CONTAMINANTS OF EMERGING CONCERN TREATMENT BY MICROALGAE: THE STATUS AND FUTURE OF PHYCOREMEDIATION

by

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Abstract

Chemicals of emerging concern (CECs) including pharmaceutical and personal care products are being discharged into aquatic environments at rates causing downstream ecological damage. The removal of these CECs by traditional wastewater treatment plants is insufficient given these facilities were mainly designed prior to the advent of analytical capacities that detect these contaminants at their microtitre concentrations. As sustainable alternatives to wastewater treatment are being considered and developed, these novel technologies must address the traditional demands of wastewater treatment as well as the new challenges posed by CECs. Research has highlighted microalgae as an ideal class of organisms to meet the new challenges in wastewater remediation. Their light-driven metabolism of the primary constituents of wastewater, namely dissolved organic, nitrogenous, and phosphorous material, is well-documented, as is their potential re-use as biofuels, nutraceuticals, or as fertilizers. The opportunities to treat CECs using microalgae is a new research field spanning the past decade, and literature-reported conclusions about their potential is promising.

This thesis summarizes and expands on the current understanding of microalgal-mediated CEC treatment. CECs considered in this text include those from the following classes: antibiotics, antifungal/antimicrobial agents, nonsteroidal anti-inflammatory drugs, anticonvulsants & antidepressants, artificial sweeteners, beta-adrenoceptor blocking agents, lipid regulating drugs, endocrine-disrupting chemicals, X-ray contrast media, UV filters, stimulants, anti-itching drugs, insect repellents, and plasticizers. The first section presents a meta-analysis of the reported treatment of CECs by eukaryotic and prokaryotic microalgae. This analysis highlights the phylogenetic trends in CEC treatment as well as the chemical and biological effects that drive the primary mechanisms of CEC removal. The second section of this thesis proposes a new avenue of microalgae-mediated CEC treatment through genetic modification. In the form of a review article, this section outlines the potential to heterologously express cytochrome P450 enzymes, primarily responsible for human drug metabolism, to amplify the capacity for microalgae to treat CECs. Considerations regarding enzyme selection, microalgal expression, and

potential hindrances to this technology are discussed, serving as a theoretical basis for future experimental validation. Finally, the third section encompasses the preliminary research conducted in the Beaty Water Research Centre. Experiments investigating the capacity to improve CEC treatment using different biological (natural and synthetic) interventions are described and initial results are discussed. The document broadly addresses the past and potential future of microalgal CEC treatment and may serve as a guiding document for future researchers.

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List of Abbreviations

ABC
ATP-binding cassette
ANOVA
Analysis of variance
ATP
Adenosine triphosphate
BPA
Bisphenol A
CEC
Contaminant of emerging concern
CW
Centrate wastewater
DNA
deoxyribonucleid acid
EDC
Endocrine-disrupting chemical
EPS
Extracellular polymeric substance
ETR_{Max}
Maximum electron transport rate
FAD
Flavin adenine-dinucleotide
FMN
Flavin mononucleotide
GC
gas chromatography
HPLC
High performance liquid chromatography
HRT
Hydraulic retention time
HSM
High-salt media
Km
Michaelis constant
LED
Light-emitting diode
logP
Partition coefficient
NADPH
Nicotinamide adenine dinucleotide phosphate
NSAID
Non-steroidal anti-inflammatory
PCR
Polymerase chain reaction
pKa
Dissociation constant
RBG
Red Blue Green
RNA
ribonucleic acid
SDS
Sodium dodecyl sulfate
TAP
Tris-acetate phosphate
UV
Ultraviolet
Vmax
Maximum rate of enzyme activity
WWTP
Wastewater treatment plant

Chapter 1

Introduction

1.1 Preamble

Medical advancements leading to the improved quality of life for billions of people has simultaneously resulted in the advent of emerging crises including the widespread contamination of aquatic ecosystems by pharmaceutical products as well as other contaminants of emerging concern (CECs). As this pressing issue receives increased attention due to these CECs' myriad negative environmental and human-health effects, technologies have emerged by which the contaminants can be removed by one of their greatest avenues of delivery to aquatic systems, treated wastewater effluent. These technologies include artificial irradiation, filtration, and chemical treatments, all of which consume vast amounts of energy. While solutions to CEC contaminantion are known, their widespread use is incompatible with managing the simultaneous crisis of global climate aberrations resulting from the combustion of fossil fuels. Novel solutions to wastewater CECs are required that repurpose waste products and use natural energy sources to combat these paired issues. Bioremediation is an opportunity to take advantage of the natural capacity of plants to employ sunlight-driven biochemistry that can treat contaminants in an environment. In the context of wastewater CECs, microalgae may serve as an ideal agent of bioremediation as they are documented to flourish in wastewater environments and have a recorded capacity to treat CECs contained therein. This instance of bioremediation has the potential to mitigate the issue of aquatic environmental contamination without directly contributing to the equally pressing issue of global climate change. In this thesis, different avenues of microalgal-based bioremediation will be investigated and discussed under the context of its potential as a sustainable alternative to traditional wastewater treatment.

1.2 The need for sustainable biotechnologies

Environmental engineering, the discipline under which this thesis is submitted, concerns itself with developing technical solutions to mostly anthropogenic issues affecting the atmosphere, pedosphere, and hydrosphere. With the advent of the 21st century, these technical solutions are increasingly developed to not only manage pressing issues, but to apply solutions that minimize further environmental harm. This focus on 'sustainable' solutions is being driven by increasing concerns over global climate change, and its deleterious effects concerning both humans and their environment alike.

This environmental change is driven by the combustion of fossil fuels, a process that became instrumental to global development during the 18th century when the coal-powered steam engine was commercialized, triggering the industrial revolution (Smil, 2017). Subsequent technological breakthroughs including the invention of the generator and the discovery of petroleum and liquefied natural gas in geological deposits have triggered an unprecedented 300-year era of technological advancement allowing mainstream air-travel, the expansion of potable water availability, modern medicine, global communication networks, and countless other breakthroughs that have radically altered human understanding of the world around us (Smil, 2017). These carbon-rich based fuels can be efficiently converted to other sources of energy through combustion, thus releasing carbon dioxide and other gases into the atmosphere. These gases contribute to an atmospheric greenhouse effect, altering equilibration of incoming and radiated solar energy such that less energy is returned to space. The strength of the greenhouse effect is dependent on the concentration of greenhouse gases, including those released through the combustion of fossil fuels, and atmospheric concentrations of these greenhouse gases have increased markedly since the 18th century (IPCC, 2019). The most important greenhouse gas, carbon dioxide, had not surpassed 280 parts per million (ppm) in millions of years prior to 1700,

yet its average concentration during 2020 was 412.5 ppm (Tans & Keeling, 2021). This increase has been linked to increasing global surface temperatures, which has subsequently affected climates across the planet, posing new risks to human health and well-being through extreme weather, changing precipitation patterns, biodiversity loss, among others (IPCC, 2019). These global effects are collectively referred to as climate change.

1.3 Contaminants of emerging concern

A similar technological achievement in the past century has been the development of modern medicine, thanks in part to the development of myriad pharmaceutical compounds and the increased accessibility of personal care products that improve human hygiene. Medications are commonly prescribed to limit blood pressure and blood sugar, prevent pregnancies and seizures, limit infection and acid reflux, or treat cancer, depression or other common diseases affecting humans, pets and livestock. Consequently, the global average life expectancy has increased by 29 years since 1945, an encouraging testament to the benefits of these compounds (Roser et al., 2019). The increased analytical capacity of scientific equipment, however, has indicated a troubling trend; concentrations of many of these chemicals are present and increasing in aquatic ecosystems, leading to their classification as contaminants of emerging concern (CECs). Many of these beneficial CECs are introduced to ecosystems by agricultural runoff or through municipal wastewater (Stefanakis & Becker, 2015).

Traditional wastewater treatment plants collect a community's wastewater (usually including both black and gray water), transported through sewage networks, and filter out solids and particulates above a specific size. The wastewater is then pumped into a settling tank, where smaller particulates and suspended solids are collected and undergo anaerobic digestion. The remaining nutrient-rich wastewater is then treated in a secondary process (e.g., activated sludge system) where microbial activity converts the dissolved organic into biological material through the encouragement of bacterial growth. The now nutrient-deficient water may undergo further

treatment by sterilizing agents or may be ejected into an aquatic ecosystem at this point. These treatment strategies and facilities were mostly designed prior to any understanding of CECs or their potential for ecological damage, and thus there are few opportunities in the process described above to treat CECs given their susceptibility to metabolism is greater in lipophilic, not hydrophilic, environments (Bolong et al., 2009).

Environmental concentrations of CECs have increased since they were first detected, and the quantity and variety of a given CEC being introduced to ecosystems varies temporally and geographically. Interestingly, the concentration of recreational drugs in London, England's wastewater streams is higher on the weekends than during weekdays (Sulej-Suchomska et al., 2020). By the same token, some geographic areas experience higher concentrations of pharmaceuticals, namely those close the healthcare facilities (Kleywegt et al., 2016). Understanding what CECs are present in a community's wastewater, and therefore probably in its treated effluent, is critical to designing *in situ* treatment options tailored for a community. These methods of analytical detection are prohibitively expensive, however, as hundreds or even thousands of compounds may need to be screened for (Dulio et al., 2018).

There exist technologies that can effectively remove CECs from wastewater, with relative ease. Sorbing material like activated carbon can be added to the nutrient-depleted wastewater (i.e. secondary wastewater) and can remove compounds with chemistries conducive to sorption (Bolong et al., 2009; Wintgens et al., 2008). Apart from sorption, natural irradiation and UV irradiation have the effect of photodegrading some compounds, particularly the recalcitrant and otherwise difficult to remove anticonvulsant pharmaceutical carbamazepine (Chu et al., 2021; Dai et al., 2012; Wang et al., 2020).

While these strategies for CEC removal are effective, a pragmatic approach to CEC remediation may not support the implementation of these programs. The material costs associated with bulk sorbing materials, pH-adjusting chemicals, and the capital expense of installing an energy-

intensive UV remediation system at every wastewater treatment plant are prohibitive (Vasilachi et al., 2021). The infrastructure and spatial demands may also prevent the establishment of extra treatment tanks and holding wastewater for any longer duration could overload the treatment capacity of a facility. Furthermore, these treatment options may effectively resolve the issue of CECs in aquatic ecosystems, but their energetic demands and added waste production may run counter to a mandate to implement treatment options that contribute minimally to climate change (Baruah et al., 2021).

1.4 Microalgal solutions

Biological avenues have been explored in the past to remove deleterious compounds and mitigate their negative effects on an environment. Modern bioremediation has a 70-year history that has resulted in processes like bioventing (microorganisms degrading organics in groundwater), composting (degrading contaminants in agricultural waste), bioaugmentation (addition of archaea or bacteria to speed up remediation processes at on site), rhizofiltration (root-based soil or water remediation) and biostimulation (adding growth-limiting nutrients to a contaminated site to induce biological activity) (Azubuike et al., 2016). Removing contaminants from soil is the most common application of bioremediation technologies, but novel technologies are emerging to treat plastic waste by microbial activity using natural and heterologous enzymes (Austin et al., 2018; Ru et al., 2020), monitoring air pollution using lichen (Pescott et al., 2015), and removing heavy metals from soil using fungi (Kapahi & Sachdeva, 2019). Bacteria are already commonly applied to municipal wastewater treatment via aerobic and anaerobic strategies (Samer, 2015), however, their activity does not adequately treat CECs (Tran et al., 2018). Bioremediation of CECs will need to be sustainably driven and hopefully universal in its application, thus microalgae are emerging as a potential class of organisms suitable for CEC bioremediation in wastewater.

1.4.1 Microalgae and other phototrophs

Microalgae constitute a vast and informal organization of unicellular, photosynthesizing organisms from different evolutionary histories. Cyanobacteria are gram-negative prokaryotes containing thylakoids originating from the earliest group of photosynthesizing organisms; stromatolites (Mazard et al., 2016) . Their prokaryote nature sometimes precludes them from being categorized as microalgae, but their role as photosynthetic drivers of bioremediation will justify their inclusion as microalgae for the purposes of this report. Eukaryotic phytoplankton do not share a common ancestor, but their defining characteristic as a polyphyletic group is their use of chlorophyll pigments to drive the photosynthetic process. Some classes (like the chlorophytes and rhodophytes) originated through the endosymbiosis of cyanobacteria, whereas some originated through the endosymbiosis of other algae, leading organisms like diatoms to partition their cellular photosynthetic infrastructure from the remainder of their cells by multiple membranes (Friedl et al., 2012).

Microalgae are globally relevant as their roles of primary producers are engaged throughout the world in myriad habitats. Marine microalgae produce almost 50% of global oxygen, surpassing the contributions of the Amazon rainforest. Other microalgae form the base of trophic relationships in coral reefs, habitats renowned for their biodiversity and productivity. Microalgae even inhabit terrestrial and cryospheric habitats, contributing to symbiotic relationships with lichens and with snow bacteria (Gray et al., 2020; Rindi, 2007; Schubert et al., 2020). Microalgae not only contribute to global ecology, but they are also directly relevant to human industries as their blooms can form anoxic ‘dead zones’ that result in massive fish die-offs. Some cyanobacteria produce toxins that can cause respiratory issues for humans or bioaccumulate in shellfish and other organisms causing public health distresses (Stauffer et al., 2019). The huge variety of microalgae stems from their evolutionary histories, their broad diversity, and their subsequent adaptations to specific, but varied environments. This diversity of light-driven

organisms presents an opportunistic ‘arsenal’ for engineered solar-powered biological interventions to industrial woes (Li, 2020).

1.4.2 Microalgae as biotechnology

Microalgae have been investigated for their industrial relevance since the 1940s when their potential as a human and agricultural feedstock was popularized by Alistar Hardy (Hardy, 1941). Their rapid growth rates and ability to biologically recycle nuisance nutrients in waste streams has been seen as an elegant link in many efforts to minimize waste. An improved understanding in microalgal physiology has unveiled strategies to improve their capacities to produce bioproducts relevant to human use (Mobin et al., 2019). Cultured microalgae are the main diet of rotifers, themselves an important feed for aquaculture practices. Proteinaceous microalgae are suitable feedstocks for industries like aquaculture, agriculture, and even human nutrition, as exemplified by the rise of algal supplements in the health foods sector (Caporgno & Mathys, 2018). While many claims about microalgal nutrition can be contested, many microalgae do produce nutraceutical compounds like the lipid DHA (an omega-3 fatty acid essential for some human function) or the carbohydrate chrysolaminarin (storage polysaccharide that can help reduce cholesterol levels) (Wells et al., 2017).

Manipulating the nutrient regime of these cultured microalgae can alter their nutritional value, triggering trophic effects on the organisms that consume them (Sterner & Elser, 2002). Cells that grow without traditional Leibniz limitation (i.e. growth is not limited by any nutrient deficiency) are generally referred to as growing ‘exponentially’. These cells are characteristically protein-rich as they incorporate nitrogenous and phosphorous nutrients into their cellular infrastructure required for reproduction and growth. When a nutrient becomes growth limiting, cells begin entering the ‘stationary’ phase of growth wherein cells must direct photosynthetic energy towards macromolecules not containing the limiting nutrient, normally nitrogen or phosphorous, and thus

they produce greater amounts of carbohydrates and lipids (Liefer et al., 2019; Sterner & Elser, 2002).

The production of carbohydrates and lipids in microalgae is of industrial interest as alternatives to fossil fuels become integral to a planet experiencing climate change. Microalgal carbohydrates can be extracted and distilled to produce bio-ethanol, and the cell's lipids, which are mainly stored as triacylglycerols (a very dense source of energy), can be converted to biodiesel (Brennan & Owende, 2010). One important limitation of the mass-production of algal feeds or fuels is the infrastructure demands required for their cultivation at mass scale. Wastewater treatment, however, may provide a concentrated source of both water and nutrients to allow this kind of cultivation. Microalgae can readily grow in traditional wastewater effluent, producing proteins, lipids, and carbohydrates at industrially relevant rates (Ge et al., 2018; Ge & Champagne, 2016). Any implementation of novel wastewater treatment technologies, however, should consider at the forefront issues surrounding CECs in wastewater effluent (Collotta et al., 2018).

Algal wastewater treatment has been investigated academically for decades, but new literature from the past 10 years has expanded to investigate algal capacities to remove both macro-pollutants (dissolved nutrients, organic compounds) and micro-pollutants (CECs) from wastewater (Matamoros et al., 2015). The former serves as convenient ingredients to a growth media resulting in algal proliferation that can result in a breakdown of CECs. This elegant conversion of a waste stream into a valuable culturing media is in line with sustainable philosophy regarding the circular economy and re-using waste streams as a marketable good (Maryjoseph & Ketheesan, 2020).

1.5 Conclusion

Microalgal wastewater treatment solutions have the capacity to function in several ways to improve the wastewater treatment sector. As a sunlight-driven agent of remediation, microalgal wastewater treatment may serve to reduce the carbon emissions associated with treatment while

also producing a viable by-product that has industrial re-use potential. Second, microalgae have been shown to effectively remove some CECs from wastewater environments, providing a potential solution to an emerging issue that might otherwise necessitate the introduction of energy- and capital-intensive infrastructure. Finally, this potential for microalgae to serve as an 'all-in-one' system of wastewater treatment by removing macronutrients, dissolved organic material, and CECs, may be used in the design of wastewater treatment facilities where currently none exist. Wastewater treatment, as a semi-passive algal process, can be implemented in smaller scales than traditional wastewater treatment and therefore may lend itself to supporting smaller communities or those that hope to quickly treat a portion of their wastewater while awaiting long-term infrastructure.

This thesis centers on the opportunities for wastewater (including CEC) treatment using microalgae. The second chapter outlines technical work undergone at Queen's University to develop microalgal cultures better suited to their intended treatment. By applying principals of community ecology, algal physiology, and genetic manipulation, different microalgae were investigated to enhance their bioremediation capacities, as well as to increase their marketability as a by-product for downstream applications. The third chapter presents a meta-analysis of microalgal treatment of CECs recorded in the available literature. Discussed are how phylogenetic discrepancies, environmental conditions, and the mechanisms of removal exhibited by microalgae can be applied to the design of a microalgal wastewater remediation system. The fourth chapter investigated the emerging field of synthetic biology and its applications to algal CEC treatment. Specifically, it centers on the heterologous expression of cytochrome P450 enzymes capable of metabolizing CECs. The physiological, genetic, and practical considerations of this technology are discussed.

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Chapter 2

Meta-analysis of the chemical and biological drivers of microalgal removal of contaminants of emerging concern

2.1 Abstract

The release of chemicals of emerging concern (CECs) into aquatic ecosystems has become a pressing issue as advances in analytical capacity have uncovered this new problem. CECs are concentrated in wastewater effluent as wastewater treatment plants are incapable of removing some of these compounds. Microalgae, viewed as a sustainable alternative to traditional wastewater treatment, have been shown to also remove select CECs from their environments. The opportunity to treat wastewater and the CECs contained therein reinforces their suitability for broad implementation, but there is limited knowledge about microalgal-meditated CEC removal. The phylogenetic, environmental, and chemical drivers of CEC removal are quantified the available literature. A further emphasis on the mechanisms of CEC removal is discussed. The conclusions drawn from this chapter will better inform wastewater managers as to the appropriate cultures and environmental conditions that might best remove a CEC in question. This chapter further highlights the gaps in knowledge in this field and recommends that the mechanism of removal always be elucidated in further studies so as to better understand the conditions that drive CEC removal.

2.2 Introductions

2.2.1 Contaminants of Emerging Concern

The increased application of pharmaceutical and hygienic compounds over the past century has markedly improved overall human and livestock health (Lichtenberg, 2019). Owing to advances in analytical capacity, these myriad compounds are being detected in aquatic environments at

increasing concentrations, resulting in their classification as chemicals of emerging concern (CECs) (Tran et al., 2018). Medications prescribed for their physiological effects in people and animals can act on other organisms, when they accumulate or are present at bioactive concentrations, leading to ecological effects that threaten ecological and human health. Antibiotics in aquatic environments can perpetuate antimicrobial resistance genes that can be transferred to humans through drinking water supplies (Aslam et al., 2018). Endocrine-disrupting chemicals, at very trace concentrations, can have effects on fish populations by triggering hermaphroditism and can lead to species collapse through trophic cascades (Kidd et al., 2007; McCallum et al., 2019). Other compounds can bioaccumulate in organisms, leading to trophic biomagnification that can be life-threatening to people reliant on aquatic organisms for their diet (Choo et al., 2020). CECs constitute a broad range of compounds, numbering in the tens of thousands of unique products, that differ in their chemical makeup; their membrane-permeability, solubility, susceptibility to sorption, and their overall environmental prevalence can all determine the degree to which they enact downstream effects (Dulio et al., 2018). With the increased spotlight on these pollutants with varying chemistries, solutions are being developed to treat these contaminants at their point sources.

Many CECs are introduced into aquatic environments via municipal wastewater effluent. Pharmaceuticals are consumed in doses that account for the wastage that naturally occurs through hepatic clearance (Hilmer et al., 2005; Tolboom et al., 2019). For example, amoxicillin tends to be prescribed at higher than bioactive doses because it is assumed that 40% of the compound will be taken up and have a desired medical effect, while the remainder will be excreted (Stephens et al., 2018). The vast proportion of consumed chemicals are excreted, usually alongside some form of metabolic by-product. Under contexts wherein excreta are transferred to a municipal wastewater treatment plants (WWTPs), the case for roughly 52% of the global population, bulk nutrients and solids are removed from the wastewater without accounting for the micropollutants

that remain in solution (Barbu et al., 2017). The design of conventional wastewater treatment largely predates the consensus that CECs should be targeted for removal, and thus any remediation of CECs in these systems is predominantly accidental and incomplete. The range of influent wastewater concentrations of CECs is largely dependent on geography, seasonality, and even based on a weekly pattern, as is the case for illicit drugs (Sulej-Suchomska et al., 2020; Tran et al., 2018). In some instances, the effluent concentration of some CECs exceeds their lethal concentration causing immediate consequences for the aquatic environments directly downstream of WWTPs (Li & Lin, 2015). Thus, emerging contaminant remediation may be best implemented as an added process combined with traditional wastewater treatment to treat would-be effluent from any remaining micropollutants contained therein.

2.2.2 Microalgal wastewater treatment

For more than 50 years, photosynthetic organisms have been investigated for their role in wastewater remediation, given their potential to sustainably convert the bulk nutrients in wastewater into bio-products that can be tailored for specific downstream effects (biofuels, fertilizers, etc.) (Collotta et al., 2018). The practice of integrating plants to treat contaminants in an ecosystem began in terrestrial contexts, where certain species of trees and grasses have been cultivated to remove heavy metals or industrial by-products from contaminated soils (Chibuike & Obiora, 2014; Pulford & Watson, 2003). Microalgae provide a more convenient class of organisms for wastewater treatment given their growth suspended in solution, and because of their rapid growth rate (Collotta et al., 2018; Ge et al., 2018). Whereas microalgae have long been considered as an amendment or alternative to traditional wastewater treatment, their capacity to assist in the removal of CECs has received academic interest in the past decade (Tolboom et al., 2019). Algal removal of CECs can be enlisted by several mechanisms that either remove the compound from wastewater or transform it into a new, and usually more benign, compound.

2.2.3 Biosorption

Biosorption is a common mechanism of CEC removal and has been investigated using biological substrates other than microalgae-like bacteria, nanoparticles and lignocellulosic biomass (Asemave et al., 2021; Kokkinos et al., 2020). Biosorption refers to two independent processes, the attraction of compounds to the exterior of a cell (adsorption) and the accumulation of a compound within the cell (absorption). Functionally, these are two distinct processes, but they collectively represent those processes wherein the compound remains chemically untransformed and is removed physically from the solution (Kaplan, 2013). This is important when considering the economic effects of microalgae CEC treatment as there is a capacity to recover oftentimes high-value pollutants from biomass after treatment, a common process in the sorptive treatment of heavy metals (Kaplan, 2013). Adsorption is a mechanism that comprises multiple chemical relationships between CECs and sorbents; ion exchange, complexation, and electrostatic interactions are potential routes of adsorption depending on the chemical properties of the interacting materials (Bilal et al., 2018). The cell surfaces of microalgae contain matrices of proteins and carbohydrates that provide additional functional groups to supplement the naturally occurring sites on cell walls leading to increased adhesion to the cells (Suresh Kumar et al., 2015). Oftentimes, CECs adsorbed onto cell walls will be taken up by the cell where it can bioaccumulate in cytosolic or lipid vacuoles (Swackhamer & Skoglund, 1993).

2.2.4 Biodegradation

Biodegradation is the metabolic catabolism of CECs that occurs by enzyme activity either extracellularly or intracellularly. Biodegradation is the primary process by which current biological wastewater treatment operations remove CECs as bacterial communities use organic pollutants as an energy source (Tran et al., 2018). CECs that are biodegradable by bacteria are largely also biodegraded by microalgae, but the phylogenetic diversity of microalgae includes some groups capable of broad substrate metabolism using cytochrome P450 enzymes (Mthakathi

et al., 2015). It has been reported that microalgal contributions to biodegradation are not limited to direct metabolism but that their presence in a solution can increase rates of bacterial degradation of CECs, though this remains relatively unexplored (Xiong et al., 2020).

2.2.5 Photodegradation

Algal wastewater treatment can be a heterotrophic process, but its appeal as a novel sustainable process lies in its capacity to use light as an energy source, which provides an added avenue for CEC removal by algae enabled photodegradation. Lagoon and activated sludge wastewater treatment strategies do allow for daylight exposure, but the opacity of wastewater usually prevents any meaningful light penetration through the fluid, limiting this mechanism of removal in traditional wastewater treatment options (Rühmland et al., 2015). Photodegradation is classified based on the agent of degradation. In direct photodegradation, a photon enacts chemical changes on a compound resulting in ionization, transformation to an excited state, or the breaking of chemical bonds (Díaz-Quiroz et al., 2020; Wei et al., 2021). Indirect photodegradation occurs when a photosensitizer is energized by a photon, creating a reactive species that reacts with a compound to degrade it (Wei et al., 2021). Like biodegradation, photodegradation transforms contaminants into new compounds, lessening their capacity to be recaptured and reused. Further, the transformed compounds are usually less concerning ecologically, but exceptions do exist wherein the product of photodegradation is more harmful than its original constituent (Li & Lin, 2015). Thus, care must be taken when designing an illuminated algal system for wastewater treatment such that the targeted compounds are monitored through their degradation to ensure the safe discharge of effluents.

The degree and mechanism of CEC removal by microalgae varies based on the species used, the target compounds, and the physiochemical conditions of the remediation setup, suggesting that an ideal operation would require screening and experimenting with biological and environmental factors. Wastewater effluent is not monolithic, it varies in its content geographically and

temporally; thus algal-mediated CEC removal may be required to be tailored on a site-specific basis. This paper seeks to provide early evidence of the phylogenetic and environmental trends in microalgae-driven CEC treatment. A census of the available literature was performed, exploring the microalgal removal of common nuisance CECs. The effects of biological and physiochemical design on the degree of removal via phycoremediation was also discussed. In addition, the different algal mechanisms for CEC removal were investigated, as well as the potential factors influencing their susceptibility to algal treatment. Finally, recommendations are provided as to where the research focus on this technology should be directed to realize the integration of this sustainable wastewater treatment with effective removal of CECs.

