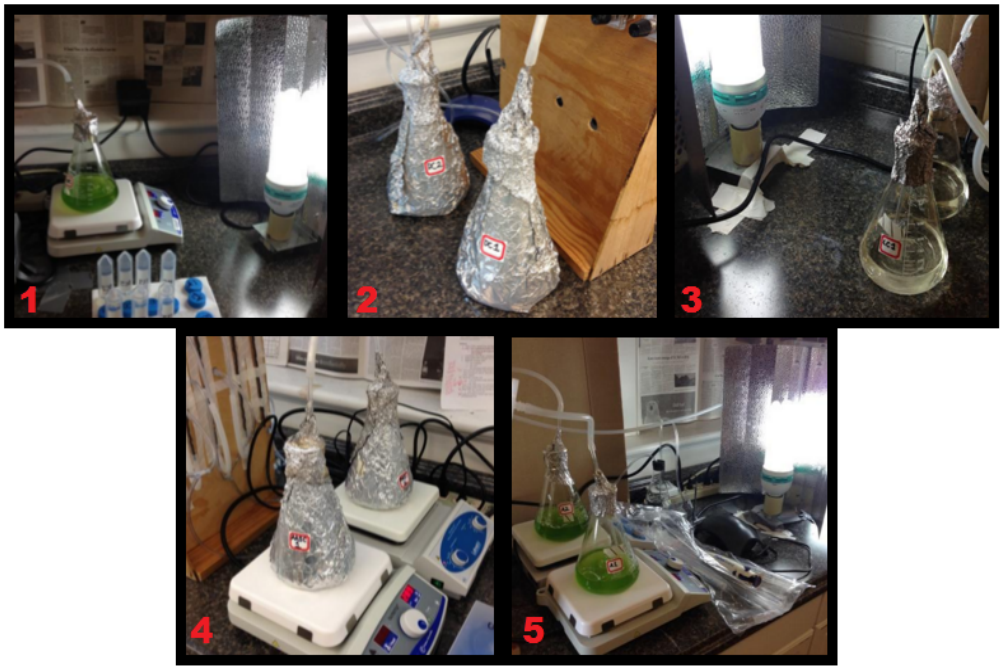


Figure 2.1. Experimental setup for duplicate reactors of: (1) negative control (NC), (2) dark control (DC), (3) light control (LC), (4) autoclaved algae sorption control (AASC), and (5) experimental algae (EA).

25-mL samples were collected from the NC, DC, LC, and EA reactors at 0, 6, 24, 48, 96, 144, and 192 hours and 25-mL samples for the AASC reactors were collected at 0 and 192 hours. Samples containing algae (EA, AASC, and NC) were analyzed for OD as described in Section 2.2 and then filtered through 0.7-μm pore size glass microfiber filters (Millipore) to remove

algae cells. NC, EA, and AASC filtrates and also unfiltered DC and LC samples were then cleaned up and concentrated via solid phase extraction (SPE), using a procedure modified from two previously validated methods (Zhou and Jiang, 2012; Zhang et al., 2014). In brief, Oasis® HLB SPE 3cc (160 mg) cartridges were pretreated with 3 mL of methanol and equilibrated with 5 mL of HPLC-grade water. 25-mL samples were then loaded onto the cartridges at 5 mL/min. Loaded cartridges were washed with 2 mL of a 50:50 (v/v) HPLC-grade methanol and water solution, vacuum dried for 15 minutes, and eluted with 4 mL of a 2/49/49 formic acid-methanol-acetonitrile (v/v/v) solvent mixture. Eluted samples were evaporated to dryness under a gentle stream of air overnight. Finally, samples were reconstituted into 1.67 mL of a formic acid-methanol-acetonitrile (2/49/49 v/v/v) solvent mixture to achieve an SPE concentration factor of 15x and transferred to sealed, amber crimp-top vials. These were refrigerated for up to one month.

CIPX concentrations for experimental and control samples were measured via high performance liquid chromatography (HPLC), using an approach based on previously validated methods (Idowu and Peggins, 2004; Lee et al., 2007; Muchohi et al., 2011; Piñero et al., 2013). The instrument was a Shimadzu 2010-AB HPLC with both fluorescence and UV detectors. CIPX's fluoroquinolone structure renders it highly fluorescent; therefore, the fluorescence detector was used as the primary detector. Excitation and emission wavelengths were set at 280 and 445 nm, respectively. The mobile phase comprised a mixture of two solutions: A) 1% formic acid in DI water, which accounted for 74% of the total flow; and B) 50:50 methanol and acetonitrile, which accounted for 16% of the total flow. This mixture was pumped isocratically at 0.5 mL/min. Separation was achieved on a 125 mm x 3.2 mm C18 column (Phenomenex). Injection volume was 20 µL. Under these conditions, retention time for CIPX was 1.9 minutes.

Appendix A presents an example chromatogram for a CIPX standard in the solvent mixture (formic acid/acetonitrile/methanol, 2/49/49) and a sample from an experimental reactor containing algae in MB3N and CIPX and also the CIPX calibration curve achieved using this method, Figures A2 and A3, respectively.

3. Results and Discussion

3.1 Assessing the Impact of CIPX on Algae Growth Rate

In order to evaluate the usefulness of algae-mediated WWTP effluent polishing systems in removing not only estrogenic steroid hormones (as previously demonstrated by Zhang et al. [2014]), but also potent antibiotic pharmaceuticals, it was essential to first demonstrate that environmentally relevant concentrations of CIPX do not significantly impede algae growth. This is critical given that it and other antibiotic pharmaceuticals are specifically designed to kill certain microorganisms.

Axenic cultures of the model alga *S. dimorphus* were exposed to different initial CIPX concentrations over the range 0 - 5000 ng/L for 8 days. Individual concentrations were selected to encompass concentrations of experimental and environmental relevance. The lowest concentration, 0 ng/L, was used as a negative control. The highest concentration, 5000 ng/L, corresponds to the initial CIPX concentration used in subsequent experiments probing algae-mediated CIPX removal; wherein it was necessary to start with a high enough initial concentration to allow for accurate measurements over time as the parent compound was removed. Concentrations of 600, 300, and 100 ng/L correspond to the measured ranges of CIPX in municipal wastewater treatment plant (WWTP) influents (250-600 ng/L) and effluents (75-375 ng/L) (Jelić et al., 2012). Samples with 25 and 50 ng/L concentrations were included to capture any inhibition effects caused by CIPX after it is released from the WWTP and diluted into a receiving water.

Figure 3.1 presents algae biomass concentrations over time in the presence of selected CIPX concentrations. All measurements are normalized using the initial (t = 0) biomass concentration for their respective initial CIPX concentration, in order to allow for direct

comparison among data from the different concentrations studied. The initial algae biomass concentration for all tested CIPX concentrations was roughly 130 mg/L.

