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Evaluation of a Microalgal Bioassay Based on Community Level Physiological Profiling (CLPP)

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Abstract

This thesis examined the use of commercially available Biolog 96-well plates containing different carbon sources for a microalgal bioassay. Quaternary ammonium compounds (QACs) were used as potentially toxic model compounds to demonstrate the applicability of the assay. Toxicity of dodecyl trimethyl ammonium chloride (DTAC) and didecyl dimethyl ammonium bromide (DDAB) on the growth of *Scenedesmus obliquus* varied significantly under autotrophic, heterotrophic and mixotrophic growth conditions. The concentration for 50% growth inhibition effect (EC_{50}) of DTAC followed the order: autotrophic (0.48 ± 0.03 mg of DTAC/L) > heterotrophic (1.46 ± 0.04 mg of DTAC/L) > mixotrophic (2.11 ± 0.06 mg of DTAC/L), whereas for DDAB, the order of inhibition was different as: heterotrophic (0.52 ± 0.02 mg of DDAB/L) > autotrophic (1.18 ± 0.08 mg of DDAB/L) > mixotrophic (1.35 ± 0.02 mg of DDAB/L). Moreover, EC_{50} values were a function of carbon source and growth regime, showing that only EC_{50} values do not fully capture the toxic effects of a potentially toxic compound might have on algal communities. Therefore, a new assay was developed based on community level physiological profiling (CLPP). Five different mixtures of artificially defined microalgal communities were employed and the changes in substrate utilization patterns by the treatment of hexadecyl trimethyl ammonium chloride (CTAC) were quantified using principal component analysis (PCA). The toxic effect of CTAC was significant ($P < 0.05$), showing 58% inhibition compared to the control and the effect was more pronounced for the treatment than that obtained by varying the initial composition of the defined algal communities. The newly developed assay was further applied on wetland water samples, wastewaters (i.e. primary and secondary), river water, and activated carbon treated and untreated oil sand process waters (OSPWs). The assay was able to generate a distinguishable response among these samples, measuring small differences within the respective water groups and larger differences between them.

Keywords: Mixotrophic and heterotrophic microalgal bioassay, quaternary ammonium compounds (QACs), freshwater algae, Biolog plates, community level physiological profiling (CLPP)

Contributions of Authors

All experimental designs, data collections, analyses and interpretation of the results were performed by Jun-Woo Kim. The written manuscripts were carried out in collaboration with my supervisors, Drs. Lars Rehmann and Madhumita B. Ray.

Dedicated to my family:

Father: Dong-Suk Kim

Mother: Eun-Ok Kim

Sister: Katie Mi-Kyung Hong

Brother: James Tae-Yeul Kim

Spouse: Chloe Nan-Joo Choi

Son: Sol Chan-Seul Kim

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List of Abbreviations

ANOVA   Analysis of variance

AWCD   Average well color development

AWFD   Average well florescence development

BAC   Benzalkonium chlorides

BDHAC   Benzyl dimethyl hexadecyl ammonium chloride

BDTAC   Benzyl dimethyl tetradecyl ammonium chloride

BDDAC   Benzyl dimethyl dodecyl ammonium chloride

CLPP   Community level physiological profiling

CPCC   Canadian phycological culture centre

CC   Chlamydomonas center

CCAP   Culture collection of algae and protozoa

CPRG   Chlorophenol red-β-D-galactopyranoside

CSUP   Carbon source utilization pattern

CTAB   Hexadecyl trimethyl ammonium bromide

CTAC   Hexadecyl trimethyl ammonium chloride

CPB   Hexadecyl pyridinium bromide

DTDMAC   Ditallow dimethyl ammonium chloride

DTAB   Dodecyl trimethyl ammonium bromide

DTAC	Dodecyl trimethyl ammonium chloride
DDAB	Didecyl dimethyl ammonium bromide
DDAC	Didecyl dimethyl ammonium chloride
DNA	Deoxyribonucleic acid
EDDAB	Ethyl dodecyl dimethyl ammonium bromide
EHDAB	Ethyl hexadecyl dimethyl ammonium bromide
ED	Euclidean distance
EDCs	Endocrine disrupting compounds
HS	High salt
MS	Molecular structure
MW	Molecular weight
OD	Optical density
OECD	Organization for economic cooperation and development
OC	Organic carbons
OSPW	Oil sand processed water
ONPG	O-nitrophenyl- β-D-galactopyranoside
PICT	Pollution-induced community tolerance
PCA	Principal component analysis
PC	Principal component

QACs	Quaternary ammonium compounds
RNA	Ribonucleic acid
SD	Standard deviation
STAB	Stearyl trimethyl ammonium bromide
STAC	Stearyl trimethyl ammonium chloride
TTAB	Tetradecyl trimethyl ammonium bromide
TOC	Total organic carbon
UTEX	University of Texas at Austin
UV	Ultra-violet
YES	Yeast estrogen screen
YAS	Yeast androgen screen

Nomenclature

EC_{50} Concentration of a toxicant that induces 50% of maximal response (mg/L)

EC_{10} Concentration of a toxicant that induces 10% of maximal response (mg/L)

Chapter 1. Background

1.1 Introduction

Contaminated sites are managed by the Canadian federal government by a risk-based approach. Several components to the risk-based approach include site identification and characterization, detailed site investigations and risk assessment, evaluation of different risk management strategies, implementation of a selected management strategy, assessment and monitoring [1]. Moreover, the Canadian environmental quality guidelines have been established by the Canadian Council of Ministers of the Environment (CCME) and are available for the effective management of federal contaminated sites. The guidelines are important as they are used for evaluating the degree of contamination at the site to decide if further site investigations and management actions are required.

The guidelines are set based on the scientific information on toxicological studies. Biological assays are commonly employed in toxicity assessment of various contaminants. Bioassays are defined as lab cultures of organisms where growth conditions are manipulated by input of specific chemicals. They complement a chemical analysis in defining the concentration of contaminant by providing a direct measurement of toxic effects of a specific compound and detect impacts from many sources for which chemical criteria are poorly suited to determine synergistic, intermittent and chronic pollutant effects [2]. Moreover, this is an efficient way of confirming the cause and effect relations between parameters for which only observational correlations can be achieved from field surveys [3].

Bioassays can work in two ways, *in vitro* and *in vivo*. *In vitro* methods are based on specific modes or cellular mechanism towards the detection of certain chemicals, while *in vivo* methods assess the integrative effects of a toxicant on whole organisms, providing direct information about the ecological effect. Commonly applied *in vitro* bioassays include the *umuC* and comet for genotoxicity, Ames for mutagenicity and YES and YAS for estrogenicity and androgenicity detections [4], while *in vivo* assays use test species from different trophic levels, i.e. bacteria, algae, duckweed, crustaceans and fish, to measure the effects of a

potential toxic compound on growth, reproduction, feeding activity and mortality [5]. Commonly used *in vivo* standardized tests include the bacteria luminescence inhibition assay [6], green microalgae growth assay [7], duckweed growth assay [8], Daphnia reproduction assay [9], and fish egg assay [10].

Among several existing *in vivo* standardized test systems, microalgae as model organisms are being used increasingly for bioassay [11,12,13]. They are photosynthetic organisms with an ability to convert light energy into organic carbon and serve as food source for organisms of higher trophic levels, such as crustaceans, mussels, other filtering and detritus feeding invertebrates or vertebrates and small fishes. In addition to their value as food for higher trophic organisms, algal photosynthesis and respiration can strongly influence water column oxygen dynamics [14]. Furthermore, they even play a role in nutrient cycling and serve as habitat for other organisms [15]. The well-being of microalgae is crucial for the maintenance of a healthy aquatic ecosystem. More importantly, their relatively higher sensitivity to micropollutants over fish and crustaceans make them popular in environmental toxicity studies [16]. Other advantages of algal assay's include simplicity, speed and cost effectiveness [2].

Despite of the advantages and recent popularity of application of microalgal bioassay on toxicity assessment, limitations still exist. Therefore, in this Ph.D. research, the gaps in the current algal toxicity experiment were identified and a new assay based on "community level physiological profiling (CLPP)" technique was developed. Quaternary ammonium compounds (QACs) were selected as a model micropollutant stress inducer. The potential of developed assay was further tested using water samples with complex compositions.

1.2 Literature Review and Research Gaps

1.2.1 Approach to Contaminated Sites

The risk-based approach is acknowledged in more detail through a 10-step process known as the steps for addressing a contaminated site, which is briefly explained in Figure 1.1 [1]. In addition, the Canadian environmental quality guidelines make important aspects in the approach as they set goals for the success of the remediation or risk management strategy.

The environmental quality guidelines exist for soil, water and sediments. Generic soil remediation criteria have been developed for four land uses: agricultural, residential/parkland, commercial and industrial. Similar, groundwater and surface water criteria have been established for four water uses: freshwater supporting aquatic life, water used for irrigation, livestock watering and human drinking water. Freshwater and marine/estuarine sediments' guideline present for a variety of contaminants. Toxicological studies based on bioassays using different organisms are playing a crucial role setting guideline baselines for different contaminants which are initially accessed by the environmental engineers for step 3 in Figure 1.1.

STEP 1: Identify Suspect Site – Identifies a potential contaminated site
STEP 2: Historical Review – Gathers past information pertaining to the site
STEP 3: Initial Testing Program – Provides a preliminary characterization of contamination and site conditions
STEP 4: Site Classification – Prioritizes the site based on the severity
STEP 5: Detailed Testing – Focuses on specific areas of concern identified in STEP 3 and performs in-depth investigation
STEP 6: Re-Site Classification – Updates severity ranking based on information from STEP 5
STEP 7: Remediation/Risk Management Strategy Development – Develops a plan to address contamination issues
STEP 8: Remediation/Risk Management Strategy Implementation – Implements a plan to address contamination issues
STEP 9: Final Sampling and Reporting – Confirms and documents the success of the management strategy
STEP 10: Long-Term Monitoring – Ensures the success of long-term management strategy

Figure 1.1 Steps for addressing a contaminated site (redrawn from, [1]).

1.2.2 Types of Bioassays

Commonly applied *in vitro* bioassays include the *umuC* and comet for genotoxicity, Ames for mutagenicity and YES (yeast estrogen screen) and YAS (yeast androgen screen) for estrogenicity and androgenicity detections [4]; these are briefly described here. The *umuC* was developed and published in 1985 [17]. A plasmid (pSK 1102) containing the *umuC* gene in association with the *lacZ* receptor gene was constructed and introduced into a gram-negative bacteria *Salmonella typhimurium* strain. The DNA damaging agents induce the

expression of *umuC* operon and therefore, the *lacZ* gene. The gene product of *lacZ* is β-galactosidase and the expression level reflecting genotoxicity was determined using a colorimetric dye by converting a colourless substrate called ONPG (o-nitrophenyl- β-D-galactopyranoside) into a yellow solid called o-nitrophenyl. The comet assay, also known as the single cell gel-electrophoresis assay, is commonly applied for identifying genotoxicity. It uses eukaryotic cells and involves the encapsulation of cells in a low-melting point agarose cell suspension, the lysis of the cell membrane in a neutral or alkaline (pH > 13) buffer and the electrophoresis of lysed cell samples [18]. By the exposure to a genotoxic agent, the damaged cells take an elongated form resembling the comet, unlike an intact round shape. The Ames test is based on the cell stains from *Salmonella typhimurium* auxotrophic mutants, which are characterized by a gene mutation that prevents the synthesis of L-histidine, an amino acid that is necessary for bacterial growth. By exposure of mutants to mutagens, a reversal of the mutation occurs and synthesize L-histidine for normal growth. The level of mutagenicity is indicated by the colonies counted following incubation [19]. Yeast estrogen and androgen screenings (YES and YAS) are based on a genetically modified *Saccharomyces cerevisiae* containing human estrogen and androgen receptors coupled to a reporter gene, *lacZ*. Endocrine disrupting compounds (EDCs) bind to a receptor and induce the synthesis of β-galactosidase, which can be detected using a colorimetric dye (chlorophenol red-β-D-galactopyranoside; CPRG) changing a color from yellow to purple [20].

Commonly used *in vivo* standardized tests are mainly bacteria luminescence inhibition assay [6], green microalgae growth assay [7], duckweed growth assay [8], Daphnia reproduction assay [9], and fish egg assay [10]. Most bacterial tests are based on the gram-negative bacillus *Vibrio fischeri*. It bioluminescences when it is metabolically active. The bacteria are obtained in a freeze-dried form and a solution of NaCl is added to create an osmotic pressure. The acute toxicity of a substance can be determined after incubation, bioluminescence level measurement and the calculation of the EC_{50} parameter by software. MICROTOX® (Modern Water, UK) is currently the most popular kit of its type in the market. Other types employ photosynthetic organisms, such as microalgae and duckweed. Toxicity experiments are most often based on the green algae and duckweed (*Lemna minor*) and determine a growth inhibition concentration EC_{50} of the toxic chemical. Another organism that

is frequently used as indicator is *Daphnia magna*. It is within the group of invertebrates and crustaceans and plays a very important role in the trophic chain, bridging the gap between the producers and consumers of higher orders [21]. Toxicity tests are conducted in a small container and the reproductive outputs are measured within a relatively short period of time, typically, 21 days. Lastly, ecotoxicological testing can be performed with fish. Among approximately 150 species, the most common is zebrafish (*Brachydanio rerio*). This is because it is a vertebrate model organism in genetics, neurophysiology and biomedicine and it is one of the first vertebrates whose genome was sequenced. Females can lay eggs every 2 to 3 days, at several hundred at a time and they are transparent making them easy to visualize their development in real time. Their development from embryo to adult usually takes 3 to 4 months [22].

1.2.3 Microalgal Bioassay

Bioassays using a single algal species are by far the most commonly used in which highly culturable algae are grown in toxicant media and growths are monitored over a predefined period [23]. Some of the single algal genera commonly used are *Chlorella*, *Scendedesmus*, *Selenastrum*, *Navicula*, *Spirulina*, *Anabaena* and *Microcystis* among the microalgae [2]. Alternatively, planktonic or benthic algal communities of natural origin are assessed in contrast to the unialgal approach [24]. The use of unialgal experiments is favored because of greater control and reproducibility; although, the validity of selecting one algal species to represent the response of a community can be questionable. The use of a natural algal community is better in a sense that it considers more than one species as even within a genus, some species may be more responsive indicators than others [25] but, more importantly, the natural site of concern is also taken into consideration [24].

While there is always a dose and response relationship with an effective concentration (e.g., EC_{50}, the concentration of a toxicant that gives half-maximal response), the response relationship may not produce conclusive evidence on the algal tolerance level based on their background. For example, Wang [24] noted similar range of EC_{50} values (1.8, 2.7 and 2.1 mg/L) after 24 hours exposure to Zn for natural algal communities collected from the Illinois River, Peoria (US) sewage treatment plant, and Farmington (US) sewage treatment

plant, respectively. Also, two chemically similar quaternary ammonium compounds produce almost equal EC_{50} levels of 0.041 and 0.021 mg/L for (*Pseudokirchneriella subcapitata*; formerly known as *Selenastrum capricornutum*) after 72 hours exposure to benzalkonium chlorides and didecyl dimethyl ammonium bromide, respectively [26]. Therefore, the need for improvement on EC_{50} as a measure of toxicity of a compound on a single species can be considered or else, measuring the change of a defined microbial community due to an environmental stress can be developed as a better diagnostic tool.

Algal assemblages have been successfully applied to monitor the impacts of aquatic stressors and aquatic toxicity [27]. For example, a shift in a naturally occurring microalgal community composition reflects an environmental change that can be used as an indicator to evaluate the changes in environmental conditions as had been tested earlier in marine [28], freshwater [29], and wetland ecosystems [30]. However, traditional ways of identifying an isolate and quantifying a species composition using cell morphology and molecular level RNA/DNA amplification techniques have limitations as they can be both time and cost intensive and require specialized expertise [31]. To overcome this limitation, a potential way to assess an impact on a microalgal community due to an environmental stress is to use functional or metabolic potential characterization employing the method of community level physiological profiling (CLPP).

1.2.4 Community Level Physiological Profiling (CLPP)

A functional or metabolic characterization of a microbial community is well developed after a method was first published by Garland and Mills [32]. The method is called community level physiological profiling (CLPP) and it uses a commercially available Biolog 96-well plate containing 95 different carbon sources. Different microbial communities are compared and classified based on carbon source utilization patterns (CSUPs) [32]. The relatively simple protocol and ease of use make it very practical for various applications. Based on the ISI database search using key words such as Biolog and community, about 1363 publications (prior to July 2017) on CLPP with an increasing trend were found.

CLPP has been applied on a variety of study fields and expanded from prokaryote

bacteria to eukaryotic fungi [33]. A perturbation or change in the microbial community has been observed in both terrestrial and aquatic environments, caused by a plant interaction [34], root secretion [35], spill of hydrocarbon [36], metal contamination [37], water pollution with acid mine drainage [38], and differently fed bluegills in guts [39]. Moreover, this technology has been further upgraded by the simultaneous use of antibiotics or cycloheximide to repress signal interferences from bacteria or fungi, respectively [40,41] and modified as an assay to study the community tolerance to an antibiotic sulfachloropyridazine using the pollution-induced community tolerance (PICT) concept [42] and the community toxicity to gold nanoparticles and ciprofloxacin [43].

Not only the community level, but, the metabolic profiling technology has also been established on single strains. Previously, it was used for the identification purpose on bacteria, yeast and fungi with databases present for different Biolog plates (available from Biolog, CA, USA). More recently, it has been applied on the isolates of microalgal strains from wastewater for the comparisons of organic carbon metabolic potentials [44] and on mammalian cells where it was found that human cancer cell lines from different organ tissues produced distinct profiles of metabolic activity [45]. The potential of the functional or metabolic characterization of individual or microbial community is enormous and continuously growing.

1.2.5 Quaternary Ammonium Compounds (QACs)

An emerging and not yet fully studied class of pollutants are so-called micropollutants. Micropollutants are chemicals present at very low concentrations and easily found in aquatic systems. Their effects on the aquatic environment have been very challenging to assess [46]. Among other micropollutants, surfactants are a diverse group of chemicals with high annual global production of about 14 million tons in 2008 with 2.8% annual increase to 2012 [47]. This makes surfactants a ubiquitous class of organic contaminants that are commonly found in aquatic ecosystems [48]. Recently, more attention was given to cationic surfactants, quaternary ammonium compounds (QACs). This is because they are known for the higher toxicity to living organisms than other anionic and nonionic ones [47].