2.3 Methods

2.3.1 Data collection

Literature was scanned and screened by Google Scholar (scholar.google.com) using the search terms “microalg*” AND (“removal” OR “treatment”) AND “contaminant name”. The search was conducted for each of the emerging contaminants listed by Tran et al. (2018) given their prevalence globally and their potential for environmental damage. They were broadly categorized by the following classes:

(i) Antibiotics.

(ii) Antifungal/antimicrobial agents.

(iii) Nonsteroidal anti-inflammatory drugs (NSAIDs).

(iv) Anticonvulsants & antidepressants.

(v) Artificial sweeteners.

(vi) Beta-adrenoceptor blocking agents.

(vii) Lipid regulating drugs.

(viii) Endocrine-disrupting chemicals (EDCs)

(ix) X-ray contrast media.

(x) UV filters.

(xi) Stimulants

(xii) Anti-itching drugs.

(xiii) Insect repellents.

(xiv) Plasticizers.

The abstracts for the first 45 papers of each search were surveyed and those papers that cultivated eukaryotic microalgae or cyanobacteria in batch cultures under experimental conditions were included in the dataset. Literature pertaining to the outdoor cultivation of microalgae (i.e., in high-rate algal ponds) were excluded from the review for their uncontrolled growth conditions. For each publication, the contaminants investigated for removal were recorded; their initial media concentration and final concentration were noted, as were the environmental and biological conditions of the experiment (Supplementary material T1). These experimental factors included the genus of the microalgae cultivated, the media type and its sterility, whether cells were maintained in suspension through aeration or shaking, temperature and light regimes, and length of cultivation. Removal efficiency is defined as the percentage of a CEC that was removed from a culture by the final day of the experimental period. Chemical attributes for each contaminant were recovered using the online database Chemspider (ver. 2021.0.13.0).

2.3.2 Data analysis

Data visualization and statistical analyses were performed using the statistical program R (ver. 4.0.4). Linear regression models were considered without data transformation when a variable's residuals were normally distributed. Normality plots for the residuals of the four main environmental factors (HRT, temperature, photoperiod, and light intensity) are presented in Appendix 2 and reinforce that linear regressions are appropriate since there is no clear heteroscedasticity. Prior to an analysis of variance (ANOVA), however, data was tested for normality using the Shapiro-Wilks test and log10 transformed when it was not initially normally distributed. Relationships and correlations are described below when the p-value of significance for the statistical result is less than 0.05. Compounds were separated into their respective classes for broader pattern identification, though contaminant-specific trends are identified when noteworthy. Unless otherwise noted, analyses discussed are inclusive of the entire dataset to better understand large-scale trends when there is insufficient data to perform phylogenetic- or

contaminant- specific trends. Patterns within genera are discussed for those which are populous enough within the dataset to perform robust analysis. When assessing the degree of removal, the emphasis in this analysis is the percentage of compound removed, as opposed to the bulk removal, to better account for the broad range of initial concentrations used in the literature.

2.3.3 Bibliometrics

The completed dataset comprised 61 papers published since 2013 to May of 2021 (earlier papers were unavailable or didn't conform to the qualifications above), representing 651 recorded instances of algal-mediated removal of chemical of emerging concern. Research in this technique has grown over the past decade, as evidenced by the 5-fold increase in publications between 2015 and 2021 (Figure 2.1).

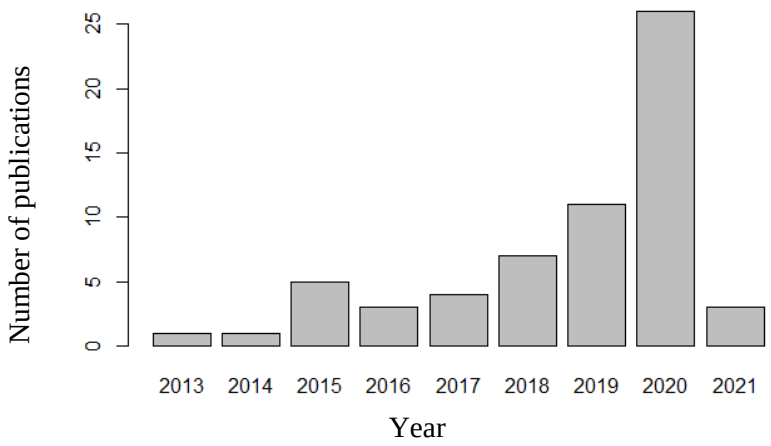

Figure 2-1: Number of publications including the batch cultivation of microalgae with the intent to remove CECs. Data collected via Google Scholar.

2.4 Phylogenetic Effects

Microalgal physiology is a key attribute to both the mechanisms and degree of removal of CECs. Given the enormous range of evolutionary trajectories and life-histories included under the catch-all phrase ‘microalgae’, it was expected that there would be a non-uniform relationship between the experimental microalgal genus and the amount of a CEC removed from a culture. An article by Wang et al. (2018) outlines this discrepancy as *Scenedesmus obliquus*, *Desmodesmus* sp., *and Chlorella pyrenoidosa* removed varying amounts of the antimicrobial triclosan under the same growth conditions. The data collected from the literature review was analyzed for the phylogenetic trends associated with each CEC. To account for the limited data from specific species and CECs, the analysis considers trends more broadly, classifying organisms based on their genus and grouping CECs by their associated class. While there was a great number of genera and different compounds included in the literature, the majority of data was concentrated to a select number of algae (Figure 2-2). This presents an early conclusion for the analysis that further effort should be directed at understanding the various dynamics between genera and different CECs such that we develop a more rounded sense of the potential for this technology to advance.

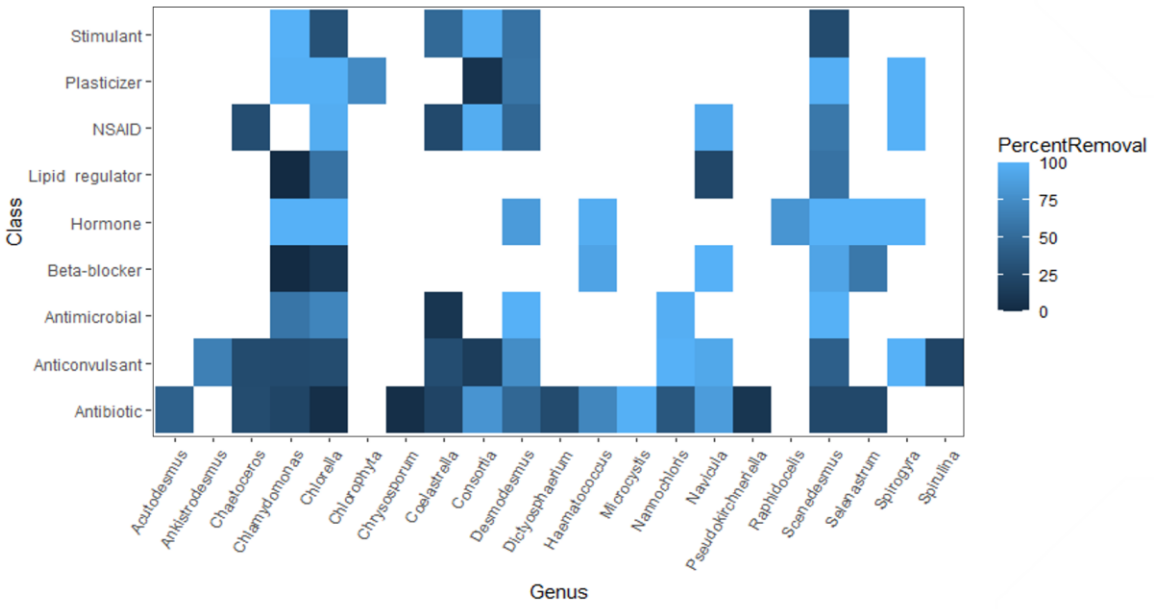

Figure 2-2: Summary of the data contained in the meta-analysis. Of note are the gaps in the dataset. Data was concentrated to select genera and there remaind opportunity to explore novel CEC - genera pairings to improve the efficiency of this technology.

2.4.1 NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs), hormonal compounds, and beta-blockers had median removal efficiencies greater than 80%, indicating their susceptibility to microalgal treatment. The NSAID class constituted 4 contaminants; acetaminophen and naproxen were removed at efficiencies of 100 ± 0% and 94.8 ± 2.1%, respectively, whereas ibuprofen and diclofenac were removed at efficiencies of 76.2 ± 19.9% and 70.3 ±21.1%. The genus *Navicula* removed NSAIDs about 37.8% more than *Chlorella* and 41.6% more than *Scenedesmus* based on a Tukey multiple comparisons of means. There are limited data with respect to the use of *Spirogyra* and *Navicula* (n=7, respectively), however, indicating avenues for further investigation (Ding et al., 2020; Garcia-Rodríguez et al., 2015). NSAID removal was found to vary in the

presence of polycultures of microalgae, with a median removal efficiency of 73.9 ± 32.8% in cultures comprising at least *Chlorella* sp. and varying other genera.

2.4.2 Endocrine-disrupting compounds

Endocrine-disrupting compounds (EDCs) are a well-studied target class of CECs and thus have generally received quite a bit of research attention in the last decade. However, this review could only account for 41 noted instances of their removal by microalgae.

Algal culture median removal efficiency for estradiol was 86.9±15%, while that for estrone and ethinylestradiol was 76.9±29.9% and 63.6±36.5%, respectively. This discrepancy indicates a complexity in the susceptibility of contaminants to algal removal given the chemical structure and properties of these compounds are highly conserved. Nonetheless, estradiol has also been shown to be more susceptible to biodegradation in soil removal experiments than estrone and ethinylestradiol, indicating a consistency in this behaviour (Ying & Kookana, 2005). The most representative algal genera in the dataset, *Chlamydomonas, Scenedesmus, Selenastrum,* and *Chlorella* were responsible for removal efficiencies of 86.8±12.2%, 80.2±18.0%, 78.4±35.4%, and 69.0±36.3%, respectively. While median removal efficiencies for EDCs are high, abiotic factors may dictate the success of microalgal CEC treatment given the phylogeny of the microalgae does not explain the wide range of reported removal reported above.

2.4.3 Beta-blockers

Beta-blocking pharmaceuticals in wastewater that have been reported to be removed by microalgae include metoprolol and atenolol. There is limited evidence ($n<15$) to suggest *Scenedesmus* and *Haematococcus* species may remove up to 80% of beta-blockers from wastewater, however, more data suggests that *Chlorella* has a median removal efficiency of

64.9±42.6% for these compounds. A mean metoprolol removal efficiency of 21.6±12.4% greater than atenolol was noted from the analysis likely owing to its increased susceptibility to photolysis (Marothu et al., 2019).

2.4.4 Antimicrobials

The antimicrobial class of CECs includes the antifungal and antibacterial agents triclosan and fluconazole. Up to 98% of triclosan was removed from cultures containing *Nannochloris* (Bai & Acharya, 2017), while *Scenedesmus* and *Chlorella* species removed 61.4±24.6% and 55.5±10.4% of triclosan, respectively. Fluconazole was removed at a median removal efficiency of 7.7±7.7%. This is consistent with its negligible rates of sorption and biodegradation in wastewater treatment operations, though some photodegradation has been observed in some studies (Chen et al., 2014; Kahle et al., 2008).

2.4.5 Stimulants

Caffeine is a stimulant and a well-recognized CEC owing to its global ubiquity in wastewater streams. Its median removal by microalgae was 64.9±31.3%, with *Chlamydomonas* being responsible for 99±0.5% removal of the compound. *Coelastrella*, *Scenedesmus*, *Chlorella*, and *Desmodesmus* genus accounted for caffeine removals between 48% and 59%. These trends in the microalgal removal of antimicrobials and stimulants reinforce the notion that across varying physicochemical conditions, phylogenetic effects of removal remain integral to the design of efficient algal-mediated CEC removal systems. That some organisms are better suited to one type of removal may indicate an efficient route to broad removal is through the use of polycultures (Godwin et al., 2017).

2.4.6 Plasticizers

Plasticizers leach out of materials and enter waste streams where they can disrupt hormonal function in organisms (Corrales et al., 2015). BPA is the most widely recognized plasticizer, and its removal in microalgal cultures ranged from less than 20% to complete removal with a median removal efficiency of 61.2±26.2%. Literature reported removal by the *Chlorella* genus, where its median removal is 63.8±36.9%, is greater than the other most reported genus, *Chlorophyta*, responsible for a median removal of 56.6±21.3%. There are limited data (n<5) that support 95-99% removal of BPA from cultures by *Spirogyra, Scenedesmus,* and *Chlamydomonas*. The plasticizer tris(2-chloroethyl) phosphate was investigated for its removal by a polyculture containing the genera *Chlorella* and *Scenedesmus* and had limited removal efficiencies of 7.1±0.2%.

2.4.7 Antibiotics

Antibiotic compounds are the most studied class of CECs, representing almost two thirds of all data collected in this analysis. The antibiotic tetracycline consistently exhibited the greatest removal by microalgae, with 99±0.1% removal. Macrolide antibiotics like clarithromycin, azithromycin and roxithromycin were all removed near 75% efficiency at 75.7±18.5%, 74.8±13.1%, and 74.5±9%, respectively. That these similar compounds were reportedly removed in similar quantities is indicative that their similar chemical structures renders them susceptible to the same removal mechanisms. The predominant mechanism of removal for macrolide antibiotics is biodegradation, unlike the majority of antibiotics which are generally removed by sorption (Xiong et al., 2018). Ciprofloxacin, ofloxacin, norfloxacin, and enrofloxacin are fluoroquinolone antibiotics included in this analysis. Despite their similar class, ciprofloxacin showed greater removal by microalgae, a removal efficiency of 78.5±29.4%, compared to ofloxacin (48.9±28.0%), norfloxacin (25.9±21.3%), and enrofloxacin (23.6±28.5%). Although the

macrolide compounds show subclass-specific consistency in their microalgal-mediated removal, the pattern appears to be inconsistent across antibiotic groups. The sulfonamide antibiotics, sulfamethoxazole and sulfadiazine, have removal efficiencies of 47.7±31.7% and 42.0±26.3%, respectively, though reported removals ranged from 3% to 95%. Sulfamethazine had a median removal of 23.4±12.9% and was not found to exceed 62% in the literature. The wide range of removal exhibited by the sulfa drugs indicates that their removal may be more linked to environmental determinants of removal like temperature and light intensity (the most variable abiotic factors contained in the dataset), as will be explored below. Regarding the phylogenetic determinants of antibiotic removal, standout genera include *Navicula, Haematococcus,* and *Chlamydomonas* with removal efficiencies of 85.6±0.1%, 84.5±11.0%, and 77.0±25.8%. Common genera in the treatment of CECs, *Desmodesmus, Chlorella,* and *Scenedesmus,* exhibited removal efficiencies less than 50%, suggesting that the biological determinants of the removal of antibiotics by microalgae merit further investigation.

The anticonvulsant carbamazepine is an often researched CEC owing to its recalcitrance in aqueous environments, making it difficult to remove from wastewater streams (Hai et al., 2018). Its reported removal efficiency by some microalgal strains disrupts the conventional notions that the genera *Chlorella* and *Scenedesmus* could be the standard strains for CEC treatment. In fact, studies involving *Navicula, Nannochloris,* and *Ankistrodesmus* species noted median removal efficiencies greater than 70%, whereas *Chlorella* and *Scenedesmus* genera had median removal efficiencies of 12.6±11.2% and 25.8±16.2%. The lipid regulator gemfibrozil also exhibited varied removal efficiencies by the genera *Scenedesmus (*37.3±25.2%) and *Chlorella* (26.7±25.2%). However, it should be noted that reported removals by these two genera ranged between 1% and 68%, suggesting environmental conditions may be important to the removal of gemfibrozil by microalgae.

The analysis presented above indicates potential phylogenetic determinants of CEC removal. The four most common genera in the dataset were *Chlorella* (n=186), *Scenedesmus* (n=119), *Chaetoceros* (n=90), and *Chlamydomonas* (n=52). The remaining 17 genera have less representation our data, with a median of 11.5 noted instances of removal, indicating a dearth of research outside the traditional experimental strains that dominate the literature. Despite the multifaceted conditions that can lead to algal removal of CECs, certain genera appear to be better suited to removing certain classes of chemicals. This conclusion paired with the limited number of genera investigated for CEC removal, and hence included in this study implies that there is a need to further screen other microalgal strains for their removal efficiencies for different types of CECs. A better understanding of which organisms are best suited for specific CEC removal could assist wastewater managers with implementing specialized algal treatment systems designed for the unique CEC composition of wastewater. Some efforts have been made to screen a greater diversity of microalgae, Gojkovic et al. (2019) investigated the removal capacity for 8 species of microalgae native to Northern Sweden and discovered a broad range of removal for 19 contaminants. Identifying organisms for specific CECs could provide the basis for using polycultures to customize algal remediation efforts. The screening effort suggested should emulate in scale the testing performed to investigate algal lipids suitable for biofuels and other nutraceuticals.

2.5 Environmental Effects

While microalgal phylogeny can dictate some CEC removal, there were instances in our dataset where removal varied greatly after cultivation under the same experimental conditions. Microalgal physiology can be greatly impacted by environmental conditions, and many organisms are tolerant to a diversity of conditions. Given that microalgal-mediated CEC removal is driven by biological processes, stimulating the greatest removal may be driven not only by the

appropriate species selection, but also by cultivating that species under culturing conditions that are conducive to improved CEC removal.

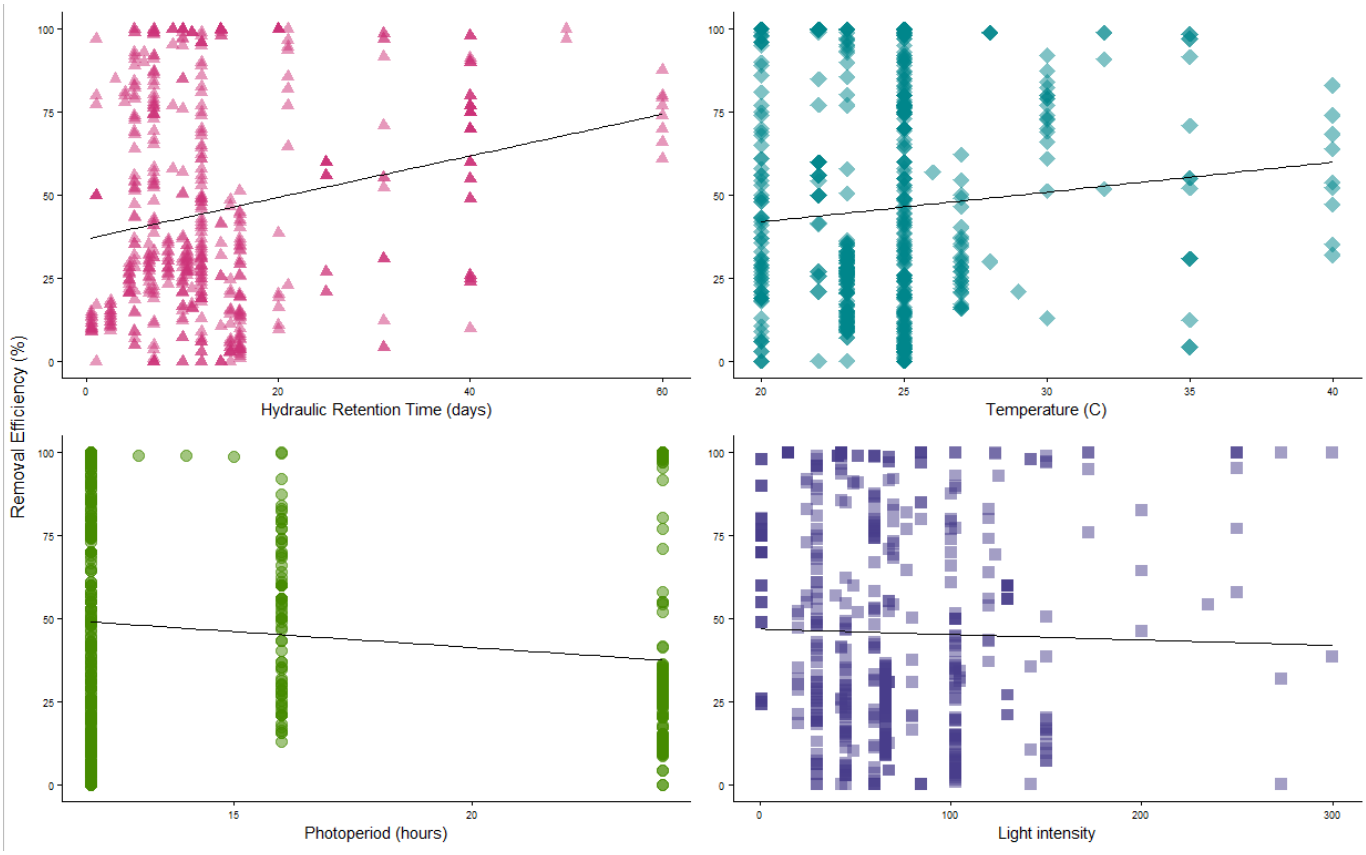

Figure 2-3 Linear regression analyses for four environmental factors affecting CEC removal. Regressions include all available data collected across all CEC classes and microalgal genera. Plot A depicts the relationship between HRT and CEC removal, showing a positive correlation. Plot B depicts the relationship between temperature and CEC removal, again with a positive correlation. Plots C and D depict the relationship between photoperiod or light intensity with CEC removal, both showing negative correlations between light delivery and removal.

2.5.1 Hydraulic retention time

HRT marks the number of days microalgae are cultured. HRT in the dataset ranged from several hours to 60 days, with a range of removal efficiencies across that timeframe. HRT can be correlated, in this context, as a representation of culture density; cultures grow exponentially during the first few days of the culturing then slowly taper into stationary growth after about 7 days (depending on the initial culture density) (Andersen, 2005). A linear regression comparing percentage removal to HRT demonstrated a significant increase in removal, about 0.4%, for each day remaining in culture (Figure 2-2 A) (r^2=0.05). More removal was correlated with longer HRT, though the degree to which that correlation may benefit wastewater managers may be limited as the increase could be marginal compared to the operating costs associated with an increase in the holding capacity of such facilities. An ANOVA revealed that HRT had a strong positive correlation to the percentage removal and that both CEC class and microalgal genus affected the degree to which HRT predicts removal. Despite the positive correlation, complete CEC removal in microalgal cultures could, at times, be attained quickly. *Chlorella vulgaris* removed up to 99% of amoxicillin after 12 hours in culture (Shi et al., 2018). A breakdown of the effect of HRT on different classes of CEC elucidated which compounds may benefit from extending cultivation HRT. Antibiotics, anticonvulsants, and EDCs had improved removal efficiencies of 0.46%, 0.77%, and 0.42% per day. Despite the significance to this relationship, it may be concluded that manipulating HRT is of limited use to managers designing microalgal CEC remediation strategies given the marginal benefits of increasing HRT and incurring costs as a result.

2.5.2 Temperature

Culture temperature exhibited a similar relationship to HRT with regards to its effect on algal removal of CECs. A series of ANOVAs between temperature and removal revealed a significant interaction between temperature and both class and genus concerning the percentage removal of

CECs. The phylogenetic specific relationships between removal and temperature can be explained by the varying ideal temperatures exhibited by different microalgae, where growth can be impaired at temperatures both higher and lower than a species' thermal ideal. The class-specific relationship may be explained by different CEC removal mechanisms which vary by class. Temperature is more important in predicting rates of biodegradation and in predicting rates of sorption of photolysis (Melo et al., 2014). Overall, increasing temperature resulted in an increase in the amount of contaminant removal. This trend's rate of improved removal, 0.73% per degree Celsius, is minimal compared to the overall removal requirements of the system (Figure 2-2 B) (r^2=0.08). This trend represents the entire dataset, however as noted there are class- and phylogenetic-specific trends in the effect of temperature on removal.

Antibiotics, anticonvulsants, hormones, NSAIDs, plasticizers, and beta-blockers showed no relationship in their microalgal-mediated removal relative to temperature. This could prove advantageous to wastewater managers as lower temperatures are conducive to more energy efficient operational costs. Antimicrobial compounds were removed at a rate of an added 9.6% reoval for each increase in degrees Celsius.

Meanwhile, lipid regulators exhibited a negative relationship, decreasing in removal by 14.1% for every increase in degrees Celsius. This relationship for lipid regulators merits further investigation given data were only available for removal between 22°C and 29°C.

Limited phylogenetic patterns of CEC removal relative to temperature could be identified. Organisms from the genus *Chlamydomonas* were negatively affected by increasing temperatures, decreasing in CEC removal by 6.80% for each increase in degrees Celsius. Broadly, a limited relationship between temperature and CEC removal could be discerned likely due to confounding factors. For example, this relationship is probably influenced by the increased growth rates of microalgae at higher temperatures. Additionally, temperature does increase rates of carbohydrate exudation, important to several mechanisms of removal (Maršálek & Rojíčková, 1996; Singh &

Singh, 2015). In this study, 83% of reported removal investigations presented in this analysis were conducted between 25°C and 30°C which is consistent with traditional phycological practices, however wastewater is traditionally between 10 and 15 degrees Celsius (Andersen et al., 2005; Xu et al., 2019). Further research is required to establish whether microalgal CEC bioremediation is possible at lower temperatures or whether heating might be required, thus contributing to the operational requirements of these facilities.

2.5.3 Light effects

Photoperiod is commonly considered to be positively associated with removal (Cuellar-Bermudez et al., 2017). Appropriate lighting encourages photodegradation and algal growth, and thus intuitively the expectation would be that an increase in light exposure should improve removal. This generalization, however, was challenged in the analysis of the dataset, which exhibited a negative relationship between photoperiod and removal. Between 12 and 24 hours, each added hour of photoperiod saw a 0.92% decrease in overall CEC removal (Figure 2-2 C) (r^2=0.02). Different classes of CECs responded differently to a lengthened photoperiod with anticonvulsants and NSAIDs showing decreased removal (-1.63% and -2.76% per added hour, respectively) and antimicrobials and stimulants showing increased removal (8.5% and 3.8% per added hour, respectively). Other classes showed no specific relationship between photoperiod and removal. This conclusion is reinforced by the absence of a relationship between algal genus and photoperiod, which implies limited biological effects on removal mechanisms that are light-driven.