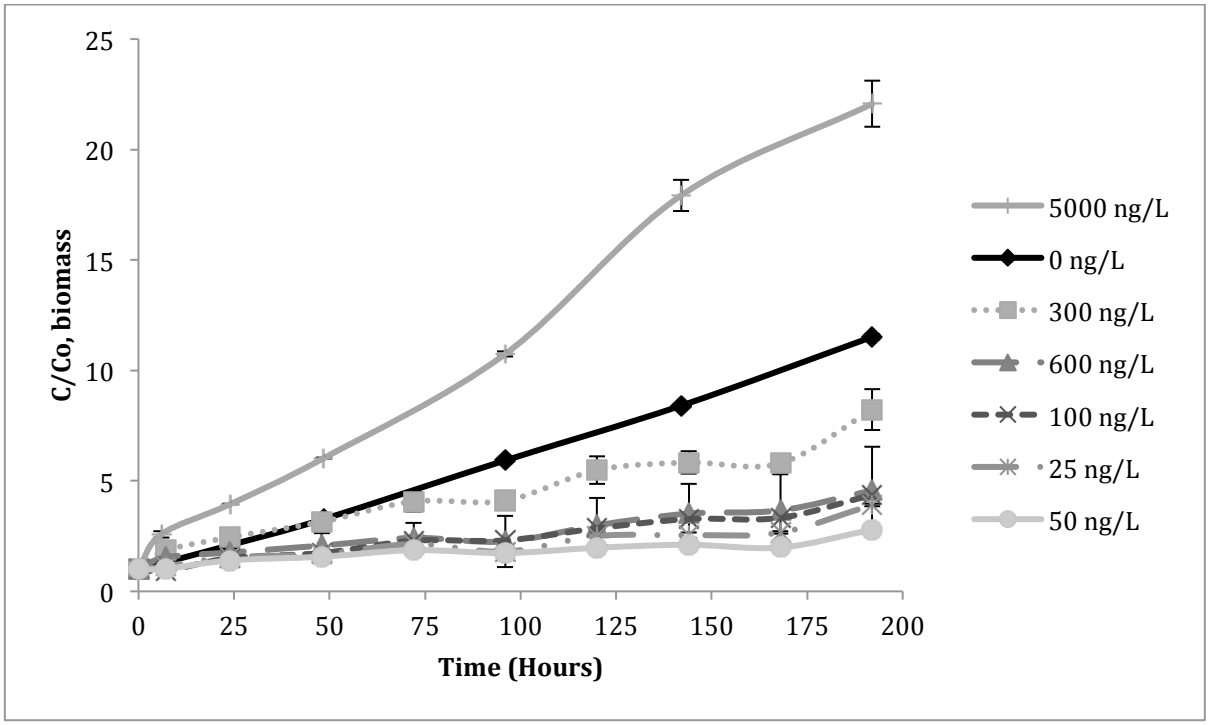


Figure 3.1. Algae biomass concentrations over time during exposure to different CIPX concentrations. Error bars correspond to averages ± standard error from duplicate (n = 2) measurements.

There are four key observations to be made from Figure 3.1. The first is that all reactors exhibit fairly exponential growth over roughly 8 days. This is not unexpected based on classical microbial growth models and previous measurements in our lab (Zhang et al., 2014). Thus, there is no indication that CIPX has an immediately drastic adverse impact on the algae's growth, even though it and other antibiotics are designed to kill microorganisms, specifically Gram-positive and Gram-negative bacteria (Norvill et al., 2016).

The second noteworthy observation from Figure 3.1 pertains to a seemingly contradictory effect of CIPX on algae growth, whereby the environmentally relevant concentrations (25-300

ng/L) appear to slightly inhibit algae growth compared to the control (0 ng/L), but 5000 ug/L appears to dramatically stimulate algae growth. This was an unexpected result; however, Zhang, et. al. (2014) also reported unexpectedly enhanced growth for the same algae species in the presence of the steroid hormone 17β-estradiol at much-higher than environmentally-relevant concentrations (e.g., 5-100 ug/L). They did not provide any conclusive rationale for why this was observed, but they speculated that at high concentrations, the estrogens became an organic carbon source for the algae and stimulated its growth "mixotrophically" or "heterotrophically" as has been previously demonstrated from some algae species (Apt and Behrens, 1999; Greca et al., 1997; Faramarzi et al., 2008). To the best of our knowledge, this is the first observation of algae exhibiting a dual response of inhibition at low concentrations and stimulation at high concentrations in the presence of an antibiotic drug.

A third observation from Figure 3.1 addresses the engineering application of this data. Although there are dramatic differences in growth rate for algae samples exposed to different CIPX concentrations over 8 days, these are not clearly evident until after 72 hours. Up until that point, all series corresponding to environmentally-relevant CIPX concentrations grow at roughly the same rate as the control, as made evident by the overlapping error bars. This is noteworthy because relevant algae LCA studies make reference to anticipated hydraulic retention times (HRTs) of 24-48 hours (Resurreccion, 2013; Colosi et al., 2015). Therefore, it can be inferred from Figure 3.1 that environmentally relevant concentrations of CIPX will not mediate significant algae growth inhibition in anticipated integrated algae-WWTP systems.

Finally, the growth experiment summarized in Figure 3.1 fails to capture one additional effect that long-term exposure to CIPX may have on the algae; namely, acclimation. The algae cells had not been previously exposed to CIPX for any duration, but in a real flow-through

system, it is expected that the algae may become acclimated to the presence of CIPX at low concentrations. Such acclimation, as made evident by development of antibiotic resistance, has been widely observed for various bacteria (Norvill et al., 2016; Al-Ahmad et al., 1999; Berendonk et al., 2015; Rizzo et al., 2013). If the algae could become similarly acclimated, it could further reduce the impact of CIPX on algae growth rate at environmentally-relevant concentrations. This should be explored in future work. Regardless, the first objective of this study was achieved, and it seems safe to conclude that algae is not appreciably inhibited by environmentally relevant concentrations of CIPX.

3.2 Assessing the Impact of Algae on CIPX Concentrations

3.2.1 CIPX Apparent Removal

To reiterate from Section 1, the principal goal of this project was to evaluate algae-mediated removal of potent antibiotic pharmaceuticals, as had been previously observed for estrogenic steroid hormones by Zhang, et. al. (2014). This was done by measuring apparent removal of CIPX in the presence of active algae biomass over 8 days. Initial concentrations of algae and CIPX were 20 mg/L and 5000 ng/L, respectively. Experimental results of the apparent removal of CIPX are presented in Figure 3.2.