Quaternary ammonium compounds (QACs) are large organic molecules having

molecular weights between 300 and 400 g/mol. They are typical cationic surfactants as they have a positively charged central nitrogen atom giving hydrophilicity. In case of hydrophobic regions, four functional groups are attached covalently to the positively charged central nitrogen atom (R4N+). These functional groups (R) include at least one long hydrocarbon chain and the rest are mostly short chain substituents such as methyl (in alkyl) or benzyl (in aryl) groups [49]. The length of a long alkyl chain confers QACs distinctive physical or chemical properties and led their widespread uses in agricultural, domestic, healthcare and industrial applications as pesticides, detergents, fabric softeners, personal care products, disinfectants, corrosion inhibitors, antistatic agents, biocides, emulsifiers and asphalt [50,51].

The extensive use of QACs in everyday applications make them ubiquitous contaminants and they are commonly found in sewage, sewage sludge, industrial wastewater, wastewater effluents, laundries and hospitals effluents, surface waters, and aquatic sediments [26,52]. The measured QACs concentrations are in the ranges of µg/L (Table 1.1) and were toxic to microalgae (Table 1.2). Although, the precise mechanisms are unclear [53], surfactant toxicity on algae appeared to work on outside of the cell by interfering with the phospholipid bilayer and altering the organization, stability and permeability of the membrane [54] and more importantly, on inside of the cell by corrupting thylakoid organization and chlorophyll synthesis with consequential impairment of photosynthetic capacity [55], leading to the cell death [56].

1.3 Research Objectives

Use of Biolog microplate for functional or metabolic characterization is prevalent in bacteria, yeast and fungi, however, it has not been well utilized in microalgal species and especially in toxicity studies. Because of their ecological importance and sensitivity, microalgae were selected as model organism and a single and community of microalgae were employed with a functional or metabolic characterization technique to assess the toxicity of a class of micropollutants, quaternary ammonium compounds (QACs) and the effects of various water sources with complex compositions. The range of concentrations for QACs present in various effluents and aquatic environment (Table 1.1) and the toxicity of QACs on microalgae (Table 1.2) are considered. The potential of a developed assay was further tested

using water samples with complex compositions collected from natural water, wastewater effluents and industrial wastewater. Often, it is difficult to differentiate the effects of waters from different water bodies using a standard single algae growth inhibition test. The need for improvement on EC_{50} as a measure of toxicity is better assessed using a community level physiological profiling (CLPP) technique. In addition, the toxicity effects under mixotrophic and heterotrophic growth conditions also will be evaluated, given the fact that the aquatic environment contains a mixture of organic and inorganic carbon sources. The specific objectives, which constitute the respective chapters of this thesis, are:
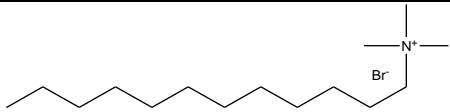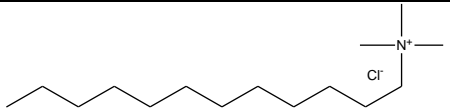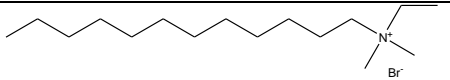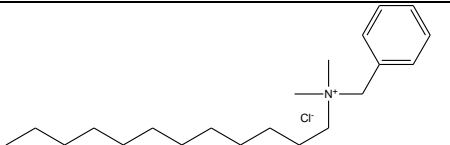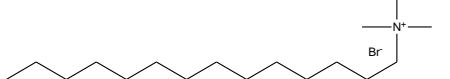
I. Assess the effects of quaternary ammonium compounds (QACs) on *Scenedesmus obliquus* using Biolog plates under light and dark conditions (Chapter 2)

II. Develop microalgal bioassay based on the community level physiological profiling (CLPP) (Chapter 3)

III. Assess water samples with complex compositions using microalgal bioassay based on the community level physiological profiling (CLPP) (Chapter 4)
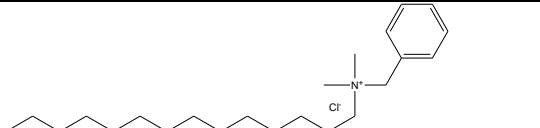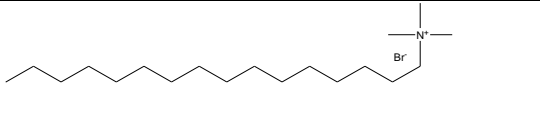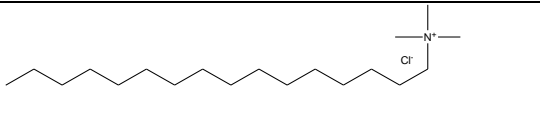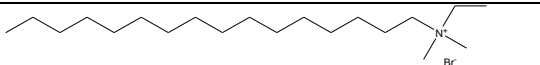
Table 1.1 Concentrations of QACs in influent and effluent of wastewater, effluent of hospital, surface, river, sea water were found from different literatures.
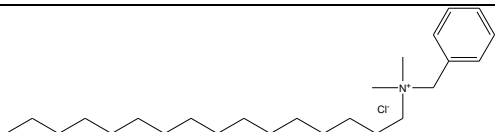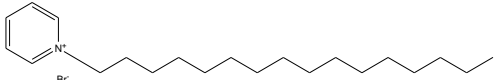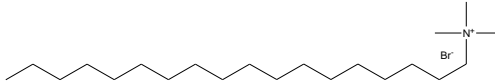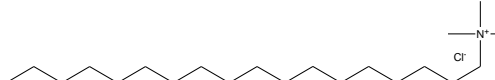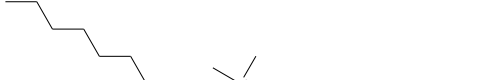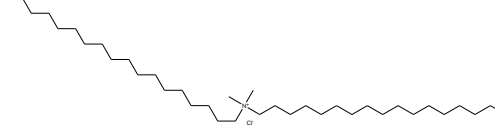
QACs	Concentration (mg/L)	Details	Reference
DTDMAC	0.01	in the river Rhine	[57]
	0.006	at Lobith	[57]
	0.017 - 0.025	in the river Meuse	[57]
	2	influents of Dutch sewage water	[57]
	0.7	in sewage	[58]
	0.062	sewage effluents	[59]
Monoalkonium chloride	< 0.002	several rivers in the United States	[60]
Dialkonium chlorides	0.35 - 0.48	in sewage collected in Germany	[61]
	0.006 - 0.012	surface water in Germany	[61]
	0.002	Millers River (MA); at various distances downstream from wastewater treatment facilities	[62]
	0.024	Otter River (MA); at various distances downstream from wastewater treatment facilities	[62]
	0.017	Blackstone River (MA); at various distances downstream from wastewater treatment facilities	[62]
	0.033	Rapid Creek (SD); at various distances downstream from wastewater treatment facilities	[62]
Benzalkonium chloride	4 - 5	hospital effluent water	[63]
	2.8	hospital	[26]
	0.17	influent	[26]
	0.0041	effluent of the treatment plants	[26]
Mixed QACs	1	in sewage	[58]
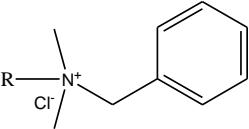
	0.05 - 0.1	in municipal sewage	[63]
	n.d. - 0.075	surface, river, sea water	[47]
	0.005 - 0.02	Main river in Germany	[64]
	0.05	river	[65]
	0.01 - 0.02	river	[66]

Table 1.2 Effective concentrations of 50% inhibition (EC$_{50}$) and 10% inhibition (EC$_{10}$) were obtained from other studies for microalgal species under different quaternary ammonium compounds (QACs) and exposure duration. QACs are ordered in terms of hydrophobic chain length (C$_{12}$ top to C$_{18}$ bottom).

QACs	Molecular structure	Duration (hours)	EC$_{50}$ (mg/L)	EC$_{10}$ (mg/L)	Algal species	Reference
DTAB		96	1.50	-	Chlorella pyrenoidosa	[67]
		96	0.55	-	Scenedesmus quadricauda	[67]
		96	0.19	-	Chlorella vulgaris (FACHB-6)	[68]
DTAC		96	1.36	-	Chlorella pyrenoidosa	[67]
		96	0.50	-	Scenedesmus quadricauda	[67]
EDDAB		96	0.20	-	Chlorella vulgaris (FACHB-6)	[68]
BDDAC		96	0.20	-	Chlorella vulgaris (FACHB-6)	[68]
TTAB		96	0.38	-	Chlorella pyrenoidosa	[67]
		96	0.32	-	Scenedesmus quadricauda	[67]
		96	0.18	-	Chlorella vulgaris (FACHB-6)	[68]

BDTAC		96	0.17	-	*Chlorella vulgaris* (FACHB-6)	[68]
CTAB		96	0.17	-	*Chlorella pyrenoidosa*	[67]
		96	0.36	-	*Scenedesmus quadricauda*	[67]
		144	0.32	0.034	*Chlorella vulgaris*	[69]
		96	0.16	-	*Chlorella vulgaris* (FACHB-6)	[68]
		96	0.09	-	*Pseudokirchneriella subcapitata*	[70]
		96	0.03	-	*Microcystis aeruginosa*	[70]
CTAC		96	0.17	-	*Chlorella pyrenoidosa*	[67]
		96	0.22	-	*Scenedesmus quadricauda*	[67]
		96	0.15	0.022	*Chlorella vulgaris*	[71]
		96	0.14	-	*Chlorella vulgaris* (FACHB-6)	[68]
		240	2.80	0.41	*Dunaliella bardawil* (UTEX 200)	[72]
		72	0.78	-	*Pseudokirchneriella subcapitata*	[73]
		96	0.20	-	*Chlorella vulgaris* (FACHB-6)	[74]
EHDAB		96	0.12	-	*Chlorella vulgaris*	[68]

					(FACHB-6)	
BDHAC		96	0.16	-	*Chlorella vulgaris* (FACHB-6)	[68]
CPB		96	0.13	-	*Chlorella vulgaris* (FACHB-6)	[68]
STAB		96	0.10	-	*Chlorella pyrenoidosa*	[67]
		96	0.15	-	*Scenedesmus quadricauda*	[67]
		96	0.11	-	*Chlorella vulgaris* (FACHB-6)	[68]
STAC		96	0.19	-	*Chlorella pyrenoidosa*	[67]
		96	0.58	-	*Scenedesmus quadricauda*	[67]
DDAB		72	0.021	-	*Pseudokirchneriella subcapitata*	[26]
DTDMAC		4	0.51	0.04	Natural community	[57]
		96	0.06	-	*Pseudokirchneriella subcapitata*	[70]
		96	0.05	-	*Microcystis*	[70]

					aeruginosa	
		96	0.07	-	*Navicula pelliculosa*	[70]
BAC		72	0.041	-	*Pseudokirchneriella subcapitata*	[26]

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Chapter 2. Assessment of the effects of quaternary ammonium compounds (QACs) on *Scenedesmus obliquus* using Biolog plates under light and dark conditions

[A revised version of this chapter has been prepared for submission to Journal of Aquatic Toxicology]

Abstract

Toxicity effects of two quaternary ammonium compounds (QACs), dodecyl trimethyl ammonium chloride (DTAC) and didecyl dimethyl ammonium bromide (DDAB), were assessed using a freshwater microalgal strain, *Scenedesmus obliquus*. The toxicity experiments were conducted under three different conditions; autotrophic, mixotrophic and heterotrophic. Mixotrophic and heterotrophic conditions were carried out by utilizing YT Biolog plates. Data were analyzed using effective concentration at 50% (EC_{50}) for all growth conditions and the effects of DTAC and DDAB were further analyzed for the differences in the growth inhibitions for different individual organic carbons, as well in the generated profiles of effective concentrations (EC_{50}) for mixotrophic and heterotrophic conditions. In all the growth regimes, the significant toxicity effects of DTAC and DDAB were conclusive with EC_{50} values of 0.48 ± 0.03 mg of DTAC/L and 1.18 ± 0.08 mg of DDAB/L for autotrophic conditions, 2.11 ± 0.06 mg of DTAC/L and 1.35 ± 0.02 mg of DDAB/L for mixotrophic, and 1.46 ± 0.04 mg of DTAC/L and 0.52 ± 0.02 mg of DDAB/L for heterotrophic conditions on fifth day of treatment (120 hours). If compared, DTAC inhibited the growth of *Scenedesmus obliquus* most in autotrophic followed by heterotrophic and mixotrophic conditions. However, for DDAB, the order was different as heterotrophic being the most sensitive followed by autotrophic and mixotrophic conditions. In addition, the toxicity effects were significantly different ($P < 0.001$) depending on the organic carbon that was utilized by *Scenedesmus obliquus*. Moreover, in the presence of organic carbon, the light made *Scenedesmus obliquus* less sensitive to DTAC and DDAB. Finally, the differences in the toxicity effects of DTAC and DDAB under mixotrophic and heterotrophic conditions were further tested by forming four different profiles of effective concentrations (EC_{50}) for different individual organic carbons.

2.1 Introduction

The presence of micropollutants in aquatic systems is receiving attention of scientific community as their effects on the aquatic environment are very challenging to assess [1]. Among the diverse groups of micropollutants, quaternary ammonium compounds (QACs) deserve special attention due to their many applications as surfactants, emulsifiers, asphalt, fabric softeners, disinfectants, pesticides, corrosion inhibitors and personal care products used in agricultural, domestic, healthcare and industrial applications [2, 3, 4]. QACs present a low degree of selectivity in their biocidal action against different types of microorganisms; i.e. bacteria, viruses, fungi, protozoa, and algae [5]. In addition, QACs promote bacterial strains to be resistant to clinically important antimicrobial agents, a potential risk for human health [6].

Typically, surface water is mainly exposed to QACs-containing residual products by the discharge of sewage effluents [7, 8] and they are found in the various aquatic environments at µg/L to mg/L levels [9, 10]. This could pose negative effects on aquatic ecosystems [7] as toxicity tests using standard test organisms revealed that concentration ranges found in different environments are toxic to a wide variety of aquatic organisms, including algae, fish, mollusks, barnacles, rotifers, starfish and shrimp [11].

Among standard test specimens, microalgae are being used increasingly for bioassay in QACs' toxicity studies [12, 13, 14]. As a primary producer and also as a food source for organisms of higher trophic levels, well-being of microalgae is crucial for the maintenance of a healthy aquatic ecosystem. More importantly, their relatively higher sensitivity over daphnids, rotifers, protozoans and fish make them popular in various toxicity studies [9, 15].

Microalgal toxicity studies in the past on QACs were conducted primarily on the growth inhibition tests under autotrophic conditions following the standard guidelines [16, 17] and the toxicity effects were evaluated by means of effective concentration (EC) [12, 13, 14]. Although, it seems to be a relatively simple protocol, a significant drawback is that equal EC levels could be derived from two different compounds within the same group of QACs [9].

Moreover, toxicity effects of QACs under mixotrophic and heterotrophic growth conditions were not evaluated, given the fact that the aquatic environment contains a mixture of organic and inorganic carbon sources.
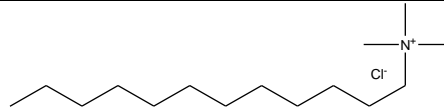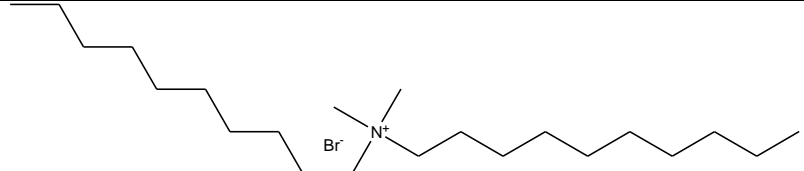
Therefore, in this study, microalgae toxicity experiments were conducted not only under autotrophic growth condition but also, mixotrophic and heterotrophic conditions by the use of Biolog plates. The use of Biolog plates allowed to quantify EC_{50}'s for 57 different carbon sources. The effects of two quaternary ammonium compounds (QACs), dodecyl trimethyl ammonium chloride (DTAC) and didecyl dimethyl ammonium bromide (DDAB), were evaluated in various ways using a freshwater algae strain, *Scenedesmus obliquus*. This species was selected as it is a standard test species used in several occasions for the algal toxicity experiments [18, 19, 20, 21].

2.2 Materials and Methods

2.2.1 Chemicals

Dodecyl trimethyl ammonium chloride (DTAC; purity ≥ 99%; CAS No. 112-00-5) and didecyl dimethyl ammonium bromide (DDAB; 98% purity; CAS No. 2390-68-3) were purchased from Sigma-Aldrich (MO, USA). These chemicals were selected among the other quaternary ammonium compounds (QACs) due to their distinct molecular structures, DTAC with one and DDAB with two long hydrocarbon chains (Table 2.1). The solutions were prepared using Milli-Q water (Millipore, Toronto, ON, Canada).

Table 2.1 Quaternary ammonium compounds (QACs), synonyms, molecular structures (MS) and molecular weights (MW) are shown. Molecular structures were drawn by ChemDraw® Ultra (version 8.0).

Quaternary ammonium compounds (QACs)	Synonym	MS	MW (g/mol)
Dodecyl trimethyl ammonium chloride	DTAC		263.89
Didecyl dimethyl ammonium bromide	DDAB		406.54

2.2.2 Microalgal strain and maintenance

An axenic culture of freshwater green algae, *Scenedesmus obliquus* (CPCC 5), was obtained from the Canadian Phycological Culture Centre. The culture was maintained in a modified HS (high salt) minimal medium [22] with 0.8 g of NaHCO$_3$/L [23] at 25°C on shaking (150 rpm) with a light intensity of 140 µE m^{-2} s^{-1} (16 hrs. Light: 8 hrs. dark) using an incubator (Infors HT Multitron, Basel, Switzerland). The algal growth was determined routinely measuring optical density (OD) at 600 nm with a 200 pro infinite series microplate reader (Tecan, Männedorf, Switzerland). Cells in the exponential growth phase were used to calculate the specific growth rate (0.82 ± 0.08 d^{-1}, n = 3) and to use for the toxicity experiment. The cell density (cells mL^{-1}) was measured using a hemocytometer (Hausser Scientific, PA, USA) and the correlation of optical density versus cell count was generated (Appendix A). To maintain axenic condition, the culture was checked regularly for the bacterial contamination by streaking onto nutrient agar plate and no contamination was found throughout the course of experiments.