Similar relationships were evident with regards to light intensity. Increasing the light intensity by 1 µmol photons/m^2 s^2 generally resulted in a decreased removal by 0.14%. This trend was exacerbated in one classes of CECs, with antibiotics showing a 0.2% decrease in removal for each µmol photons/m^2 s^2 increase in light intensity (Figure 2-2 D) (r^2=0.01). Comparing removal

efficiency with the multiplication of photoperiod and light intensity gives an overall relationship between light and removal (i.e. the sum total of light delivered in a day). Only antimicrobial compounds showed a significant relationship between light and removal, with increased removal related to greater light delivery. Despite this relationship between light and CEC removal, microalgal illumination remains integral to efficient CEC removal. Larsen et al. (2019) cultivated microalgae from the genera *Chlorella* and *Scenedesmus* under both light and dark conditions, and quantified the removal of the antibacterial CEC triclosan. Across different types of media (synthetic wastewater, distilled water, growth media), illuminated cultures almost completely removed the CEC, whereas dark cultures could remove up to 63% of the compound. This apparent contradiction between light and CEC removal may be explained by an increase in algal biomass associated with increased lighting, thus shading the interior of cultures and limiting the action of photodegradation.

2.5.4 Media constitution

The constituents of microalgal growth media can influence algal physiology, thus impacting its ability to treat CECs. Most experiments outlined in this analysis were conducted using either diluted wastewater or specialized algal media (i.e. BG-11 or high-salt media). The largest differences between these two categories is the degree of control over algal nutrient delivery (wastewater nutrients are often unbalanced, resulting in a more rapid nutrient-induced growth limitation), and the high organic carbon content in wastewater (Ge et al., 2017; Kim et al., 2013; Shu et al., 2018). Nonetheless, no relationship was observed between media type and CEC removal. Media preparation, however, was noted to affect CEC removal. Autoclaving media prior to culturing renders the liquid axenic, thus inhibiting opportunities for microalgae to interact with bacteria to co-metabolise CECs. There was no genus-specific relationship between autoclaving media and removal, however, there was a CEC class-specific response. NSAIDS were removed

42% more when media was not autoclaved, while plasticizers were removed 26% more. This is consistent with findings by Xiong et al. (2017) who showed that cultivating microalgae with a bacterial inocula could improve removal in CECs susceptible to biodegradation.

2.6 Mechanisms of Removal

Algal CEC removal is facilitated by several independent mechanisms that are biologically and chemically driven. Liu et al. (2021) investigated the recorded mechanisms of removal for 114 CECs, which will be used to further investigate the phylogenetic and environmental factors that contribute to CEC removal.

Table 2-1 Summary of the physicochemical conditions that contribute to the removal of CECs by different mechanisms of removal. Values are reported in terms of the percent removal associated with the written unit of each variable. The value for aeration, as a binary variable, represents the percent removal associated with aeration compared to no aeration.

	Environmental Condition					Compound Chemistry	
Mechanism	Temperature [per degree Celsius]	Photoperiod [per hour]	Volume [per L]	HRT [per day]	Aeration [yes]	Molar mass [per g/mol]	LogP [per unit]
Biosorption	*insig.*	*insig.*	*insig.*	-6.80%	*insig.*	-2.30%	7.50%
Biodegradation	0.81%	*insig.*	*insig.*	0.55%	*insig.*	*insig.*	*insig.*
Direct Photodegradation	*insig.*	-0.75%	-2.76%	0.94%	*insig.*	-0.10%	4.92%
Indirect Photodegradation	*insig.*	-0.63%	-2.73%	*insig.*	10.80%	-0.04%	7.60%

2.6.1 Biosorption

Biosorption is a valued mechanism of removal because it is not light-dependent, and thus can be applied to heterotrophic cultures of microalgae. Also, in cultures growing under natural light regimes, sorption can continue to treat CECs at night. The process is also idealized from a wastewater management perspective because of its rapidity and consistency in speed and removal efficiency given known equilibrium constants for environmental conditions (Pomiès et al., 2013). During wastewater treatment, sorption is also a mechanism of removal by other solid surfaces in cultures, but the analyses below will consider the effects of sorption to be negligible for the glass walls, aeration equipment, and other surfaces inside culture vessels.

Sorption of pollutants is dependent on the physical and chemical properties of the cultivation environment, as well as the contaminant itself; temperature, pH, and salinity can affect the rate of sorption for many compounds (Norvill et al., 2016). Biological effects must also be considered when the sorbing material is a living organism. Membrane composition can vary phylogenetically, diatoms have a cell wall made of silica, coccolithophores have calcium carbonate walls, whereas most chlorophytes have a traditional plant cell wall (Popper et al., 2014). The physiology of cells will also affect removal as the production of extracellular polymeric substances (EPS) is highly conducive to increased pollutant removal by sorption (Soto-Ramírez et al., 2021; Zhou et al., 1998). An ANOVA indicated that biosorption and molecular mass are related in contaminant removal from microalgal cultures. Specifically, compounds with molecular masses between 250 and 375 g/mol are susceptible to biosorption, and removal across those compounds is greater when the compounds are lighter.

The process is metabolically passive, but nonetheless a biological pattern of removal was apparent with microalgae from the genera *Selenastrum*, *Haematococcus*, and *Chlamydomonas* (generally 10+ µm in diameter) removing more than 75% of the CECs that are susceptible to biosorption, whereas the median removal for the genera *Chlorella* and *Scenedesmus* (generally 1-10 µm in diameter) were less than 60%. Gadd (2001) predicted that cultures containing smaller

cells would be more susceptible to CEC removal by sorption because of their increased ratio of surface area to culture volume. The relationship presented above suggests there may be validity to this convention given the low removal from the genera *Chlamydomonas*. Conversely, however, larger cells like those from the genera *Haematococcus* also exhibited great removal capacities, meanwhile smaller cells like from the genera *Chlorella* exhibited less removal. This may be explained by the increased exudation of carbohydrates by larger microalgae, which provide added binding sites on cell walls and through the increased development of extraceullular polymeric substances (EPS) (Finkel, 2007; Passow, 2002). The effect of cell size was not quantified for this analysis, but merits further investigation depending on the type of adsorption targeted by wastewater managers.

There was a negative relationship between hydraulic retention time and removal for those compounds susceptible to biosorption. Removal decreased by 6.8% for each day in culture beyond 6 days. Although pH data was unavailable for this analysis, time in culture could serve as a proxy for pH given the gradual pH increase of algal cultures as they photosynthesize and utilize dissolved carbon dioxide (Abdel-Raouf et al., 2012). As pH increases, the cellular capacity for adsorption on cells decreases, as shown with the removal of some heavy metals by microbial sorbents (Furuhashi et al., 2019; Zhou et al., 1998). The relationship between algal-mediated CEC adsorption and pH merits further investigation to elucidate these effects under controlled experimental conditions more relevant to this technology.

Absorption, the uptake of contaminants into the cell, requires crossing cellular membranes, and thus a positive relationship between the octanol-water partitioning coefficient (logP) was expected (Swackhamer & Skoglund, 1993). As logP increased by 1 log, removal increased by 7.5%. Despite the high percentage of total removal exhibited by some compounds that are susceptible to sorption, the total removal used in this analysis may include removal by multiple mechanisms. While sorption is a mechanism of CEC removal, in some instances sorption is not

the only mechanism of removal and thus it merits mentioning that these analyses may be clouded by other underlying mechanisms. An experiment investigating the removal of sulfamethoxazole, trimethoprim, carbamazepine, and triclosan lead to sorption being responsible for 11%,11%, 13% and 27% of total removal in *Nannochloris* sp. cultures (Bai & Acharya, 2017).

2.6.2 Biodegradation

The degree of CEC transformation by microalgae can vary based on the organism's physiological action on that compound, but ranges from the addition of functional groups to the complete mineralization and removal of a compound (Katagi, 2010; Matamoros et al., 2016). Generally, the effect of biodegradation on a compound results in its increased polarization owing to enzymatic activity that oxidizes the compound, which usually reduces its ecotoxicity (Avila et al., 2021; Wang et al., 2019). The product of biotransformation exhibited by microalgae varies phylogenetically and is affected by the cultivation environment because some enzymes are expressed in different organisms and have different ideal operating temperatures (Wang et al., 2019). Biodegradation in microalgae is enzymatically driven, and thus determining the ideal species for the removal of a specific compound requires an understanding of cell metabolomics to discover the potential avenues available to cells for degrading that compound (Ufarté et al., 2015). There are also adaptive effects exhibited in the microalgal biodegradation of CECs; an increased exposure to compounds can increase a species' ability to biodegrade a compound experimentally (Xiong et al., 2017). For example, the exposure of *Desmodesmus* sp. to BPA upregulated genes encoding oxidoreductases that oxidated BPA (Wang et al., 2017)
Biodegradation is also affected by the algal cultivation environment. Substrates in the microalgal growth media can encourage or discourage CEC catabolism, and a growth environment that encourages a mixotrophic growth strategy has resulted in increased rates of CEC biodegradation. Conversely, an increased concentration of organic carbon in the media (whether by addition or through microalgal exudates) can increase the biodegradation of CECs by co-metabolism, the

process by which a bacterial-microalgal symbiosis increases CEC removal (Tan et al., 2015; Xiong et al., 2017). This biodegradation, therefore, is not primarily performed by the microalgae, but instead microalgae support and enhance the bacterial removal of CECs. This difference is largely captured in the analyses presented below regarding the effect of media sterilization prior to experimentation.

For those microalgae reported to remove compounds susceptible to biodegradation, there were few standout genera. The genera *Haematococcus* and *Navicula* had CEC removal in the range of 84% to 96% and had the greatest median removal efficiencies for genera with $n > 10$. The other biological determinant of biodegradation in algal cultures is the presence of bacteria that can act symbiotically with microalgae to co-metabolise CECs (Xiong et al., 2017). Data was collected regarding media preparation, and notably whether it was autoclaved or otherwise sterilized to prevent contamination by bacteria. Whether media was prepared axenically was insignificant in its effect on CEC removal, contradicting the notion that the presence of bacteria enhances CEC removal.

Nonetheless, media type was significant in its effect on CEC removal and a Tukey multiple comparisons of means showed that wastewater cultivation (axenic or not) resulted in 25% greater removal than traditional algal growth media. The increased content of organic material in diluted wastewater relative to algal growth media may drive this relationship (Tan et al., 2015).

In our analyses, temperature positively correlated with CEC removal for compounds susceptible to biodegradation at a rate of 0.81% for every increase in degrees Celsius. Conversely, there was no relationship between temperature and removal for those compounds not susceptible to biodegradation, suggesting that increased temperature may not be a proxy for increased biomass and its associated effects on removal, but rather on the enhanced microbial activity associated with increased temperatures. Notably, our analyses included cultures between 20°C and 40°C. As such the repercussions for non-biodegradation removal at lower temperatures merits further

investigation. HRT was also positively correlated with CEC removal at a rate of 0.55% per day. The analysis of other cultivation and environmental conditions including light intensity and photoperiod, bubbling, agitation, and initial contaminant concentration were found to be insignificant in their effects on algal removal of CECs susceptible to biodegradation.

2.6.3 Photodegradation

Direct photodegradation is not solely dependent on the delivery of photons to properly degrade compounds. There is a compound-specific wavelength that is required to enact the photodegradation, which can be determined based on the chemistry of the contaminant. Light availability for photodegradation can also be hindered between the light source and its target contaminant. Algal cultures can affect rates of photodegradation when biomass and other suspended material like tannins and humic acids inhibits light penetration (Martínez-Zapata et al., 2013). Furthermore, a contaminant can be susceptible to direct photodegradation based on its aquatic chemistry. When the pH of a culture is greater than the dissociation constant of the contaminant, the greater proportion of the compound is anionic, and thus more susceptible to photodegradation (Koumaki et al., 2015).

Compound chemistry was noted to affect CEC removal for those contaminants that can be removed by direct photodegradation. There was a negative correlation between molar mass and removal, with 0.1% less removal as molar mass increased by 1 g/mol. Conversely, there was an increased removal efficiency of 4.92% for each increase of its water:octanol coefficient (logP). Beiknejad & Chaichi (2014) found that dyes were more susceptible to photodegradation as their lipophilicity increased, validating the negative correlation above. A literature review yielded no information on the relationship between molar mass and photodegradation, thus suggesting that the negative correlation between mass and removal is an artefact of mass being inversely correlated to logP.

Some culture conditions were also important in governing the rate of removal for compounds that are removed by direct photodegradation. An ANOVA demonstrated that media type was significant in dictating removal, and a Tukey multiple comparison of means test concluded that wastewater cultivation yielded better removal efficiencies than traditional algal growth media. Culture volume was inversely correlated with CEC removal at a rate of -2.76% per liter of culture. This was likely due to the increased surface area to volume ratio in smaller cultures. which promote light penetration through the culture.

Hydraulic retention time increased CEC removal at a rate of 0.94% per day. This relationship is evident given the added opportunities for photodegradation that increase with exposure to more light. Light intensity, however, was insignificant in its relationship with CEC removal. Further, photoperiod was inversely correlated with CEC removal, albeit at a relatively small (0.75%) decrease in removal per hour of light between 12 hours and 24 hours. These two findings regarding light intensity and photoperiod do not suggest that a light-driven process might be independent of light delivery. Rather, it is hypothesized that an abundance of light can lead to the growth of microalgae that ultimately competes for light in the culture. This reveals a balance that must be considered, when designing microalgal wastewater treatment operations, between the biological and photolytic effects of CEC removal that will be prioritized.

2.6.4 Indirect photodegradation

Indirect photodegradation is a more complex process because of the class of photosensitizers required to act along with the compound for the photodegradation to occur. These dissolved chemicals vary widely and include humic and fulvic acids, iron, dissolved oxygen, and dissolved ions (Hom-Diaz et al. 2015). These compounds are readily available in wastewater, implying indirect photodegradation could occur in microalgal wastewater treatment. However, its unpredictability as a mechanism should be highlighted prior to inducing indirect photodegradation. Different photosensitizers act on compounds differently, meaning that in a

treatment environment, different spectra of light could induce photosensitizers to transform CECs into different by-products. Thus, ensuring the results of indirect photodegradation are not more harmful than the original compounds requires monitoring of a multivariate process (Chiron et al., 2006; Werner et al., 2005).

The results discussed above for direct photodegradation are consistent with those of indirect photodegradation, and given that those compounds susceptible to direct photodegradation are predominantly also susceptible to indirect photodegradation, this was an expected conclusion. By the same statistical analyses, wastewater cultivation leads to greater removal than media cultivation. Culture volume is inversely related to removal at almost the same rate (2.73%). The relationship between logP and CEC removal is stronger as removal increases by 7.6% for each increase of logP. Light intensity remains insignificant in predicting CEC removal and photoperiod remains negatively correlated at a rate of -0.63% per hour between 12 and 24 hours. The main distinction between direct and indirect photodegradation is the effect of culture aeration on removal. An aerated culture resulted in 10.8% more removal than a culture without aeration, which was likely due to the increased dissolved oxygen concentrations that act as a photosensitizer (Ren et al., 2016; Werner et al., 2005).

Although photodegradation can remove compounds from wastewater, caution must be exercised since the transformation products of photodegradation can continue the deleterious effects of their parent compounds. For example, in the case of carbamazepine, its photodegradation products have been recorded as presenting a greater acute toxicity than carbamazepine itself (Donner et al., 2013).

2.7 Conclusion

This study outlined some of the myriad of factors that dictate the degree of microalgal treatment of CECs. The phylogenetic trends in removal for the main CEC classes were presented. Predominantly, the oft-studied genera *Chlorella* and *Scenedesmus* underperform compared to

other genera assessed in our dataset. These two genera are often considered model organisms in their capacity to remediate wastewater in the traditional sense. Whether other genera could remove more CECs merits further exploration given their limited representation in our dataset. An increased effort in microalgal screening for CEC removal would elucidate the validity of these trends, though care should be taken to design experiments such that the abiotic factors affecting removal are kept relatively consistent. With an increased incidence or reporting on these genera, an improved index may be generated that can pinpoint the specific organisms best suited to a particular CEC. With that index, the design of microalgal consortia may efficiently treat wastewaters containing multiple CECs with different strategies for removal.

This report has highlighted the importance of understanding the relative contributions of different mechanisms of removal to microalgal CEC treatment. Whereas some publications partition removal by their concomitant mechanisms, most report the bulk removal of a CEC and comment on their expected pathways of removal. To design microalgal CEC treatment operations more strategically, mechanisms of removal must be clearly identified to better understand how to improve culture removal capacity. The relationship with pH was not explored in this analysis because not enough data was available to adequately examine trends, however the chemical basis for many of the effects described in this report are affected by pH. Hence, it is recommended that the pH of the culture be reported to better elucidate how the mechanisms of removal are affected by culture chemistry.

The environmental conditions that affect CEC removal are multivariate, though some trends have emerged through this analysis. For instance, increasing temperature tends to improve removal, and microalgae grown in wastewater are more successful at treating CECs than microalgae grown in algal growth media. The mechanisms of microalgal CEC treatment are varied in their actions and the eventual fates of CECs. Sorption is predominantly reserved for compounds within a certain range of molar mass, and these compounds' lipophilicity is a strong indicator of their

degree of absorption. Biodegradation is greatly improved in cultures grown with symbiotic organisms and sources of organic carbon. Photodegradation does not increase with light intensity and photoperiod, rather a balance between fostering algal growth and light delivery must be reached to maximize light-driven removal.

While better understanding the mechanisms of removal and the phylogenetic- and contaminant-specific trends in CEC removal by microalgae is an important step in the development of robust CEC-targeted phycoremediation, it may be concluded that the capacity for natural remediation necessitates artificial intervention. Currently, the average CEC removal capacity of the surveyed articles suggests less then half of CECs are removed. To improve the efficiency of this technology, biological engineering may serve to broaden the quantity of CECs microalgae can remove from a system. They may also serve to target compounds not currently removed by microalgae. The recombinant expression of CEC-metabolizing enzymes has the capacity to move this technology from a sporadic process to one that is targeted on the removal of select deleterious compounds.

Overall, microalgae-based strategies for CEC treatment of wastewater present a promising avenue for mitigating the deleterious ecological and human-health effects of contaminants in aquatic environments. A more thorough investigation of the underlying causes and variables affecting removal has the potential to further improve microalgal efficiency, increasing the likelihood that this technology can be applied broadly.

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Chapter 3

Heterologous cytochrome P450 expression in microalgae for the treatment of chemicals of emerging concern

3.1 Abstract

Pharmaceuticals and personal care products known collectively as contaminants of emerging concern (CECs) in wastewater effluent are gaining increased attention as their limited removal in wastewater treatment plants are becoming known. Additive treatment options tend to require expensive or energy intensive processes, thus there is a need to develop sustainable technologies that can efficiently remove CECs from wastewater. Because of their reported effectiveness in the treatment of the traditional constituents of wastewater, microalgae have emerged as a potential agent of bioremediation. The study of their removal of CECs is in its infancy but it is acknowledged that their removal is insufficient through traditional microalgal cultivation. Synthetic biology has the potential to improve CEC removal by microalgae through the transgenic expression of CEC-catabolizing enzymes. Enzymes from the cytochrome P450 (CYP) superfamily show promise as a preliminary realization of this concept based on their role in human pharmacokinetics. This paper considers the potential to pair human CYP enzymes with the photosynthetic apparatus of microalgae to develop a light-driven infrastructure for CEC removal. Considerations involving the selection of an appropriate CYP, added transformations to improve enzyme activity, and repercussions on microalgal growth and cultivation are discussed. This paper ultimately advocates for the realization of this concept in a proof-of-concept model.

3.2 Introduction

Synthetic biology is expanding the repertoire of biocatalytic industries, and microalgae are emerging as sustainable alternatives to traditional non-photosynthetic hosts. Transgenic

microalgae have been cultured for the purpose of high-value compound synthesis, but little work has been attempted to apply transgenic microalgae to compound degradation. This review investigates the opportunities to express recombinant enzymes to develop cultures capable of high efficiency phycoremediation of pollutants in wastewater streams.

The development and application of personal care and pharmaceutical compounds has dramatically expanded in the past century to the benefit of global human health, but their accumulating concentrations in municipal wastewater streams pose significant and growing health and environmental risks (Tran et al., 2018). Traditional wastewater treatment plants, which filter solids, remove macro-nutrients, and sterilize effluent prior to discharge, have not been designed to remove these contaminants of emerging concern (CECs) and their deleterious ecological and human health downstream effects are increasing (He et al., 2018; Sharma et al., 2019). Effluent antibiotics can promote antimicrobial resistance in ecosystems, steroidal estrogens can damage fish stocks by feminization, and myriad other xenobiotics including non-steroidal anti-inflammatory drugs, anticonvulsants, lipid regulators or beta-blockers can bioaccumulate (Adeel et al., 2017; Gomes et al., 2020). As such, there is a demand for novel technologies that can be implemented into traditional wastewater operations to remove CECs, without costly or resource-intensive infrastructures. Biological treatment options are being explored as a replacement for chemical and physical treatments, which can be unsustainable in their energy and material requirements (Kumar et al., 2018). Bioremediation is a growing research field, as species-contaminant interactions are continually explored, and plant-based strategies are sought after for their natural ability to convert solar energy into chemistry. While terrestrial plants have been employed to treat dissolved, soil-bound, and even atmospheric pollutants (Agarwal et al., 2019; Lone et al., 2008), the rapid growth rate and environmental tolerance of microalgae make these organisms an attractive alternative for wastewater treatment (Ferro, 2019; Shukla et al., 2017; Stockenreiter et al., 2016). Opportunities for genetic

manipulations of plants for bioremediation have been exploited in higher plants (Doty, 2008; Maestri & Marmiroli, 2011), but are only beginning to be explored in microalgae.

Microalgae have long been considered as an alternative or complementary agent to traditional wastewater treatment as they convert dissolved nutrients into macromolecules with downstream industrial applications like biofuels or fertilizers (Collotta et al., 2018; Goldman & Stanley, 1974; Hintz et al., 1966). Researchers have used tailored growth environments to improve microalgal capacity to treat wastewater macronutrients (Collotta et al., 2018; Ge et al., 2017), but improving algal capacity in the treatment of micropollutants remains integral to developing sustainable phycoremediation strategies for wastewaters. Normal microalgae have been shown to assist in the removal of CECs from municipal wastewaters. Under conditions simulating algal wastewater growth, consortia of microalgae were reported to instigating the removal of >70% of 23 common pharmaceuticals at relevant contaminant concentrations (Matamoros et al., 2015). Mechanisms of CEC removal by microalgae vary depending on the chemical structure of the xenobiotic, but most CECs are removed by physicochemical (adsorption, absorption); photochemical, or metabolic (co-metabolism, or intracellular metabolism) processes (Tolboom et al., 2019; Xiong et al., 2018). The rates of removal by various processes are on par with those recorded in traditional wastewater treatment operations, highlighting the treatment opportunity that enhancing algal removal abilities might provide (Gentili & Fick, 2017). Microalgal metabolism of CECs presents a potentially more complete processing capability because, unlike sorption and uptake, compounds can be rendered inert and thus encounter fewer downstream complications pertaining to subsequent biomass disposal (Tiwari et al., 2017).

Algal biodegradation of CECs can occur intracellularly or extracellularly by leveraging a suite of Phase I and Phase II enzymes (Sutherland & Ralph, 2019). Phase I biotransformations increase the lipophilicity of a xenobiotic and, in microalgae, these reactions are catalyzed mainly by enzymes of the Cytochrome P450 (CYP) superfamily (Mthakathi et al., 2015; Nelson et al., 2008;

Pflugmacher & Sandermann, 1998). Biodegradation is responsible for most reported CEC removal by microalgae (Sutherland & Ralph, 2019), but recombinant protein expression has the potential to improve the degree of removal and the range of compounds as it has in higher plants (Hussain et al., 2018). By leveraging the unique electron-transfer infrastructure of the photosynthetic apparatus, the expression of heterologous CYP enzymes known to metabolize xenobiotics may prove to be an elegant biocatalytic treatment option.

3.3 Previous Efforts at CEC Removal by Microalgae

Natural organisms have been identified and selected for targeted bioremediation by virtue of that species' response to particular environments and/or contaminants with screening efforts regularly discovering promising additional species (Fabris et al., 2020; Gojkovic et al., 2019). Different species can be better adapted to specific nutrient environments, temperatures, or light intensities, all of which impact CEC catabolism by affecting cellular physiology (Kube et al., 2018; Larsen et al., 2019). Meanwhile, different strains of the same species are compared in their abilities to treat CECs owing to random mutations that might affect their tolerance to an CEC-rich environment or some downstream mechanism (i.e., lipid production) that might make a strain more suitable to these applications (Sweiss, 2017). Adaptation to environments containing large concentrations of pollutants can be encouraged by direct exposure to a would-be toxic environment, allowing mutations in a culture to persist and colonize the new environment (García-Balboa et al., 2013; González et al., 2012; Sánchez-Fortún et al., 2009). Alternatively, a more gradual exposure to contaminants in an environment drives an acclimatization process that, rather than relying on random mutations, drives genetic changes in the culture resulting in robust cultures capable of better responding to contamination in their environment (Romero-Lopez et al., 2012). While these directed evolutionary strategies have yielded success, engineering genetic improvements in microalgae biocatalytic capacity may yield more targeted and robust solutions.

3.4 Transgenic phycoremediation

There has been great advancement in the realm of microalgae genetic modification in the past decade. Dozens of recombinant proteins have been expressed and extracted from microalgae for therapeutic (Barrera & Mayfield, 2013; Specht & Mayfield, 2014), aquaculture (Kiataramgul et al., 2020; Maneenin et al., 2018), and value-added purposes (Braun-Galleani et al., 2015; Wannathong et al., 2016). Algae can even be transgenically modified to manufacture high-value compounds requiring multiple sequential enzymes (Laursen et al., 2015). Employing these strategies for biocatalysis requires, in most cases, only one enzyme per target compound (Lin & Tao, 2017). For example, early attempts at transgenic biocatalysis involved the successful secretion of laccases into the growth medium by *Chlorella* strains, albeit with comparatively low rates of removal (Chiaiese et al., 2011; Lauersen et al., 2013). There have been few attempts to express membrane-bound enzymes that redirect metabolic energy to continually operate as 'algal cell factories' (Fu et al., 2016). Fusions with thylakoid-bound proteins have the capacity to redirect photosynthetic energy to improve efficiency of enzyme function, although these transformations can have negative implications for plastid metabolism (Fabris et al., 2020; Saxena et al., 2014). Heavy-metal contaminant removal by microalgae was improved after thylakoid expression of proteins conveying greater arsenic and cadmium resistance (Piña-Olavide et al., 2020; Ramírez-Rodríguez et al., 2019), and dehydrogenases and monooxygenases for biocatalysis have been expressed in cyanobacteria (Böhmer et al., 2017; Sengupta et al., 2019). In contrast, CECs are metabolized hepatically by enzymes with large redox potentials, which are difficult to replicate in transgenic bacteria, but not in microalgae and cyanobacteria (D'Adamo et al., 2019; Jodlbauer et al., 2021). Equipping microalgae with hepatic enzymes capable of metabolizing these pollutants may present a novel strategy for wastewater treatment.