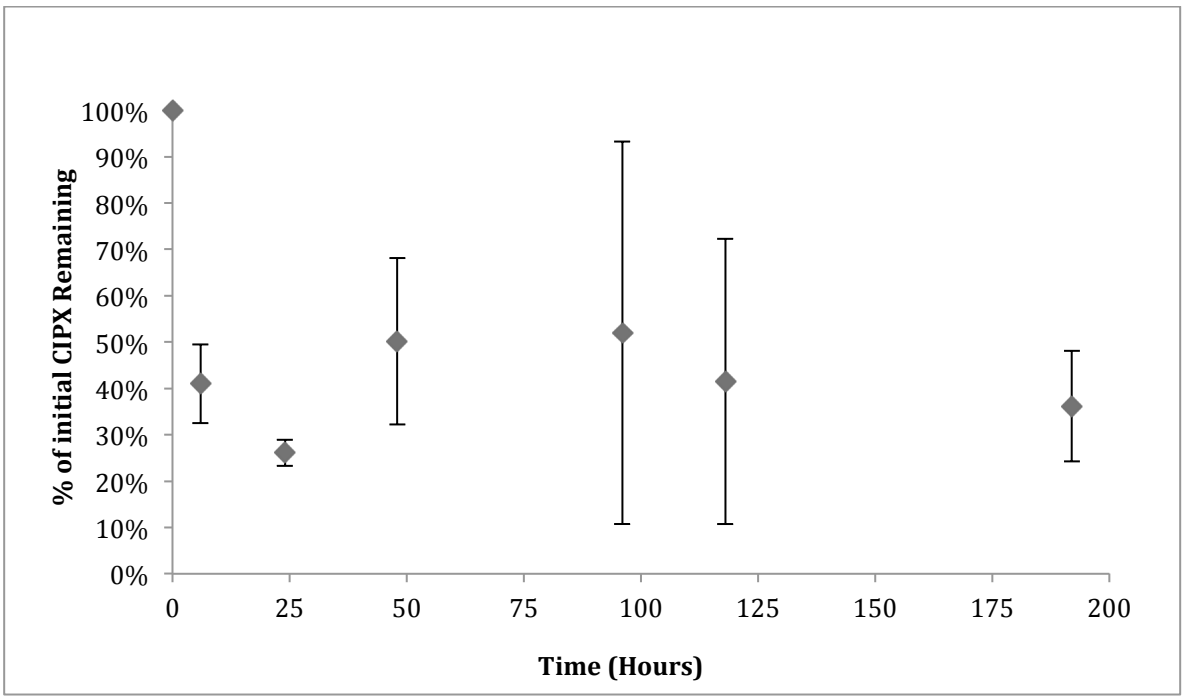


Figure 3.2. Time series of CIPX concentration measured in an experimental reactor normalized by dividing each measurement by the initial (day 0) concentration. Error bars correspond to averages ± standard error from duplicate (n = 2) measurements.

Figure 3.2 reveals that algae mediate rapid, effective removal of CIPX. Roughly 60-75% of the initial CIPX is removed from the system within the first 24 hours, after which the concentration remains relatively unchanged, within experimental error, for the rest of the 8-day interval. Measurements at 48, 96, and 122 hours exhibit larger than desired standard errors. This is attributed to experimental artifacts (e.g., stirring interruptions). Regardless, the error bars for these measurements do encompass the estimated CIPX removal efficiency (60-75%) for this system, based on the first few and last measurements (as noted above). Additionally, this removal percentage is higher than biological CIPX removal percentages for conventional WWTPs reported by Jelic et al., 2012, whereby only 40% of influent CIPX was removed during the biological treatment step. It is noteworthy that the removal of CIPX appears to occur mostly within the first 24 hours of contact with *S. dimorphus*, given that common HRT values for the

proposed algae-treatment system are expected to be around 24-48 hours (as noted in Section 3.1). The ability of the algae treatment system to significantly reduce the CIPX concentration over such a short period of time contributes to its overall viability as an effective WWTP technology.

3.2.2 Analysis of CIPX Removal Mechanisms

Based on previous work (Zhang et al., 2014; Norvill et al., 2016; Batchu et al., 2014; Ren et al., 2016; Challis et al., 2014) it was anticipated that CIPX might be removed by some combination of sorption, biotransformation, and photo-transformation. The extent to which each of these mechanisms contributes to CIPX apparent removal is of interest, because they result in different outputs that are then released to the environment. A series of control reactors was thus used to analyze the various pathways by which CIPX concentration could be reduced over time. Figure 3.4 presents the final CIPX concentrations (t = 192 hours) for each type of control, as normalized to their respective starting CIPX concentrations.

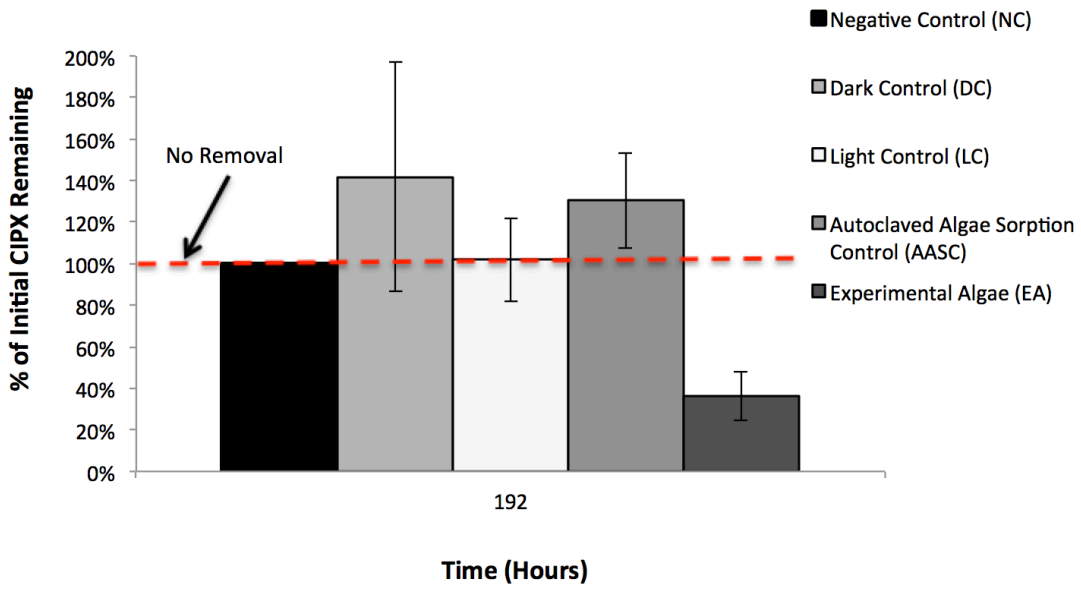


Figure 3.4. Normalized final CIPX concentrations (t = 192 hours) in each control. *negative control (NC):* algae in medium with no CIPX and exposed to light; *dark control (DC):* CIPX-spiked medium without algae and wrapped in foil; *light control (LC):* CIPX-spiked medium without algae and exposed to light; *autoclaved algae sorption control (AASC)*: autoclave-deactivated algae biomass in CIPX-spiked medium and wrapped in foil; *experimental algae (EA):* algae in CIPX-spiked medium and exposed to light. Error bars correspond to averages ± standard error from duplicate (n = 2) measurements.