2.2.3 Toxicity tests on microalgae

The toxicity test was conducted under three different conditions; autotrophic, mixotrophic and heterotrophic; autotrophic condition, according to the standard protocol of Organisation for Economic Cooperation and Development [16] with modifications on the cell density and use of 96 microplates and mixotrophic and heterotrophic conditions using Biolog plates.

2.2.3.1 Autotrophic toxicity experiments

Inhibition efficiency tests in growth (%) were conducted using DTAC and DDAB concentrations 0, 400, 800, 1200, 1600, 2000 and 2400 µg/L. The tests were performed in 96 microplates with a final volume of 150 µL and incubated as described above. *Scenedesmus obliquus* in exponential phase was transferred to wells containing different concentrations of DTAC and DDAB. Wells without DTAC and DDAB served as a control. A cell density of 1×10^6 cells/mL was used and experiments were repeated eight times (n = 8). The growth inhibition

was calculated using the difference in fluorescence readings (excitation at 470 nm and emission at 650 nm; [24]) using a M1000 pro infinite series microplate reader (Tecan, Männedorf, Switzerland) on the fifth day of treatment.

2.2.3.2 Mixotrophic and heterotrophic toxicity experiments

The toxicity tests were performed using Biolog plates. Prefilled 96-well microtiter plates (YT microplate; Biolog, CA, USA) were used under different treatment conditions. The YT Biolog plates were selected as they have both sections of wells with or without included tetrazolium dye (Biolog YT microplate; available from Biolog, CA, USA) and only 57 bottom wells without tetrazolium dye were used for the analysis as tetrazolium dye can be light sensitive (Biolog redox dye mixes; available from Biolog, CA, USA). Organic carbons and sources in the Biolog plates were described in Table 2.2. The same cell density of 1×10^6 cells/mL was exposed to final concentrations of 0, 400, 800, 1200, 1600, 2000 and 2400 µg/L of DTAC and DDAB for mixotrophic and without two higher concentrations for heterotrophic toxicity test. The Biolog plates contained multichannel pipetted 100 µL of mixed *Scenedesmus obliquus* in 50 µL of modified HS media with a compound, either DTAC or DDAB. The Biolog plates were kept in dark at 25°C for heterotrophic condition and incubated as above for mixotrophic condition. Measurements of growths were determined using a M1000 pro infinite series microplate reader (Tecan, Männedorf, Switzerland) measuring fluorescence (excitation at 470 nm and emission at 650 nm; [24]). The fluorescence measurement reflecting the growth was successfully used in this study for their known sensitivity to the quaternary ammonium compound in microalgae [25], even at a lower pigment concentration per cell under dark than light conditions [26]. Toxicity on the fifth day of treatment was used for further analysis as it showed the prominent differences in growth from the control in mixotrophic conditions and allowed the growth of the heterotrophic ones for the toxicity effects to be effective. Moreover, the growth was in actively growing conditions on fifth day of treatment. Experiments were conducted in triplicates (n = 3).

Table 2.2 Organic carbons and sources in YT Biolog plates in 57 bottom wells are presented. Total represents the number of organic carbons in each group of sources. One well is for negative control included water.

Carbon source	Chemical	Total
Carbohydrate	D-Cellobiose, Gentiobiose, Maltose, Maltotriose, D-Melezitose, D-Melibiose, Palatinose, D-Raffinose, Stachyose, Sucrose, D-Trehalose, Turanose, α-D-Glucose, D-Galactose, D-Psicose, L-Rhamnose, L-Sorbose, α-Methyl-D-Glucoside, β- Methyl-D-Glucoside, Amygdalin, Arbutin, Salicin, Maltitol, D-Mannitol, D-Sorbitol, Adonitol, D-Arabitol, Xylitol, i-Erythritol, Glycerol, L-Arabinose, D-Arabinose, D-Ribose, D-Xylose	34
Carboxylic acid	Fumaric Acid, L-Malic Acid, Bromo-Succinic Acid, γ-Amino-Butyric Acid, α-Keto-Glutaric Acid, 2- Keto-D-Gluconic Acid, D-Gluconic Acid	7
Amino acid	L-Glutamic Acid	1
Fatty acid	Tween 80	1
Polymer	Dextrin, Inulin	2
Ester	Succinic Acid Mono-Methyl Ester	1
Combination	Succinic Acid Mono-Methyl Ester plus D-Xylose, N-Acetyl-L-Glutamic Acid plus D-Xylose, Quinic Acid plus D-Xylose, D-Glucuronic Acid plus D-Xylose, Dextrin plus D-Xylose, α-D-Lactose plus D-Xylose, D-Melibiose plus D-Xylose, D-Galactose plus D-Xylose, m-Inositol plus D-Xylose, 1,2-Propanediol plus D-Xylose, Acetoin plus D-Xylose	11

2.2.4 Analysis

The raw well fluorescence development measured through fifth day of treatment was corrected by subtracting the initial fluorescence readings at t = 0 day and corrected data on fifth day of treatment were used for further analyses for mixotrophic and heterotrophic conditions. Inhibition efficiency tests in growth (%) and effective concentrations at 50% (EC_{50}, the concentration of a toxicant that gives half-maximal response) were calculated according to the standard protocol of Organisation for Economic Cooperation and Development [16]. EC_{50} parameters were estimated using the resulting sigmoidal dose-response curve. The data was analyzed using the Hill equation ($\% \, Inh. = \, a + \frac{a-b}{1+(X_{QAC}/EC_{50})^{-m}}$) following the method described by Huber [27]; where % inh. is the percentage of growth inhibition, X_{QAC} is the QAC concentration [mg/L] and a, b, m and EC_{50} are fitting parameters representing the low response, high response, Hill-slope, and the half maximal effective concentration [mg/L]. The parameters were estimated using nonlinear regression analysis implemented in Matlab (Matlab 2016b MathWorks, Natick, MA), see Appendix B for code.

2.3 Results and Discussion

2.3.1 Autotrophic toxicity experiments

Initial toxicity experiments on *Scenedesmus obliquus* were conducted in an autotrophic condition and the growth inhibitions in percentage were determined following the guideline [16]. The growth inhibitions (%) were plotted against the varying DTAC and DDAB concentrations (Figure 2.1) and EC_{50} of 0.43 ± 0.03 mg of DTAC/L and 1.10 ± 0.08 mg of DDAB/L were derived on fifth day of treatment (120 hours) by fitting the data using Hill equation. Similar levels of EC_{50} were noted previously; for example, EC_{50} of 0.50 mg of DTAC/L of *Scenedesmus quadricauda* and 1.36 mg of DTAC/L of *Chlorella pyrenoidosa* were found after 96 hours [13], although, a much lesser amount for DDAB of 0.021 mg/L for *Pseudokirchneriella subcapitata* was found after 72 hours [9]. The values are within the findings of other freshwater algae with similar molecular structures of quaternary ammonium compounds (QACs); 0.19, 0.55 and 1.50 mg of DTAB (bromide attached instead of chloride)/L for *Chlorella*

vulgaris, *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*, respectively, after 96 hours [13, 14] and 0.51 mg of DTDMAC/L for natural community after 4 hours [10]. The variations in EC_{50} could be due to several reasons such as variations in species tested, as some are more sensitive than others [28], duration, cell density used [29], and a matrix used for EC_{50} calculation. Typically, EC_{50} values based on final yield were generally lower and could differ by a factor of 2 compared with those based on growth rate [30].
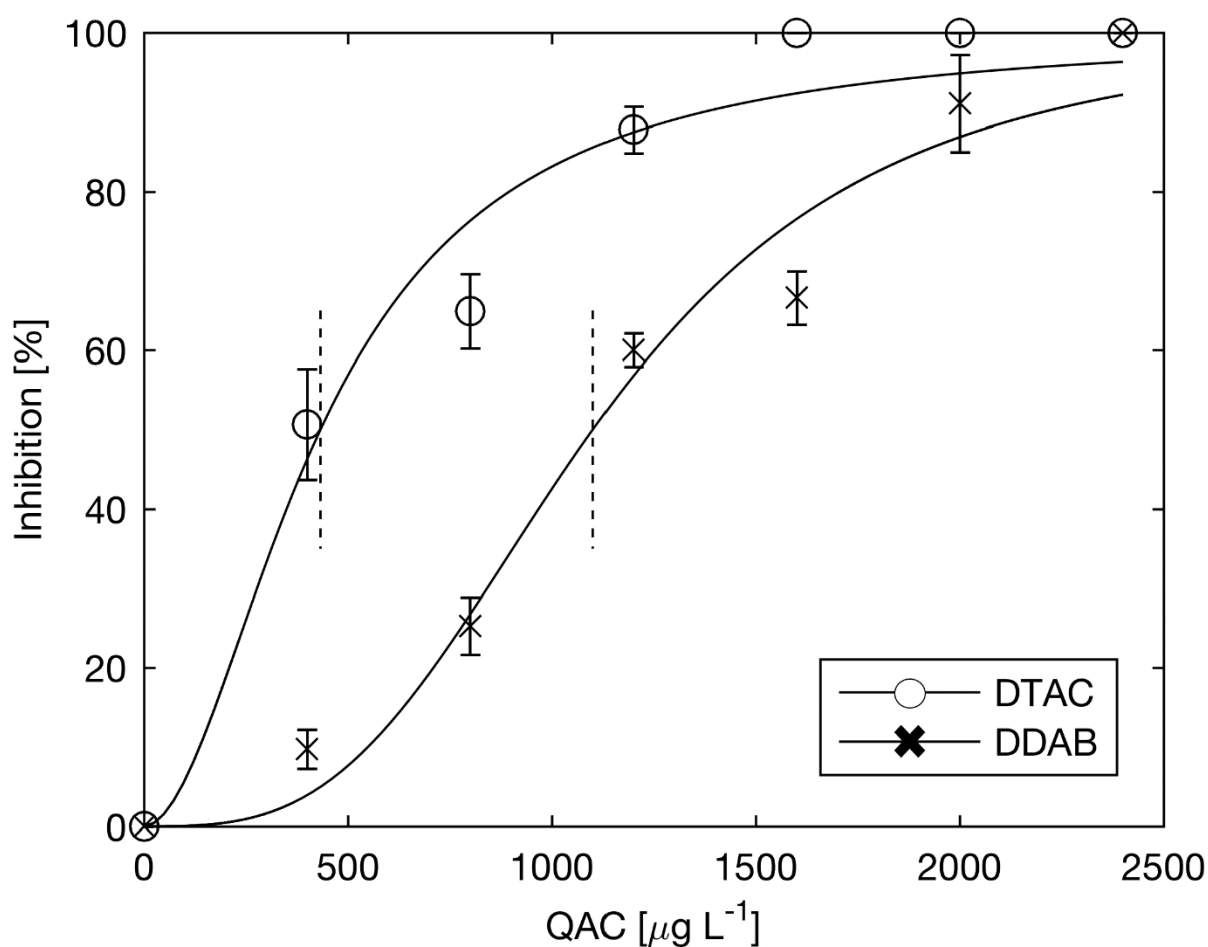
Figure 2.1 The percentage growth inhibition for *Scenedesmus obliquus* in an autotrophic condition. Cells were grown with 400, 800, 1200, 1600, 2000 and 2400 µg/L of DTAC and DDAB. Fluorescence readings measured on the fifth day of treatment were used for the calculations. Experiments were replicated eight times (n = 8). The error bars represent the standard deviations. The solid lines represent the best fit solution of the Hill equation and the vertical dashed lines indicate the estimated EC_{50} concentration.

2.3.2 Mixotrophic and heterotrophic toxicity experiments

Data collected using Biolog plates were analyzed for the differences in the response to the varying DTAC and DDAB concentrations in several different levels. Toxicity effects were assessed by using (i) average well fluorescence development (AWFD), which gives an average growth response over total wells in general, and calculated effective concentration (EC_{50}), (ii) different individual organic carbons and their growth inhibitions and (iii) profiles of effective concentration (EC_{50}). The results of three different analyses are presented below.

2.3.2.1 Average well fluorescence development (AWFD) and EC_{50}

Toxicity effects were first assessed by using an average well fluorescence development (AWFD) as a function of time in order to estimate a suitable time to conduct dose-response studies with goal to capture the effect on a maximum number of carbon sources (Figure 2.2). Gradual growth inhibitions were apparent with higher concentrations of DTAC and DDAB, although greater inhibitions occurred with DDAB in both mixotrophic (Figure 2.2b) and heterotrophic conditions (Figure 2.2d). Similar to the autotrophic condition, fifth day of treatment (120 hours) was selected and growth inhibitions in percentage were determined using AWFDs and plotted against the varying DTAC and DDAB concentrations for mixotrophic (Figure 2.3a) and heterotrophic conditions (Figure 2.3b). The data clearly do not follow a sigmoidal trend, as the presented curves are simply the average of 57 sigmoidal curves. The key assumption of the mechanism of the response needed for the Hill equation is therefore not met and the Hill model cannot be used to estimate a 'global' EC_{50}. Polynomial interpolation was therefore used to estimate apparent EC_{50} values of 2.11 ± 0.06 mg of DTAC/L and 1.35 ± 0.02 mg of DDAB/L for mixotrophic, and 1.46 ± 0.04 mg of DTAC/L and 0.52 ± 0.02 mg of DDAB/L for heterotrophic conditions (Figure 2.3). These results present that *Scenedesmus obliquus* is more sensitive to DDAB than DTAC (EC_{50} is 1.6 to 3 fold lower) and the toxicity effect is greater under heterotrophic than mixotrophic conditions (EC_{50} is 3 fold lower). In addition, the EC_{50} values vary significantly with the metabolic conditions and are very compound specific. For example, the inhibition effect of DTAC on the growth of *Scenedesmus obliquus* followed the order: autotrophic (EC_{50} of 0.48 ± 0.03 mg of DTAC/L) > heterotrophic (1.46 ± 0.04 mg of DTAC/L) > mixotrophic (2.11 ± 0.06 mg of DTAC/L). Whereas

for DDAB, the order of inhibition is different as: heterotrophic (0.52 ± 0.02 mg of DDAB/L) > autotrophic (1.18 ± 0.08 mg of DDAB/L) > mixotrophic (1.35 ± 0.02 mg of DDAB/L). This difference could be due to the distinct molecular mechanisms behind DTAC and DDAB toxicity. Although, the precise mechanisms are unclear [31], surfactant toxicity on algae appeared to work on outside of the cell by interfering with the phospholipid bilayer and altering the organization, stability and permeability of the membrane [32], and more importantly, on inside of the cell by corrupting thylakoid organization and chlorophyll synthesis with consequential impairment of photosynthetic capacity [33], leading to the cell death [34]. We speculate that the toxicity effects are more on inside of the *Scenedesmus obliquus* for DTAC and on outside for DDAB and in fact, this corresponds with the molecular structures and weights of relatively smaller DTAC (molecular weight (MW) of 263.89 g/mol) with one and larger DDAB (MW of 406.54 g/mol) with two long hydrocarbon chains (Table 2.1).