3.5 Target enzymes

The cytochrome P450 enzymes (CYPs) are heme-thiolate proteins named for their typical absorbance band at 450nm. The CYP superfamily is divided into families and subfamilies depending on the amino acid sequence of the protein. Enzymes are identified by nomenclature indicating family, subfamily, and subfamily isoform such that CYP2A1 refers to a cytochrome P450 belong to family "2" and subfamily "A" and is the first isoform of that family to be named (Nelson, 2006). More than 41,000 CYPs have been named, with that number increasing as more enzymes are identified or synthetically designed for biotechnological applications (Nelson, 2018). This class of proteins typically catalyzes terminal monooxygenations critical for cellular function including carbon assimilation, biosynthesis of cellular infrastructure, and the degradation of xenobiotics (Werck-Reichhart & Feyereisen, 2000). CYP structure is a peptide chassis supporting an iron-containing heme group that forms the catalytic site adjacent to the substrate binding site and is typified by cysteine ligation protected by a β-bulge that allows for two H-bonds that regulate its heme iron redox potential (Ueyama et al., 1996; Poulos & Johnson, 2015). Prokaryotic CYPs are free-floating, whereas most eukaryotic CYPs are membrane-bound, often located in the endoplasmic reticulum or mitochondrial membranes. Membrane-bound proteins are structurally similar to those that are free-floating except for a hydrophobic 20 amino-acid transmembrane helix that tethers the structure to a membrane (Johnson & Stout, 2013). Recombinant CYPs have been expressed in eukaryotic microalgae and cyanobacteria for myriad purposes, and examples are reported in Table 1.

Table 3-1 Heterologous CYP expression has been recorded for eukaryotic microalgae and cyanobacteria. The known instances of expression are presented below, with the details and

the purpose of expression described. There have been no published transgenic microalgae designed to treat CECs in wastewater.

Phytoplankter	Enzyme	Origin	Purpose	Reference
Synechococcus sp. PCC 7002	CYP79A1	*Sorghum bicolor*	-Dhurrin pathway	(Lassen et al., 2014)
Chlamydomonas reinhardtii	CYP79A1	*Sorghum bicolor*	-Dhurrin pathway	(Gangl et al., 2015)
Synechocystis sp. PCC 6803	CYP79A1 CYP71E1	*Sorhum bicolor*	-Dhurrin pathway	(Wlodarczyk et al., 2016)
Synechococcus sp. PCC 7002	CYP1A1	*Rattus norvegicus*	-Electron sink	(Berepiki et al., 2016)
Chlamydomonas reinhardtii	CYP79A1	*Sorghum bicolor*	-Dhurrin pathway -Algal growth in bioreactor	(Zedler et al., 2016)
Chlamydomonas reinhardtii	CYP79A1	*Sorghum bicolor*	-Dhurrin pathway -Fused to *Arabidopsis* ferredoxin	(Mellor et al., 2016)
Chlamydomonas reinhardtii UVM4	CYP76AH16	*Coleus forskolin*	-13*R*(+) manoyl oxide hydroxylation	(Lauersen et al., 2018)
Synechococcus sp. PCC 7002	CYP1A1	*Rattus norvegicus*	-Electron sink	(Berepiki et al., 2018)
Chlamydomonas reinhardtii	CYP79A1-Fd1	*Sorghum bicolor*	-Dhurrin pathway	(Mellor et al., 2019)

			-Fused to *Arabidopsis* ferredoxins and *Sorgghum* CYP reductase	
Synechocystis sp. PCC 6803	CYPCHX	*Acidovorax* sp. CHX100	-Cyclohexane hydroxylation	(Hoschek et al., 2019)
Phaeodactylum tricornutum	CYP716A12	*Medicago truncatula*	-Betulin pathway	(D'Adamo et al., 2019)
Synechoccous elongatus PCC 7942	CYP701A3	*Stevia rebaudiana*	-*ent*-kaurenoic acid synthesis	(Ko & Woo, 2020)

The CYP catalytic cycle is mostly conserved throughout the superfamily (Ortiz de Montellano, 2015) (Figure 1). When the substrate enters the binding site, it displaces an axial water molecule. This triggers an electron transfer from a reductase protein, reducing the heme-bound iron(III) center to its iron(II) state. Triplet dioxygen then reacts with the iron(II) heme, forming an iron(III)-oxygen bond. The heme is further reduced, forming a negatively charged iron(III)-peroxo complex which is rapidly protonated to an iron(III)-hydroperoxy complex (known as Compound I). A second protonation of the complex assists an oxygen-oxygen scission, releasing water and forming a ferryl/porhyrin radical cation. This reactive species oxidizes the substrate, which is released, and the enzyme returns to its iron(III) state (Meunier et al., 2004). The large oxidation-reduction potential of Compound I implies that it can also be involved in other reaction types, but its specific activity for substrate hydroxylation and other reactions is not yet fully understood (Guengerich, 2018).

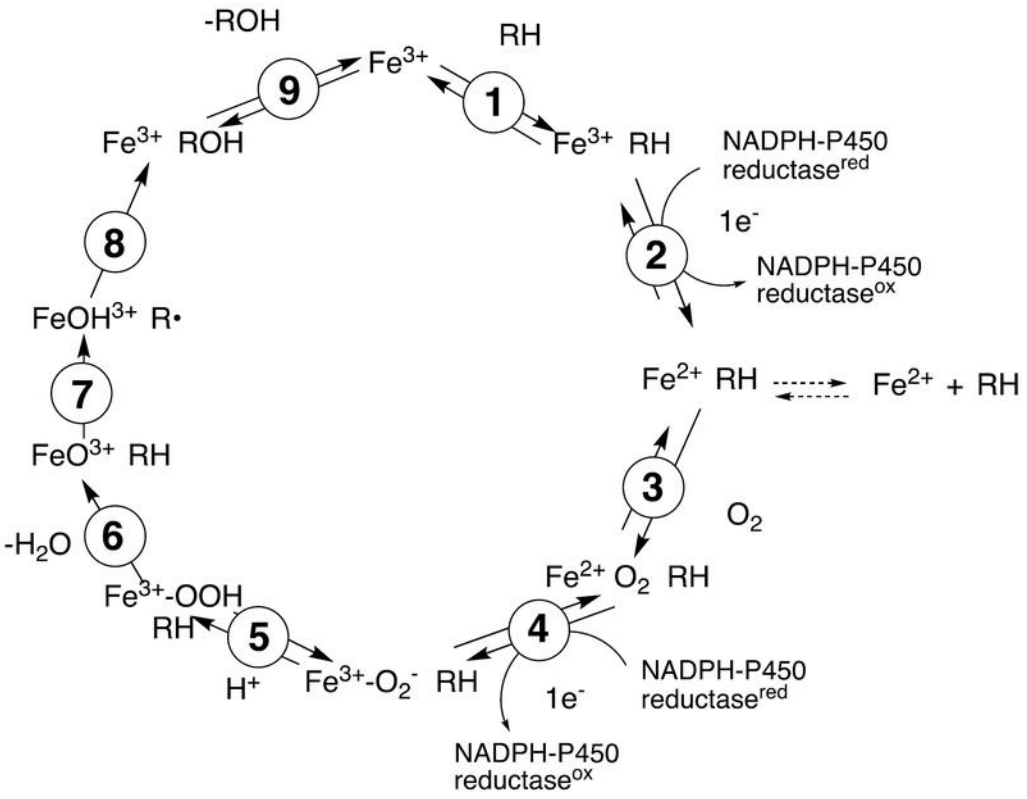

Figure 3-1 The cytochrome P450 catalytic cycle, courtesy of Guengrich (2018).

In humans, Phase I biotransformation occurs mainly within cells of the liver and gastrointestinal tract and is critical to pharmacological principles given its predominant role in liver drug clearance (Sevior et al., 2012). Through Phase I biotransformation, an ingested active metabolite can be deactivated, an inactive metabolite can be activated, or an active metabolite can transform into a new active metabolite. Hence, understanding how a drug will be transformed can inform metabolic fate of drugs administered. CYPs are the most important class of enzymes responsible for human Phase I biotransformation, metabolizing 78% of the 200 most consumed hepatically-cleared pharmaceuticals prescribed (Zanger et al., 2008). Humans encode 57 CYPs, most of which target multiple xenobiotic compounds (Rendic & Guengerich, 2021). Those most common in human tissues include CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 (Lewis, 2004; Zanger et al., 2008). CYP3A4 has the broadest substrate affinity, acting on more than 60% of commercially-available pharmaceuticals (Zhou et al., 2005). While

microalgae express dozens of different CYP proteins, some of which are capable of contaminant-specific biodegradation, no protein to date has been identified capable of broad pharmaceutical metabolism like the human proteins listed above (Stravs et al., 2017; Mthakathi et al., 2015). Compounds regularly detected in wastewater effluent globally that can be metabolized by human CYPs are presented in Table 2.

Table 3-2 CECs of high ecological concern are listed alongside the CYP that metabolizes it. Compounds may be metabolized by more than one CYP, though its breakdown products and enzyme kinetics are subject to vary between CYPs for the same CEC.

Contaminant	Enzyme	Reference
17beta-ethinylestradiol	CYP1A1	(Badawi et al., 2001)
	CYP1A2	(Badawi et al., 2001)
	CYP1B1	(Badawi et al., 2001)
	CYP3A4	(Badawi et al., 2001)
Amoxicillin	CYP2C19	(Furuta et al., 2001)
Atenolol	CYP3A4	(Reeves et al., 1978)
Benzafibrate	CYP3A4	(Miller & Spence, 1998)
Caffeine	CYP1A2	(Chainuvati et al., 2003)
	CYP1B1	(Shimada et al., 1997)
	CYP2C8	(Rendic & Guengerich, 2021)
	CYP2C9	(Utoh et al., 2013)
	CYP2D6	(Ha et al., 1996)

	CYP2E1	(Tassaneeyakul et al., 1994)
	CYP3A4	(Ha et al., 1996)
	CYP3A5	(Tassaneeyakul et al., 1994)
	CYP3A7	(Tassaneeyakul et al., 1994)
Carbamazepine	CYP1A2	(Masubuchi et al., 2001)
	CYP2B6	(Pearce et al., 2002)
	CYP2C8	(Mesdjian et al., 1999)
	CYP3A4	(Pearce et al., 2002)
	CYP3A5	(Furukori et al., 1998)
	CYP3A7	(Nakamura et al., 2003)
Clarithromycin	CYP2C19	(Saito et al., 2005)
Clindamycin	CYP3A4	(Wynalda et al., 2003)
Codeine	CYP2D6	(Williams et al., 2002)
	CYP3A4	(Yue & Säwe, 1997)
	CYP3A7	(Rendic, 2002)
DEET	CYP1A2	(Usmani et al., 2002)
	CYP2A6	(Usmani et al., 2002)
	CYP2B6	(Usmani et al., 2002)
	CYP2C19	(Usmani et al., 2002)
	CYP2D6	(Usmani et al., 2002)
	CYP2E1	(Usmani et al., 2002)
	CYP3A4	(Usmani et al., 2002)
	CYP3A5	(Usmani et al., 2002)

Diclofenac	CYP1A1	(Mancy et al., 1999)
	CYP1A2	(Mancy et al., 1999)
	CYP2B6	(Bort et al., 1999)
	CYP2C8	(Mancy et al., 1999)
	CYP2C9	(Obach & Reed-Hagen, 2002)
	CYP2C18	(Bort et al., 1999)
	CYP2C19	(Bort et al., 1999)
	CYP3A4	(Shen et al., 1999)
Erythromycin	CYP1A1	(James & Boyle, 1998)
	CYP1A2	(James & Boyle, 1998)
	CYP1B1	(James & Boyle, 1998)
	CYP2B6	(James & Boyle, 1998)
	CYP3A4	(Tanaka, 1998)
	CYP3A5	(Ronis et al., 1999)
Estriol	CYP3A7	(Sarkar et al., 2003)
Estrone	CYP1A1	(Ohe et al., 2000)
	CYP1A2	(Ueno et al., 2000)
	CYP1B1	(Shimada et al., 1997)
	CYP2B6	(Ohe et al., 2000)
	CYP2C9	(Modugno et al., 2003)
	CYP2E1	(Ohe et al., 2000)
	CYP3A4	(Yamazaki et al., 1998)
	CYP3A5	(Huang et al., 1998)

Gemfibrozil	CYP3A4	(Miller & Spence, 1998)
Ibuprofen	CYP2C8	(Backman et al., 2016, p. 8)
	CYP2C9	(Bell et al., 2007)
	CYP2C19	(McGinnity et al., 2000)
Indomethacin	CYP2C9	(Nakajima et al., 1998)
	CYP2C19	(Nakajima et al., 1998)
Metoprolol	CYP2C19	(Rendic, 2002)
	CYP2D6	(Belpaire et al., 1998)
Miconazole	CYP3A4	(Rendic, 2002)
Naproxen	CYP1A2	(Rodrigues et al., 1996)
	CYP2C8	(Tracy et al., 1997)
	CYP2C9	(Miners et al., 1996)
Propranolol	CYP1A2	(McGinnity et al., 2000)
	CYP2C19	(McGinnity et al., 2000)
	CYP2D6	(McGinnity et al., 2000)
	CYP3A4	(Yoshimoto et al., 1995)
Sulfamethoxazole	CYP3A4	(Rendic, 2002)
Trimethoprim	CYP2C8	(Rendic, 2002)
	CYP2C9	(Rendic, 2002)
	CYP3A4	(Rendic, 2002)

Pharmaceuticals can act not only as substrates for CYPs, but also as enzyme inhibitors or inducers. (Lin & Lu, 1998). Inhibition of CYPs can be reversible, quasi-reversible, or irreversible based on the mechanism of inhibition. Instances where substrates compete for access to the

activation site, but inhibitors do not react with the enzyme, are reversible and can arise based on different concentrations or affinities to the enzyme (Deodhar et al., 2020). These inhibitors do not prevent the enzyme from metabolizing a substrate, instead they slow the rate of enzyme activity (Kamel & Harriman, 2013). Instances where the CYP catalytic cycle begins and metabolite formation from an inhibitor interrupts enzyme function are considered quasi-reversible or irreversible inhibition. Quasi-reversible inhibition can generally be corrected by irradiation at 400-500nm (Correia & Hollenberg, 2015; Ullrich & Schnabel, 1973). Given the convenience of the algal photosynthetic spectra peaking in the 400-500nm range, quasi-inhibition of CYPs *in situ* may be inconsequential. Irreversible inhibition occurs when an intermediate is catalyzed by an enzyme that alters the conformation of that enzyme permanently or destroys it (Deodhar et al., 2020). Induction of CYP activity by a compound is less dependent on compound interaction with the enzyme, but rather induction occurs at the translational level wherein specific receptor-ligand interactions induce specific CYP production (Tompkins & Wallace, 2007). CYP inducers and inhibitors that are often quantified in municipal wastewater are presented in Table 3.

The induction system of CYP in plants is not yet well understood, largely because plants do not express those receptors that induce CYP activity in humans (Pandian et al., 2020). As such, the effect of inducers on recombinant CYPs remains a knowledge gap.

Table 3-3 CYPs may also be inhibited or induced by CECs, suggesting that a wastewater environment laden with different CECs may affect CYP activity. Proper characterization of the CEC environment in the target media may elucidate the degree to which other CECs might affect enzyme activity.

Contaminant	Inhibits	Reference	Induces	Reference
Azithromycin	CYP2A6	(Xia et al., 2002)		
	CYP3A4	(Mae et al., 2000)		

Benzafibrate	CYP2C8	(Kajosaari et al., 2005)	CYP1A1	(Sérée et al., 2004)
	CYP7A1	(Roglans et al., 2004)		
Caffeine	CYP1A1	(Tassaneeyakul et al., 1993)		
	CYP1A2	(Tassaneeyakul et al., 1993)		
Carbamazepine	CYP1A2	(Masubuchi et al., 2001)	CYP1A2	(Parker et al., 1998)
	CYP2C19	(Lakehal et al., 2002, p. 19)	CYP2A6	(Williams et al., 2010)
			CYP2B6	(Faucette et al., 2004, p. 2)
			CYP2C8	(Daily & Aquilante, 2009)
			CYP2C9	(Rendic, 2002)
			CYP2C19	(Rendic, 2002)
			CYP3A4	(Ketter et al., 1995)
			CYP3A5	(Usui et al., 2003)
Chloramphenicol	CYP2C9	(Rendic, 2002)		
	CYP2C19	(Park et al., 2003)		
	CYP3A4	(Park et al., 2003)		
Ciprofloxacin	CYP1A2	(Hersh & Moore, 2004)	CYP2E1	(Peterson et al., 2004)
	CYP3A4	(McLellan et al., 1996)		
Clarithromycin	CYP2C19	(Furuta et al., 2005)	CYP3A4	(Jacobson, 2004)
	CYP3A4	(Hersh & Moore, 2004)		
Clindamycin	CYP3A4	(Wynalda et al., 2003)		
Codeine	CYP2D6	(Rendic, 2002)		
Diclofenac	CYP1A2	(Ohyama et al., 2014)		
	CYP2C9	(Monostory et al., 2004)		
	CYP2E1	(Monostory et al., 2004)		

	CYP3A4	(Masubuchi et al., 2002)		
Erythromycin	CYP1A2	(Fisman et al., 1996)	CYP3A4	(Yasuda et al., 2008)
	CYP3A4	(Hersh & Moore, 2004)		
	CYP3A5	(Watanabe et al., 1999)		
Estriol	CYP1A2	(Elskus, 2004)	CYP1A1	(Son et al., 2002)
Gemfibrozil	CYP1A2	(Wen et al., 2001)	CYP2C8	(Prueksaritanont et al., 2005)
	CYP2C8	(Wang et al., 2002)	CYP3A4	(Prueksaritanont et al., 2005)
	CYP2C9	(Wen et al., 2001)		
	CYP2C19	(Wen et al., 2001)		
	CYP3A4	(Wang et al., 2002)		
Ibuprofen	CYP2C8	(Daily & Aquilante, 2009)		
Indomethacin	CYP2C8	(Daily & Aquilante, 2009)		
Ketoprofen	CYP2C8	(Główka et al., 2011)		
	CYP2C9	(Kappers et al., 1996)		
Metoprolol	CYP2D6	(Yue & Säwe, 1997)		
Miconazole	CYP1A2	(Niwa et al., 2005)		
	CYP2A6	(Draper et al., 1997)		
	CYP2B6	(Zhang et al., 2002)		
	CYP2C9	(Venkatakrishnan et al., 2000)		
	CYP2C19	(Niwa et al., 2005)		
	CYP2D6	(Niwa et al., 2005)		
	CYP2E1	(Niwa et al., 2005)		

	CYP3A4	(Monostory et al., 2004)		
Ofloxacin	CYP1A2	(Fuhr et al., 1992)		
Propranolol	CYP1A1	(Tassaneeyakul et al., 1993)		
	CYP1A2	(Tassaneeyakul et al., 1993)		
	CYP2D6	(Kudo et al., 1997)		
			CYP2E1	(Sampol et al., 1997)
Sulfamethazine	CYP2C9	(Zweers-Zeilmaker et al., 1997)	CYP3A4	(Rendic, 2002)
Sulfamethoxazole	CYP2C8	(Wen et al., 2002)		
	CYP2C9	(Wen et al., 2002)		
	CYP3A4	(Wen et al., 2002)		
Sulpiride	CYP1A2	(Rane et al., 1996)		
Tetracycline	CYP3A4	(Zhao & Ishizaki, 1997)		
Thiabendazole	CYP1A2	(Bapiro et al., 2001)	CYP1A1	(Fratta et al., 1994)
Triclosan	CYP1A1	(Hanioka et al., 1996)		
	CYP2B6	(Hanioka et al., 1996)		
Trimethroprim	CYP2C8	(Wen et al., 2002)		
	CYP2C9	(Wen et al., 2002)		

3.5.1 CYP selection for microalgal CEC treatment

There are myriad CYP enzymes which could be employed to biologically treat municipal wastewater effluent. Selecting which enzymes to express should be driven by first delineating the purpose of remediation. Prioritizing the removal of one or two CECs would narrow the options outlined above. Thus, a complete analysis of CECs in the target wastewater would highlight the compounds of greatest priority while also assist in understanding whether inducers and inhibitors

are present which could act on an expressed enzyme. For broad-scale CEC removal, such as healthcare infrastructures like hospitals and nursing homes where effluent concentrations of pharmaceuticals are elevated and varied, it would be possible to create a polyculture of strains expressing different CYPs, or express multiple CYPs in the same host (Santos et al., 2013). The matrix effects of different compounds in a treatment environment will need to be established for transgenic microalgae as their growth conditions are different from those regularly used to study CYP pharmacokinetics.

Human CYPs perform optimally under conditions similar to those in the human body, therefore, some aspects of recombinant CYP expression and performance may be impacted by its intended application. Human enzymes have evolved to operate at standard body temperatures, and hypothermia inhibits CYP3A4 activity in humans by 11.1% for every degree Celsius below 36.5°C (Hostler et al., 2010). Hypothermia from 37°C to 33°C in mice reduced the activity of CYP3A but not CYP2C, CYP2D, and CYP2E isoforms, indicating an isoform-specific reaction to temperature fluctuation (Zhou et al., 2011). Further, some reduction in CYP activity during hypothermia is likely affected by mammalian physiological responses to stress (transporters, membrane permeability, oxygenic delivery) and thus might not affect recombinant human CYPs (Smits et al., 2020). While some microalgal treatment systems may maintain cultures near body temperature, others are likely to provide minimal heating in order to improve the energetic efficiency of the treatment operation (Xu et al., 2019). CYP from some organisms have been observed to be very adaptable, for example CYP119A1 from *Sulfolobus solfataricus*, an archaea that grows at 80°C, was shown to operate well at 25°C, suggesting the thermal range of non-mammalian CYP may not be as limited it is in mammals (Rabe et al., 2010). Hence, the effect of temperature should be explored for optimizing rates of biocatalysis in microalgae by comparing CYP activity when expressed in species and strains tolerant to different temperatures.

Variations in pH can also affect the activity of recombinant enzyme activity. Human CYP systems in the liver operate at the ideal intrahepatocyte pH of 7.0 (Hall, 2016). Meanwhile, the pH of irradiated microalgal stroma (the fluid matrix inside a chloroplast) may fluctuate between 7.6 and 8.0 to ensure an appropriate proton gradient to drive ATP synthesis (Takizawa et al., 2007; Werdan et al., 1975). In irradiated cyanobacteria, cytoplasmic pH is generally recorded between 7.7 and 8 (Belkin et al., 1987; Mangan et al., 2016). In CYP2D6, enzyme kinetic parameters were investigated across a pH gradient for the metabolism of bufuralol and dextromethorphan (Rougée et al., 2016). Between pH of 7.0 and 8.0, the Michaelis constant (Km) of the enzyme decreased by 53.6% and 40.0%, while the maximum rate of enzyme activity (Vmax) increased by 49.9% and decreased by 44.3% for the two compounds, respectively (Rougée et al., 2016). Similar parameters have been explored for CYP3A4 between pH of 7.0 and 8.0 in the enzyme's activity on midazolam, testosterone, and dextromethorphan. The Km increased by 21.1% and 60.7% for midazolam and testosterone, respectively, and decreased by 35% for dextromethorphan; while the Vmax decreased by 67% and 78%, and increased by 142% for the three compounds, respectively (Rougée et al., 2017). CYP activity does not uniformly decrease with increased alkalinity, but it should be expected that CYP behavior in microalgae may not reflect that of pharmacological literature given the discrepancies of intracellular makeup between these cells and the enzyme's host cells. Other non-plant organisms have similar CYP systems that metabolize the same compounds as humans, but their pharmacokinetic traits may be better suited to wastewater treatment environments (Burkina et al., 2017). Teleost fish are unable to regulate their body temperature and have CYP systems adapted to lower and more varied internal temperatures in the range of 5°C to 20°C (Koch et al., 1992; Snegaroff & Bach, 1990). CYP systems in teleosts may also be better suited to chloroplast stroma since their intrahepatocyte pH is more alkaline than in humans (Furimsky et al., 1999; Nikinmaa et al.,

1999). CEC-metabolizing CYPs in piscine and other non-human animals are reviewed extensively by (Burkina et al., 2015) and (Lewis et al., 1998).

3.5.2 Enzyme engineering

Enzyme engineering affords the opportunity to improve protein function and may be a beneficial strategy for improving heterologous CYP activity in microalgae. Directed evolution depends on random and iterative changes in protein structure paired with screening mutants for improvements in a desired quality. In a brute force mutagenesis approach to improve thermostability in CYP2B1, 3000 mutant variants were screened for their activity at reduced temperature, resulting in a 1.4-fold increase in activity at the researcher's target temperature (Kumar et al., 2006). Rational expansion engineering relies on specific alterations in protein structure informed by its chemistry (Xu & Du, 2018). A CYP from the gram-positive bacterium *Bacillus megaterium* had its axial cysteine ligand replaced with serine, leading to a class of enzymes capable of improved C-H alkylization (Chen et al., 2018). Further directed evolution on this new class of cytochrome enzyme has resulted in proteins capable of forming chiral bicyclobutanes, reactions very rarely encountered naturally (Zhang et al., 2019). *De novo* enzyme design is becoming a tangible reality as computational power improves the capacity to design catalytic sites for specific reactions (Huang et al., 2016; Linder, 2012). The dimensional relationship between protein sequence and structure presents logistical hurdles for protein engineers and machine-learning methods have emerged to organize amino acids such that a novel catalytic site can be maintained by a stable proteinaceous architecture (Greener et al., 2019). Protein engineering efforts to improve the activity, stability, and specificity of proteins enhanced by machine-learning approaches presents a new frontier in research to generate highly efficient human CYPs within photoautotrophic hosts.

3.5.3 Improving transgenic CYP efficiency

Once the appropriate CYP enzyme has been selected and perhaps altered to benefit a microalgal host, further consideration must be placed on how to most effectively harness the power of the CYP to metabolize CECs. The site of transformation is important given the varying opportunities when genetically modifying microalgae. Transforming microalgal chloroplasts is more convenient than nuclear transformation owing to the chloroplast's tendency to integrate genes by homologous recombination rather than by random integration (Barahimipour et al., 2016). Glass bead agitation, biolistic delivery, and electroporation are established methods of transformation in chloroplasts (Boynton et al., 1988; Economou et al., 2014; Xie et al., 2014). Cyanobacterial genetic transformation is a more established field with transformations possible both passively and by emerging techniques including conjugation, ultrasonic transformation, and CRISPR/Cas9 based methodologies (Lin et al., 2019).

Microalgal expression of human CYPs provides an elegant coupling of the need for electron transfer mechanisms with the reducing power provided through oxygenic photosynthesis (Figure 2). The light-dependent reactions of photosynthesis comprise a series of redox reactions initiated by pigment absorption of photons, the energy which can be transferred through systems that reduce NADP+ to NADPH and generate a proton gradient used to produce ATP. The large redox potentials of photo-oxidized photosystems II and I drive the metabolism of plant cells through downstream pathways that direct metabolic energy which fortuitously can be coupled to the needs of heterologously expressed enzymes in these systems (Löwe et al., 2018; Nielsen et al., 2013). Ensuring balance between efficient functioning of transgenic products without compromising cellular metabolism is a critical consideration when such systems are coupled to microalgal energetics. If a recombinant CYP could be sufficiently powered, further manipulation may be required to ensure that enough enzyme is expressed, and that CECs can be delivered to the thylakoid such that meaningful efficiency of remediation can be achieved. The following section outlines how these potential bottlenecks may be resolved.