Working through Figure 3.4 from least to most complicated control makes it possible to roughly allocate overall apparent removal among the various hypothesized mechanisms. First, the CIPX concentration in the dark control (DC) remains largely unchanged over the entire duration. This rules out loss of CIPX via sorption to the reactor walls, volatilization, or any other experimental artifact. Next, comparison between the ending DC and light control (LC) CIPX concentrations reveals the extent to which photolysis contributes to CIPX removal, since exposure to light is the only difference between these reactors. From these data, photolysis does not contribute to overall CIPX removal. Third, since neither reactor is exposed to light, comparison of DC and the autoclaved algae sorption control (AASC) would give an idea of how sorption to algae cells contributes to CIPX removal; however, there is no appreciable change in

CIPX concentration in the AASC reactors over 192 hours. Therefore, it can be concluded that sorption to algae cells does not contribute to algae-mediated removal of CIPX. Finally, comparison of AASC (dead algae) and EA (live algae) reactors gives an indication of how algae-mediated biotransformation contributes to CIPX removal. In the EA reactor, 35% of the initial CIPX concentration remained after 192 hours, compared with 100% remaining in the AASC. This demonstrates that it is biotransformation rather than sorption that mediates the observed reduction in CIPX. As a whole, the EA reactors were the only reactors in which an observable reduction in CIPX concentration occurred. Therefore, since removal via sorption and photolysis were ruled out in the AASC and LC controls, the CIPX apparent removal observed can be fully attributed to algae-mediated biotransformation.

Figure 3.5 presents a graphical recap of how the evaluated removal mechanisms contribute to removal of CIPX versus estrogens, based on data from this thesis and Zhang, et. al. (2014). This presentation of the removal data emphasizes the importance of biotransformation and sorption reactions for emerging contaminants evaluated to date. It also reaffirms visually that direct photolysis contributes very little if at all to algae-mediated contaminant removal. These similarities and differences can be attributed to the differences in chemical properties (Table 1.2) between CIPX and the steroid hormone estrogens.

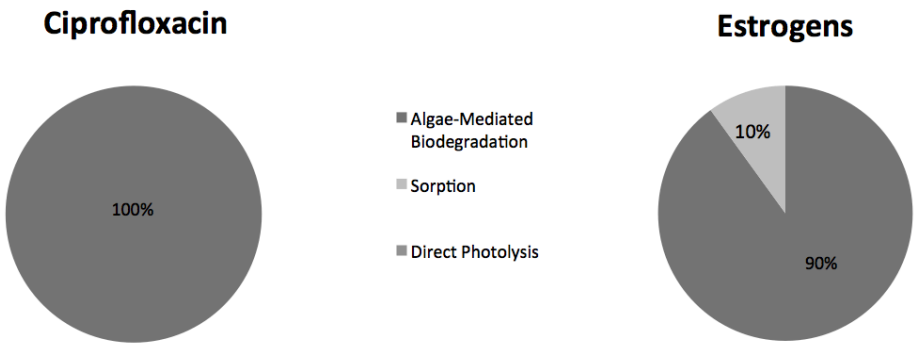


Figure 3.5. Schematic representation of the percent contribution to removal that can be allocated to each of the three major removal mechanisms studied for both CIPX and estrogens.

It was interesting that CIPX did not exhibit any sorption-based removal when sorption accounted for roughly 10% of algae-mediated estrogen removal (Zhang et al, 2014). However, this result is not unexpected since previous studies have shown that the sorption of a chemical to algal biomass and activated sludge varies greatly in different systems and is largely dependent on minute deviations in physical parameters such as temperature, pH, and light that affect the chemical parameters of the sorbents, solids, and medium (Norvill et al., 2016). However, known chemical parameters of the compounds can be used to speculate reasons for the removal observations.

First, the small difference in sorption removal allocation between CIPX and steroid estrogens is likely due to differences in their chemical structures and parameters at the pH of the culture medium and may also be attributed to slight deviations in environmental conditions due to challenges associated with exact reproducibility of the experimental setup. The structure of CIPX (Table 1.2) is such that it exists primarily in the form of a zwitterion (one positive charge plus one negative charge) at pH 7 (Ma et al., 2015). This renders the molecule neutral as a whole, but theoretically makes it less likely to sorb due to the opposing charges at each end. Further, Gultom and Hu, 2013 report that most species of algae typically have negatively charged cells,

although there is some variation between different species. Another study performed by Ives, 1959 analyzing the surface electric charge at different pH on *Chlorella* sp. reported that algae biomass exhibits roughly zero surface charge at pH 7 (see Appendix B, Figure B1.). Thus, it repels both the positively and negatively charged sections of the CIPX molecule. In contrast, estradiol and the other steroid hormones exhibit neutral charges at pH 7 (Lorphensri et al., 2006; Neale et al., 2009), which slightly increases their capacity to sorb onto neutral algae cells. Whether *S. dimorphus* is negatively or neutrally charged at pH 7, the neutral estrogens have a greater potential of sorbing to the algae biomass than the zwitterionic CIPX. The observed lack of sorption-based CIPX removal could be beneficial for engineered implementation of algae-based WWTP effluent polishing; in so far as sorption is the least desirable removal mechanism for any type of emerging contaminant, given that sorbed compounds in WWTPs often end up in the environment through land application of sludge fertilizers and even volatilization when anaerobically digested.

Second, it was somewhat unexpected that none of the observed removal for CIPX or the estrogens could be attributed to photolysis. Some previous studies had reported photolytic degradation of structurally related compounds under various seemingly relevant conditions (e.g., simulated solar light, circumneutral pH, etc.) (Batchu et al., 2014; Challis et al., 2014; Matamoros et al., 2015; Norvill et al., 2016; Ren et al., 2016). However, CIPX and the selected estrogens do have relatively low values of quantum yield, which indicates the ability of a compound to degrade as photons are absorbed: 0.158 mol/Einstein for CIPX at pH 7 (Batchu et al., 2014), and 0.07-0.0246 mol/Einstein for estrogens at pH 7 (Ren et al., 2016). Other studies have also theorized that degradation of emerging contaminants via direct photolysis, in which the contaminant itself absorbs photons and then undergoes light-induced degradation, is greatly

hindered in the presence of algae and algal growth medium, due to decreased light penetration and absorbance of light energy by other organic compounds (Norvill et. al. 2016). Beyond this, it is also possible that indirect photolysis, in which other "photo-sensitizing" compounds absorb photons and then undergo degradation via initiation of a chain reaction that then also degrades the contaminant of interest, may contribute to CIPX and/or estrogen removal under certain conditions. It is very possible that indirect photolysis occurred but was not captured by the experimental setup of the LC reactors used in this study and Zhang et al. (2014). As such, future work should focus on examination of direct and indirect photolysis of CIPX and estrogens in actual WWTP effluent instead of algae medium.