Moreover, it was interesting to note that under the same condition of light, EC_{50} of mixotrophic settled above that of autotrophic for DTAC and DDAB. This suggests that the toxicity effects might have been alleviated by gaining energy through organic carbon sources in mixotrophic conditions. Similarly, *Chlamydomonas reinhardtii* cells under atrazine stress conditions (causing photosynthesis inhibition) maintained viability by changing their photosynthetic metabolism to a heterotrophic one by upregulating genes involved in the respiratory electron transport of the mitochondria [35]. Moreover, less susceptibility to photoinhibition for mixotrophic cultures might have been an extra advantage, when photosynthetic capacity was impaired. It was shown that tris–acetate–phosphate grown mixotrophic *Chlamydomonas reinhardtii* cultures were less susceptible to photoinhibition than photoautotrophic cultures when subjected to high light as thylakoids from mixotrophic *C. reinhardtii* produced less O_2 than those from photoautotrophic cultures [36].
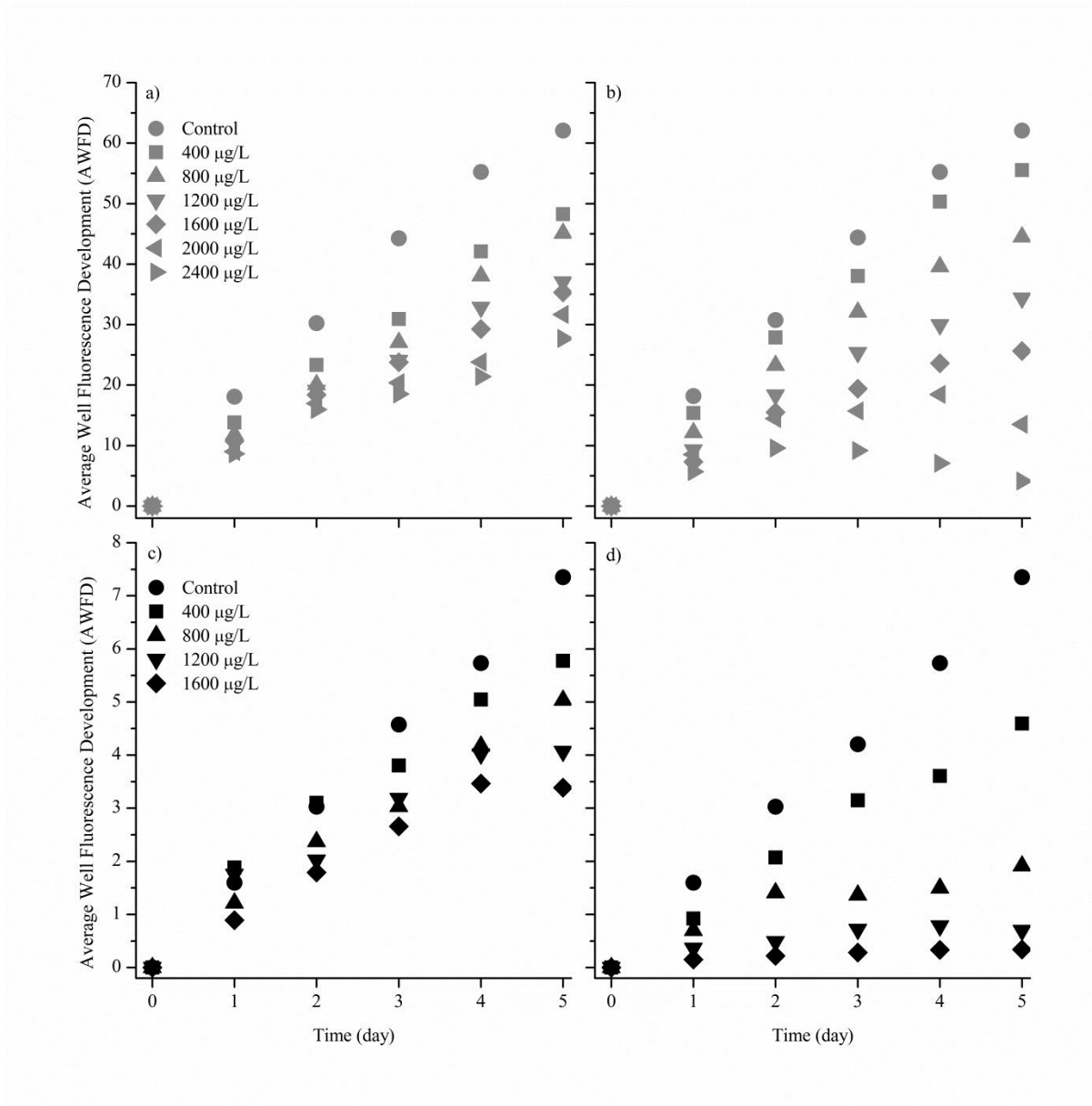
Figure 2.2 Toxicity tests on *Scenedesmus obliquus* using Biolog YT plates were conducted under 400, 800, 1200, 1600, 2000 and 2400 µg/L of DTAC and DDAB for mixotrophic and without two higher concentrations for heterotrophic conditions. Average well fluorescence developments (AWFD) are shown through fifth day. a) and c) are shown for the treatment with different concentrations of DTAC under mixotrophic and heterotrophic regimes, respectively, and likewise b) and d) are for DDAB. The average readings are of triplicates (n = 3) and standard deviations are within 10%. The control represents no treatment.
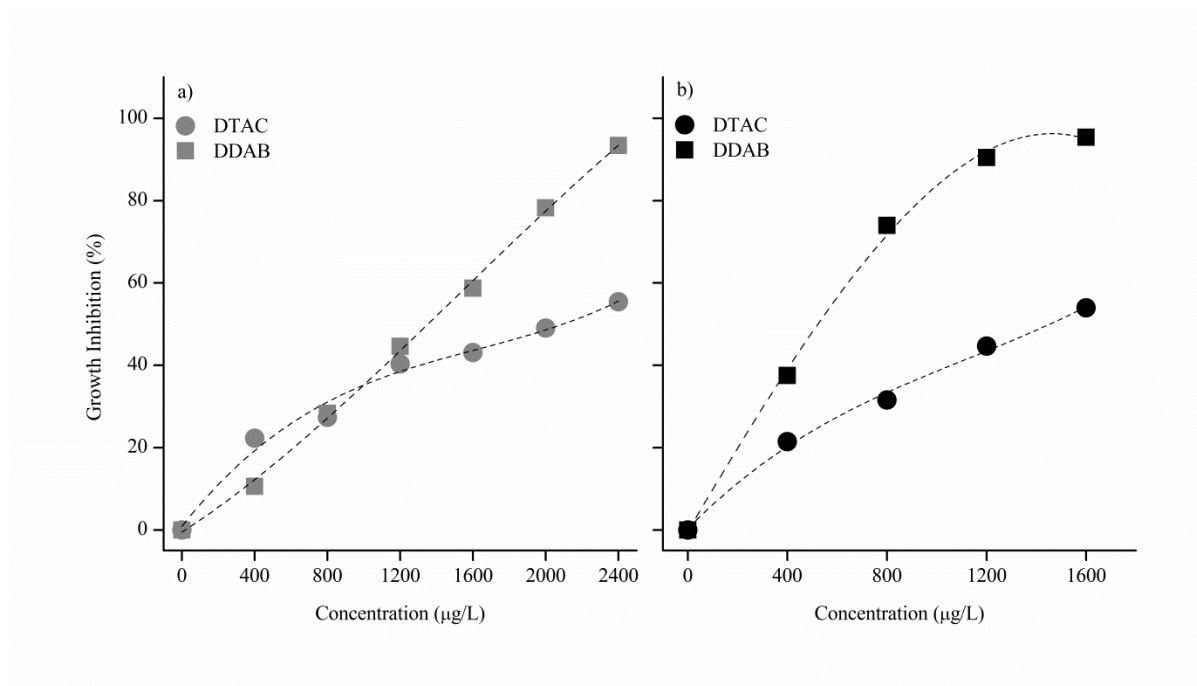
Figure 2.3 Toxicity tests on *Scenedesmus obliquus* using Biolog YT plates were conducted under 400, 800, 1200, 1600, 2000 and 2400 µg/L of DTAC and DDAB for mixotrophic and without two higher concentrations for heterotrophic conditions. The growth inhibitions in percentage are shown for different growth regimes a) mixotrophic and b) heterotrophic. Average well fluorescence developments (AWFD) on fifth day of treatment were used for the calculations. The average of triplicates are shown and standard deviations are within 10%. The dash lines were obtained by fitting the data using a polynomial function in Origin 8.

2.3.2.2. Different individual organic carbons and their growth inhibitions

Toxicity effects were assessed in more detail by estimating EC_{50} values of the two QACs for heterotrophic and mixotrophic growth on different individual organic carbons as shown in Figure 2.4. It can be seen that the individual dataset can be approximated well with the Hill equation. In addition, the degrees of inhibitions were different upon which organic carbon substrates were used by *Scenedesmus obliquus*. A similar finding was observed on the toxicity test of metals of aluminium and copper on two bacterial strains of *Pseudomonads*, *P. pseudoalcaligenes* KF707 and *P. fluorescens*, under different carbon sources [37]. It was found that quality of carbon source greatly influences the amount of metal that each bacterium can tolerate and increases in carbon source complexity resulted in a decrease in growth when

metal concentrations approached inhibitory levels, the investment required to enter central metabolism decreases with less complex carbons [37]. Likewise, in yeast cells, the toxicity effect of mercaptoethanol was shown to be dependent on the tested carbon sources (methanol, glucose and yeast extract) for the wild-type and mutant strain ecr1 of *Pichia methanolica* [38].

b)

Growth Inhibition (%)

Concentration (μg/L)

c)

Growth Inhibition (%)
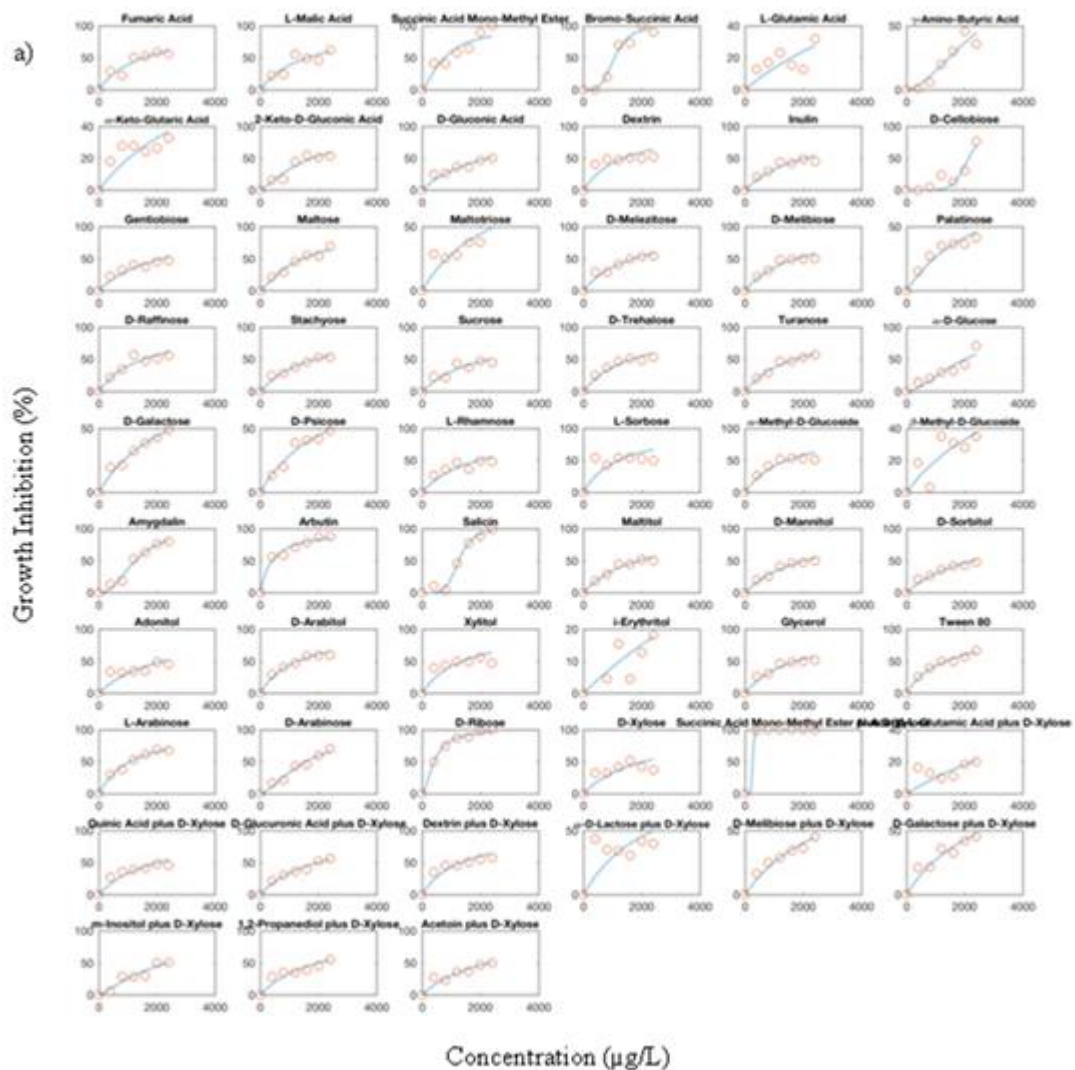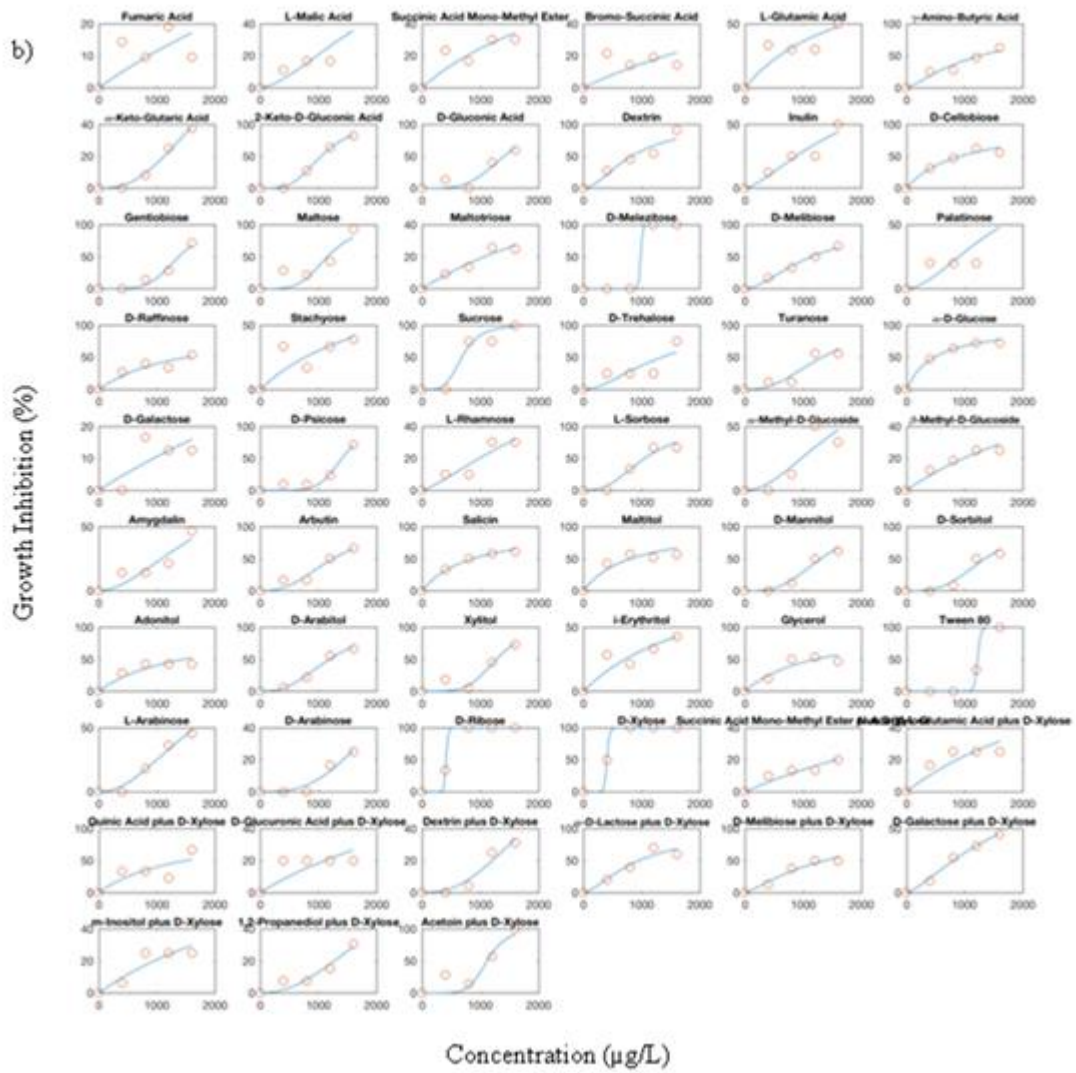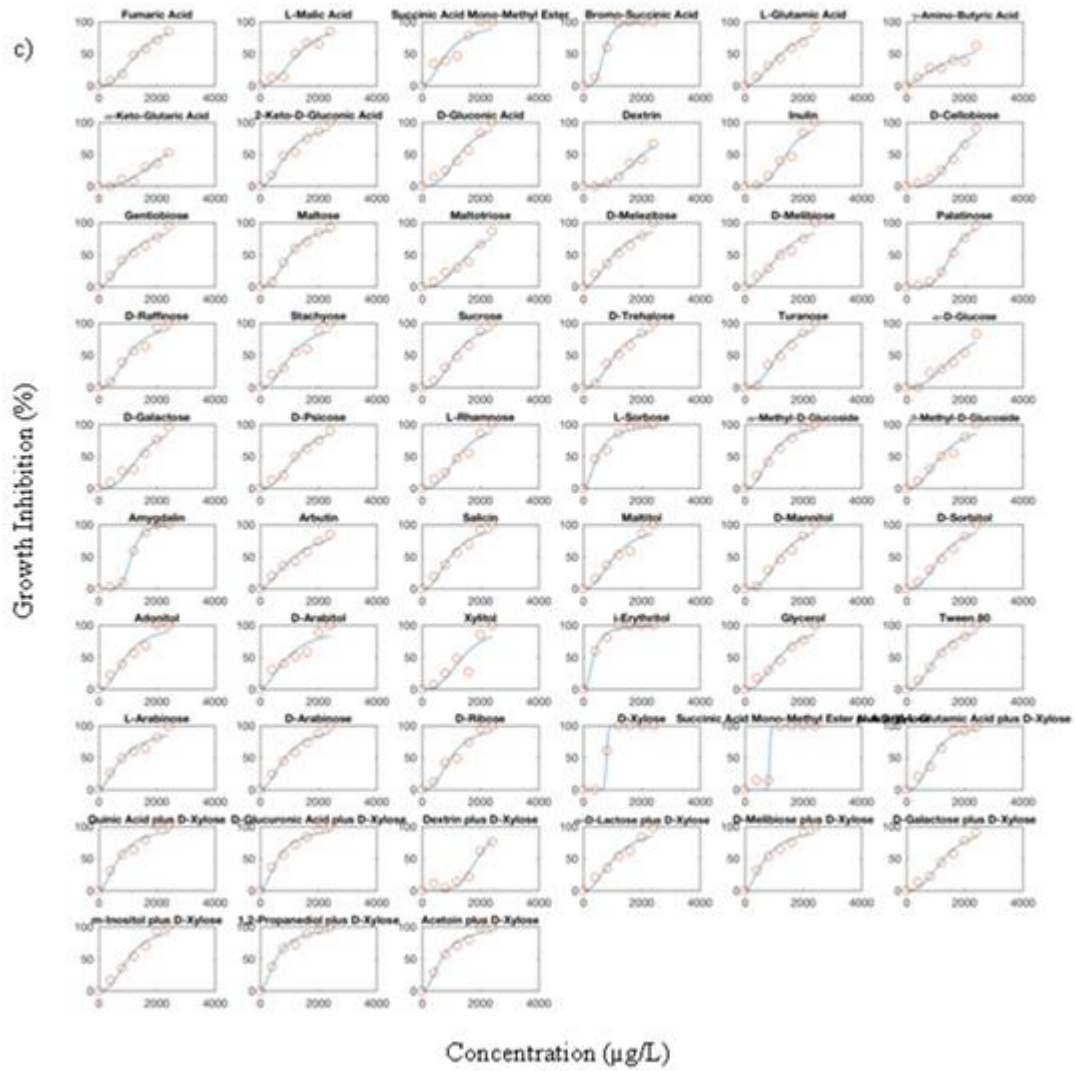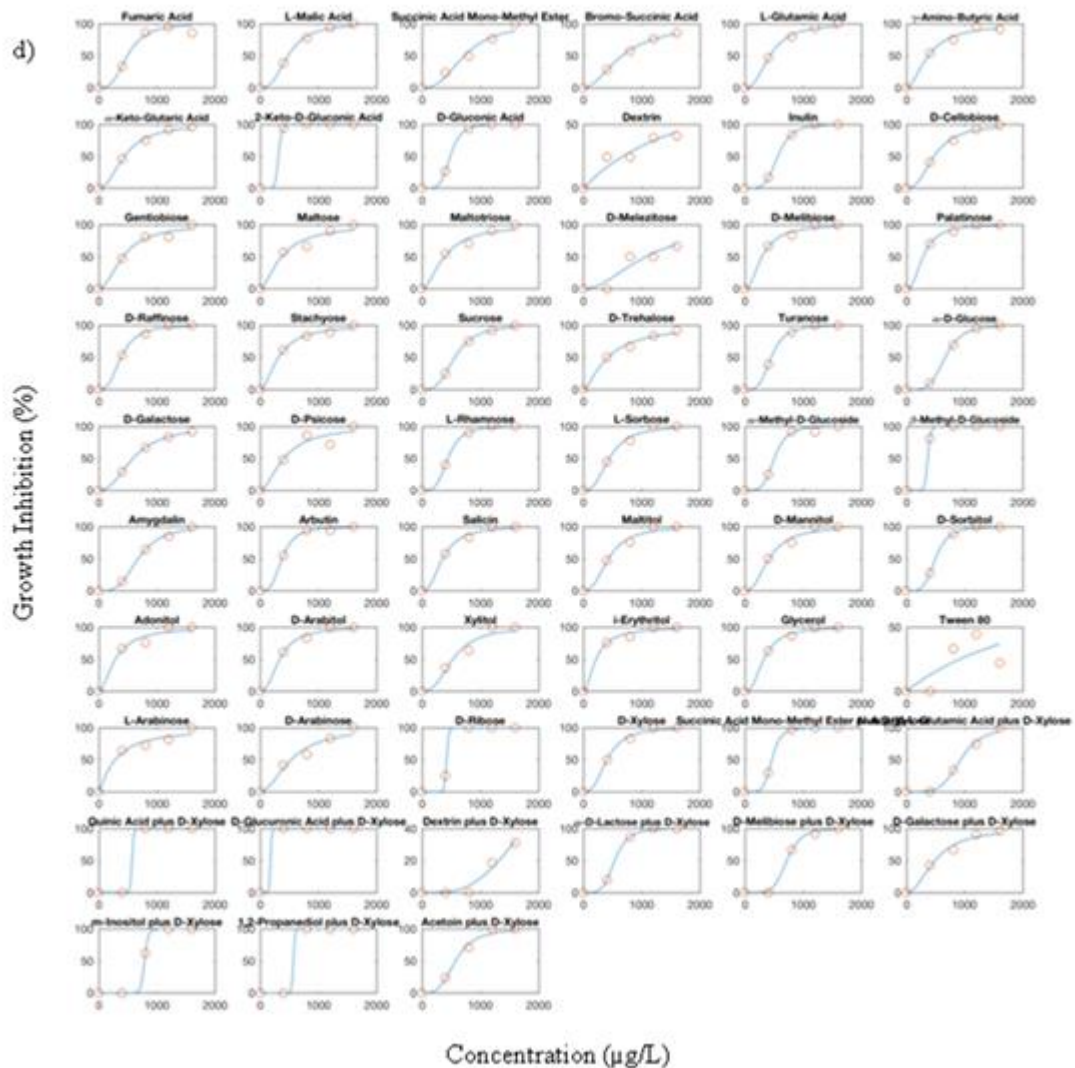
Concentration (µg/L)