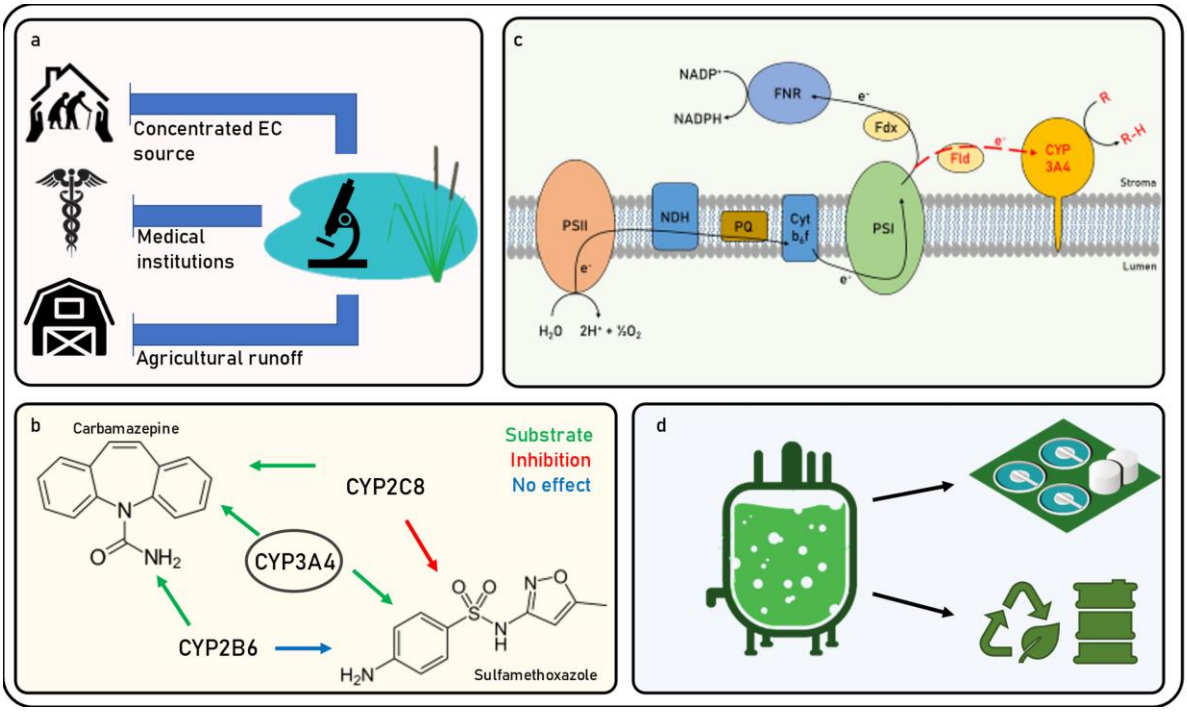

Figure 3-2 A model of transgenic CYP in microalgae to remove CECs from wastewater. First, wastewater must be investigated in order to characterize its CEC content to identify those which will be targeted for removal (a). Then, the appropriate CYP(s) for expression are selected based on the contaminant-enzyme effects that might result based on the CEC environment of the wastewater (b). Once expressed, light-driven hydrolysis will provide the reducing power to the heterologous CYP(s) at rates that may meaningfully draw down CEC concentrations (c). With the transgenic cultures designed, the growth conditions of the culture will be investigated so as to improve CEC removal while ensuring the safe re-use of algal biomass (d).

3.5.4 Improving Protein Expression

There are many biological considerations that must be considered when attempting to improve protein expression in transgenic microalgae. Species selection may be informed by total protein content, since there are phylogenetic and species-specific trends in cellular macromolecular composition (Finkel et al., 2016; Liefer et al., 2019). Cyanobacteria are generally the most

protein-rich class of microalgae, with *Synechococcus elogatus* UTEX 2973 being exceptionally protein-rich owing to its rapid growth rate (Yu et al., 2015). The eukaryotic microalgae *Chlorella vulgaris* is also relatively protein-rich and is a common host for heterologous protein expression (Guccione et al., 2014). Genetic regulatory strategies including the use of promoters, untranslated regions, codon optimization, and metabolic engineering can increase the yield of recombinant proteins and are reviewed for eukaryotic microalgae and cyanobacteria (prokaryotic microalgae) by Doron and colleagues and Santos-Merino and colleagues, respectively (Doron et al., 2016; Santos-Merino et al., 2019).

Effective CEC treatment will require high level heterologous enzyme expression sufficient to impart meaningful metabolism of target CECs to below toxicity threshold levels. Achieving appropriate levels of expression has proven to be challenging. For example, CYP1A1 expressed in the cyanobacteria *Synechococcus* sp. reached a maximum concentration of 6.2 pmol mg^{-1} of total protein, which is approximately 30-fold less than recombinant CYP1A1 expression in heterotrophic bacterial hosts (Berepiki et al., 2016). By comparison, CYP content in human microsomes ranges from 19 to 260 pmol mg^{-1} of total protein (Venkatakrishnan et al., 2000). Between a 3- and 47-fold decrease in protein expression was noted when CYP79A1 was fused to electron transfer partners and expressed in *Nicotiana benthamiana*, highlighting a further need to improve protein stability when expressed in recombinant hosts (Mellor et al., 2019).

3.5.5 Metabolite Movement

While CYP expression and activity in microalgae can be improved by judicious strain selection and linkage with appropriate electron transfer partners, ensuring the proper delivery of substrates to the enzymes with efficient disposal of metabolized by-products is also critical to maximizing CYP activity. Most chemical CECs found in wastewater are pharmaceuticals and personal care products whose deleterious environmental effects are owed to their lipophilicity which results in easier passage across membranes (Pereira et al., 2015; Zenker et al., 2014). Some CEC delivery

to heterologous enzymes may not require further genomic intervention as many CECs are already absorbed and metabolized by cells, suggesting CECs will passively concentrate intracellularly (Hena et al., 2021; Maryjoseph & Ketheesan, 2020; Tolboom et al., 2019). Lipophilic compounds readily bioaccumulate in lipid vacuoles in microalgae (Coogan et al., 2007; Mason et al., 1995), but their transfer from vacuoles to thylakoids merits consideration. Other contaminants readily sorb to the extracellular matrix of microalgae then are gradually transported intracellularly, perhaps owing to their unconfirmed role in preventing reactive oxygen species accumulation by offsetting oxidative stress onto the contaminants rather than on cellular macromolecules (D'Alessandro et al., 2018; Yu et al., 2017). The active transport of CECs within microalgae is not well understood, many sequenced transporters operate differently than expected and the diversity of CECs confound transport predictability (Facchinelli & Weber, 2011; Rea, 2007). The phylogenetic diversity of microalgae may also affect passive delivery of CECs to heterologous CYPs; cyanobacteria have only one membrane separating the extracellular environment from biocatalyzing enzymes, whereas some microalgae like diatoms have undergone endosymbiosis twice, meaning that CECs have to cross extra membranes to access the chloroplast, perhaps requiring more cellular energy for CEC delivery (Facchinelli & Weber, 2011).

In those instances where CECs do not accumulate intracellularly, further genetic modification may be required to deliver compounds to their corresponding CYP. P-glycoprotein (P-gp) is an important ATP-Binding Cassette (ABC) transporter in pharmaceutical metabolism in humans, with analogs in the genome of the model plant species *Arabidopsis thaliana* (Rojas-Pierce et al., 2007). Human P-gb transports roughly half those compounds listed in Table 1 (Kim, 2002). Many ABC transporters have been characterized for their pharmacological effects in humans, and their complementary expression in microalgae could be tailored to target compounds in wastewater. As an example, the anticonvulsant carbamazepine does not accumulate in microalgae (Bai & Acharya, 2017; Larsen et al., 2019). It is transported variably by some human ABC transporters,

but most consistently by RLIP76, a non-ABC transporter which could be expressed in microalgae to accumulate intracellularly and be metabolized by CYPs 1A2, 2B6, 2C8, 3A4, 4A5, or 3A7 (Awasthi et al., 2005). After CECs have been oxidized by a recombinant CYP, its logP will increase and may render to compound unable to cross lipid bilayer membranes (Bennion et al., 2017). Should intracellular concentrations of these products prove toxic or debilitating to the cell, further transformations could include hepatic efflux transporters, which are proteins designed to excrete these metabolites from metazoan liver cells (Li et al., 2008). Multi-protein expression in microalgae could allow researchers to further tailor transgenic strains to improve their capacity to remediate CECs in wastewater.

3.5.6 Electron Delivery

In a typical CYP catalytic cycle, electrons must be transferred to the enzyme by electron donors. Without a dedicated electron donor, reducing power can still be delivered to CYPs, although the rate of electron delivery is very low (Lauersen et al., 2018). Microsomal CYPs rely on the electron donor Cytochrome P450 Reductase (CPR) that catalyzes the electron transfer between NADPH and the CYP monooxygenase. CPR is comprised of two subunits, the flavin mononucleotide (FMN) domain and the flavin adenine dinucleotide (FAD) domain. NADPH interacts with CPR by donating a hydride ion to the FAD contained in the FAD domain (Waskell & Kim, 2015). Interflavin electron transfer from FAD to FMN is sequential and made rapid by the conformity of CPR that allows the orbitals of the molecules to overlap. The conformation of CPR then changes to favor electron donation by FMN to a substrate-bound CYP (Hamdane et al., 2009; Murataliev et al., 2004). CPR is integral to mammalian drug metabolism as it is the main reducing agent of all hepatic CYPs (Gu et al., 2003; Wang et al., 1997). Pairing a CYP with its CPR has yielded increases of efficiency by up to two orders of magnitude in some bacterial CYP expression systems (Hirakawa & Nagamune, 2010). Heterologous CYP expression has been paired with the cognate CPR of the enzyme to increase enzyme efficiency in microalgae, however

enzyme activity has not been found to increase as dramatically as in its bacterial counterparts (D'Adamo et al., 2019; Fuentes et al., 2016; Saxena et al., 2014).

CPR co-expression with a CYP counterpart requires competing with entrenched cellular metabolism for NADPH, and therefore the energy directed to heterologous proteins may not be optimal *without further manipulation* other possibilities for heterologous CYP reduction have been explored in plants to avoid bottlenecks involving other cellular biopathways. A NADPH-independent system of CYP reduction was engineered by fusing ferredoxin, the main electron acceptor from PSI, to CYP79A1 in *N. benthamiana* using an inert linker (Mellor et al., 2016). *In vivo*, the ferredoxin-fused CYP outperformed the native unfused CYP 1.5-fold. Further experimentation compared three different *Sorghum bicolor* ferredoxin isoforms fused to CYP79A1 with the flavodoxin-containing domain of the same CPR of the plant. *In vivo* activity of CYP79A1 fused with the CPR domain was five times greater than those fused with ferredoxins, and twenty-five times greater than the unfused CYP (Mellor et al., 2019). These fusions direct energy partitioning towards CYP by transferring electrons directly from PSI to the target protein. Other potential electron carriers for heterologous enzyme reduction including plastocyanin and cytochrome c have been described (Mellor et al., 2017), although their specific redox potentials limit their capacities to reduce some CYPs.

3.6 Metabolic effects

With the insertion of a CYP paired to an electron transfer partner, native microalgae cellular metabolic activity would be expected to decline as the cell compensates for energy being shunted to a new pathway (Lin & Tao, 2017). The microalgae *Chlamydomonas reinhardtii* expressing CYP79A1 grew to a maximum density 69.5% less than its wild-type counterpart after 7 days in growth media (Zedler et al., 2016). The cyanobacteria *Synechococcus elongatus* PCC 7942 strains expressing CYP701A3 and kaurene synthase matched the growth rate of wild-type cells, but further expressions of CPR and kaurene oxidase in the strain lowered its maximum cell

density to 90.1% that of the wild-type strain (Ko & Woo, 2020). Notably, the order of expression in the transgene affected growth.

3.6.1 Light-based tradeoffs

With the insertion of a non-native electron sink into the photosynthetic complex, there may be further opportunities to improve CYP activity by forcing electron flow towards that sink. The diatom *Paeodactylum tricornutum* expressing CYP716A12 and two other heterologous proteins designed to produce betulin from lupeol showed no apparent growth inhibitions after 5 days of growth (D'Adamo et al., 2019). The cyanobacteria *Synechococcus* sp. PCC 7002 expressing rat CYP1A1 grew to the same density as its wild-type counterpart, while its maximum electron transport rate (ETR_{Max}) from the reaction centre of photosystem II was 31.3% greater than the wild-type strain (Berepiki et al., 2016). Knocking out circular electron transport genes in this transformed strain lead to a further 16% increase in the ETR_{Max} of *Synechococcus* sp. PCC 7002 (Berepiki et al., 2018). This suggests that, rather than competing with cellular metabolism, recombinant CYP activity may act to enhance native photosynthesis. Guided by this hypothesis, the impact of photo-irradiation on enzyme activity merits further exploration as the levels of irradiation that could be attained have not yet been achieved.

3.6.2 Ideal growth conditions

When considering microalgae metabolism as it pertains to CEC treatment, the culturing strategy for microalgae will require optimization for highest removal rates. When prioritizing the production of protein, phycologists maintain microalgal cultures in the exponential growth phase since it constitutes the greatest proportion of cellular macromolecules (Liefer et al., 2019). Assuming a constant supply of nutrients (common in wastewater environments), cells can remain in the exponential phase at high densities, maximizing the concentration of CYP in the culture.

Allowing cells to reach the stationary phase, however, may serve to improve overall bioremediation in the culture as cells accumulating lipids and exuding carbohydrates may encourage further transport of CECs intracellularly (Liefer et al., 2019; Yu et al., 2017). Nonetheless, stationary growth brought about by nutrient limitation causes cellular pigmenta and Photosystems I and II to decrease in concentration, thereby reducing the available reducing power for transgenic CYPs (Liefer et al., 2018). CEC removal rates will need to be assessed *in situ* to determine the best culturing practices for transgenic strains.

3.7 Infrastructure considerations

Owing to the ecological constraints impact large-scale cultivation of genetically-modified microalgae, and to conform to these limitations cultures will likely be applied in closed bioreactors rather than high-rate algal ponds (Beacham et al., 2017). This mitigates concern for other algal species to colonize the culture and dilute the concentration of CYP in the treatment process, but will increase the costs associated with these efforts (Richardson et al., 2012). The implementation of these technologies will require the buy-in of local stakeholders and governments, and therefore an added consideration before pilot studies are the social ramifications of genetic engineering for environmental causes. In Europe, where genetically-engineered crops are less socially accepted than in other parts of the world, Villaeral et al. (2020) investigated the social acceptance rates of genetically modified microalgae for the purposed of biofuel production. Surveys reported broad support for the technology given its potential side-effects are further explored and that cultures are maintained in secure, closed, systems. Similar results could be expected from another industrial application of geneticall modified microalgae given it is not being considered as a food additive or replacement, where the greatest hesitation to genetically modified crops lies (Villareal et al., 2020).

Given the scale of wastewater requiring treatment, large-scale photobioreactor design will need to overcome issues associated with efficient illumination, sufficient mixing, and proper nutrient

delivery and removal (da Silva & Reis, 2015). Despite the increased capital and recurring expenses associated with closed bioreactor cultivation, there are increased opportunities to further enhance CEC removal under these conditions. Culturing transgenic microalgae expressing biocatalytic CYPs may tolerate irradiation at higher levels than traditional cultivation given its increased effects on CYP activity, which may have the added consequence of improving rates of photodegradation (Díaz-Quiroz et al., 2020) Closed bioreactors provide an added opportunity to implement further CEC-removing mechanisms like UV-irradiation into the treatment system (Du et al., 2015), and will also reduce the risk of concentrated emerging contaminants being released into receiving environments. Proper monitoring of CECs will need to be established to ensure their removal is maximized. Further identifying by-products of CYP metabolism will assist in efforts to determine downstream applications of microalgal biomass (i.e., intracellular by-products may affect microalgae reuse as a fertilizer).

3.8 Conclusion

Transgenic microalgae are emerging as excellent platforms for heterologous enzyme activity based on the large redox potential associated with oxygenic photosynthesis and their ability to grow to great densities in wastewater environments. Genetic transformations of microalgae and cyanobacteria are reported to produce pharmaceutical, nutraceuticals, and other value-added products by expressing enzymes from the Cytochrome P450 family. Product synthesis by CYPs can require careful implementation of strings of recombinant proteins organized in metabolons, whereas xenobiotic catalysis by CYPs requires only that the CYP is expressed and sufficiently supplied with reducing power and substrate. Most polluting xenobiotics are metabolized by CYP3A4, CYP2C6, CYP2D6, and CYP2E1, though dozens more are available for specific compound removal. Selection of an appropriate CYP demands consideration not only of its substrates, but also its inhibitors present in the medium, and the CYP origin may depend on the physiochemical conditions of the treatment. For this technology to meaningfully neutralize

deleterious compounds in wastewater, their electron delivery systems, protein expression, and substrate transport infrastructure will require optimization and further fundamental research. Microalgae already present a novel cost-effective solution to persistent and emerging contaminants in wastewater, and further equipping these organisms with recombinant CYPs may constitute an exciting future for such technologies which merit exploration.

3.9 Works Cited

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Chapter 4

Adaptation of microalgae for improved CEC removal

4.1 Abstract

Microalgal-induced bioremediation of contaminants of emerging concern (CECs) requires improvements in its overall capacity to treat these compounds in order to be developed into a realized technology. There are several avenues by which microalgal CEC removal can be enhanced and this chapter discusses ecological and transgenic approaches to improving CEC removal. Cultivating polycultures of microalgae allows for more efficient nutrient and resource distribution, and may improve overall CEC removal as different cultures may be specialized to remove different CECs. Here, a native polyculture originating from a Kingston, ON wastewater treatment plant is compared to a monoculture of *Chlorella vulgaris*, a model microalgal species used in this field. The two cultures did not exhibit significant differences in their CEC removal, though they did treat municipal wastewater effluent at high efficiencies. Inducing organic carbon-supplemented mixotrophy in the polyculture did not meaningfully alter its treatment of pollutants. Also discussed is the design and development of a transgenic strain of *Chlamydomonas reinhardtii* expressing the human cytochrome P450 enzyme CYP3A4. The recombinant protein is fused to an electron transfer partner that allows the enzyme to redirect photosynthetic energy towards catabolizing pharmaceutical compounds. Its transformation is not realized but improvements to the concept are discussed.

4.2 Introduction

4.2.1 CEC treatment by microalgae

Municipal wastewater treatment relies on the separation of solids, the bacterial degradation of nutrients, and the sterilization of wastewater before it satisfies appropriate water quality

guidelines and is discharged into surrounding water bodies or redirected for reuse. Myriad pharmaceuticals, personal care products, and other chemicals are often detected in effluent streams at wastewater treatment plants because they are not treated by the existing physical, biological, and chemical treatment processes embedded in these infrastructures (Tran et al., 2018). The presence of these contaminants of emerging concern (CECs) and their innumerable deleterious effects on aquatic ecosystems are increasingly reported in academic literature and water quality assessments (Clarke & Smith, 2011). As awareness and concern about poor CEC treatment grow, so do the calls for the implementation of new technologies capable of sustainably removing these pollutants from wastewaters.

Microalgal wastewater treatment may serve as a passive, low-energy, low-cost substitute to existing treatment options like ultraviolet (UV) -irradiation. Literature-reported conclusions around the algal removal of CECs, and by proxy industrial interest, have expanded in the past decade with research groups investigating the effects of different species and environmental conditions on the ability for algae to treat wastewater contaminated with those pharmaceuticals and personal-care products most commonly reported in effluent streams (Tolboom et al., 2019). Mixotrophic growth, a burgeoning growth strategy for industrial algal applications wherein cells derive energy both photoautotrophically and heterotrophically, has yet to be investigated explicitly in the context of CEC removal research. Its overall effects on cellular physiology; increased transparent exopolymeric substance and dissolved organic matter production, rapid growth, and improved co-metabolism are all conducive to improving CEC removal (Kim et al., 2013; Zhan et al., 2017). Mixotrophic algal growth also improves the economic budget of industrial algal growth since it presents an opportunity for algae to consume products generally considered waste products of other industrial processes (Ge et al., 2018). An ideal algal treatment system would use nutrients available in wastewater to proliferate, actively and passively remove

the CECs of concern in the system, while simultaneously producing biomass conducive to industrial re-use (biofuels, biofertilizers etc.)(Kumar et al., 2020).

Polycultures of algae, comprising two or more species, may be more efficient in their removal of CECs as different species with different physiologies and non-overlapping niches may allow a greater diversity of removal processes. Locally-adapted consortia of microalgae are less susceptible to viral infections and other unexpected crop failures, which are a weakness to the full-scale application of bioremediation projects (Godwin et al., 2017, 2018). The mixotrophic growth model presented by Ge et al (2018) promised wastewater nutrient depletion, while encouraging lipid-rich algal growth. Mixotrophic growth in algal cultures has also improved the ability for algal cultures to remove emerging contaminants from wastewater (Xiong et al., 2017). This project combines these two concepts by investigating a mixotrophic growth model's effect on a local polyculture.

4.2.2 Synthetic biology interventions in CEC removal

Genetic engineering has advanced in the past decades owing to new technologies and improved understanding of genetic material. The opportunity to apply microbial cells as bio-factories has long been practiced, as exemplified using *E. coli* to produce insulin in 1978 (Goeddel et al., 1979). Photosynthetic organisms are paramount to the future of sustainable bio-factories given their ability to direct solar energy to the production of high-value molecules (Cuellar-Bermudez et al., 2015; D'Adamo et al., 2019). Microalgae have been genetically transformed using the established methods of particle bombardment, bead agitation, viral transmission, and electroporation targeting nuclei, mitochondrial, and chloroplast genetic material (Ortiz-Matamoros et al., 2018). This project investigates the application of genetically transformed *Chlamydomonas reinhardtii* to express human hepatic cytochrome P450 (CYP) enzymes capable of catabolizing myriad pharmaceuticals. Given the CYP transformation in the chloroplast leverages the phosphorylation of NADPH+ through photosystem I, the effects of this

transformation on photosynthetic efficiency and cellular metabolism will be important to understand as this technology is applied to pilot-scale test systems. This project seeks to validate the development of a transgenic strain of microalgae designed to treat pharmaceuticals and other personal care products as presented in Chapter 3.

4.3 Experimental Methods

4.3.1 Microalgal cultivation

Centrate wastewater (CW), the liquid product of the dewatering process after anaerobic digestion, was also collected from the Ravensview WWTP in Kingston, Ontario, Canada. The CW was left to settle for 24 hours and decanted into glass bottles and autoclaved, after which the solution had a transparency resembling herbal tea. The CW was diluted tenfold with ultra-pure (18.2MΩ-cm) water, leading to the following nutrient concentrations: ammonia-N, 35.06 mgL^{-1}; nitrate-N, 1.54 mgL^{-1}; phosphate-P, 4.52 mgL^{-1}. The media's pH was amended to a value of 7 prior to experimentation. CW dilution was determined based on previous experimentation indicating that at greater concentrations of CW cucltures were inhibited by concentration of ammonia (Pazmino, 2020).

Cultures were cultivated in triplicate 2L glass bottles using red-blue-green light-emitting diode light arrays (Atlantik V1, Orphek). Light intensity was measured between 100 and 120 umol $m^{-2}s^{-1}$ in the array and all bottles which were circulated through the array daily to account for lighting discrepancies. Cultures were illuminated at a 24-hour photoperiod, and microalgae were maintained in suspension using 0.2um filtered air pumped through a sterile airstone set to the minimal flow required to maintain suspension (about 1.5L/min). Cultures were maintained between 23°C and 25°C throughout the experiment. Three experimental conditions were executed for this preliminary experiment, a culture containing *Chlorella vulgaris* in autotrophic growth, a

consortia of native microalgae in autotrophic growth, and a consortia of native microalgae in mixotrophic growth.

4.3.2 Autotrophic *C. vulgaris*

The autotrophic media was comprised of centrate wastewater diluted 1:10 in ultra-pure water. *Chlorella vulgaris* was supplied by the National Research Council's Aquatic and Crop Resource Development Research Centre.

4.3.3 Autotrophic consortia

The autotrophic media was comprised of centrate wastewater diluted 1:10 in ultra-pure water. A locally sourced microalgal consortia was collected from secondary wastewater at the Ravensview wastewater treatment plant (WWTP) near Kingston, ON.

4.3.4 Mixotrophic consortia

The mixotrophic media was the same including the addition of 1.5mL 99% glycerol per liter of media. Glycerol was selected as an organic carbon amendment due to it being a by-product of downstream microalgal lipid processing in the production of biofuels.

Into both media (10% CW and 10%CW+glycerol) was added three CECs (carbamazepine, ibuprofen, ciprofloxacin) dissolved in methanol such that their final concentrations in the media was 15 parts per million (ppm). Cultures were acclimated to these growth conditions for 10 generations of growth, roughly 7 days, prior to the beginning of the experiment.

4.3.5 Nutrient drawdown and biomass qualification

Given the multi-species nature of the experimental polyculture, cellular enumeration and chlorophyll concentration were inappropriate proxies for culture growth, thus dry cell weight was employed to monitor growth. 1.2um glass fiber filters (GF/F, Whatman USA) were dried at 60C

and pre-weighed. 50mL of algal culture was passed through the filter and filters were dried for 18 hours prior to re-weighing.

Nutrient concentrations and biomass accumulation were monitored at 48-hour intervals. Ammonia concentrations in culture filtrate were quantified using an ion-selective electrode (Cole-Parmer, USA) and phosphorus concentrations were quantified spectrophotometrically at 710nm using the ammonium molybdate method (EPA) after organic phosphate conversion to orthophosphate (Hach, USA).

Algal macromolecular content was analyzed after cultures had removed nutrients from their media and they had been in stationary phase for at least 3 days. Cultures were centrifuged in 50 mL tubes at 10,000xg for 10 minutes. The supernatant was discarded, and the remaining biomass was flash-frozen using liquid nitrogen and stored at -80°C. The frozen biomass was subsequently lyophilized and stored until macromolecular analysis.

Cellular lipids were quantified using a gravimetric analysis after a solvent extraction (Folch et al., 1957). 30mg of biomass was measured into glass centrifuge tubes and resuspended in 0.15mL of ultra-pure water and frozen to assist in cell lysis. 3mL of 2:1 v/v chloroform:methanol was added to tubes which were vortex mixed for 30 seconds, sonicated for 2 minutes, and centrifuged at 5000xg for 5 minutes. The supernatant was collected in a new tube and the process of vortex-mixing, sonicating, and centrifuging was repeated two more times. 1mL 0.88% KCl was added to the combined supernatant which was vortex-mixed and centrifuged, after which the lower phase was transferred by glass pipet to a weigh-boat and dried at 50°C in a fume hood until dry and ready for weighing.

Carbohydrates were quantified using the established phenol-sulfuric acid method (DuBois et al., 1956). 30mg of dry biomass was measured into a glass tube and resuspended in 1mL of 12M sulfuric acid. Tubes were placed in the darkness for 1 hour, after which samples were redissolved in 9mL ultra-pure water and set in a bath of boiling water for 1 hour. Tubes were centrifuged at

10000 x g for 10 minutes and 2.7mL of the supernatant were transferred to clean tubes. 5mL of concentrated sulfuric acid was added to the aliquots and vortex-mixed immediately. 0.05mL 80% phenol was added to tubes, which were vortex-mixed immediately. Tubes sat for 20 minutes to allow for color development, and sample absorbance was quantified at 490nm and compared to a D-glucose standard curve.