Overall, the ability of the proposed system to remove such a large portion of the initial CIPX concentration primarily through algae-mediated biotransformation is noteworthy. The predominance of chemical reactions (i.e., biotransformation) over physical removal (i.e., sorption or volatilization) for estrogens and CIPX is significant in so far as degradation reactions create aqueous-phase products that are then released into the environment. Zhang, et al. (2014) evaluated the residual estrogenic potency of the biotransformation and photo-transformation products arising in their algae system. They concluded that rapid removal of an individual steroid hormone did not directly translate into equally rapid removal of the associated estrogenic toxicity, because the reaction products were as or more potent than the parent compounds. Analogously, it is of interest to know what products are produced during algae-mediated CIPX removal and whether or not their potency matches that of the parent compound. The threat of antibiotic resistance (AR) and antibiotic resistant bacteria (ARB) in nature is a major source of motivation for the development of treatment systems that will remove antibiotics from WWTP effluents before they are discharged into receiving waters. Therefore, it is of utmost importance

to ensure that the proposed algae treatment system will not bio-transform CIPX into byproducts that are as or more potent than the parent compound and will strongly promote AR downstream. The hope is that either: 1) CIPX is converted into a small number of principle metabolites that are too dissimilar to the parent product to foster spread of AR to CIPX; or 2) CIPX is converted into a large variety of metabolites, such that no single product exists at a high enough concentration to foster spread of AR to CIPX. Future research will focus on determining the identities of biotransformation byproducts arising from algae-mediated CIPX removal and assessing their potency in dissemination of AR.

3.2.3 Kinetic Analysis of CIPX Apparent Removal

Once it had been determined that biotransformation predominates algae-mediated CIPX removal, it was of interest to perform a kinetic analysis so that apparent removal rate could be quantified and then compared to existing data (e.g., Zhang et al [2014]). This comparison was based on determination of a pseudo-first order rate constant (*k*). This parameter was computed based on fitting of experimental data from Figure 3.2 to the pseudo-first order rate model summarized by Eqn. 1.

$$\frac{d[C]}{dt} = k[C] \qquad \text{Eqn. 1}$$

After integration, Eqn. 1 becomes:

$$\ln([C]) = kt + \ln([C]_0) \qquad \text{Eqn. 2}$$

Eqn. 2 can be rearranged according to Eqn. 3:

$$\ln([C/C_0]) = kt \qquad \text{Eqn. 3}$$

For the purpose of simplification, Equations 1, 2, and 3 only account for the effects of biodegradation-based removal without specifically isolating removal via sorption. Since no sorption was observed for algae-mediated CIPX removal and only 10% for estrogens removal,

generalizing the equations for biodegradation allowed for ease of comparison between the two studies. When accounting for sorption, an additional term known as the retardation factor would need to be incorporated into Eqn. 1:

$$\frac{d[C]}{dt} = \frac{k[C]}{R} \qquad \text{Eqn. 4}$$

As presented in Equation 4, the retardation factor, which represents effects of sorption, works to reduce the removal rate, *k*. If Zhang et al. 2014 had accounted for sorption effects of the estrogens, they would have calculated lower *k* values. Depending on the magnitude of their *R* value, this could have a major impact on their reported pseudo-first order analysis results. Therefore, future work will involve singling out the effects of sorption on removal rates for the estrogens study.

From Eqn. 3, a value for *k* can be determined by computing the slope for a plot of $\ln([CIPX_t/CIPX_0)$ versus time. This plot is presented in Figure 3.3A, wherein it is evident from the low R^2 (18%) that this rate model does a poor job fitting the data over the measured 8-day duration. Accordingly, the data were re-plotted for just the first 24 hours, yielding a much higher R^2 value (75%, Figure 3.3B). This window of time corresponds to the expected HRT for engineered algae-WWTP systems. The best-fit value of the *k* parameter for CIPX is -0.047 $hours^{-1}$. Interestingly, Zhang, et al. (2014) also found that they could not fit the pseudo-first order rate model for the entire duration of their algae-mediated estrogen removal reactions (Figure 3.3C), and that the fit was much better for just the initial 24 hours (Figure 3.3D). It is therefore possible to directly compare the *k* value from this study with the *k* values reported by Zhang, et. al. (2014).

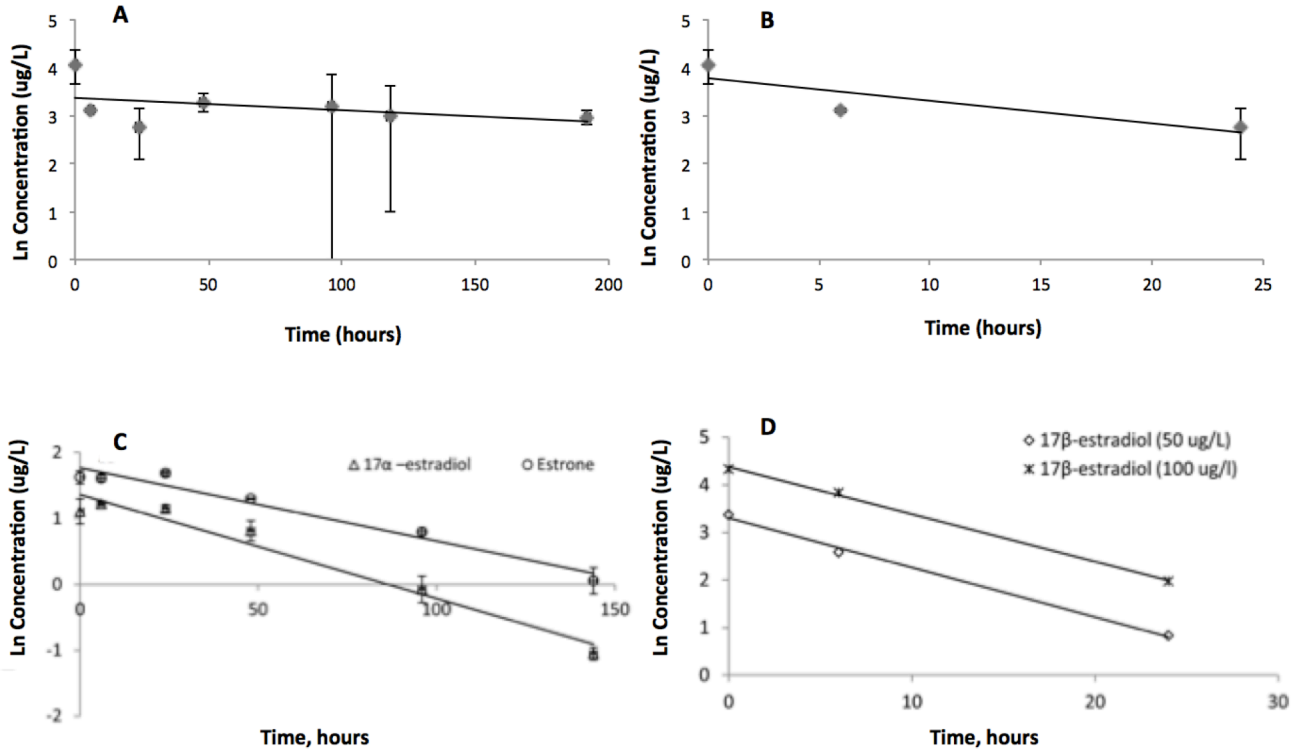


Figure 3.3. The natural logarithmic transformations of CIPX concentration and estrogen concentration versus time in algae medium. (A) CIPX with an initial concentration of 5000 ng/L over full time series; (B) CIPX with an initial concentration of 5000 ng/L over the first 24 hours; (C) 17α-estradiol and estrone with initial concentrations of 5000 ng/L; (D) 17β-estradiol with concentrations of 50,000 and 100,000 ng/L. Error bars correspond to averages ± standard deviation from duplicate (n = 2) measurements.