Figure 2.4 Toxicity tests on *Scenedesmus obliquus* using Biolog YT plates were conducted under 400, 800, 1200, 1600, 2000 and 2400 µg/L of DTAC and DDAB for mixotrophic and heterotrophic conditions (two higher concentrations were not tested). The growth inhibitions in percentage for different individual organic carbons are shown in 2.4a and 2.4b for DTAC and 2.4c and 2.4d for DDAB for mixotrophic and heterotrophic regimes, respectively. The average readings (n = 3) on fifth day of treatment are used and standard deviations are within 10%. The solid lines represent the model fit of the Hill equation.

2.3.2.3 Profiles of effective concentration (EC$_{50}$)

The estimated EC$_{50}$ values on each carbon source are shown in Figure 2.5. Moreover, it was interesting to note that, in the presence of organic carbon, the light made *Scenedesmus obliquus* less sensitive to DTAC and DDAB as can clearly be seen in the histogram shown in Figure 2.6. This might be to the fact that the cells are actively transporting organic carbon into the cells. This effect was more apparent for DDAB (Figure 2.7). This figure further shows a wide distribution of EC$_{50}$ values, indicating that focusing on a single carbon source or a single mode of cultivation is not sufficiently capturing the effect a pollutant might have on an algae species in its natural environment. It can further be speculated that the presence of microbial and algal consortia in natural environments will further influence the ability of individual species to react to the presence of pollutants.

For instance, Wang [29] noted similar range of EC$_{50}$ values (1.8, 2.7 and 2.1 mg/L) after 24 hours exposure to Zn for natural algal communities collected from Illinois River, Peoria (US) sewage treatment plant, and Farmington (US) sewage treatment plant, respectively. Also, two chemically similar quaternary ammonium compounds produce almost equal EC$_{50}$ levels of 0.041 and 0.021 mg/L for *Pseudokirchneriella subcapitata* after 72 hours exposure to benzalkonium chlorides and didecyl dimethyl ammonium bromide, respectively [9]. This way of evaluating the toxicity effects could be more powerful as varying sensitivities over the number of various organic carbons making a unique profile.

Figure 2.5 Effective concentrations (EC$_{50}$) are plotted for different individual organic carbons. a) and b) are shown for the treatment with DTAC under mixotrophic and heterotrophic regimes, respectively, and likewise c) and d) are for DDAB.
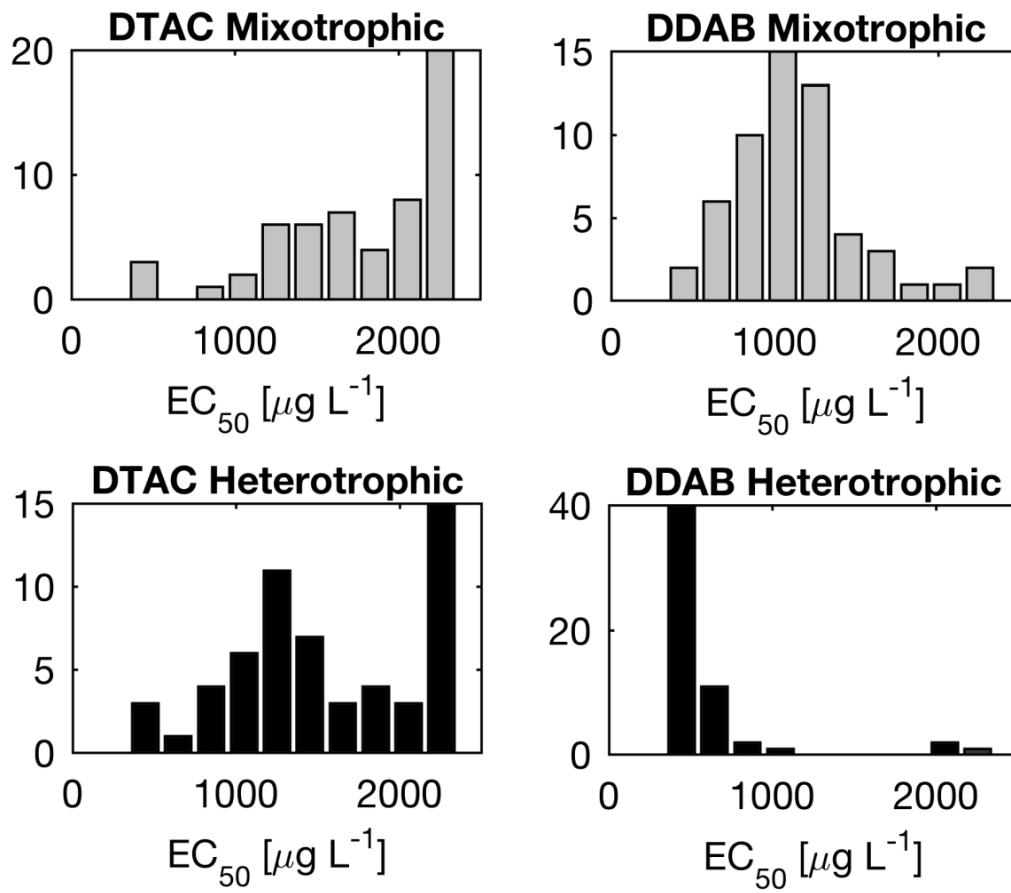
Figure 2.6 Effective concentrations (EC$_{50}$) in range are shown for different conditions.
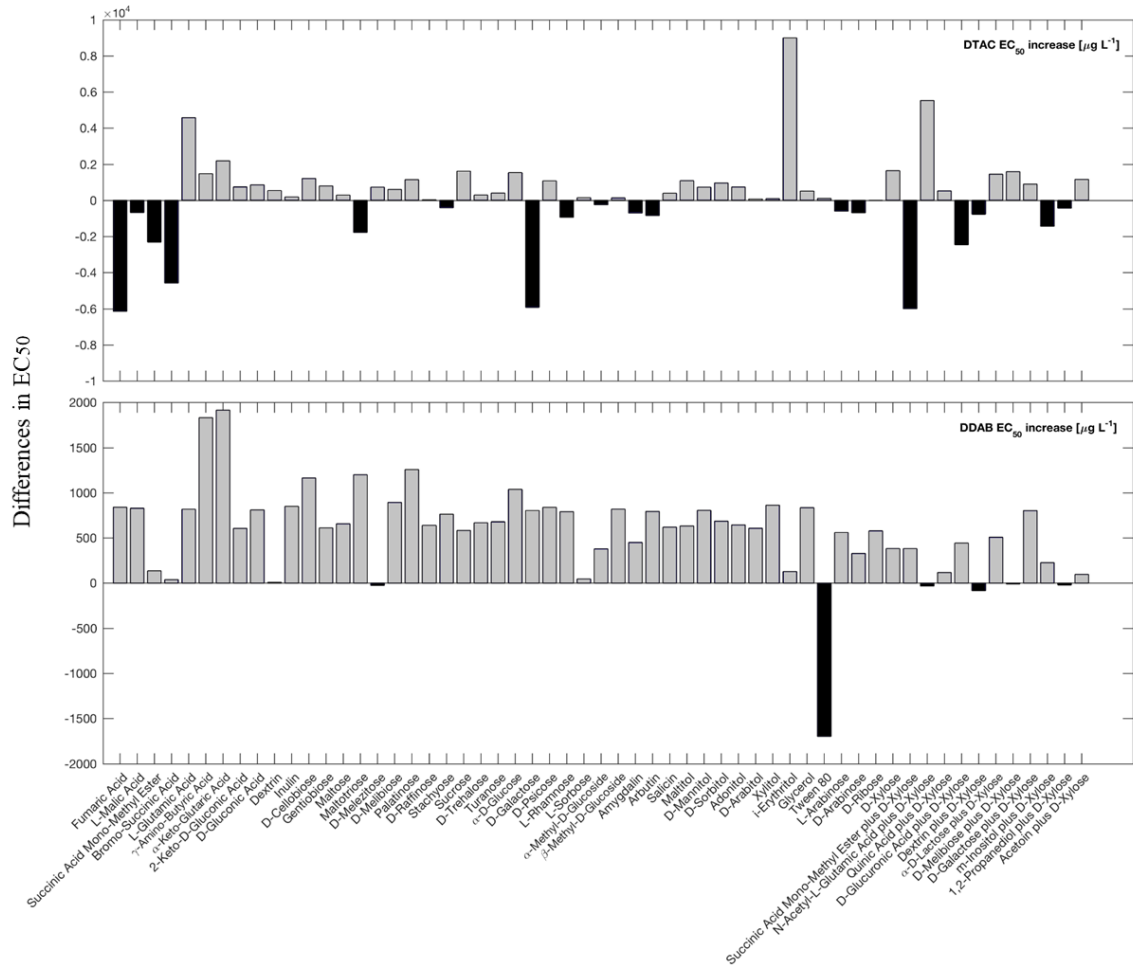
Figure 2.7 Differences in effective concentrations (EC$_{50}$) between mixotrophic and heterotrophic conditions are shown for DTAC and DDAB treated.

2.4 Conclusions

These data show that the selected microalgae species reacts very different to the presence of micropollutants depending on its growth conditions. Therefore, the common practice of quantifying toxicity of given compounds based on an EC_{50} value under well-defined conditions might not fully capture the true impact that such a chemical might have in the environment and further assays development should be undertaken.

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Chapter 3. Development of microalgal bioassay based on the community level physiological profiling (CLPP)

Abstract

Microalgal bioassay was developed based on community level physiological profiling (CLPP) to assess the effect of environmental stressors such as micropollutants. ECO Biolog plates were used to determine the changes in substrate utilization patterns caused by external disturbances such as the presence of antibiotics (a mixture of streptomycin sulfate and penicillin GT sodium), hexadecyl trimethyl ammonium chloride (CTAC), a micropollutant, and their combination (antibiotics and CTAC). The performance of the bioassay was assessed using artificially defined communities made up of five freshwater algae strains (*Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Desmodesmus subspicatus*, *Selenastrum capricornutum*, and *Scenedesmus obliquus*) at five different compositions with varying ratios of the test algae. Differences in the response as indicated by substrate utilization patterns to induced stressor by five defined microalgal communities were quantified using principal component analysis (PCA). The changes in substrate utilization patterns are probably due to the changes in metabolic potentials of the individual strains. The effects were more pronounced for the treatments than that obtained by varying the initial composition of the defined algal communities. The effects of the external factors were found to be consistent in the highest cell density of 300,000 cells per well to the lowest of 25 cells per well.

Keywords: Bioassay, micropollutants, freshwater algae, Biolog plates, community level physiological profiling (CLPP)

3.1 Introduction

The application of bioassays has recently become popular in toxicity assessment of various contaminants including micropollutants in water. Bioassays can provide a direct measurement of toxic effects of a specific compound at a very low concentration in environmental metrics. Due to the higher sensitivity of biological system over the chemical system, the potential implications of micropollutants can often be determined principally through the biosystem approach [1]. Biosystem can work in two ways, *in vitro* and *in vivo*. *In vitro* methods are based on specific modes or cellular mechanism towards the detection of certain chemicals, while, *in vivo* methods assess the integrative effects of a toxicant on whole organisms, providing direct information on the ecological effect. Commonly applied *in vitro* bioassays include the *umuC* for genotoxicity, Ames for mutagenicity and YES for estrogenicity detections [2], while, *in vivo* assays use test species from different trophic levels, i.e. bacteria, algae, duckweed, crustaceans and fish, to measure the effects of a potential toxic compound on growth, reproduction, feeding activity and mortality [3]. Commonly used *in vivo* standardized tests are mainly bacteria luminescence inhibition assay [4], green microalgae growth assay [5], duckweed growth assay [6], Daphnia reproduction assay [7], and fish egg assay [8].

Among several existing *in vivo* standardized test systems, microalgae as model organisms are being used increasingly for bioassay [9,10,11]. As a primary producer and also as a food source for organisms of higher trophic levels, well-being of microalgae is crucial for the maintenance of a healthy aquatic ecosystem. More importantly, their relatively higher sensitivity over fish and crustaceans to micropollutants make them popular in environmental toxicity studies [12].

Earlier studies conducted on algal toxicity have used both single algal species and natural algal population containing many species as the test organisms [3,13,14]. Commonly, the use of unialgal experiments is favored because of greater control and reproducibility, however, the validity of selecting one algal species to represent the response of a community can be questionable. On the other hand, the use of a natural algal community also can determine the effect of their natural habitat on the test outcome [15]. While, there is always

a dose and response relationship with an effective concentration (e.g., EC_{50}, the concentration of a toxicant that gives half-maximal response), the response relationship may not produce conclusive evidence on the algal tolerance level based on their background. For example, Wang [15] noted similar range of EC_{50} values (1.8, 2.7 and 2.1 mg/L) after 24 hours exposure to Zn for natural algal communities collected from Illinois river, Peoria (US) sewage treatment plant, and Farmington (US) sewage treatment plant, respectively. Also, two chemically similar quaternary ammonium compounds produce almost equal EC_{50} levels of 0.041 and 0.021 mg/L for (*Pseudokirchneriella subcapitata*; formerly known as *Selenastrum capricornutum*) after 72 hours exposure to benzalkonium chlorides and didecyl dimethyl ammonium bromide, respectively [16]. Therefore, instead of using EC_{50} as a measure of toxicity of a compound on a single species, measuring the change of a defined microbial community due to an environmental stress can be developed as a better diagnostic tool.

Algal assemblages have been successfully applied to monitor the impacts of aquatic stressors and aquatic toxicity [17]. For example, a shift in naturally occurring microalgal community composition reflects an environmental change that can be used as an indicator to evaluate the changes in environmental conditions as had been tested earlier in marine [18], freshwater [19], and wetland ecosystems [20]. However, traditional ways of identifying an isolate and quantifying a species composition using cell morphology and molecular level RNA/DNA amplification technique have limitations as they can be both time and cost intensive and require specialized expertise [21].

A potential way to assess an impact on microbial community due to an environmental stress is to use functional or metabolic potential characterization employing the method of community level physiological profiling (CLPP). Different microbial (bacterial) communities can be compared and classified based on carbon source utilization patterns (CSUPs) gathered using Biolog microplates containing 95 different carbon sources [22]. The advantages of CLPP are its relatively simple protocol and ease of use [23], allowing it to be used with environmental samples containing complex communities made of numerous bacteria as well as using it to investigate a single tissue *in vitro*. Its application has been well described on variety of research areas including toxicological studies [24], e.g., acid mine drainage [25], hydrocarbon pollutants [26], heavy metals and antibiotics [27,28]. The test species expanded

from prokaryote bacteria to eukaryotic fungi and algae [22,29,30,31]. In addition, recently, metabolic profiling technology has been established on mammalian cells and it was found that human cancer cell lines from different organ tissues produced distinct profiles of metabolic activity [32].