Proteins were quantified using the Lowry colorimetric method using bovine serum albumin as a standard (Liefer et al., 2019). 10mg of dried biomass was placed in microtubes containing ceramic beads and 1.5mL 2% sodium-dodecyl-sulfate (SDS) buffer. Tubes were agitated using a bead mill (Next Advance, USA) in 4 cycles with tubes placed on ice in between agitations to prevent overheating. Tubes were centrifuged at 10000 x g for 20 minutes and supernatant was collected for protein quantitation using the Bio-Rad DC protein assay (Bio-Rad Laboratories, USA).

4.3.6 CEC Quantitation

CECs were quantified via high-performance liquid chromatography (HPLC). Sample filtrate at the end of the experiment was extracted by liquid-liquid extraction using 80mL of filtrate and 80mL of 2:1 methanol:hexane. The titrated solvents were dried under a nitrogen gas stream and dissolved in 4mL of acetonitrile. Quantitation was performed on an Agilent 1260 HPLC equipped with YMC Triart C-18 (150mmx3mm, 3um, 12nm) column running at a temperature of 35°C and a flow rate of 0.4mL/min. CECs were analyzed at 280nm, 254nm, 225nm, and 263nm and compared to standard curves of CECs dissolved in acetonitrile.

4.3.7 Statistical analyses

The statistical program R (ver. 4.0.4) was used for the statistical analyses presented below. Linear regression models were considered without data transformation. Prior to an analysis of variance

(ANOVA), however, data was tested for normality using the Shapiro-Wilks test and log10 transformed if it was not initially normally distributed.

4.3.8 Design of genetic expression

The experimental cultures *C. reinhardtii* CC-1568, CC-620 mt+, and CC-620 mt- were purchased from the Chlamydomonas Resource Centre at the University of Minnesota. The strain CC-1568 lacks the protein psbH, which renders the microalgae incapable of photosynthesis. A complementary plasmid, pASapl, encoding the psbH gene was also purchased such that the microalgae could be transformed with the return of photosynthetic ability as a selection marker (Economou 2014) limiting the proliferation of antimicrobial resistance. CC-1568 was cultured in tris acetate phosphate media (TAP) at 26°C prior to transformation. Cellular density was measured my microscopy after cells were stained using Lugol's solution (Liefer et al., 2019). The sequence encoding human cytochrome P45 3A4 (CYP3A4) (UniProt# P08684)(Molowa et al., 1986) was codon optimized for *C. reinhardtii* using the program SnapGene. The sequence was further manipulated by the addition of a 12-AA linking sequence that attached the codon-optimized FAD domain of cytochrome p450 reductase (Mellor et al., 2019) due to the domain's affinity for photosynthetic energy (Figure 4.5). This sequence, termed *CYPalg*, was synthesized and inserted into the pASapl plasmid using *sapI* and *hindIII* as restriction enzyme cloning sites (Genscript, USA). Using heat-shock transformation, the plasmid was taken up by DH5-alpha competent cells and ampicillin-resistant cultures were grown overnight in LB media at 37°C. The plasmid was extracted using a standard extraction kit (Thermo Fisher, USA).

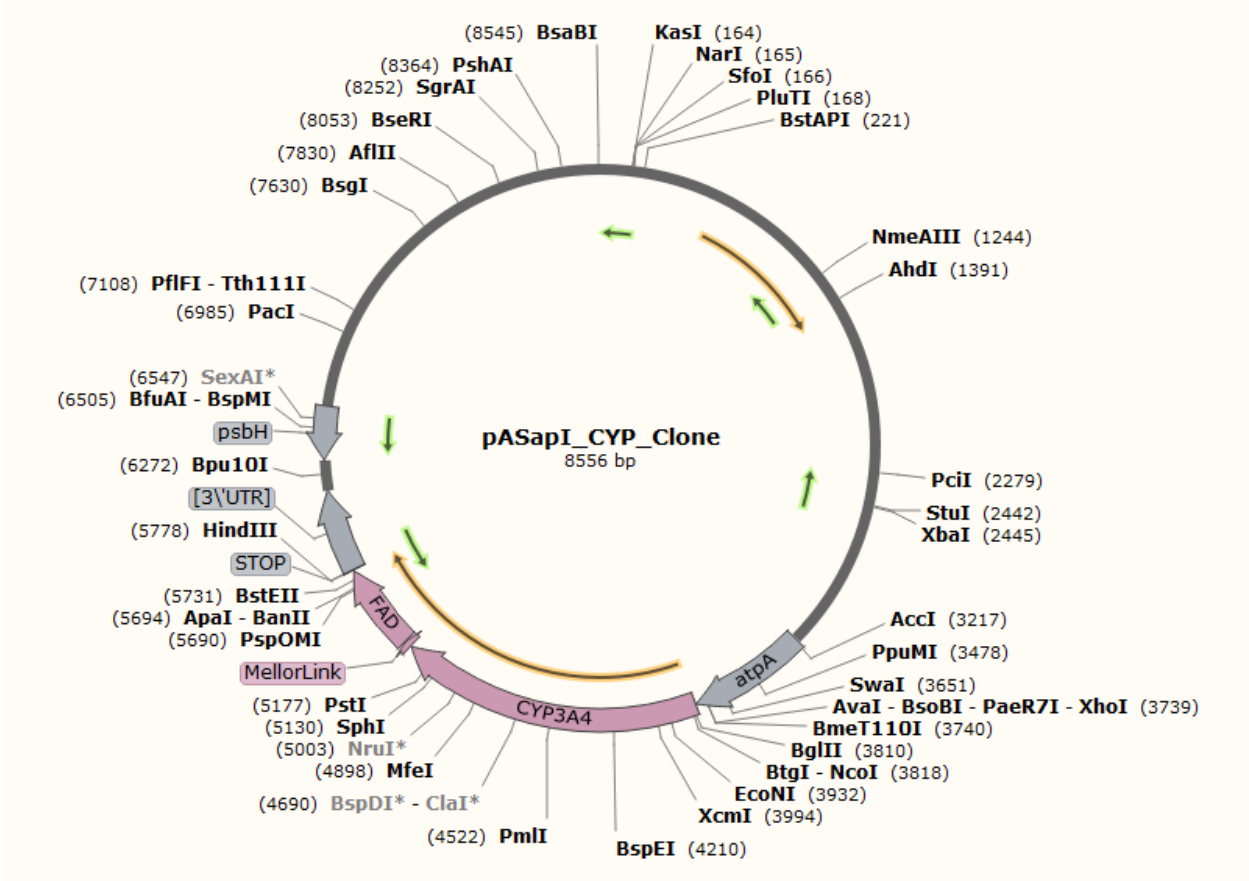

Figure 4-1 pASapI plasmid for expression in *psbH*-deficient *Chlamydomonas reinhardtii*

4.3.9 Genetic transformation

Algal transformation was based on the methods of Economou et al. (2014) . The strains of *C. reinhardtii* CC-620 and CC-621 are termed mt+ and mt-, respectively, regarding their mating type. The two strains were grown in 300mL tris-acetate phosphorus medium until they reached the stationary phase of growth. They were then centrifuged at 3000xg for 5 minutes and their pellets were resuspended in 1.2L of high salt media (HSM) lacking nitrogen (by not adding ammonium chloride to the media) overnight. Cultures were centrifuged at 3000xg for 5 minutes, supernatant was discarded, and the biomass resuspended in 120mL HSM lacking nitrogen. The mt+ and mt- cultures were combined for 2 hours, after which the culture was centrifuged at 10000xg for 10 minutes. The supernatant was collected and passed through a 0. 22uL membrane

filter. The combination of the two opposite mating types triggers sexual reproduction in *C. reinhardtii*, which results in the cells exuding the enzyme gametolysin into the media which temporarily weakens the algal cellular wall making it more susceptible to glass bead permeabilization (Cronmiller et al., 2019).

The psbH-lacking culture was grown in 400mL TAP medium until it reached the mid-logarithmic stage of growth. The culture was centrifuged at 3000xg for 5 minutes and the pellet was resuspended in 3mL of gametolysin solution for 1 hour. 0.3g of glass beads were added to test tubes and autoclaved. 300uL of the gametolysin-*C. reinhardtii* solution was added to each tube and 5ug of plasmid DNA (dissolved) was added to 8 of the 10 tubes. The test tubes were vortex-mixed at maximum speed for 15s after which the solution was dissolved in 3.5mL HSM 0.5% w/w agar kept at a temperature of 42°C. The agar solution was immediately decanted onto HSM 2% w/w agar plates, left in the dark for 20 minutes, and incubated at 26°C under 20 umol $m^{-2}s^{-1}$ for 24 hours then the light intensity was increased to 100 umol $m^{-2}s^{-1}$. After 3 weeks, green colonies were streaked across fresh plates and the resulting colonies were dissolved in 100uL ultra-pure water to confirm transformation. Tubes were centrifuged, the supernatant discarded, after which the cells were prepared for DNA extraction (Economou et al., 2014).

Cells were lysed by the addition of 1mL Trizol reagent (Invitrogen, USA). Cells were resuspended by pipetting and incubated for 5 minutes. 0.2mL of chloroform was added and tubes were incubated for 2 minutes then centrifuged at 10000xg for 5 minutes. The interphase was isolated and 0.3mL of ethanol was added to the solution and mixed. After 2 minutes, tubes were centrifuged at 10000xg for 5 minutes. The supernatant was discarded, and the remaining DNA pellet was dissolved in a sodium citrate ethanol solution for 30 minutes, centrifuged, and the supernatant discarded. The pellet was again redissolved in 1.5mL 75% ethanol and incubated for 10 minutes. Tubes were centrifuged and the supernatant discarded while the remaining pellet was dried for 10 minutes in a biosafety cabinet (Bo et al., 2021).

The purified DNA was redissolved in 300uL 8mM NaOH and the solution was used for PCR amplification and visualization. The primers F, R1, and R2 were designed by the authors of Economou et al. (2014) to confirm the return of the *psbH* gene to *C. reinhardtii* thus confirming the successful transformation of the chloroplast with the pASapI plasmid. The untransformed *C. reinhardtii* contains DNA which produces a 1.0kb band using the F and R1 primers while the *C. reinhardtii* expressing *psbH* from the pASapI plamids produces a 1.2kb band using the F and R2 primers (Figure 4.6). DNA, primers, and mastermix (Bio-Rad, USA) were combined and placed in a thermocycler. Amplified bands were added to a 1.2% agarose gel with SYBR Safe and quantified alongside a DNA ladder.

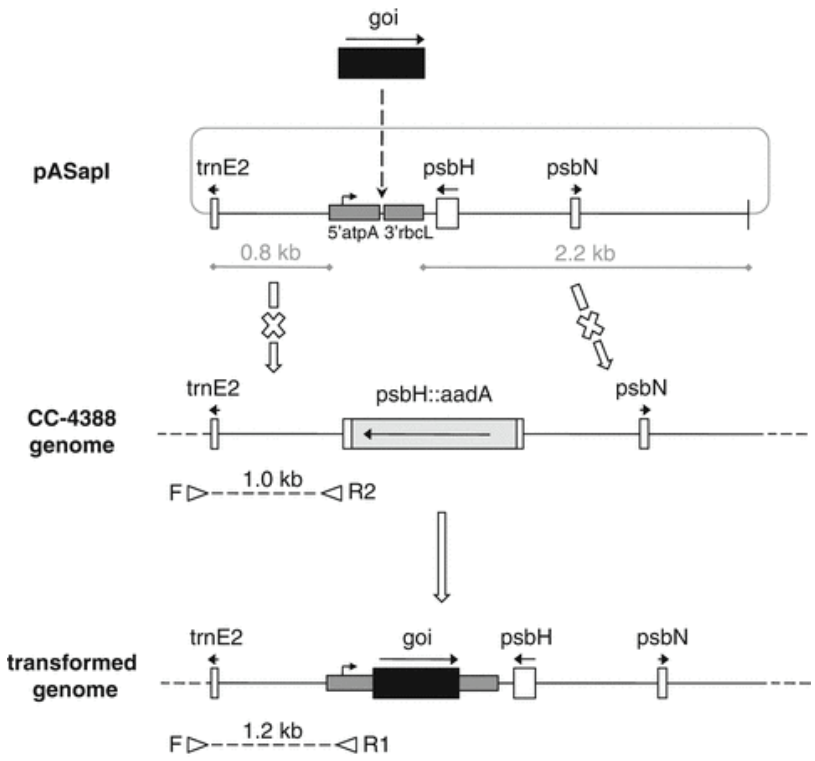

Figure 4-2 Confirmation of algal transformation using the primers F, R1, and R2. Figure courtesy of Economou et al. (2014)

4.4 Results

4.4.1 Consortia experiment results

Microalgae were cultivated for 13 days in CEC-spiked dilute centrate wastewater, and their growth dynamics were investigated. Microalgal density was not found to be statistically significantly different between the autotrophic and mixotrophic polycultures with a final density almost 2/3 less than the autotrophically-grown *C. vulgaris* monoculture ($p<0.05$). The maximum density of the cultures did not exceed 400mg/L for any culture. The same polyculture could attain densities between 600 and 1200mg/L in greater concentrations of centrate wastewater after acclimation (Pazmino, 2020), indicating that the polycultures have the potential to achieve greater densities and therefore improve their biological removal of nutrients and pollutants. The negligible difference between the autotrophic and mixotrophic polyculture may be due to the relatively short acclimation period to the added glycerol that was too short to enact physiological changes in the mixotrophic culture (Cecchin et al., 2018). Alternatively, the community dynamics within the cultures may have shifted to favor more mixotrophic-tolerant species with potentially different responses to the growth environment (Olofsson et al., 2019). The change in pH exhibited as the cultures grew into stationary phase mostly followed the traditional acidification of media as cells metabolize ammonium and release protons to maintain cellular neutrality (Eustance et al., 2013). Once again, the final pH between the autotrophic and mixotrophic cultures was not significantly different. Traditionally, mixotrophic cultures become more acidic given the increased respiration in a mixotrophic environment (Li et al., 2014).

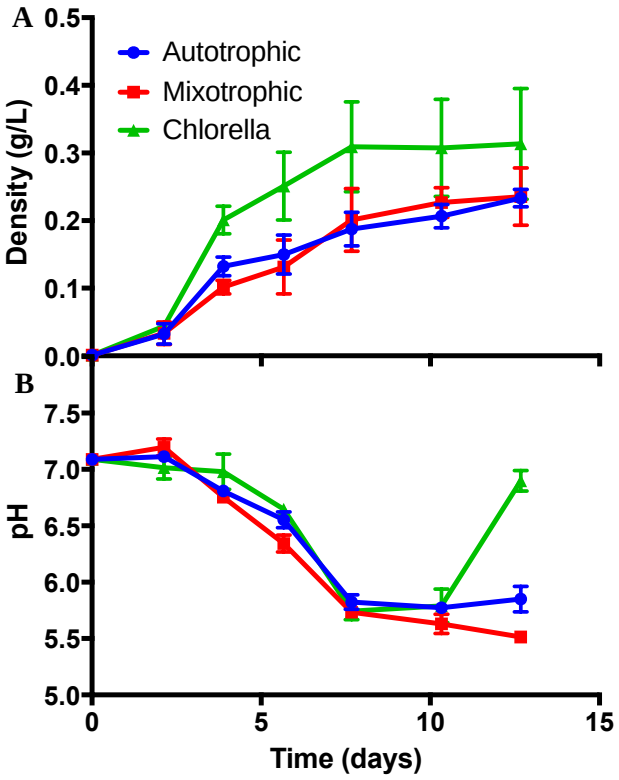

Figure 4-3 Microalgal density (A) and media pH (B) across the experimental period.

Given the closeness in growth dynamics across the three cultures, a similarly uniform response to the treatment of nutrients in the culture was expected. Nitrate concentrations were reduced quickly as cells used its bioavailable nitrogen to build the macromolecular infrastructure of growth: proteins, and nucleic acids. Ammonium was drawn down by almost two thirds at the same rate by all cultures. Phosphorus from organic phosphate was noted to emerge as the limiting nutrient after 7 days of growth, as shown by the levelling of growth (Figure 4-3) within the same timeframe. Improving the efficiency of algal nutrient removal will require an understanding of the nutrients limiting algal growth so as to maximize the exponential growth stage thus increasing cellular density and further increasing the biological effects of microalgae on CEC removal. With an appropriate period of acclimation, the polyculture was shown to remove most nutrients from

the same centrate wastewater grown in 30% and 50% CW media, thus the benefits of added biodiversity may be more important at higher concentrations (Pazmino, 2020).

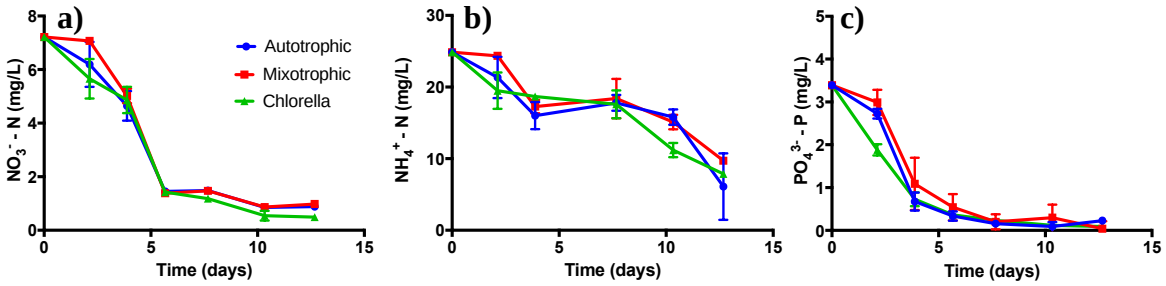

Figure 4-4 Nutrient drawdown by microalgae grown in centrate wastewater. The removal of nitrate (a), ammonium (b), and phosphate (c) was near complete in all cultures following 13 days of cultivation.

Macromolecular content was quantified in cells after 13 days of culturing when they had been in the stationary growth phase for six days. There was no significant difference between the means of lipid, carbohydrate, or protein content between the autotrophic and mixotrophic consortia. The *C. vulgaris* monoculture had a greater carbohydrate content than either of the consortia ($p<0.05$), which accounted for the major difference in cellular biomass between the cultures after their growth into stationary phase. That the polycultures did not become more carbohydrate-rich or lipid-rich than the *C. vulgaris* was not unexpected given that genus is often studied because of its tolerance of wastewater environments and its quick accumulation of macromolecules with potential for industrial reuse (Ge et al., 2018; Ikaran et al., 2015). The opportunity for niche diversification afforded to the polycultures, however, did not readily improve their nitrogen or phosphorus accumulation, and thus their slower growth combined with their equal removal of nutrients compared to *C. vulgaris* would suggest a limited benefit to wastewater managers. An increased tolerance to varying environmental conditions (i.e. temperature & light) is presumed for consortia, however, and their benefits merit continued investigation (Hietala et al., 2017; Narwani

et al., 2016). Additionally, their community resilience to contamination may be a further benefit to this approach (Steiner et al., 2006).

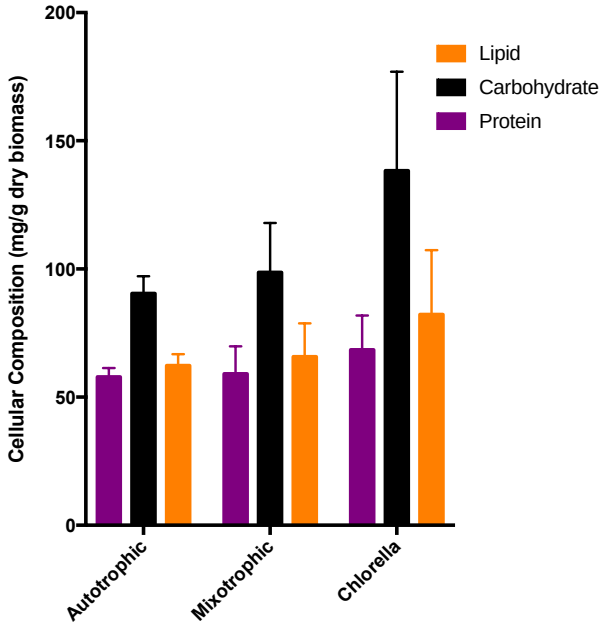

Figure 4-5 Macromolecular content of the three biological treatments cultured in centrate wastewater. Carbohydrates differed significantly between the monoculture of *C. vulgaris* and the two consortia cultures. The effect of a mixotrophic growth environment did not affect the macromolecular content of the microalgae.

Contaminant removal by the three biological treatments was the same after two weeks of cultivation. Carbamazepine was almost completely removed in all instances, an unprecedented rate of removal by microalgae alone (see Chapter 2). This removal could likely be attributed to the low-intensity UV light included in the full spectrum aquarium lights used, as carbamazepine is recalcitrant and traditionally its removal is made possible due to its UV-sensitivity (Dai et al., 2012; Xiong et al., 2016). Ciprofloxacin removal averaged 70.42% +/- 1.52 which is high relative to traditional removal of the antibiotic (Chapter 2). This removal efficiency may also have been enhanced by the effects of UV degradation on the contaminant (Milh et al., 2021). Comparing the

effects of the monoculture and the polycultures, there was no significant difference between treatments. An abiotic control would validate this hypothesis but was not completed in this pilot experiment.

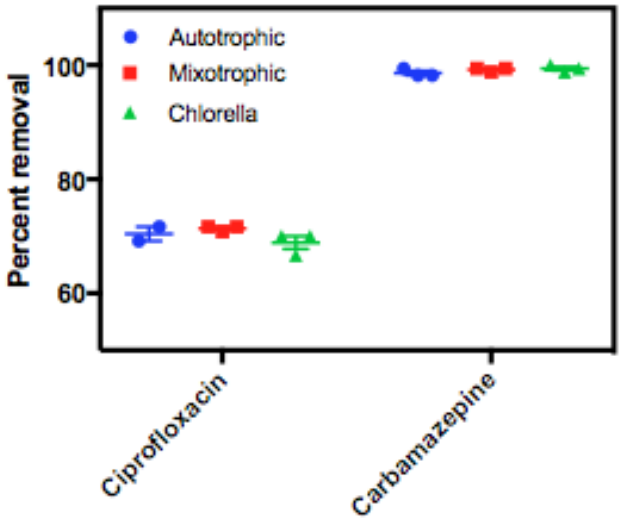

Figure 4-6 Contaminant of emerging concern removal by the biological treatments.

The importance of introducing biodiversity into biological and environmental engineering processes is important for the diversification of function and the increased tolerance to community collapse (Corcoran & Boeing, 2012). In the treatment of macropollutants (nitrogenous and phosphorous chemicals) and micropollutants (ciprofloxacin and carbamazepine), however, there was not a noticeable improvement in remediative capacity with increased species diversity. The effect of a mixotrophic growth environment did not tangibly affect the density of the culture nor its pollutant removal dynamics in the polyculture. The added organic carbon to culture would theoretically increase cellular density as well as increase its rate of carbohydrate exudation which would both contribute to the removal of some CECs (Subashchandrabose et al., 2013). Thus, the acclimation period used to introduce mixotrophy to the polyculture may have been too short, and further study should confirm a physiological response to mixotrophy.

4.4.2 CYP3A4 microalgae results

Colonies developed on the HSM agar plates, and upon re-streaking grew under illumination. Polymerase chain reaction (PCR) investigation, however, indicated that none of the colonies that appeared to grow photosynthetically had been transformed with the pASapI plasmid (Figure 4-7). The transformation process was repeated four times using the original plasmid and original psbh-lacking *C. reinhardtii* (gametolysin was prepared each time) and colonies developed, but did not carry the genetic signature of transformation.

This may be explained by the plasmid correctly encoding the psbh gene but not properly encoding the CYP3A4 gene. Further investigation on this project merits re-evaluating the plasmid design, enzyme selection, or the codon optimization methods employed given that problems may exist in the design of genetic constructs. To confirm that transformation of cells occurred, and that the issues experience are plasmid design-derived, plasmids encoding green fluorescent proteins may be employed to confirm the glass bead transformation is effective. Should this

method demonstrate limited or no transformation, other methods of transformation like biolistic delivery may be explored.

Figure 4-7 Agarose gel demonstrating no transformation of cells.

4.5 Limitations and Next Steps

The unique circumstances concerning the COVID-19 pandemic prevented further exploration in both the environmental and the genetic factors affecting algal treatment of emerging contaminants. The experimental results concerning the use of mixotrophic and polyculture strategies to enhance wastewater treatment merit further exploration by investigating those opportunities where each strategy has the potential to enhance the traditional wastewater treatment by microalgae outlined in Chapter 2 of this thesis.

As such, research pursuing this matter should consider first confirming the transformation method is appropriate for the strain of *Chlamydomonas* employed (described above). With that confirmation, a series of plasmids should be designed that encode different CYP enzymes, and their expression should be confirmed using CYP quantitation methods like the carbon monoxide method of Choi (2003). Finally, different electron transfer partners should be encoded and expressed to operate in conjunction with a given CYP. CYP3A4 remains the ideal candidate for proof-of-concept experimentation due to its broad substrate affinity. Once this expression is confirmed, its capacity to remediate carbamazepine, a model recalcitrant CEC, will serve to validate this technology and ensure the ultimate paper's broad dissemination.

4.6 Conclusions

The genetic manipulation of microalgae aimed at enhancing algal metabolism of CECs represents an exciting development in the field of phycoremediation, and the experimental work outlined in this chapter establishes a potential strategy for validating these hypotheses. Despite its inconclusive nature, these projects will serve as a resource to future experimentation and will contribute to the development of novel wastewater treatment strategies to sustainably treat wastewater and the emerging contaminants contained therein.

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Chapter 5

Contributions to Engineering and Science

5.1 Summary of conclusions

This thesis outlines two distinct opportunities to improve the capacity for microalgae to treat and ultimately remove CECs from municipal wastewater. First, this project considered the traditional mechanisms of microalgal-induced CEC removal and investigated the contributions of chemical, biological, and environmental conditions to contaminants removal. Then, novel adaptations to microalgae that may further enhance their removal of CECs by harnessing photosynthetic energy to power heterologous enzymes were considered. In each instance, both experimental results and summarizing contemporary literature have led to conclusions and proposals that may enhance microalgal removal of CECs in future implementations of this technology.

5.2 Advances in the field of environmental engineering

Chapters 3 and 4 focused on the capacity for photosynthetic organisms to remediate municipal wastewater. With an understanding of the compounds targeted for removal, researchers could tailor the growth environment to favor the mechanism of removal most conducive to a specific contaminant's chemistry. Further, phylogenetic considerations were reinforced by the findings outlined in Chapter 3 which demonstrated a biological trend in genera affinities for removal. Finally, the conclusions of Chapters 2 & 4 reinforced the potential to leverage non-traditional growth strategies to further enhance microalgal CEC treatment. Mixotrophic growth could be employed, particularly when co-metabolism is a common mechanism of removal. Culturing multiple species may have the benefit of more broadly treating nutrients in the wastewater, while

allowing for the tailoring of consortia to target the CECs in their media and while improving the stability of the culture.