Zhang, et. al. (2014) also computed a half-life ($t_{1/2}$) parameter for each of their compounds of interest, using Eqn. 5.

$$t_{1/2} = \ln(2)\,/k \qquad \text{Eqn. 5}$$

Table 3.1 presents a comparison of k and $t_{1/2}$ values from this study and Zhang, et. al. (2014). From these data, it is evident that the k value for algae-mediated CIPX removal is within the range of previously observed k values for algae-mediated estrogen removal. This is interesting, given the differences in structure between CIPX and estrogenic steroid hormones, presented in

Table 1.2. It is also interesting that the algae exhibit rapid initial removal of all target compounds without an observable lag phase, even though they did not have any previous exposure to each drug. This adds to the appeal of the proposed algae-based treatment system.

Table 3.1. The pseudo-first-order removal rate constants (*k*), R^2 values, and half-life ($t_{1/2}$) for CIPX (24 hour removal) and the estrogenic compounds studied by Zhang, et. al. (2014) mediated by interaction with *S. dimorphus*.

Compound	***k* (hours^{-1})**	**R^2 (correlation coefficient for *k*)**	**$t_{1/2}$ (hours)**
17β-estradiol	-0.1039	0.99	6.8
Ciprofloxacin	-0.0470	0.75	14.75
17α-estradiol	-0.0158	0.96	45.7
Estrone	-0.0111	0.96	64.1

4. Conclusions

The overarching goal of this project was to explore whether integrated algae and wastewater treatment systems can help with removal of other emerging contaminants in addition to estrogens. The scope of chemicals of interest was narrowed down to focus on antibiotics, or more specifically, ciprofloxacin (CIPX) as a highly prescribed model antibiotic. Removal of antibiotics is of particular interest due to their potential to promote antibiotic resistance in humans and other organisms as well as stimulate the presence of antibiotic resistant bacteria (ARB) when entering natural systems through wastewater discharge.

Since antibiotics are intended to deactivate bacteria and the proposed treatment system is grounded in the use of live algae cells to mediate removal, the preliminary objective of this research was to ensure that exposure to CIPX would not hinder the biomass production of the proposed algae treatment system. By way of bench-scale experiments, reactors containing algae species, *S. dimorphus*, in MB3N medium were spiked with different concentrations of CIPX and biomass was analyzed over time. Overall, it was found that CIPX concentration has little effect on biomass production, especially at a hydraulic residence time (HRT) of 24 hours anticipated for the proposed treatment system.

The second objective of this study was to determine whether or not CIPX could be removed by *S. dimorphus* and through which possible removal pathways. Through bench-scale experiments involving isolated control reactors and HPLC analysis to measure CIPX concentration over time, an average removal of 65% was observed, indicating that the proposed algae treatment system is an effective method for removal of CIPX. Rapid removal of CIPX was observed within the first 24 hours and a pseudo-first order analysis revealed a removal rate of 0.047 hr^{-1} for algae-mediated removal of CIPX. Compared with the removal rates observed for

the estrogens study, CIPX has the second-fastest removal of the four compounds analyzed. Of the removal mechanisms studied (sorption, direct photolysis, and biotransformation), algae-mediated biotransformation constituted 100% of the total removal of CIPX and no significant removal was observed for any of the other removal pathways.

5. Future Work

Future work in this realm of research will consist of two stages of experiments, as described below and presented schematically in Figure 5.1:

First, I would like to gain a better understanding of sorption and photodegradation of CIPX to confirm the conclusions made from the original removal experiment. Therefore, it will be beneficial to perform experiments that are specifically designed to evaluate the sorption and photodegradation removal mechanisms. To better analyze removal via sorption, either a sorption isotherm experiment or desorption experiment will be performed. The setup of the sorption isotherm experiment would consist of adding a known concentration of CIPX to a beaker containing both algae biomass and a solvent, shaking it, and analyzing the solvent portion for CIPX concentration via HPLC. The amount of CIPX sorbed to the algae biomass is the amount not accounted for in the solvent portion. Although the original removal experiment ruled out direct photolysis as a removal mechanism, it is possible that indirect photolysis played a role in the overall CIPX removal. Therefore, development of an experiment to analyze indirect photodegradation of CIPX would also be of interest. The setup of an experiment to analyze photodegradation of CIPX should involve actual wastewater rather than algae medium. In order to reaffirm the conclusiveness of my removal time series results, I would like to repeat the second experiment and add more replicates to decrease the standard errors evident in the removal graphs. Further evaluation of the role of sorption in the pseudo-first order analysis of algae-mediated estrogen removal will also be performed.

Second, ongoing work will reveal to what extent the by-products of algae-mediated CIPX transformation retain their antibiotic potency. A recent review by Norvill et al., (2016) laid out many of the research gaps in this area indicating that there is significance in continuing in this

vein of work during pursuit of my PhD. Specifically, there is interest in detoxifying potent contaminants in a way that doesn't promote antibiotic resistance. This is of critical importance for assessing the extent to which this treatment strategy could inadvertently contribute to development of widespread CIPX resistance. The photodegradation and algae mediated biodegradation products of CIPX in the proposed treatment system are uncertain. Therefore, further analyses into the by-products generated by the system as well as an assessment of their efficacy and efficiency in regards to minimizing potency, and thereby reducing possibility of widespread antibiotic resistance will be performed.