The objective of the present work was to explore the possibility of developing a microalgal bioassay for environmental pollutant based on CLPP using Biolog microplates. Defined and heterogeneous algal communities were used as test species and hexadecyl trimethyl ammonium chloride (CTAC; CAS No. 112-02-7), a quaternary ammonium compound (QAC), was selected as a model stress inducer. QACs are widely used as disinfectants, fabric softeners and surfactants, and can be found in detectable amounts (4 to 75 µg/L) in wastewater effluents and aquatic environment [16,33].

3.2 Materials and Methods

3.2.1 Microalgae strains

Five axenic cultures of freshwater green algae (*Chlorella vulgaris* (UTEX 2714), *Chlamydomonas reinhardtii* (CC 125), *Desmodesmus subspicatus* (CCAP 276/20), *Selenastrum capricornutum* (CCAP 278/4), and *Scenedesmus obliquus* (CPCC 5)), were obtained from collections at University of Texas at Austin, Chlamydomonas Center, Culture Collection of Algae and Protozoa and Canadian Phycological Culture Centre. These species are selected as they are recognized as standard test species (*Chlorella vulgaris*, *Desmodesmus subspicatus* and *Selenastrum capricornutum*) and were used in several occasions for the algal toxicity experiments [34-40]. In addition, these species presented a relatively even distribution of phylogenetic distances among inland algal species [41].

3.2.2 Culture maintenance

Cultures were maintained in a HS (high salt) minimal medium [42] at 25°C on a shaker (150 rpm) with a light intensity of 140 µE m^{-2} s^{-1} (16 hrs. Light: 8 hrs. dark) using an incubator (Infors HT Multitron, Basel, Switzerland). The algal growth was quantified routinely measuring optical density at 600 nm with a 200 pro infinite series microplate reader (Tecan, Männedorf,

Switzerland). Cells in the exponential growth phase were used to calculate the specific growth rates (d^{-1}; Table 3.1) and to formulate synthetic mixed algal communities for the toxicity experiments. The cell density (cells mL^{-1}) was measured using a hemocytometer (Hausser Scientific, PA, USA). To maintain axenic condition, the cultures were checked regularly for the bacterial contamination by streaking onto nutrient agar plates [29].

Table 3.1 Growth rates of five microalgae strains were measured (± standard deviation; n ≥ 3).

Species	Strain	Growth rate (d^{-1})(n)
Chlorella vulgaris	UTEX 2714	1.01±0.10 (4)
Chlamydomonas reinhardtii	CC 125	0.75±0.07 (5)
Desmodesmus subspicatus	CCAP 276/20	0.93±0.04 (3)
Selenastrum capricornutum	CCAP 278/4	0.93±0.04 (6)
Scenedesmus obliquus	CPCC 5	0.82±0.08 (3)

3.2.3 Community Level Physiological Profiling (CLPP)

Prefilled 96-well microtiter plates (ECO microplate; Biolog, CA, USA) were used to characterize different metabolic profiles. The ECO Biolog plates were selected as they are developed for environmental applications and contain triplicates of 31 different organic substrates, which allow for increased confidence in statistical analysis [23]. Initially, experiments were conducted in Biolog plates to determine the feasibility of using the selected algal species at a cell density of 25 000 cells per well (equivalent to 166667 cells/ml) and the number of substrates utilized (metabolic richness) was determined. All wells where growth was observed were subsequently checked for bacterial contamination via microscopy and no bacterial cells were observed (based on size and morphology). After, the toxicity experiments were conducted. The toxicity tests using CLPP consist of the following steps: preparation of synthetic mixed algal communities and spiking of the potential toxicant in prepared algal communities, followed by inoculation of the mixture in Biolog plates.

3.2.3.1 Mixed algal communities

Mixed algal communities were synthesized using five algal strains at their mid-exponential phase in different ratios as shown in Table 3.2. The ratios of different algae were maintained based on the correlation of optical density versus cell count (Appendix A). The reagent reservoir (Axygen® Biosciences, CA, USA) was used for the mixture of algal strains and

the mixed cultures were brought up to the final volume of 15 mL to obtain the density of 3×10^6 cells/mL by HS media dilution. A higher inoculum cell density of 3×10^5 cells per well was used for the control and subsequent CLPP toxicity experiments as it gives a high average absorbance of maximum of 0.14. All wells where growth was observed were subsequently checked for bacterial contamination as mentioned above; no bacterial contaminations were found.

Table 3.2 Mixed algal communities were synthesized using five algal strains at their mid-exponential phase in different ratios.

Samples (ratio)	Cells (x 10^4)/well				
	Chlorella	*Chlamydomonas*	*Desmodesmus*	*Selenastrum*	*Scenedesmus*
Mixture 1 (2:1:1:1:1)	10	5	5	5	5
Mixture 2 (1:2:1:1:1)	5	10	5	5	5
Mixture 3 (1:1:2:1:1)	5	5	10	5	5
Mixture 4 (1:1:1:2:1)	5	5	5	10	5
Mixture 5 (1:1:1:1:2)	5	5	5	5	10

3.2.3.2 CLPP toxicity test

The toxicity test using CLPP was performed by exposing synthetic mixed algal communities to CTAC and antibiotics applied both individually and in combination of them. Mixed algal communities were spiked with a stock solution of 3 mg/L CTAC (purity ≥ 98%; Sigma-Aldrich, MO, USA) to yield nominal concentration of 133 μg/L, this range of concentration was shown to have a toxic effect on microalgae [43]. The Biolog plates were then inoculated with 100 μL of multichannel pipette mixed CTAC treated samples in 50 μL of HS media. For the antibiotic treatment, streptomycin sulfate (25 μg/mL, purity ≥ 95%; AMRESCO®, OH, USA) and penicillin GT sodium (100 μg/mL, purity ≥ 95%; AMRESCO®, OH, USA) were added in HS media. In case of the combined effect of CTAC and antibiotics, 100 μL of CTAC treated samples were inoculated in plates where 50 μL of antibiotic added HS media was filled. Microplates were wrapped with aluminum foil to keep microalgae under heterotrophic condition and incubated at 25°C for 11 days. Measurements of metabolic activities were determined using a 200 pro infinite series microplate reader (Tecan, Männedorf, Switzerland) measuring the absorbance at 590 nm and 750 nm. No bacterial contaminations were found after incubation for 11 days (each well was checked via light microscopy).

3.2.4 Minimum cell density requirement

A minimum cell density requirement per well for the toxicity test was investigated by a series of 5-fold dilutions of synthetic mixed algal communities. For this purpose, the ratio of 1:1:1:1:1 of *Chlorella*: *Chlamydomonas: Desmodesmus: Selenastrum: Scenedesmus* was used with the highest inoculum size of 250 000 cells per well and the lowest size of 25 cells per well. The assay was functional with inoculations as low as 25 cells per well (data not shown), however to increase robustness, accuracy and reproducibility, all further tests were conducted with 25 000 and 300 000 cells per well.

3.2.5 Analysis

An extensive dataset of approximately 100 000 absorbance readings was generated

and different fingerprints of the metabolic activities were compared by means of multivariate statistics, principal component analysis (PCA) using SPSS for Windows (SPSS, IL, USA) and one-dimensional metric of Euclidean distance (ED) followed by Weber and Legge [44]. The raw color development measured at 590 nm was corrected by subtracting the turbidity readings at 750 nm and used for further analyses. Log transformed and unscaled data were used for PC and ED analyses, respectively. The number of substrates utilized (metabolic richness) was calculated as the number of substrates with an average absorption exceeding 3 times the absorption of the negative control well at t = 8 day.

3.3 Results and Discussion

3.3.1 CLPP for individual algal species

Initial experiments were conducted to assess the metabolic profiles of the selected algal species using different organic compounds in the Biolog plates, and the results for five algal strains are shown in Figure 3.1. Each algal species has its own distinct pattern or profile which can also be used for identification purpose. Greater varieties of carbon sources were utilized by *Chlorella*, *Selenastrum* and *Scenedesmus* (Figure 3.1 a, d, and e) than *Chlamydomonas* and *Desmodesmus* (Figure 3.1 b and c, respectively). The number of substrates utilized (metabolic richness) was found to be 12 for *Chlorella*, 15 for *Selenastrum* and 13 for *Scenedesmus* compared to 4 for *Chlamydomonas* and 6 for *Desmodesmus*. Based on metabolic richness, out of all 31 carbon sources, D-galactonic acid γ-lactone ($C_6H_{10}O_6$), pyruvic acid methyl ester ($C_4H_6O_3$) and D-xylose ($C_5H_{10}O_5$) were utilized by all strains; however, α-ketobutyric acid ($C_4H_6O_3$), and α-D-lactose ($C_{12}H_{22}O_{11}$), and L-arginine ($C_6H_{14}N_4O_2$), L-asparagine ($C_4H_8N_2O_3$), L-threonine ($C_4H_9NO_3$), D-glucosaminic acid ($C_6H_{13}NO_6$), and itaconic acid ($C_5H_6O_4$) were used only by *Chlorella* and *Selenastrum*, respectively (Table 3.3). The capacity of algae to utilize and grow stably on hexoses ($C_6H_{12}O_6$; including glucose, fructose, galactose and mannose) and pentoses ($C_5H_{10}O_5$; ribose, xylose and arabinose) were noted by different species in different phyla [45]. Amino acids were preferably used by *Selenastrum* and amino acids such as glycine and glutamine were known to support slight heterotrophic growth of *Chlorogloea fritschii* [46]. Dimeric sugar with 12 carbons was utilized by *Chlorella*; likewise, sucrose and maltose were known to support algae growth heterotrophically [45]. Again higher

metabolic richness can also be concluded from the average well color development (AWCD) of 0.11 of *Chlorella*, 0.07 of *Selenastrum*, 0.06 of *Scenedesmus* to 0.01 of *Chlamydomonas* and *Desmodesmus* (Figure 3.2). The differences in metabolic pattern among the selected algal species allow the usage of these strains to form a defined algal community in which changes can be monitored.
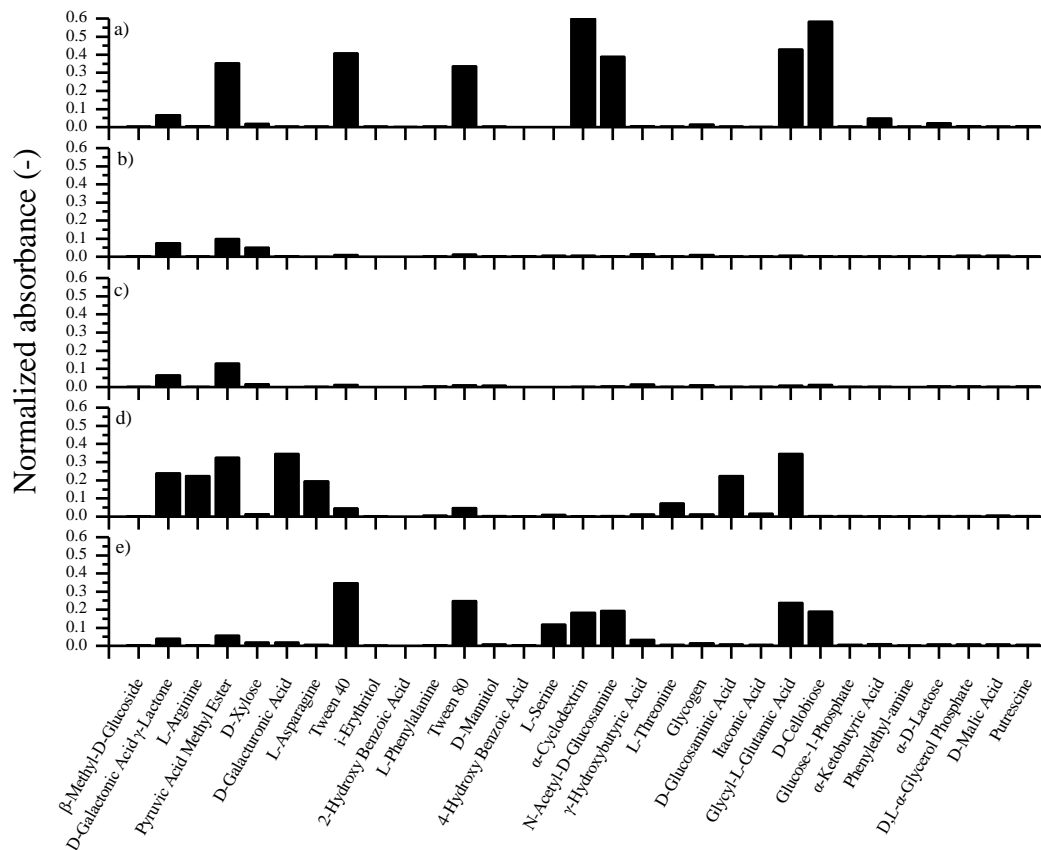
Figure 3.1 Color development over 8 days for 31 wells with different organic carbons are shown. The readings, calculated as absorbance 590-750 nm, are in triplicates and standard deviations are within 4%. a) *Chlorella*; b) *Chlamydomonas*; c) *Desmodesmus*; d) *Selenastrum* and e) *Scenedesmus*
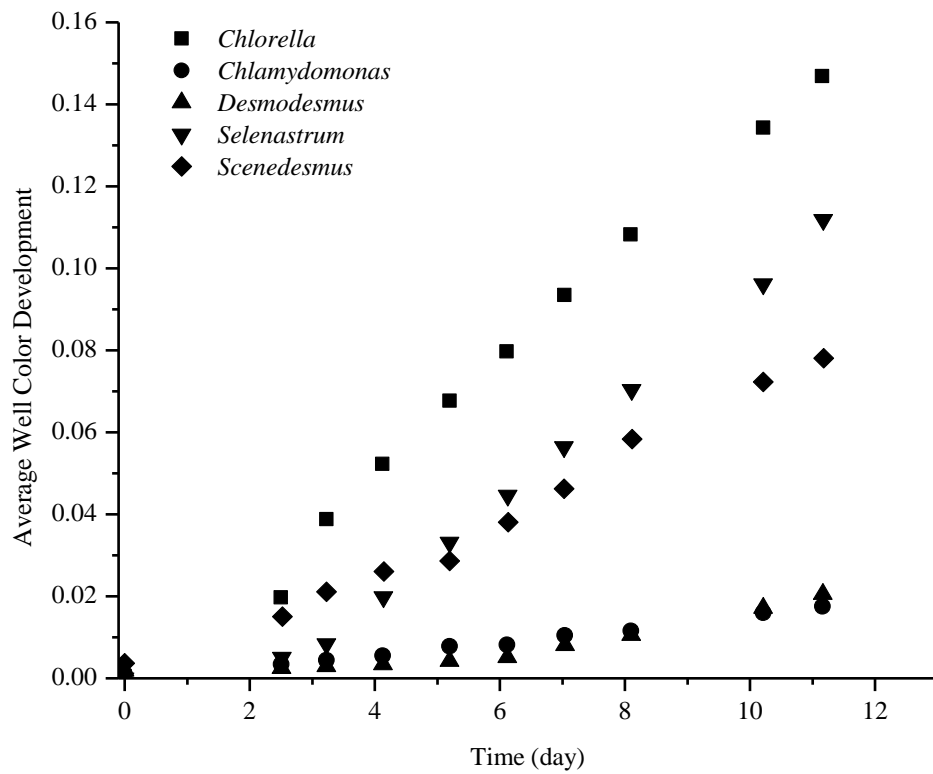
Figure 3.2 Average well color developments (AWCD) on 31 wells are shown for five algal strains through 11th day. The readings are in triplicates and standard deviations are within 4%.

Table 3.3 Substrates utilized (metabolic richness) by five individual algal strains are shown. Initial cell density of 25 000 cells per well was used and substrates utilized (metabolic richness) were calculated as substrates with an average absorption exceeding 3 times the absorption of the negative control well at t = day 8. 1 indicates when substrates were utilized.