Transgenic manipulation of microalgae has experienced increased academic interest given the potential to leverage their rapid growth rates, their ease of transformation, and their broad environmental tolerance. The heterologous expression of enzymes that are paired with photosynthetic infrastructure presents an elegant avenue to sustainable bio-factories, and Chapters 2 & 4 served as the groundwork for transgenic microalgae tailored for wastewater treatment. Presented as a roadmap to realizing this technology, Chapter 4 presents a consolidation and discussion of different considerations that should be taken into account. The details presented in Chapter 2 will further assists future research by making public protocols that were developed to realize this technology.

In short, this project will serve as an integral resource and important repository of references for those academics and industrial partners interested in improving CEC treatment using microalgal technologies. The conclusions presented in the above chapters not only provide important information regarding what is known about microalgal CEC removal, it also proposes future research in that may streamline our ability to treat these pollutants in wastewater.

5.3 Contributions to the scientific community

In the years that have culminated in the completion of this document, there have been myriad instances wherein the expertise accumulated by the author has been translated through the academic community, further enriching and rounding out others' understanding of these technologies. The author has used the skills developed in the technical section of this thesis to assist others in their cultivation, harvesting, and processing of microalgal macromolecules. Further, these skills have been translated, as methods developed for this project were shared and taught with other members of the Beaty Water Research Centre. Further, public presentations of

the material discussed above have more widely distributed this information. Presentations in Newfoundland & Labrador (PEOPLE Symposium 2019), Nova Scotia (CCEC Conference 2019), and Ontario (LEADERS Symposium 2021) have been received by public and national audiences. Indeed, some the findings from this work will be delivered by Dr. Pascale Champagne in a keynote address to the Canadian Association on Water Quality November 3rd, 2021.

This project has also set the stage for future interdisciplinary collaboration with both governmental and private partners. The author communicated with academics from many backgrounds to design experiments and ensure that the accurate considerations were made when theorizing future work. Indeed, the knowledge gained through this project was an important consideration when drafting grant proposals that were ultimately successful. The results of these chapters will have direct implications for the scientific community when they are finalized and published publicly. Chapter 3 has been written to be submitted for publication with the Journal of Environmental Systems Research, and Chapter 2 will be submitted for publication with the Journal of Hazardous Materials.

Appendix A

Meta-analysis Data

CEC [Name]	CEC Class [Name]	Microalgae Genus [Name]	Media Type [Name]	Temperature [°C]	Light [umol/m^2/s]	Photo period [h of light]	HRT [Days]	Initial CEC Concentration [ng/L]	Removal [%]	Article
Bisphenol A	Plasticizer	Chlorella	MSM	25	300	12	7	1.00E+08	38.50	(Eio et al., 2015)
Bisphenol A	Plasticizer	Chlorella	MSM	25	300	12	7	1.00E+08	100.00	
Bisphenol A	Plasticizer	Chlorella	BG11	25	2000	12	10	5.00E+07	31.58	(L. Wang et al., 2017)
Bisphenol A	Plasticizer	Chlorella	BG11	25	0	0	10	5.00E+07	14.60	
Bisphenol A	Plasticizer	Desmodesmus	BG11	26	40	12	10	1.00E+07	57.00	(R. Wang et al., 2017)
Caffeine	Stimulant	Consortia	Real wastewater	23	150	12	10	5.00E+03	99.00	(Matamoros et al., 2016)
Caffeine	Stimulant	Consortia	Synthetic wastewater	23	150	12	10	5.00E+03	17.00	
Estrone	Hormone	Haematococcus	Synthetic wastewater	25	84.7	12	50	5.00E+06	97.00	(Y. Wang et al., 2019)
Estrone	Hormone	Selenastrum	Synthetic wastewater	25	84.7	12	50	5.00E+06	80.00	
Estrone	Hormone	Scenedesmus	Synthetic wastewater	25	84.7	12	50	5.00E+06	97.00	
Estrone	Hormone	Chlorella	Synthetic wastewater	25	84.7	12	50	5.00E+06	0.00	
17-beta-ethinylestradiol	Hormone	Haematococcus	Synthetic wastewater	25	84.7	12	50	5.00E+06	100.00	
17-beta-ethinylestradiol	Hormone	Selenastrum	Synthetic wastewater	25	84.7	12	50	5.00E+06	100.00	
17-beta-ethinylestradiol	Hormone	Scenedesmus	Synthetic wastewater	25	84.7	12	50	5.00E+06	100.00	
17-beta-ethinylestradiol	Hormone	Chlorella	Synthetic wastewater	25	84.7	12	50	5.00E+06	100.00	
17-alpha-ethinylestradiol	Hormone	Haematococcus	Synthetic wastewater	25	84.7	12	50	5.00E+06	85.00	
17-alpha-ethinylestradiol	Hormone	Selenastrum	Synthetic wastewater	25	84.7	12	50	5.00E+06	0.00	
17-alpha-ethinylestradiol	Hormone	Scenedesmus	Synthetic wastewater	25	84.7	12	50	5.00E+06	85.00	
17-alpha-ethinylestradiol	Hormone	Chlorella	Synthetic wastewater	25	84.7	12	50	5.00E+06	0.00	
17-beta-ethinylestradiol	Hormone	Selenastrum	BG11+anerobig sludge digestate	25	172	12	10	5.00E+03	100.00	(Hom-Diaz et al., 2017)

17-alpha-ethinylestradiol	Hormone	Selenastrum	BG11+anerobig sludge digestate	25	172	12	10	5.00E+03	95.00	
17-beta-ethinylestradiol	Hormone	Chlamydomonas	BG11+anerobig sludge digestate	25	172	12	10	5.00E+03	100.00	
17-alpha-ethinylestradiol	Hormone	Chlamydomonas	BG11+anerobig sludge digestate	25	172	12	10	5.00E+03	76.00	
17-beta-ethinylestradiol	Hormone	Raphidocelis	BG11	25	60	12	4	1.00E+05	81.00	(Liu et al., 2018)
Diethylstilbestrol	Hormone	Raphidocelis	BG11	25	60	12	4	1.00E+05	89.00	
17-beta-ethinylestradiol	Hormone	Raphidocelis	BG11	25	60	12	4	5.00E+05	80.00	
Diethylstilbestrol	Hormone	Raphidocelis	BG11	25	60	12	4	5.00E+05	78.00	
17-beta-ethinylestradiol	Hormone	Raphidocelis	BG11	25	60	12	4	1.50E+06	78.00	
Diethylstilbestrol	Hormone	Raphidocelis	BG11	25	60	12	4	1.50E+06	60.00	
17-beta-ethinylestradiol	Hormone	Chlorella	BG11	25	ND	ND	7	1.00E+06	65.40	(Huang et al., 2019)
17-beta-ethinylestradiol	Hormone	Chlorella	BG11	25	ND		7	5.00E+05	92.10	
17-beta-ethinylestradiol	Hormone	Chlorella	BG11	25	ND		7	5.00E+04	98.80	
17-beta-ethinylestradiol	Hormone	Scenedesmus	BG11	25	200	12	12	5.00E+05	82.70	(S. Li et al., 2020)
17-beta-ethinylestradiol	Hormone	Scenedesmus	BG11	25	200	12	12	1.00E+06	64.40	
17-beta-ethinylestradiol	Hormone	Scenedesmus	BG11	25	200	12	12	2.00E+06	46.10	
Estrone	Hormone	Scenedesmus	Synthetic wastewater	32	51.3		5	5.00E+06	91.00	(Ruksrithong & Phattarapattamawong, 2019)
17-beta-ethinylestradiol	Hormone	Scenedesmus	Synthetic wastewater	32	51.3		5	5.00E+06	99.00	
Estrone	Hormone	Chlorella	Synthetic wastewater	32	51.3		5	5.00E+06	52.00	
17-beta-ethinylestradiol	Hormone	Chlorella	Synthetic wastewater	32	51.3		5	5.00E+06	99.00	
Tetracycline	Antibiotic	Microcystis	BG11	28	41	12	11	1.00E+07	99.00	(Pan et al., 2021)
Tetracycline	Antibiotic	Microcystis	BG11	28	41	13	11	5.00E+07	99.00	
Tetracycline	Antibiotic	Microcystis	BG11	28	41	14	11	1.00E+08	99.00	

Tetracycline	Antibiotic	Chlorella	BG11	28	41	15	11	1.00E+07	98.90	
Tetracycline	Antibiotic	Chlorella	BG11	28	41	16	11	5.00E+07	98.60	
Tetracycline	Antibiotic	Chlorella	BG11	28	41	17	11	1.00E+08	78.60	
Cefuroxime sodium	Antibiotic	Chlorella	BG11	25	70	24	2	1.00E+08	59.62	(Zhang et al., 2020)
Cefuroxime sodium	Antibiotic	Chlorella	BG11	25	70	12	2	1.00E+08	38.46	
Cefuroxime sodium	Antibiotic	Chlorella	BG11	25	70	0	2	1.00E+08	12.18	
Cefuroxime sodium	Antibiotic	Chlorella	BG11 w/ 500mg/L NaHCO3	25	70	24	2	1.00E+08	97.56	
Cefuroxime sodium	Antibiotic	Chlorella	BG11 w/ 500mg/L NaHCO3	25	70	12	2	1.00E+08	98.33	
Cefuroxime sodium	Antibiotic	Chlorella	BG11 w/ 500mg/L NaHCO3	25	70	0	2	1.00E+08	17.31	
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	1.00E+07	38.55	(Yang et al., 2020)
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	2.00E+07	19.36	
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	4.00E+07	20.08	
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	8.00E+07	16.31	
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	1.60E+08	9.50	
Ofloxacin	Antibiotic	Scenedesmus	BG11	25	150	12	20	3.20E+08	10.76	
Sulfamethoxazole	Antibiotic	Pseudokirchneriella	BG11	25	102.7	12	5	1.01E+10	9.04	(Q. Xiong et al., 2020)
Sulfamethoxazole	Antibiotic	Scenedesmus	BG11	25	102.7	12	5	1.01E+10	6.96	
Sulfamethoxazole	Antibiotic	Scenedesmus	BG11	25	102.7	12	5	1.01E+10	4.72	
Sulfamethoxazole	Antibiotic	Scenedesmus	BG11	25	102.7	12	5	1.01E+10	8.95	
Sulfamethoxazole	Antibiotic	Chlorella	BG11	25	102.7	12	5	1.01E+10	14.69	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 1mM glucose	25	102.7	12	5	1.01E+10	14.76	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 1mM sodium formate	25	102.7	12	5	1.01E+10	4.93	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 1mM sodium acetate	25	102.7	12	5	1.01E+10	35.29	

Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 2mM sodium acetate	25	102.7	12	5	1.01E+10	89.15	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 3mM sodium acetate	25	102.7	12	5	1.01E+10	93.15	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 4mM sodium acetate	25	102.7	12	5	1.01E+10	99.98	
Sulfamethoxazole	Antibiotic	Chlorella	BG11 w/ 8mM sodium acetate	25	102.7	12	5	1.01E+10	99.98	
Doxylamine	Antibiotic	Scenedesmus	Raw wastewater	27	45	24	10	1.00E+09	55.95	(J.-Q. Xiong et al., 2020)
Doxylamine	Antibiotic	Scenedesmus	Raw wastewater w/ 2g/L NaHCO3	27	45	24	10	1.00E+09	62.87	
Sulfadiazine	Antibiotic	Chlorella	Aquaculture wastewater	25	105	24	12	1.14E+05	32.06	
Sulfamethazine	Antibiotic	Chlorella	Aquaculture wastewater	25	105	24	12	4.70E+04	31.17	
Sulfamethoxazole	Antibiotic	Chlorella	Aquaculture wastewater	25	105	24	12	1.35E+05	34.07	
Roxithromycin	Antibiotic	Chlorella	BG11	25	77.1	12	21	1.00E+05	82.00	(J. Li et al., 2020)
Roxithromycin	Antibiotic	Chlorella	BG11	25	77.1	12	21	2.50E+05	76.93	
Roxithromycin	Antibiotic	Chlorella	BG11	25	77.1	12	21	1.00E+06	64.49	
Oxybenzone	Antibiotic	Scenedesmus	BBM	27	45	16	10	1.00E+05	24.19	(Lee et al., 2020)
Oxybenzone	Antibiotic	Scenedesmus	BBM	27	45	16	10	5.00E+05	24.05	
Oxybenzone	Antibiotic	Scenedesmus	BBM	27	45	16	10	1.00E+06	28.35	
Oxybenzone	Antibiotic	Scenedesmus	BBM	27	45	16	10	2.00E+06	23.25	
Oxybenzone	Antibiotic	Scenedesmus	BBM	27	45	16	10	3.00E+06	25.22	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.024305556	1.25E+09	64.60	(Kouchakpour et al., 2020)
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.032986111	8.75E+08	72.10	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.015625	8.75E+08	79.40	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.032986111	1.63E+09	83.20	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.024305556	1.25E+09	79.80	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.015625	1.63E+09	84.30	

Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	8.75E+08	76.40	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	1.63E+09	73.20	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	1.25E+09	78.10	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	8.75E+08	60.60	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0069 44444	1.25E+09	74.80	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0416 66667	1.25E+09	74.50	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	8.75E+08	64.50	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	1.25E+09	78.20	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	1.25E+09	79.10	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	8.75E+08	67.20	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	8.75E+08	58.70	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	1.63E+09	80.30	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	1.63E+09	78.90	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	8.75E+08	74.10	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0329 86111	1.63E+09	78.50	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	1.25E+09	75.40	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	5.00E+08	60.80	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0243 05556	2.00E+09	83.50	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	1.63E+09	83.20	
Flutamide	Antibiotic	Anabeana	Zinder	28	64.2	24	0.0156 25	1.63E+09	78.90	
Caffeine	Antibiotic	Chlamydomonas	MSM	22	43	24	14	6.60E+05	99.02	(Escudero et al., 2020)
Simvastatin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	4.76E+02	75.64	
Carbamazepine	Antibiotic	Chlamydomonas	MSM	22	43	24	14	1.35E+05	25.71	

Lidocaine	Antibiotic	Chlamydomonas	MSM	22	43	24	14	1.08E+05	18.18	
Propranolol	Antibiotic	Chlamydomonas	MSM	22	43	24	14	1.82E+04	1.05	
Erythromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	2.30E+06	21.67	
Erythromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	1.40E+04	99.25	
Erythromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	2.30E+03	99.25	
Clarithromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	8.00E+05	41.54	
Clarithromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	4.00E+04	99.79	
Clarithromycin	Antibiotic	Chlamydomonas	MSM	22	43	24	14	8.00E+03	99.79	
Sulfamethoxazole	Antibiotic	Chlorella	Synthetic wastewater	22	235	24	7	5.20E+04	54.30	(da Silva Rodrigues et al., 2020)
Sulfamethoxazole	Antibiotic	Chlorella	Synthetic wastewater	22	235	0	7	5.20E+04	42.15	
Sulfadiazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	1.00E+06	19.47	(S. Chen, Zhang, et al., 2020)
Sulfadiazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+06	33.47	
Sulfadiazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	2.00E+07	40.33	
Sulfadiazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+07	4.59	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	1.00E+06	13.88	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+06	29.32	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	2.00E+07	25.65	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+07	4.27	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	1.00E+06	3.51	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+06	12.09	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	2.00E+07	20.07	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+07	1.40	
Norfloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	1.00E+06	2.71	
Norfloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+06	14.96	
Norfloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	2.00E+07	19.47	
Norfloxacin	Antibiotic	Chlorella	BG11	25	102.7	12	16	5.00E+07	3.95	
Sulfadiazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	1.00E+06	7.02	

Sulfadiazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+06	15.12	
Sulfadiazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	2.00E+07	42.57	
Sulfadiazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+07	0.76	
Sulfamethazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	1.00E+06	8.30	
Sulfamethazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+06	13.52	
Sulfamethazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	2.00E+07	19.11	
Sulfamethazine	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+07	3.47	
Enrofloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	1.00E+06	6.54	
Enrofloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+06	9.85	
Enrofloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	2.00E+07	14.48	
Enrofloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+07	3.63	
Norfloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	1.00E+06	4.95	
Norfloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+06	7.46	
Norfloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	2.00E+07	13.36	
Norfloxacin	Antibiotic	Chrysosporum	BG11	25	102.7	12	16	5.00E+07	2.03	
Sulfadiazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	1.00E+06	36.86	(S. Chen, Li, et al., 2020)
Sulfadiazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+06	44.78	
Sulfadiazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	2.00E+07	29.18	
Sulfadiazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+07	34.72	
Sulfamerazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	1.00E+06	41.84	
Sulfamerazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+06	34.96	
Sulfamerazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	2.00E+07	35.91	
Sulfamerazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+07	30.19	
Sulfamethazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	1.00E+06	51.41	
Sulfamethazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+06	33.24	
Sulfamethazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	2.00E+07	44.44	
Sulfamethazine	Antibiotic	Dictyosphaerium	BG11	25	102.7	12	16	5.00E+07	26.02	

Sulfamethazine	Antibiotic	Chlorella	BG11	25	45	12	15	1.00E+06	20.21	(Q. Chen, Zhang, et al., 2020)
Sulfamethazine	Antibiotic	Chlorella	BG11	25	45	12	15	5.00E+06	24.46	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	45	12	15	1.50E+07	14.36	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	45	12	15	2.00E+07	15.16	
Sulfamethazine	Antibiotic	Chlorella	BG11	25	45	12	15	2.50E+07	15.64	
Sulfamethazine	Antibiotic	Scenedesmus	BG11	25	45	12	15	1.00E+06	4.11	
Sulfamethazine	Antibiotic	Scenedesmus	BG11	25	45	12	15	5.00E+06	6.38	
Sulfamethazine	Antibiotic	Scenedesmus	BG11	25	45	12	15	1.50E+07	5.85	
Sulfamethazine	Antibiotic	Scenedesmus	BG11	25	45	12	15	2.00E+07	2.93	
Sulfamethazine	Antibiotic	Scenedesmus	BG11	25	45	12	15	2.50E+07	5.53	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	45	12	15	1.00E+06	48.55	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	45	12	15	5.00E+06	21.27	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	45	12	15	1.50E+07	2.92	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	45	12	15	2.00E+07	4.26	
Enrofloxacin	Antibiotic	Chlorella	BG11	25	45	12	15	2.50E+07	2.98	
Enrofloxacin	Antibiotic	Scenedesmus	BG11	25	45	12	15	1.00E+06	46.78	
Enrofloxacin	Antibiotic	Scenedesmus	BG11	25	45	12	15	5.00E+06	11.16	
Enrofloxacin	Antibiotic	Scenedesmus	BG11	25	45	12	15	1.50E+07	2.66	
Enrofloxacin	Antibiotic	Scenedesmus	BG11	25	45	12	15	2.00E+07	2.93	
Enrofloxacin	Antibiotic	Scenedesmus	BG11	25	45	12	15	2.50E+07	0.00	
Iohexol	Antibiotic	Chlorella	Bristol	23	85	24	27	2.00E+06	41.94	(Akao et al., 2020)
Iohexol	Antibiotic	Chlorella	Bristol	23	85	24	27	5.00E+06	43.41	
Iohexol	Antibiotic	Chlorella	Bristol	23	85	24	27	1.00E+07	45.05	
Iohexol	Antibiotic	Chlorella	Bristol	23	85	24	27	1.00E+08	34.61	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	11	5.00E+04	16.25	(J.-Q. Xiong, Kim, et al., 2019)
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	11	1.00E+05	21.87	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	11	2.00E+05	30.00	

Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	11	5.00E+04	17.17	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	11	1.00E+05	15.74	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	11	2.00E+05	15.75	
Sulfamethazine	Antibiotic	Consortia	BBM	27	45	24	11	1.00E+06	27.05	(J.-Q. Xiong, Jeon, et al., 2019)
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	12	2.50E+04	31.50	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	12	7.50E+04	34.56	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	12	1.25E+05	49.14	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	12	1.75E+05	54.59	
Sulfamethazine	Antibiotic	Scenedesmus	BBM	27	45	16	12	2.50E+05	62.37	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	12	2.50E+04	27.03	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	12	7.50E+04	28.60	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	12	1.25E+05	35.34	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	12	1.75E+05	40.40	
Sulfamethoxazole	Antibiotic	Scenedesmus	BBM	27	45	16	12	2.50E+05	46.62	
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	20	4.60E+07	96.82	(Song et al., 2019)
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	20	8.60E+07	89.85	
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	20	1.26E+08	70.42	
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	20	1.59E+08	75.20	
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	12	4.60E+07	37.90	
Florfenicol	Antibiotic	Chlorella	BG11	30	128	24	12	8.60E+07	22.33	
Ciprofloxacin	Antibiotic	Scenedesmus	MSM	23	125	12	6	2.50E+04	93.00	(Grimes et al., 2019)
Amoxicillin	Antibiotic	Chlorella	f/2	25	51.4	24	0.5	1.00E+04	99.90	(Shi et al., 2018)
Amoxicillin	Antibiotic	Chlorella	f/2	25	51.4	24	1.5	5.00E+04	99.90	
Amoxicillin	Antibiotic	Chlorella	f/2	25	51.4	24	2.5	1.50E+05	99.40	
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	2.00E+05	14.90	(Mojiri et al., 2021)
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	4.00E+05	13.20	
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	6.00E+05	11.40	

Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	8.00E+05	10.10
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	1.00E+06	8.90
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	2.00E+05	17.30
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	4.00E+05	15.20
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	6.00E+05	12.80
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	8.00E+05	12.00
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	1.00E+06	9.20
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	2.00E+05	26.30
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	4.00E+05	29.60
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	6.00E+05	26.50
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	8.00E+05	23.20
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	1.00E+06	20.70
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	2.00E+05	31.90
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	4.00E+05	30.20
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	6.00E+05	28.10
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	8.00E+05	27.80
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	1.00E+06	25.60
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	2.00E+05	35.40
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	4.00E+05	33.30
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	6.00E+05	30.20
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	8.00E+05	26.30
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	1.00E+06	23.30
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	2.00E+05	31.70
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	4.00E+05	30.40
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	6.00E+05	27.50
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	8.00E+05	26.90
Carbamazepine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	1.00E+06	26.00
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	2.00E+05	12.30

Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	4.00E+05	11.60
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	6.00E+05	9.20
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	8.00E+05	9.80
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	1.00E+06	9.80
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	2.00E+05	15.20
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	4.00E+05	14.50
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	6.00E+05	10.30
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	8.00E+05	10.90
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	1.00E+06	10.40
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	2.00E+05	24.50
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	4.00E+05	27.00
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	6.00E+05	24.10
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	8.00E+05	21.80
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	1.00E+06	20.40
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	2.00E+05	27.20
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	4.00E+05	25.30
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	6.00E+05	24.00
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	8.00E+05	21.50
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	1.00E+06	20.60
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	2.00E+05	31.20
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	4.00E+05	30.30
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	6.00E+05	28.10
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	8.00E+05	26.60
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	1.00E+06	25.70
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	2.00E+05	33.10
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	4.00E+05	32.80
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	6.00E+05	30.50
Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	8.00E+05	27.20

Sulfamethazine	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	1.00E+06	26.80
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	2.00E+05	13.60
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	4.00E+05	14.30
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	6.00E+05	10.50
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	8.00E+05	9.60
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	0.5	1.00E+06	8.80
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	2.00E+05	18.10
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	4.00E+05	17.30
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	6.00E+05	14.00
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	8.00E+05	13.40
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	2.5	1.00E+06	11.90
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	2.00E+05	28.20
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	4.00E+05	28.40
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	6.00E+05	24.60
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	8.00E+05	20.80
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	4.5	1.00E+06	20.50
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	2.00E+05	35.40
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	4.00E+05	32.20
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	6.00E+05	30.50
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	8.00E+05	29.60
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	6.5	1.00E+06	28.20
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	2.00E+05	35.90
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	4.00E+05	36.50
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	6.00E+05	33.00
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	8.00E+05	28.10
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	8.5	1.00E+06	27.90
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	2.00E+05	34.80
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	4.00E+05	34.80

Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	6.00E+05	31.10	
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	8.00E+05	29.40	
Tramadol	Antibiotic	Chaetoceros	f/2	23	66	24	10.5	1.00E+06	28.10	
Ciprofloxacin	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	1.00E+07	99.89	(P. Xie et al., 2020)
Ciprofloxacin	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	5.00E+06	99.89	
Ciprofloxacin	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	1.00E+06	99.89	
Sulfadiazine	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	1.00E+07	57.94	
Sulfadiazine	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	5.00E+06	77.10	
Sulfadiazine	Antibiotic	Chlamydomonas	Synthetic wastewater	23	250	24	9	1.00E+06	95.39	
Sulfamethoxazole	Antibiotic	Consortia	Centrate wastewater	23	ND	24	7	5.00E+05	80.40	(B. Xie et al., 2020)
Sulfamethoxazole	Antibiotic	Acutodesmus	1g/L Ferty Basic 1	30	900	24	7.0833 33333	2.50E+05	43.17	(Reymann et al., 2020)
Ciprofloxacin	Antibiotic	Acutodesmus	1g/L Ferty Basic 1	30	900	24	7.0833 33333	2.50E+05	84.37	
Quetiapine	Antibiotic	Acutodesmus	1g/L Ferty Basic 1	30	900	24	7.0833 33333	2.50E+05	97.16	
Gabapentin	Antibiotic	Acutodesmus	1g/L Ferty Basic 1	30	900	24	7.0833 33333	2.50E+05	5.01	
2-Phenyl-5-benzimidazolesulfonic acid	Antibiotic	Acutodesmus	1g/L Ferty Basic 1	30	900	24	7.0833 33333	2.50E+05	12.29	
Penicillin	Antibiotic	Chlorella	BBM	23	89.9	12	6	4.00E+05	99.25	(Yazdi et al., 2018)
Penicillin	Antibiotic	Chlorella	BBM	23	89.9	12	6	8.00E+05	99.13	
Penicillin	Antibiotic	Chlorella	BBM	23	89.9	12	6	1.20E+06	99.00	
Penicillin	Antibiotic	Chlorella	BBM	23	89.9	12	6	1.60E+06	98.88	
Penicillin	Antibiotic	Chlorella	BBM	23	89.9	12	6	2.00E+06	92.75	
Penicillin	Antibiotic	Spirulina	Zarrok	23	89.9	12	6	4.00E+05	99.25	
Penicillin	Antibiotic	Spirulina	Zarrok	23	89.9	12	6	8.00E+05	98.88	
Penicillin	Antibiotic	Spirulina	Zarrok	23	89.9	12	6	1.20E+06	98.67	
Penicillin	Antibiotic	Spirulina	Zarrok	23	89.9	12	6	1.60E+06	98.69	