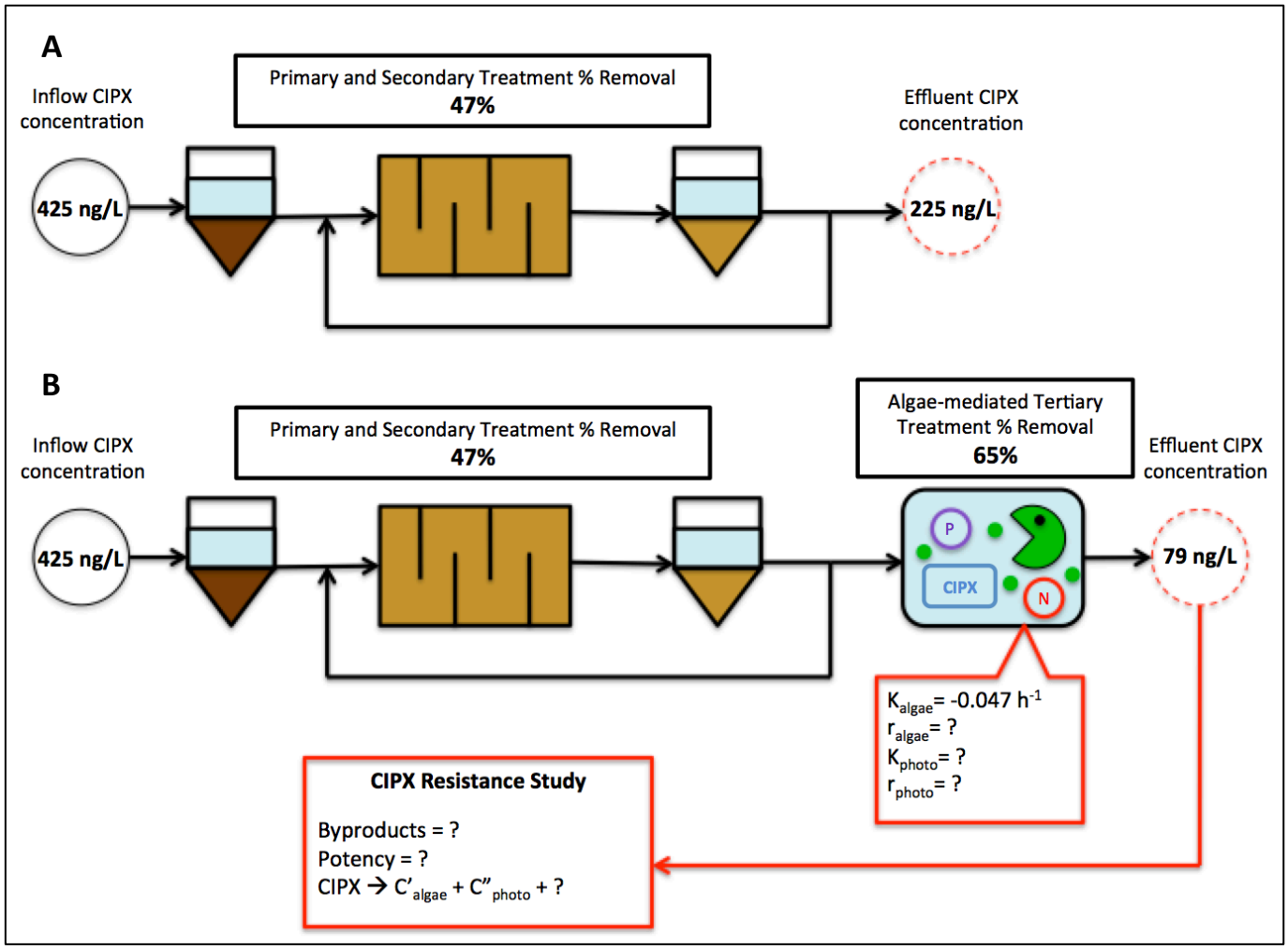


Figure 5.1. Diagram of theoretical CIPX removal in: (A) conventional WWTPs and (B) WWTP with the proposed algae tertiary treatment system. The red callout boxes in (B) represent the future work areas (i.e. indirect photolysis analyses and antibiotic resistance study). Influent and effluent concentrations are average values from (Jelić et al., 2012) and all other values presented are experimental or calculated averages.

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Appendix A.

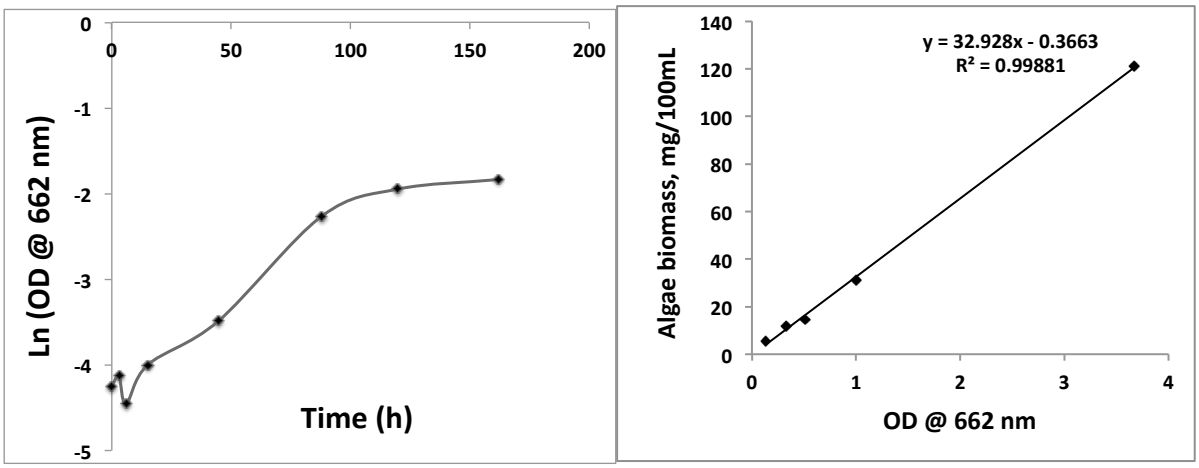

Figure A1. Algae cultivation calibration results from Zhang, (2013). *(Left):* Algae growth curve. *(Right):* A linear correlation between optical density and algae dry mass.