Well	Chemical	_Chlorella_	_Chlamydomonas_	_Desmodesmus_	_Selenastrum_	_Scenedesmus_
A3	D-Galactonic Acid γ-Lactone	1	1	1	1	1
A4	L-Arginine				1	
B1	Pyruvic Acid Methyl Ester	1	1	1	1	1
B2	D-Xylose	1	1	1	1	1
B3	D-Galacturonic Acid				1	1
B4	L-Asparagine				1	
C1	Tween 40	1		1	1	1
D1	Tween 80	1			1	1
D4	L-Serine				1	1
E1	α-Cyclodextrin	1				1
E2	N-Acetyl-D-Glucosamine	1				1
E3	γ-Hydroxybutyric Acid		1	1	1	1
E4	L-Threonine				1	
F1	Glycogen	1			1	1
F2	D-Glucosaminic Acid				1	
F3	Itaconic Acid				1	
F4	Glycyl-L-Glutamic Acid	1			1	1
G1	D-Cellobiose	1		1		1
G3	α-Ketobutyric Acid	1				
H1	α-D-Lactose	1				

3.3.2 CLPP for synthetic mixed algal communities

One goal of the assay was to determine changes in an algal community composition caused by external factors. This ability was verified first by using the assay on artificially created communities made up of the same five algae strains described above, at different relative ratios. Differences in the response of the assay to different community composition were quantified via PCA. In order to determine the appropriate time point at which to compare five different communities via PCA, the Euclidean distances of the color developments of all wells for each community between each time point and time zero were determined (Table 3.4). The time point at which the average of the Euclidian distances of the five communities exhibited the largest standard deviation was chosen to conduct PCA analysis, as it was assumed to be the time where the difference, and consequently the resolution of the assays, was maximized [44]. The maximum standard deviation occurred after nine days, however PCA was conducted with log transformed data collected after eight days, in order to avoid reaching plateau in the growth curves in some of the wells. The analysis identified three principal components. The differences in the substrate utilization patterns can then be visualized graphically in three dimensional spaces (Figure 3.3). The first principal component accounted for 41%, the second accounted for 16%, and the third accounted for 10% of the variation in the data. It can clearly be seen in Figure 3.3 that the replicates of each population cluster together in distinct sections of the plot. The five communities can therefore be distinguished from each other. Comparable results were obtained for bacterial communities comprised of four and six different soil isolates in equal proportions [47]. The quantitative way of formulating synthetic community was achieved by changing a cell density of fixed number of composition and this could be possible as it was shown that the effects of cell density on color development were clear for two bacteria strains at least for tested four well carbons [47].
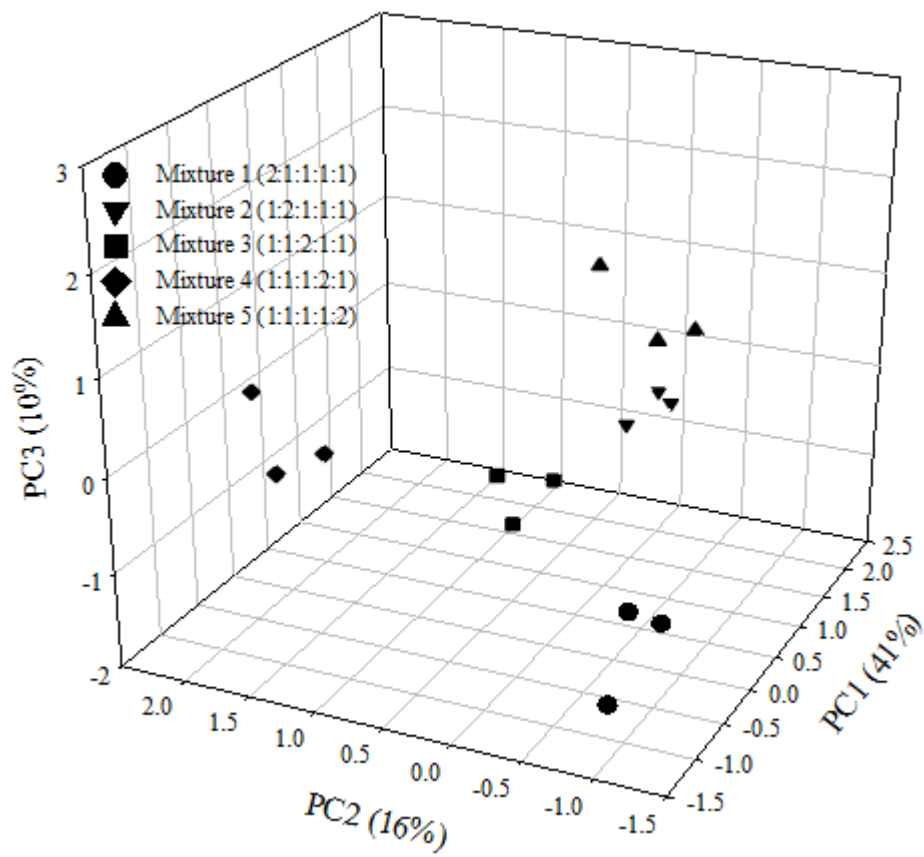
Figure 3.3 Five green algal strains in five different ratios are shown in PC analysis. The log transformed data on 8th day were used. The triplicates are shown for five ratios of differently mixed algal strains.

Table 3.4 Euclidean distances for five ratios of differently mixed algal strains are shown through 10th day. The values are the average of the triplicates and standard deviations. The standard deviations between differently mixed algal strains are also calculated.

Time (Day)	2:1:1:1:1 Euclidean Distance	1:2:1:1:1 Euclidean Distance	1:1:2:1:1 Euclidean Distance	1:1:1:2:1 Euclidean Distance	1:1:1:1:2 Euclidean Distance	Standard Deviation
0	0	0	0	0	0	0
1.4	0.088±0.002	0.103±0.005	0.096±0.002	0.090±0.006	0.121±0.003	0.013
2.4	0.259±0.001	0.260±0.014	0.267±0.009	0.258±0.011	0.283±0.008	0.011
3.3	0.406±0.002	0.383±0.014	0.391±0.011	0.391±0.011	0.391±0.009	0.009
4.3	0.499±0.007	0.494±0.013	0.505±0.010	0.501±0.016	0.504±0.005	0.004
5.3	0.598±0.001	0.598±0.017	0.610±0.016	0.610±0.013	0.611±0.021	0.007
5.8	0.646±0.004	0.647±0.015	0.684±0.009	0.666±0.011	0.673±0.007	0.016
8.3	0.890±0.004	0.885±0.022	1.063±0.054	1.026±0.017	0.906±0.010	0.084
9.5	0.996±0.001	0.998±0.029	1.216±0.071	1.117±0.019	1.025±0.007	0.095
10.3	1.079±0.002	1.075±0.035	1.291±0.065	1.187±0.024	1.114±0.010	0.091

3.3.3 CLPP for toxicity test

As the assay was able to distinguish differently composed communities, it was then used to evaluate the response of these communities to the presences of potentially toxic compounds. The same communities as before were therefore tested in the presence of CTAC, antibiotics, and a combination of both. The CLPP assay was analyzed as before and PCA identified 2 principal components (Figure 3.4). The figure shows that the four different treatments had a strong effect on the communities and the data are clustered based on the treatment, independent of the initial communities' composition. A general measure of inhibition can be seen in the average well color development in each plate. The overall average of all the values used towards PCA for the different treatments were 0.125 ± 0.005 S.E. for the control versus 0.053 ± 0.002 S.E. in the presence of CTAC, 0.116 ± 0.005 S.E. in the presence of antibiotics, and 0.049 ± 0.002 S.E. in the presence of antibiotics and CTAC. The toxic effects (quantified this way) of CTAC and of antibiotics and CTAC were significant ($P < 0.05$; One way ANOVA), showing 58% and 61% inhibition compared to the control, respectively. The effect of the external factors on substrate utilization patterns can therefore be seen as substantially more pronounced than initial permutation of the relative species' ratio (Figure 3.3). The data shown in Figure 3.3 quantify the much smaller differences based on the initial ratios, and can hence be interpreted as a magnified version of the data shown in the upper right quarter of Figure 3.4. This is in accordance with a similar finding that the pollutants, hydrocarbons, rather than the geographical origin of the samples are the main determinants affecting substrate utilization patterns in bacterial communities [26]. The selected four treatments acting as selective forces or determinants have caused all five synthetic mixtures of algal communities into four groups (Figure 3.4). It is unclear if the species selection was the cause of the different patterns created by the changes in species diversity as suggested by Wünsche et al. [48]. However, it is more likely that the changes in substrate utilization patterns between the four treatments are due to the changes in metabolic potentials of the individual strains, rather than due to a change in the composition of the (small) community. A study on microbial communities revealed that only a small number of identical species were detected in tested wells of different carbon sources after incubation with environmental samples and the few bacterial strains were responsible for significant different patterns of CLPP [49]. The changes

in plate incubation conditions by temperature and O_2 level, therefore, the physiological state of a community, were found to have an effect on overall substrate utilization patterns [29,50]. Moreover, the effects of a mixture of penicillin G and streptomycin at certain concentrations were recorded for marine dinoflagellate, *Alexandrium tamarense*, on the growth and C2 toxin production through direct effects on the physiology [51]. This indicates its role as a potential bioassay as the changes in metabolic activities induced by toxicants could be captured and had been considered as a biomarker for typical bioassays [1].

The sole effect of CTAC was still found in cells incubated in combination with antibiotics. This, again, is a promising result for the application of CLPP toxicity test where the algal communities might be at risk of contamination with prokaryotic bacteria when evaluating environmental samples. The repression of competing organisms is a necessity when aiming to focus CLPP on selected types organisms, such as algae or fungi only. The application of antibiotics is common in fungal CLPP experiments to minimize background interference from bacteria contributing to color development [29,52]. In addition, the fungicide, cycloheximide, was also used in a previous study for characterization of different bacterial communities to prevent fungal development and background interference towards the color development [53]. Although the application of cycloheximide in combination with antibiotics and toxicants was not tested in this study, it could be very useful for this technique for assessing toxicity experiments using natural algal community samples.
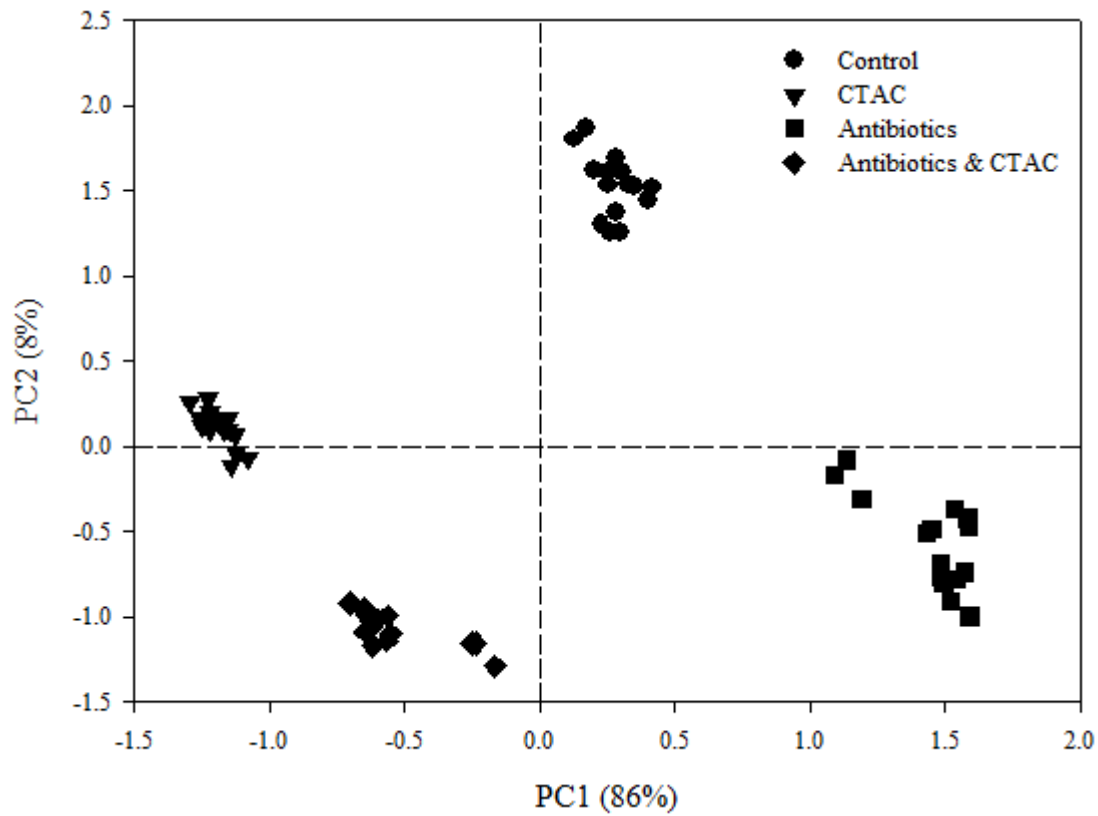
Figure 3.4 Different ratios of five species under no treatment (circle), toxic concentration of CTAC (inverse triangle), antibiotic (square), and antibiotic and toxic concentration of CTAC together (diamond) are shown in PC analysis. The log transformed data on 8th day were used for different treatments. The triplicates were used in PC analysis and shown for differently treated five ratios of differently mixed algal strains.

3.4 Conclusions

This work demonstrates the successful application of microalgal communities for CLPP assays using an easy and quick method of applying Biolog microplates containing 31 different carbon sources. The developed assay is highly sensitive and can detect small permutations in the initial community composition, however, the signal of external disturbances such as exposure to CTACs is substantially stronger. The developed assay might provide a new insight into the effects of pollutants on microalgal communities, and hence is of environmental significance.

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Chapter 4. Assessment of water samples with complex compositions using microalgal bioassay based on the community level physiological profiling (CLPP)

[A revised version of this chapter has been prepared for submission to Journal of Environmental Management]

Abstract

The ability to effectively characterize the response of microalgal communities to changes in water quality is limited. Recently, a microalgal bioassay was developed based on community level physiological profiling (CLPP). The efficacy of this assay was evaluated using three wetland water samples, a surface water sample, and two wastewater samples (i.e. primary and secondary), all collected from southwestern Ontario, Canada. In addition, the assay was applied to untreated and activated carbon treated oil sand process water (OSPW). YT Biolog plates were utilized for defined microalgal community under both heterotrophic and mixotrophic growth conditions to characterize the changes in the defined microalgal community due to the changes in water type. The response of the assay due to changes in the algal community caused by different waters was assessed by average well florescence development (AWFD), Euclidean distance (ED), metabolic/physiological profiles, and principal component analysis (PCA). In all the responses, one of the wetland samples showed a distinct difference from other water samples. The differences in algal community were plotted in profiles and tested for the effects of water samples and organic carbon substrates in the Biolog plates both individually, and also in combination. The effects were significant for all sources of variations regardless of growth conditions ($P < 0.001$; two way ANOVA). Lastly, the profiles were quantified using PCA. Although, the degrees of changes of algal community varied, all the water samples were distinguished under both growth regimes. The assay was found to be highly sensitive and could be used to differentiate different water bodies with complicated mixtures.

Keywords: Bioassay, microalgae, YT Biolog plate, community level physiological profiling

(CLPP), various waters (wetland, wastewater and oil sand process water), growth conditions (heterotrophic and mixotrophic).

4.1 Introduction

Functional or metabolic characterization of microbial community has been well developed after a method was first published by Garland and Mills [1]. Community level physiological profiling (CLPP) uses a commercially available Biolog 96-well plate containing up to 95 different carbon sources. Different microbial communities are compared and classified based on carbon source utilization patterns (CSUPs) [1]. The relatively simple protocol and ease of use make it very practical for various applications [2]. Based on ISI database search using key words such as Biolog and community, about 1363 publications (prior to July 2017) on CLPP with an increasing trend were found.

CLPP has been applied on a variety of areas and expanded from prokaryotic bacteria to eukaryotic fungi and microalgae [3,4]. The perturbation or change in the microbial community has been observed in terrestrial and aquatic environments caused by a plant interaction [5], root secretion [6], spill of hydrocarbon [7], metal contamination [8], water pollution with acid mine drainage [9], and differently fed bluegills in guts [10]. This technology has been further modified by the simultaneous use of antibiotics or cycloheximide to isolate the response of a particular group of interest in environmental samples by selectively repress signal interferences from either bacteria or fungi [11,12]. It could then be used as an assay to study the community tolerance to antibiotic sulfachloropyridazine [13] and toxicity to gold nanoparticles and ciprofloxacin [14].

Recently, a bioassay was developed based on CLPP using defined algal communities for the detection of micropollutants [4]. The objective of the current study is to determine the performance of the microalgal bioassay based on CLPP by characterizing the changes in the defined microalgal community exposed to various water sources with complex compositions. Often, it is difficult to differentiate the effects of waters from different water bodies using a standard single algae growth inhibition test [15]. In this study, wetland water samples, primary and secondary effluents from municipal wastewater treatment plant, river water, and oil sand

process water (OSPW) were assessed using YT Biolog plates in a defined microalgal community under both heterotrophic and mixotrophic growth regimes. Samples with presumably different water quality were chosen to evaluate the efficacy of a microalgae CLPP based assay.

4.2 Materials and Methods

4.2.1 Water samples

Wetland surface water samples were collected from three wetland sites in London, Ontario; Walkers pond, Pond mills and Redmond's pond in July, 2016. Primary and secondary wastewater samples were obtained from the Adelaide wastewater treatment plant located in London, Ontario in April, 2017. River water was collected from the North Thames River at a discharge point from the Adelaide wastewater treatment plant. Untreated oil sand process water (OSPW) was supplied by Suncor Energy (Calgary, AL). The OSPW was treated with granular activated carbon where most of the dissolved organic carbon (DOC) was removed. All the samples were sterilized by passing through 0.22 μm filters (Acrodisc® Pall, NY, USA), prior to addition to YT Biolog plates. The prepared samples were checked occasionally for the bacterial contamination by streaking onto nutrient agar plates and no contaminations were found for n of 5 replicates. Preliminary water assessment was conducted for different water quality. The ultra-violet (UV) spectrum of various waters was measured using a UV/Vis Spectrophotometer (Thermo Scientific, MA, USA) and shown in Figure 4.1. The total organic carbon (TOC) of the water samples was determined by dry combustion of samples at 900°C in a furnace, with the collection and detection of evolved CO_2 using a TOC analyzer (TOC-V analyzer connected with an ANSI-V auto sampler, Shimadzu, Japan). Nitrate (method 10242) and phosphate (method 10209) concentrations were analyzed using a Hach kit (Hach, CO, USA). Table 4.1 summarized the results of the analyses.
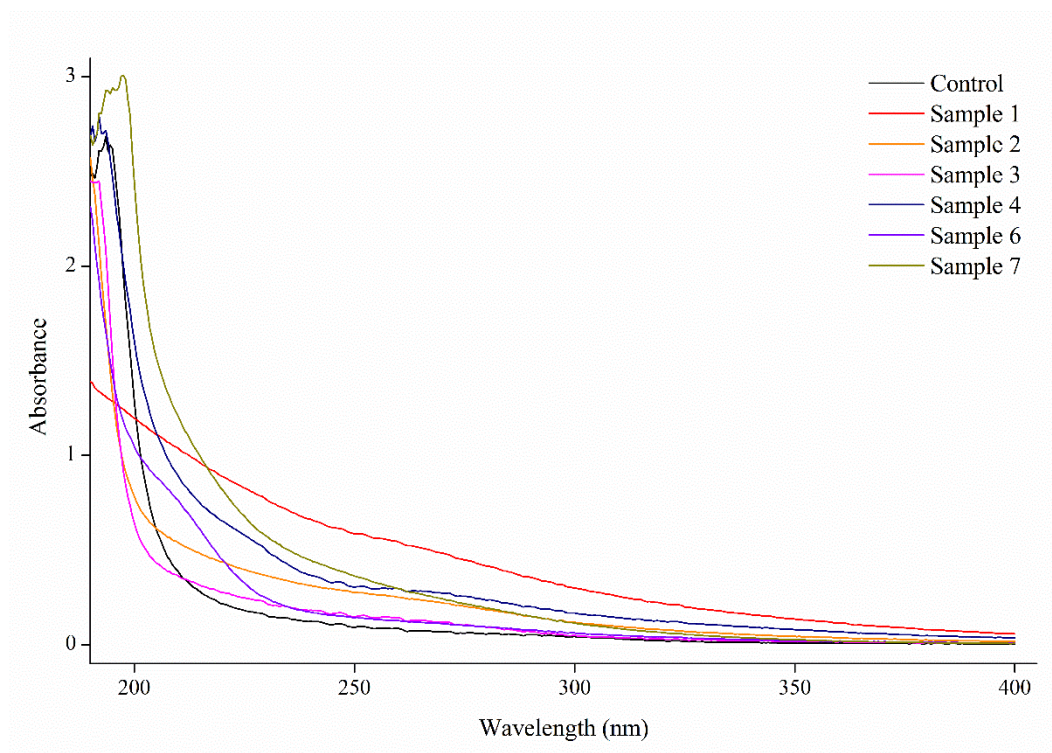
Figure 4.1 Ultra-violet (UV) spectrum of various waters. Description on samples can be referred to Table 4.1.