Penicillin	Antibiotic	Spirulina	Zarrok	23	89.9	12	6	2.00E+06	94.45	
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	1.00E+06	98.37	(X.-X. Wang et al., 2018)
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	1.50E+06	98.91	
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	2.00E+06	99.18	
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	3.00E+06	99.46	
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	4.00E+06	96.57	
Methylisothiazolinone	Antibiotic	Scenedesmus	BG11	23	900	12	6	5.00E+06	52.90	
Carbamazepine	Anticonvulsant	Chlamydomonas	raw wastewater	25	60	12	7	1.30E+02	11.77	(Zhou et al., 2014)
Carbamazepine	Anticonvulsant	Scenedesmus	raw wastewater	25	60	12	7	1.30E+02	5.00	
Carbamazepine	Anticonvulsant	Chlorella	raw wastewater	25	60	12	7	1.30E+02	3.50	
Carbamazepine	Anticonvulsant	Chlorella	raw wastewater	25	60	12	7	1.30E+02	1.00	
Estrone	Hormone	Chlamydomonas	raw wastewater	25	60	12	7	9.17E+01	84.50	
Estrone	Hormone	Scenedesmus	raw wastewater	25	60	12	7	9.17E+01	87.00	
Estrone	Hormone	Chlorella	raw wastewater	25	60	12	7	9.17E+01	87.50	
Estrone	Hormone	Chlorella	raw wastewater	25	60	12	7	9.17E+01	86.50	
Triclosan	Antimicrobial	Chlamydomonas	raw wastewater	25	60	12	7	4.17E+01	58.20	
Triclosan	Antimicrobial	Scenedesmus	raw wastewater	25	60	12	7	4.17E+01	31.40	
Triclosan	Antimicrobial	Chlorella	raw wastewater	25	60	12	7	4.17E+01	42.20	
Triclosan	Antimicrobial	Chlorella	raw wastewater	25	60	12	7	4.17E+01	53.00	
Bisphenol A	Plasticizer	Chlamydomonas	raw wastewater	25	60	12	7	2.01E+04	98.50	
Bisphenol A	Plasticizer	Scenedesmus	raw wastewater	25	60	12	7	2.01E+04	98.90	
Bisphenol A	Plasticizer	Chlorella	raw wastewater	25	60	12	7	2.01E+04	99.10	
Bisphenol A	Plasticizer	Chlorella	raw wastewater	25	60	12	7	2.01E+04	99.00	
Ciprofloxacin	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	2.93E+01	78.90	
Ciprofloxacin	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	2.93E+01	78.10	

Ciprofloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.93E+01	74.40	
Ciprofloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.93E+01	76.90	
Enrofloxacin	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	2.62E+01	74.80	
Enrofloxacin	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	2.62E+01	76.30	
Enrofloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.62E+01	76.30	
Enrofloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.62E+01	77.40	
Erythromycin	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	1.03E+03	85.90	
Erythromycin	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	1.03E+03	63.80	
Erythromycin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	1.03E+03	74.30	
Erythromycin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	1.03E+03	66.90	
Norfloxacin	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	3.23E+01	53.20	
Norfloxacin	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	3.23E+01	41.10	
Norfloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	3.23E+01	41.10	
Norfloxacin	Antibiotic	Chlorella	raw wastewater	25	60	12	7	3.23E+01	43.00	
Sulfadiazine	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	2.86E+01	74.40	
Sulfadiazine	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	2.86E+01	74.40	
Sulfadiazine	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.86E+01	52.30	
Sulfadiazine	Antibiotic	Chlorella	raw wastewater	25	60	12	7	2.86E+01	66.78	
Sulfamethazine	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	5.67E+01	48.30	
Sulfamethazine	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	5.67E+01	18.80	
Sulfamethazine	Antibiotic	Chlorella	raw wastewater	25	60	12	7	5.67E+01	22.50	
Sulfamethazine	Antibiotic	Chlorella	raw wastewater	25	60	12	7	5.67E+01	33.10	
Sulfamethoxazole	Antibiotic	Chlamydomonas	raw wastewater	25	60	12	7	5.07E+01	21.20	
Sulfamethoxazole	Antibiotic	Scenedesmus	raw wastewater	25	60	12	7	5.07E+01	20.10	
Sulfamethoxazole	Antibiotic	Chlorella	raw wastewater	25	60	12	7	5.07E+01	11.20	
Sulfamethoxazole	Antibiotic	Chlorella	raw wastewater	25	60	12	7	5.07E+01	31.30	
Carbamazepine	Anticonvulsant	Chlamydomonas	BBM	27	45	16	10	1.00E+00	37.00	(J.-Q. Xiong et al., 2016)

Carbamazepine	Anticonvulsant	Scenedesmus	BBM	28	45	16	10	1.00E+00	30.00	
Carbamazepine	Anticonvulsant	Chlamydomonas	BBM	29	45	16	10	2.50E+01	21.00	
Carbamazepine	Anticonvulsant	Scenedesmus	BBM	30	45	16	10	2.50E+01	13.00	
Carbamazepine	Anticonvulsant	Navicula	D1	23	102.7	12	6	1.79E+05	90.00	(Ding et al., 2019)
Triclosan	Anticonvulsant	Nannochloris	miliQ water	23	273	12	14	1.00E+04	100.00	(Bai & Acharya, 2016)
trimethoprim	Antibiotic	Nannochloris	miliQ water	23	273	12	14	1.00E+04	0.00	
Sulfamethoxazole	Anticonvulsant	Nannochloris	miliQ water	23	273	12	14	1.00E+04	32.00	
Triclosan	Antimicrobial	Chlorella	TAP	22	102.7	16	1	1.00E+02	50.00	(S. Wang et al., 2013)
Triclosan	Antimicrobial	Chlorella	TAP	22	102.7	16	1	2.00E+02	50.00	
Triclosan	Antimicrobial	Chlorella	TAP	22	102.7	16	1	4.00E+02	50.00	
Triclosan	Antimicrobial	Chlorella	TAP	22	102.7	16	1	8.00E+02	50.00	
Triclosan	Antimicrobial	Chlorella	TAP	22	102.7	16	1	8.00E+02	77.20	
17-beta-ethinylestradiol	Hormone	Chlorella	BG11	25	24.8	12	7	3.00E+06	92.00	(Wu et al., 2021)
17-alpha-ethinylestradiol	Hormone	Chlorella	BG11	25	24.8	12	7	3.00E+06	55.00	
17-beta-ethinylestradiol	Hormone	Scenedesmus	BG11	25	24.8	12	7	3.00E+06	73.00	
17-alpha-ethinylestradiol	Hormone	Scenedesmus	BG11	25	24.8	12	7	3.00E+06	57.00	
17-beta-ethinylestradiol	Hormone	Selenastrum	BG11	25	24.8	12	7	3.00E+06	91.00	
17-alpha-ethinylestradiol	Hormone	Selenastrum	BG11	25	24.8	12	7	3.00E+06	83.00	
Triclosan	Antimicrobial	Chlorella	TAP	25	123.3	16	7	4.00E+05	69.30	(S. Wang et al., 2018)
Triclosan	Antimicrobial	Desmodesmus	TAP	25	123.3	16	7	4.00E+05	100.00	
Triclosan	Antimicrobial	Scenedesmus	TAP	25	123.3	16	7	4.00E+05	99.70	
Carbamazepine	Anticonvulsant	Spirulina	Zarrouk	27	80	12	10	1.00E+06	30.97	(Q. Wang et al., 2020)
Carbamazepine	Anticonvulsant	Spirulina	Zarrouk	27	80	12	10	2.50E+06	20.85	

Carbamazepine	Anticonvulsant	Spirulina	Zarrouk	27	80	12	10	5.00E+06	16.43	
Carbamazepine	Anticonvulsant	Spirulina	Zarrouk + 0.3g L glucosa	27	80	12	10	1.00E+06	50.13	
Carbamazepine	Anticonvulsant	Spirulina	Zarrouk + 0.3g L glucosa	27	80	12	10	2.50E+06	37.46	
Carbamazepine	Anticonvulsant	Spirulina	Zarrouk + 0.3g L glucosa	27	80	12	10	5.00E+06	20.65	
DEET	Insect repellent	Spirulina	Standard Zarrouk	25	15	24	15	1.00E+07	70.00	(Kurashvili et al., 2018)
17-beta-ethinylestradiol	Hormone	Desmodesmus	raw domestic wastewater	22	45	12	3	1.00E+03	85.00	(R. Wang et al., 2020)
Gemfibrozil	Lipid regulator	Scenedesmus	BBM	22	130	16	25	1.00E+07	27.00	(Larsen et al., 2019)
Gemfibrozil	Lipid regulator	Chlorella	BBM	22	130	16	25	1.00E+07	24.00	
Carbamazepine	Lipid regulator	Chlorella	BBM	22	130	16	25	1.00E+07	21.00	
Ibuprofen	NSAID	Chlorella	Lagoon water	22	130	16	25	1.00E+07	60.00	
Triclosan	Lipid regulator	Scenedesmus	BBM	22	130	16	25	1.00E+07	56.00	
Triclosan	Lipid regulator	Chlorella	BBM	22	130	16	25	1.00E+07	55.00	
Triclosan	Lipid regulator	Scenedesmus	Lagoon water	22	130	0	25	1.00E+07	64.00	
Triclosan	Lipid regulator	Chlorella	Lagoon water	22	130	16	25	1.00E+07	56.00	
Bisphenol A	Plasticizer	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	(Garcia-Rodríguez et al., 2015)
17-alpha-ethinylestradiol	Hormone	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Diclofenac	Antibiotic	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
acetaminophen	NSAID	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Ibuprofen	NSAID	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Carbamazepine	Anticonvulsant	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Clofibric acid	Anticonvulsant	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Propranolol	Beta-blocker	Spirogyra	wastewater	20	15	12	20	1.00E+05	100.00	
Carbamazepine	Anticonvulsant	Ankistrodesmus	BBM	30	100	16	60	2.50E+03	80.00	(Yehya et al., 2020)

Carbamazepine	Anticonvulsant	Ankistrodesmus	BBM	30	100	16	60	1.00E+04	70.00	
Carbamazepine	Anticonvulsant	Ankistrodesmus	Proteose peptone	30	100	16	60	2.50E+03	74.00	
Carbamazepine	Anticonvulsant	Ankistrodesmus	Proteose peptone	30	100	16	60	1.00E+04	61.00	
Carbamazepine	Anticonvulsant	Ankistrodesmus	BBM	30	100	16	60	2.50E+03	87.60	
Carbamazepine	Anticonvulsant	Ankistrodesmus	BBM	30	100	16	60	1.00E+04	77.00	
Carbamazepine	Anticonvulsant	Ankistrodesmus	Proteose peptone	30	100	16	60	2.50E+03	79.20	
Carbamazepine	Anticonvulsant	Ankistrodesmus	Proteose peptone	30	100	16	60	1.00E+04	66.00	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	20	16	5	1.00E+07	28.40	(Ben Ali et al., 2021)
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	3.00E+07	82.50	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	3.00E+07	84.23	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	3.00E+07	79.11	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	120	16	5	5.00E+07	64.01	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	20	16	5	3.00E+07	47.24	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	120	16	5	1.00E+07	43.50	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	120	16	5	1.00E+07	74.10	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	20	16	5	5.00E+07	18.40	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	20	16	5	5.00E+07	32.01	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	120	16	5	1.00E+07	37.03	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	120	16	5	5.00E+07	53.81	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	20	16	5	1.00E+07	30.20	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	20	16	5	1.00E+07	52.32	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	120	16	5	1.00E+07	56.07	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	120	16	5	1.00E+07	83.10	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	20	16	5	5.00E+07	21.12	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	20	16	5	5.00E+07	35.21	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	120	16	5	5.00E+07	43.23	

Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	20	70	16	5	3.00E+07	54.24	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	40	70	16	5	3.00E+07	68.40	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	20	16	5	3.00E+07	51.23	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	120	16	5	3.00E+07	80.11	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	1.00E+07	92.12	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	5.00E+07	72.65	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	3.00E+07	69.14	
Bisphenol A	Plasticizer	Chlorophyta	Zarrouk	30	70	16	5	3.00E+07	73.25	
Bezafibrate	Lipid regulator	Navicula	D1 medium	23	43	12	21	5.00E+05	23.04	(Ding et al., 2020)
Atenolol	Beta-blocker	Navicula	D1 medium	23	43	12	21	5.00E+05	100.00	
Carbamazepine	Anticonvulsant	Navicula	D1 medium	23	43	12	21	5.00E+05	93.60	
Ibuprofen	NSAID	Navicula	D1 medium	23	43	12	21	5.00E+05	96.74	
Sulfamethoxazole	Antibiotic	Navicula	D1 medium	23	43	12	21	5.00E+05	85.65	
Naproxen	NSAID	Navicula	D1 medium	23	43	12	21	5.00E+05	94.81	
Ibuprofen	NSAID	Chlorella	Urine	35	68	24	31	3.17E+05	91.76	(de Wilt et al., 2016)
Ibuprofen	NSAID	Chlorella	Anaerobic treated black wastewater	35	68	24	31	3.17E+05	97.13	
Ibuprofen	NSAID	Chlorella	Urea-based syntetiv urine	35	68	24	31	3.17E+05	97.13	
Diclofenac	NSAID	Chlorella	Urine	35	68	24	31	1.47E+05	55.20	
Diclofenac	NSAID	Chlorella	Anaerobic treated black wastewater	35	68	24	31	1.47E+05	55.20	
Diclofenac	NSAID	Chlorella	Urea-based syntetiv urine	35	68	24	31	1.47E+05	70.97	
Trimethoprim	Antibiotic	Chlorella	Urine	35	68	24	31	2.02E+05	30.91	
Trimethoprim	Antibiotic	Chlorella	Anaerobic treated black wastewater	35	68	24	31	2.02E+05	30.91	
Trimethoprim	Antibiotic	Chlorella	Urea-based syntetiv urine	35	68	24	31	2.02E+05	30.91	
Metoprolol	Beta blocker	Chlorella	Urine	35	68	24	31	1.81E+05	52.15	

Metoprolol	Beta blocker	Chlorella	Anaerobic treated black wastewater	35	68	24	31	1.81E+05	98.63	
Metoprolol	Beta blocker	Chlorella	Urea-based syntetiv urine	35	68	24	31	1.81E+05	98.63	
Carbamazepine	Anticonvulsant	Chlorella	Urine	35	68	24	31	1.17E+05	4.31	
Carbamazepine	Anticonvulsant	Chlorella	Anaerobic treated black wastewater	35	68	24	31	1.17E+05	4.31	
Carbamazepine	Anticonvulsant	Chlorella	Urea-based syntetiv urine	35	68	24	31	1.17E+05	12.35	
Metoprolol	Beta blocker	Selenastrum	synthetic wastewater	25	49	12	40	5.00E+09	60.00	(Lv et al., 2018)
Metoprolol	Beta blocker	Chlorella	synthetic wastewater	25	49	12	40	5.00E+09	10.00	
Metoprolol	Beta blocker	Scenedesmus	synthetic wastewater	25	49	12	40	5.00E+09	91.40	
Metoprolol	Beta blocker	Haematococcus	synthetic wastewater	25	49	12	40	5.00E+09	90.70	
Sulfamethoxazole	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	2.00E+04	98	(Kiki et al., 2020)
Trimethoprim	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	2.00E+04	70.00	
Clarithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	2.00E+04	80	
Azithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	2.00E+04	90.00	
Sulfamethoxazole	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	5.00E+04	98	
Trimethoprim	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	5.00E+04	70.00	
Clarithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	5.00E+04	80	
Azithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	5.00E+04	90.00	
Sulfamethoxazole	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	1.00E+05	98	
Trimethoprim	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	1.00E+05	70.00	
Clarithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	1.00E+05	80	
Azithromycin	Antibiotic	Haematococcus	synthetic wastewater	25	ND	12	40	1.00E+05	90.00	

Sulfamethoxazole	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	2.00E+04	75.00
Trimethoprim	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	2.00E+04	24.00
Clarithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	2.00E+04	70.00
Azithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	2.00E+04	77.00
Sulfamethoxazole	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	5.00E+04	75.00
Trimethoprim	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	5.00E+04	24.00
Clarithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	5.00E+04	70.00
Azithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	5.00E+04	77.00
Sulfamethoxazole	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	1.00E+05	75.00
Trimethoprim	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	1.00E+05	24.00
Clarithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	1.00E+05	70.00
Azithromycin	Antibiotic	Selenastrum	synthetic wastewater	25	ND	12	40	1.00E+05	77.00
Sulfamethoxazole	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	2.00E+04	49.00
Trimethoprim	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	2.00E+04	25.00
Clarithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	2.00E+04	60.00
Azithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	2.00E+04	55.00
Sulfamethoxazole	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	5.00E+04	49.00
Trimethoprim	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	5.00E+04	25.00
Clarithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	5.00E+04	60.00
Azithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	5.00E+04	55.00
Sulfamethoxazole	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	1.00E+05	49.00
Trimethoprim	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	1.00E+05	25.00
Clarithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	1.00E+05	60.00

Azithromycin	Antibiotic	Scenedesmus	synthetic wastewater	25	ND	12	40	1.00E+05	55.00	
Sulfamethoxazole	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	2.00E+04	75.00	
Trimethoprim	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	2.00E+04	26.00	
Clarithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	2.00E+04	75.00	
Azithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	2.00E+04	77.00	
Sulfamethoxazole	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	5.00E+04	75.00	
Trimethoprim	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	5.00E+04	26.00	
Clarithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	5.00E+04	75.00	
Azithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	5.00E+04	77.00	
Sulfamethoxazole	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	1.00E+05	75.00	
Trimethoprim	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	1.00E+05	26.00	
Clarithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	1.00E+05	75.00	
Azithromycin	Antibiotic	Chlorella	synthetic wastewater	25	ND	12	40	1.00E+05	77.00	
Caffeine	Stimulant	Chlorella	BG11	20	30	12	12	1.00E+03	68.00	(Gojkovic et al., 2019)
Caffeine	Stimulant	Scenedesmus	BG11	20	30	12	12	1.00E+03	81.00	
Caffeine	Stimulant	Chlorella	BG11	20	30	12	12	1.00E+03	50.00	
Caffeine	Stimulant	Desmodesmus	BG11	20	30	12	12	1.00E+03	43.00	
Caffeine	Stimulant	Scenedesmus	BG11	20	30	12	12	1.00E+03	26.00	
Caffeine	Stimulant	Desmodesmus	BG11	20	30	12	12	1.00E+03	56.00	
Caffeine	Stimulant	Coelastrella	BG11	20	30	12	12	1.00E+03	70.00	
Caffeine	Stimulant	Chlore	BG11	20	30	12	12	1.00E+03	31.00	
Caffeine	Stimulant	Coelastrella	BG11	20	30	12	12	1.00E+03	49.00	
Trimethoprim	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	21.00	
Trimethoprim	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	18.00	
Trimethoprim	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	33.00	

Trimethoprim	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	31.00
Trimethoprim	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	24.00
Trimethoprim	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	48.00
Trimethoprim	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	29.00
Trimethoprim	Antibiotic	Chlore	BG11	20	30	12	12	1.00E+03	3.00
Trimethoprim	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	21.00
Fluconazole	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	6.00
Fluconazole	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	7.00
Fluconazole	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	6.00
Fluconazole	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	0.00
Fluconazole	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	13.00
Fluconazole	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	25.00
Fluconazole	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	0.00
Fluconazole	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	3.00
Fluconazole	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	9.00
Ofloxacin	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	65.00
Ofloxacin	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	49.00
Ofloxacin	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	61.00
Ofloxacin	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	58.00
Ofloxacin	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	89.00
Ofloxacin	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	77.00
Ofloxacin	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	90.00
Ofloxacin	Antibiotic	Chlore	BG11	20	30	12	12	1.00E+03	69.00
Ofloxacin	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	61.00
Carbamazepine	Anticonvulsant	Chlorella	BG11	20	30	12	12	1.00E+03	19.00
Carbamazepine	Anticonvulsant	Scenedesmus	BG11	20	30	12	12	1.00E+03	39.00
Carbamazepine	Anticonvulsant	Chlorella	BG11	20	30	12	12	1.00E+03	29.00

Carbamazepine	Anticonvulsant	Desmodesmus	BG11	20	30	12	12	1.00E+03	37.00	
Carbamazepine	Anticonvulsant	Scenedesmus	BG11	20	30	12	12	1.00E+03	42.00	
Carbamazepine	Anticonvulsant	Desmodesmus	BG11	20	30	12	12	1.00E+03	75.00	
Carbamazepine	Anticonvulsant	Coelastrella	BG11	20	30	12	12	1.00E+03	41.00	
Carbamazepine	Anticonvulsant	Chlore	BG11	20	30	12	12	1.00E+03	27.00	
Carbamazepine	Anticonvulsant	Coelastrella	BG11	20	30	12	12	1.00E+03	28.00	
Tramadol	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	20.00	
Tramadol	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	19.00	
Tramadol	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	23.00	
Tramadol	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	19.00	
Tramadol	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	19.00	
Tramadol	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	48.00	
Tramadol	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	6.00	
Tramadol	Antibiotic	Chlore	BG11	20	30	12	12	1.00E+03	53.00	
Tramadol	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	25.00	
Diphenhydramine	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	74.00	
Diphenhydramine	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	86.00	
Diphenhydramine	Antibiotic	Chlorella	BG11	20	30	12	12	1.00E+03	96.00	
Diphenhydramine	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	98.00	
Diphenhydramine	Antibiotic	Scenedesmus	BG11	20	30	12	12	1.00E+03	96.00	
Diphenhydramine	Antibiotic	Desmodesmus	BG11	20	30	12	12	1.00E+03	96.00	
Diphenhydramine	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	96.00	
Diphenhydramine	Antibiotic	Chlore	BG11	20	30	12	12	1.00E+03	99.00	
Diphenhydramine	Antibiotic	Coelastrella	BG11	20	30	12	12	1.00E+03	95.00	
Ibuprofen	NSAID	Consortia	media	23	150	12	10	5.00E+03	50.65	(Matamoros et al., 2016)
Carbamazepine	Anticonvulsant	Consortia	media	23	150	12	10	5.00E+03	12.26	

Caffeine	Stimulant	Consortia	media	23	150	12	10	5.00E+03	16.45	
Tris(2-chloroethyl) phosphate	Plasticizer	Consortia	media	23	150	12	10	5.00E+03	7.10	
Ibuprofen	NSAID	Consortia	wastewater	23	150	12	10	5.00E+03	97.10	
Carbamazepine	Anticonvulsant	Consortia	wastewater	23	150	12	10	5.00E+03	15.16	
Caffeine	Stimulant	Consortia	wastewater	23	150	12	10	5.00E+03	97.40	
Tris(2-chloroethyl) phosphate	Plasticizer	Consortia	wastewater	23	150	12	10	5.00E+03	7.10	
Sulfamethoxazole	Antibiotic	Nannochloris	media	20	142	24	14	1.00E+04	10.52	(Bai & Acharya, 2017)
Trimethoprim	Antibiotic	Nannochloris	media	20	142	24	14	1.00E+04	35.62	
Ciprofloxacin	Antibiotic	Nannochloris	media	20	142	24	14	1.00E+04	0.00	
Carbamazepine	Anticonvulsant	Nannochloris	media	20	142	24	14	1.00E+04	97.94	
Triclosan	Antimicrobial	Nannochloris	media	20	142	24	14	1.00E+04	98.00	

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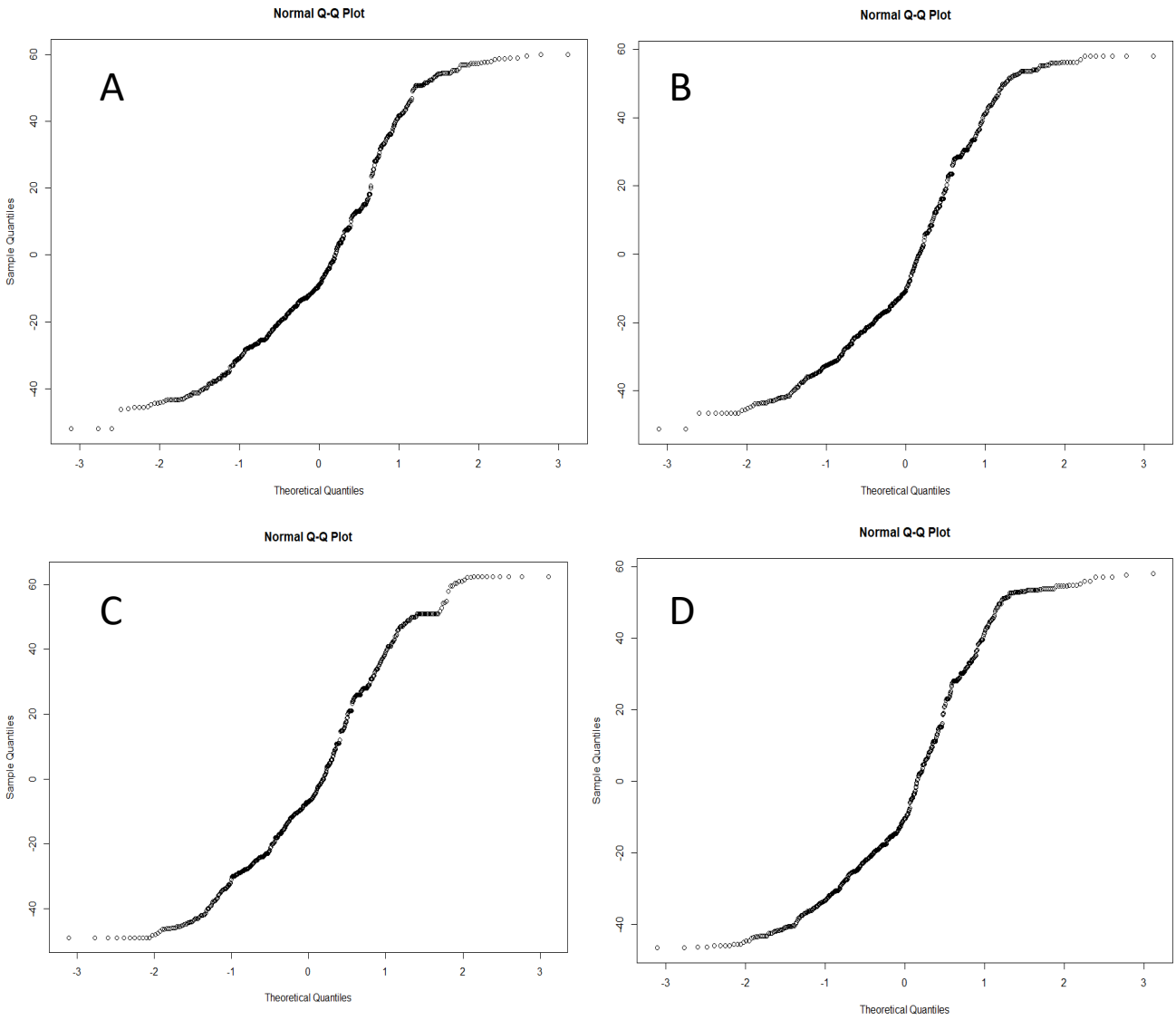

Figure 5-1: Quantile-quantile plots for HRT (A), temperature (B), photoperiod (C), and light intensity (D) . The visually linear relationship of the fitted residuals implied a linear regression is appropriate for analysis,