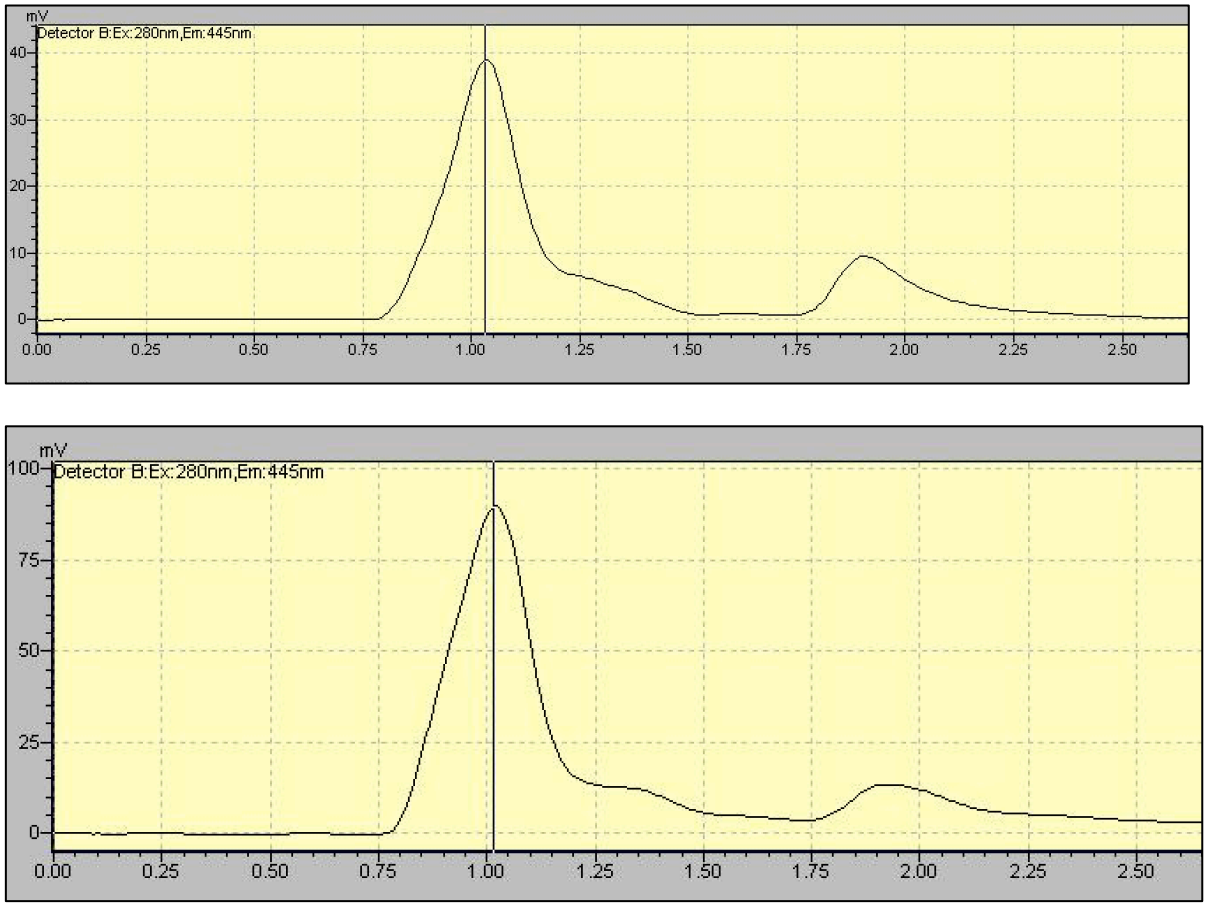

Figure A2. Example chromatograms for CIPX under fluorescence detection. (*Top*): 80 µg/L CIPX standard in formic acid/acetonitrile/methanol (2/49/49). (*Bottom*): 5 ug/L CIPX in an experimental reactor containing algae. The CIPX peaks occur at 1.9 minutes. Formic Acid in the background solvent generates a peak at 1.0 minute.

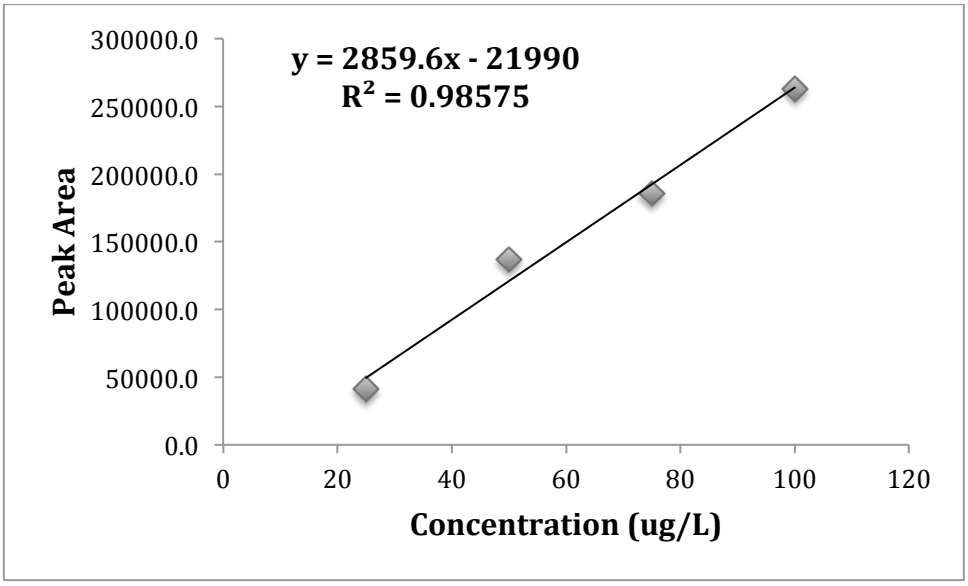

Figure A3. Calibration curve generated using CIPX standards range of 0-100 µg/L. Only standards in the range of 25-100 µg/L were found to be within the calibratable range, so standards less than 25 µg/L were not included in the calibration equation.

Appendix B.

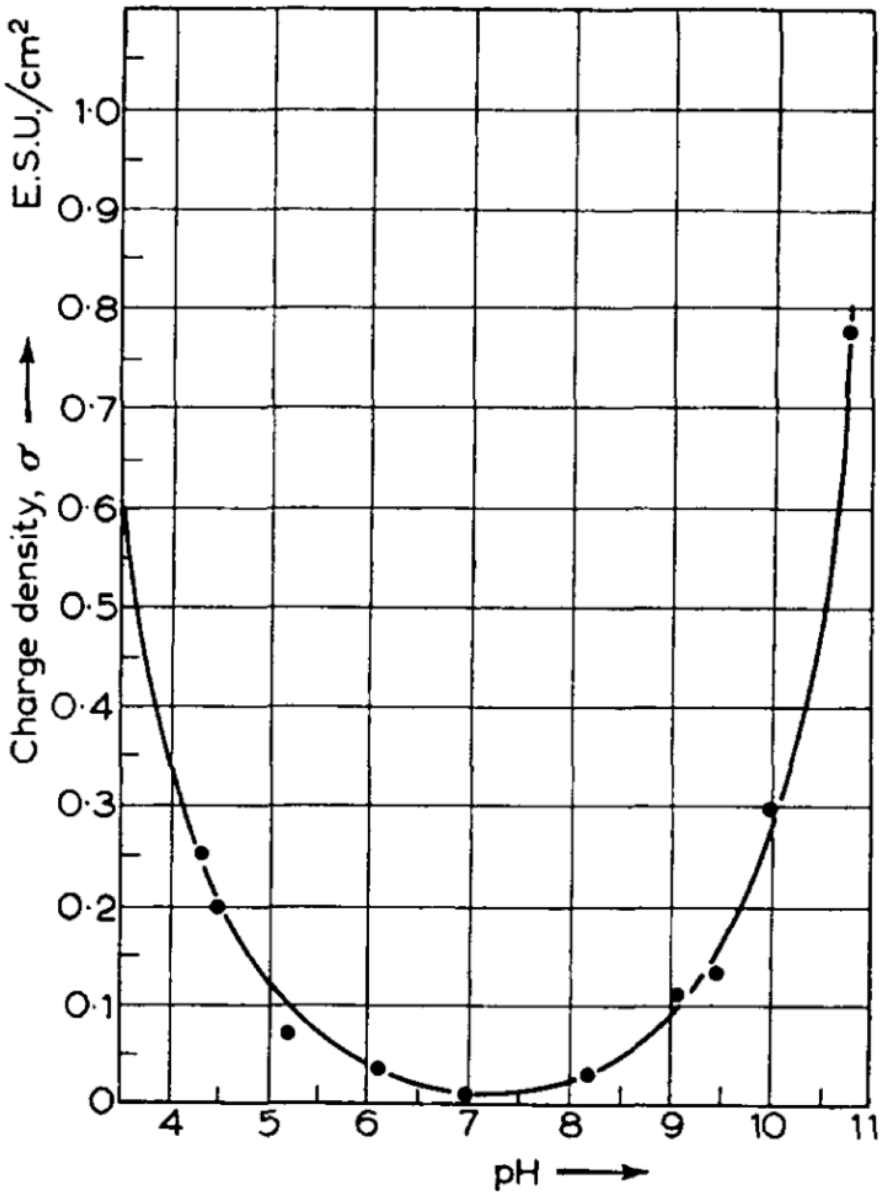

Figure B1. Surface electric charge variation with pH of *chlorella sp.* (Ives, 1959)