Table 4.1 Total organic carbon (TOC), nitrate, and phosphate concentrations were measured for various water samples.

Samples	Water	TOC (mg/L)	Nitrate (mg/L)	Phosphate (mg/L)
Control	Modified HS media	97.3	0.4	123.6
Sample 1	Walkers pond	29.9	0.4	0.004
Sample 2	Pond mills	49.6	0.4	0.04
Sample 3	Redmond's pond	46.7	0.3	0.1
Sample 4	Primary effluent	120.0	0.3	15.3
Sample 5	Secondary effluent	-	-	-
Sample 6	North Thames River	58.2	1.2	0.3
Sample 7	Untreated OSPW	81.4	0.5	0.2
Sample 8	Treated OSPW	-	-	-

4.2.2 Microalgal strains and mixed algal community

Five axenic cultures of freshwater green algae (*Chlorella vulgaris* (UTEX 2714), *Chlamydomonas reinhardtii* (CC 125), *Desmodesmus subspicatus* (CCAP 276/20), *Selenastrum capricornutum* (CCAP 278/4), and *Scenedesmus obliquus* (CPCC 5)), were obtained from

collections at University of Texas at Austin, Chlamydomonas Center, Culture Collection of Algae and Protozoa, and Canadian Phycological Culture Centre. Cultures were maintained and formulated into a synthetic mixed algal community of equal proportions as proposed by Kim et al. [4]. The reagent reservoir (Axygen® Biosciences, CA, USA) was used for the mixture of algal strains and the mixed cultures were brought up to a final volume of 15 mL to obtain the density of 1.5 x 10^6 cells/mL by a modified HS media dilution. An inoculum cell density of 1 x 10^6 cells/mL was used for the control and subsequent CLPP water sample assessment experiments. To maintain an axenic condition, the individual and mix cultures were checked regularly for the bacterial contamination by streaking onto nutrient agar plates.

4.2.3 Sample assessment using Community Level Physiological Profiling (CLPP)

Prefilled 96-well microtiter plates (YT microplate; Biolog, CA, USA) were used to characterize different microalgal growth profiles of water samples under heterotrophic and mixotrophic growth conditions. The YT Biolog plates were selected as they have both sections of wells with or without included tetrazolium dye (Biolog YT microplate; available from Biolog, CA, USA). Only sixty bottom wells without tetrazolium dye were used for the analysis as tetrazolium dye can be light sensitive (Biolog redox dye mixes; available from Biolog, CA, USA). The sample assessment using CLPP was performed by exposing a synthetic mixed algal community of equal proportions to different water samples. The Biolog plates contained 100 µL of multichannel pipette mixed algal community in 50 µL of water samples. The control represents a modified HS minimal medium. The Biolog plates were kept in dark at 25°C for heterotrophic condition and incubated at 25°C on shaking (150 rpm) with a light intensity of 140 µE m^{-2} s^{-1} using an incubator (Infors HT Multitron, Basel, Switzerland) for mixotrophic condition. The growths were determined using a M1000 pro infinite series microplate reader (Tecan, Männedorf, Switzerland) measuring a fluorescence (excitation at 470 nm and emission at 650 nm).

4.2.4 Analysis

The well fluorescence development measured through five day of treatment was corrected by subtracting the initial fluorescence readings at t = 0 day. One-dimensional metric

of Euclidean distance (ED) analysis was performed following a method suggested by Weber and Legge [16]. Ln transformed fluorescence data in profiles for different water samples were used for two way ANOVA and were compared by means of multivariate statistics, principal component analysis (PCA) using SPSS for Windows (SPSS, IL, USA).

4.3 Results and Discussion

4.3.1 Changes in the average well fluorescence development (AWFD)

The water samples were assessed on a synthetic mixed algal community of equal proportions using YT Biolog plates under heterotrophic and mixotrophic growth conditions. Growth was measured via fluorescence in each well for five days and the average well fluorescence developments (AWFD) were plotted (Figure 4.2). The main reason to evaluate the AWFD is to determine the incubation time at which the maximum difference between the respective water samples can be found [2]. Detailed data analyses are then performed for samples taken at this incubation time. The AWFD also allows for a first relative assessment of the water quality, based on the extent and rate of algae growth.
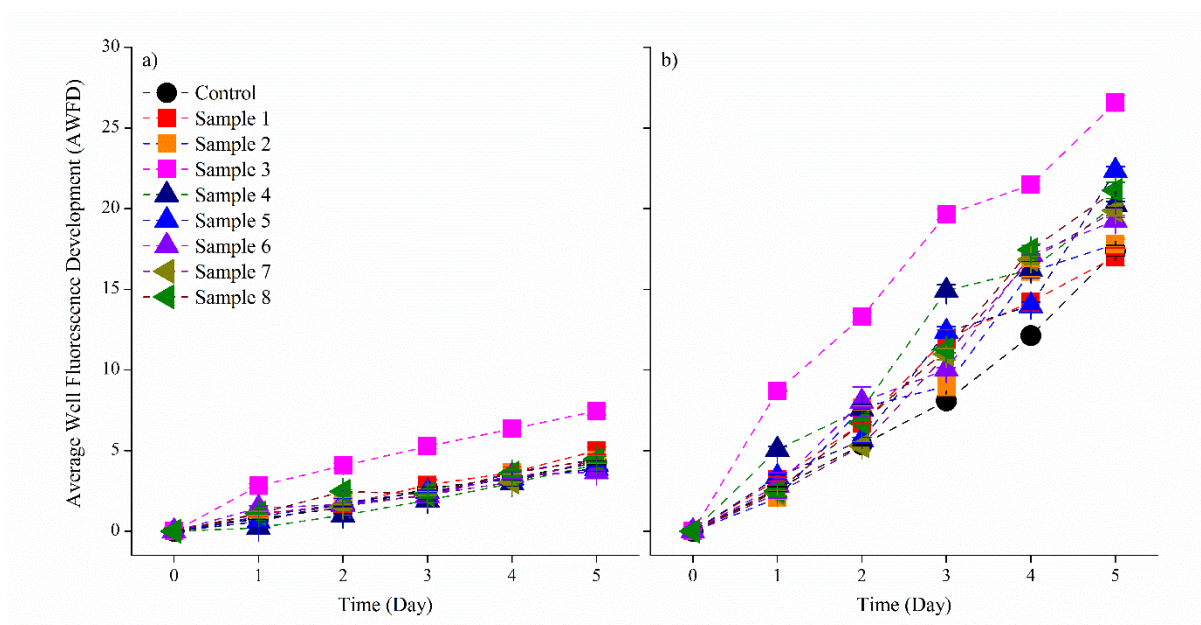

Figure 4.2 AWFD using algal community for 9 water samples (see Table 4.1) under heterotrophic (a) and mixotrophic (b) growth conditions (average of triplicates ± standard

deviations).

Three to eight times higher growth was observed in mixotrophic than heterotrophic growth conditions. This is not surprising as the growth rate in mixotrophic condition is approximately the same as the sum of the growth rate in the photoautotrophic and heterotrophic cultures, as shown for *Chlorella regularis* [17], *Chlorella vulgaris* [18], and *Spirulina platensis* [19]. The growth profiles on all water samples were very similar, a prominent difference was seen in case of Redmond's pond water compared to the other samples. The differences from the mean of the other were the largest on the fifth and third day for heterotrophic (standard deviation (SD) of 1.1) and mixotrophic (SD of 3.5) conditions [2], respectively (Figure 4.2). However, these observed differences are likely due to background fluorescence, as growth rates on all water samples appear to be identical expect for the first day on Redmond's pond water. Based on AWFD only, no difference in water quality can be detected between the samples used in this study, with the possible exception of Redmond's pond water. It is anticipated that there will be certain differences in the water quality due to their origin. Although, detailed chemical analysis of the samples was not the scope of this study, only a few characteristics such as UV absorbance, total organic carbon (TOC), nitrate, and phosphate concentrations were measured and are presented in Figure 4.1 and Table 4.1. The data show measurable differences, as do the ultra-violet (UV) spectra (Figure 4.1). Typically, larger UV absorbance in the spectral range from 190 to 350 nm indicates the presence of aromatic compounds in water, and untreated OSPW sample showed the highest UV absorbance [20]. However, these differences affect algal communities cannot be quantified through only simple AWFD comparisons. Moreover, the TOC of different water samples varied between 30-120 mg/L, nitrate varied from 0.3-1.2 mg/L, and the phosphate concentration varied from 0.004-15.3 mg/L, the effects of these variations are not clear in the growth curves, probably due to the masking effect of the control, which has high TOC, nitrate, and phosphate concentrations as shown in Table 4.1. In future, this could be rectified by having a mixed community prepared by water sample dilution instead of using a modified HS media dilution. Therefore, the data suggest that the incubation time for further analysis should be conducted.

The data obtained from the Biolog plates were further analyzed through the one-

dimensional metric of Euclidean distances (ED). A procedure developed by Weber and Legge [16] was followed for the analysis. ED can be used as a matrix to determine the best time point for detailed analysis. In order to do so, the ED of the fluorescence developments of the 59 wells containing different carbon source for each water sample can be determined. The growth on the different carbon sources deviate the most from each other at the time point when the ED goes through a maximum, hence provides the most information about the sample. The Euclidean distances of the fluorescence developments of 59 wells for each water sample between each time point and time zero are shown in Table 4.2. Though the EDs increase until day five, the relative difference between the EDs of the different water samples was the highest at day four for heterotrophic growth and at day three for mixotrophic growth (Table 4.2), which were therefore chosen for further analysis.

Table 4.2 Euclidean distances (ED) for different water samples (see Table 4.1) are shown through 5th day. The values are the average of the triplicates and standard deviations. The standard deviations between the water samples are also calculated.

		Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	
Condition	Day	ED	ED	ED	ED	ED	ED	ED	ED	ED	SD
Heterotrophic	0	0	0	0	0	0	0	0	0	0	0
	1	9.6 ± 0.7	10.7 ± 1.6	10.7 ± 0.7	34.5 ± 1.9	4.7 ± 0.6	8.9 ± 0.7	12.8 ± 1.3	10.8 ± 1.1	11.5 ± 0.3	8.5
	2	19.4 ± 0.9	19.3 ± 4.0	20.4 ± 3.9	54.9 ± 2.5	16.2 ± 1.8	20.6 ± 2.7	19.4 ± 0.6	18.2 ± 1.7	24.2 ± 0.2	11.9
	3	39.5 ± 2.7	37.5 ± 2.5	36.9 ± 3.1	83.5 ± 3.0	36.3 ± 1.7	36.3 ± 3.1	35.6 ± 1.7	36.9 ± 0.3	36.4 ± 0.1	15.6
	4	59.1 ± 1.4	62.3 ± 1.4	65.1 ± 0.1	109.0 ± 2.9	61.7 ± 3.6	57.9 ± 1.6	57.8 ± 2.7	57.3 ± 0.2	57.1 ± 2.3	16.6
	5	74.9 ± 1.5	84.4 ± 2.7	82.5 ± 2.8	119.5 ± 5.0	85.1 ± 2.4	75.9 ± 4.2	69.5 ± 2.0	80.6 ± 2.1	78.9 ± 0.3	14.4
Mixotrophic	0	0	0	0	0	0	0	0	0	0	0
	1	26.3 ± 1.5	31.4 ± 1.8	25.0 ± 1.2	81.4 ± 1.4	44.9 ± 0.9	32.4 ± 0.9	29.5 ± 5.0	25.5 ± 1.0	26.7 ± 3.0	18.1
	2	51.9 ± 0.6	63.3 ± 0.4	70.9 ± 1.1	139.4 ± 3.5	72.1 ± 2.0	54.9 ± 2.3	76.0 ± 6.4	52.9 ± 1.1	60.8 ± 0.5	27.0
	3	91.2 ± 1.8	130.0 ± 2.4	99.4 ± 4.4	204.2 ± 1.5	143.2 ± 2.3	117.7 ± 3.2	105.3 ± 3.5	113.0 ± 2.2	110.3 ± 2.9	33.9
	4	135.6 ± 2.3	152.0 ± 3.4	172.6 ± 2.6	224.5 ± 2.6	162.6 ± 3.6	144.4 ± 2.6	182.6 ± 1.2	181.2 ± 2.5	177.4 ± 2.1	26.3
	5	210.1 ± 5.0	191.8 ± 2.1	195.8 ± 1.6	276.8 ± 3.3	227.3 ± 1.6	251.2 ± 1.8	205.5 ± 7.5	216.8 ± 4.2	227.4 ± 5.8	27.3

4.3.2 Community level physiological profiles (CLPP) for various water samples

Based on the ED results, the fourth day for heterotrophic growth and third day for mixotrophic growth were chosen for further analysis. Figure 4.3 and 4.4 show the natural log of the fluorescence at these times for the algal community in the presence of the respective carbon sources for each water type under hetero- and mixotrophic growth conditions. The effects of water and organic carbon substrates were analyzed, which revealed that both were significant (two way ANOVA; Table 4.3). Moreover, the effects of water samples were dependent on the organic carbon substrates that the algal community utilized in both growth conditions (Table 4.3). This suggests a change in algal community of equal proportions due to the effects of both water type and organic carbons. The combined effect on five individual algal strains due to two external factors in a community resulted in unique profiles (Figure 4.3 and 4.4). A similar finding of growth dependency on organic substrates along with other chemicals was noted when a single strain of bacteria and yeast were tested with inorganic ions (aluminium and copper, [21]) and mercaptoethanol [22]. Here, the changes in individual five single algal strains in the community could potentially maximize the difference and consequently increase the resolution of the assay. The data as presented in Figure 4.3 and 4.4 however are not a practical tool to compare different water samples. Additional data analysis is needed to provide a convenient way to distinguish between different water samples.

Figure 4.3 Fluorescence developments after 4 days for heterotrophic growth condition in the presence of different individual organic carbons. The average readings (n = 3) are used and standard deviations are within 10%. Description on samples can be referred to Table 4.1.
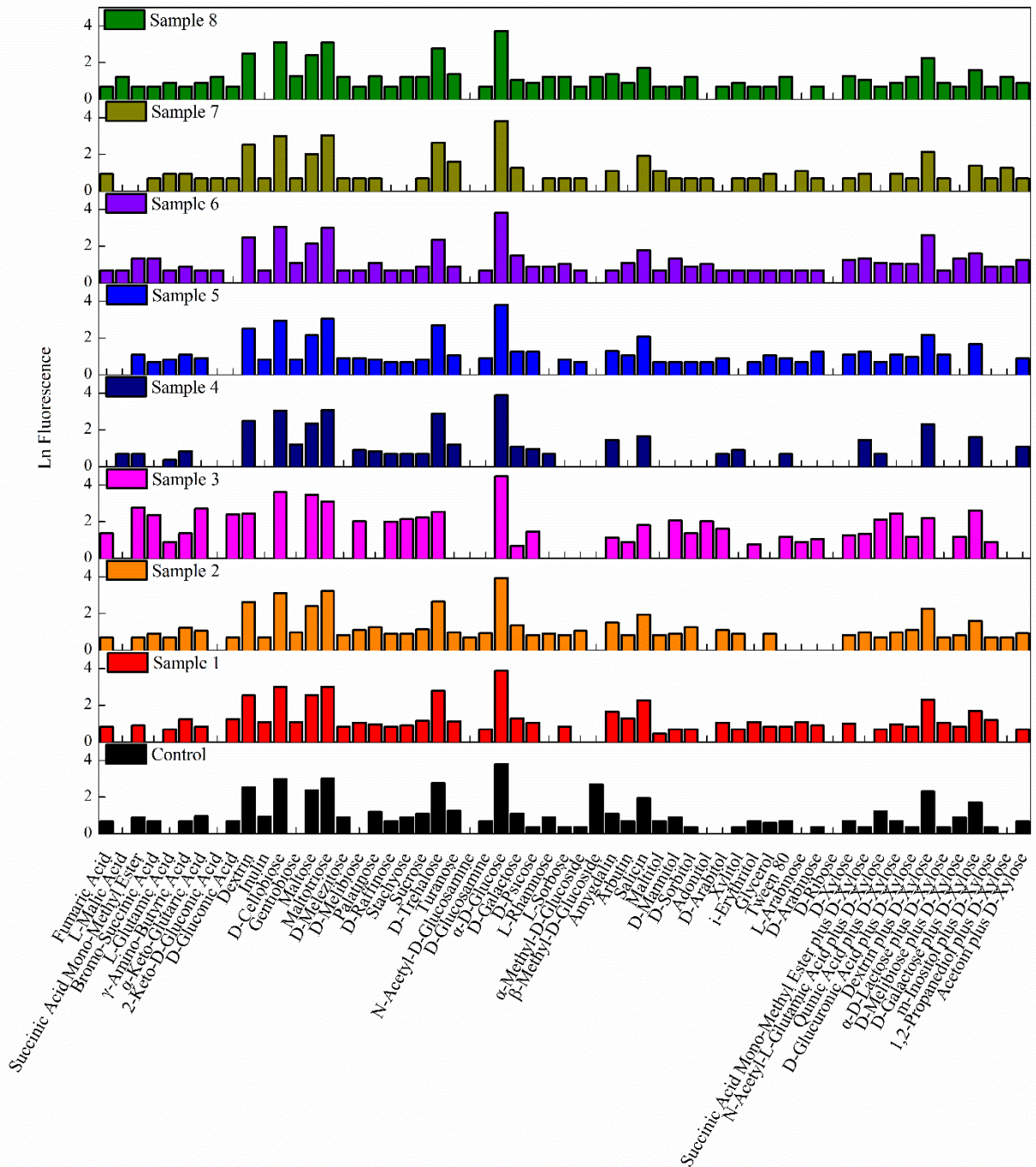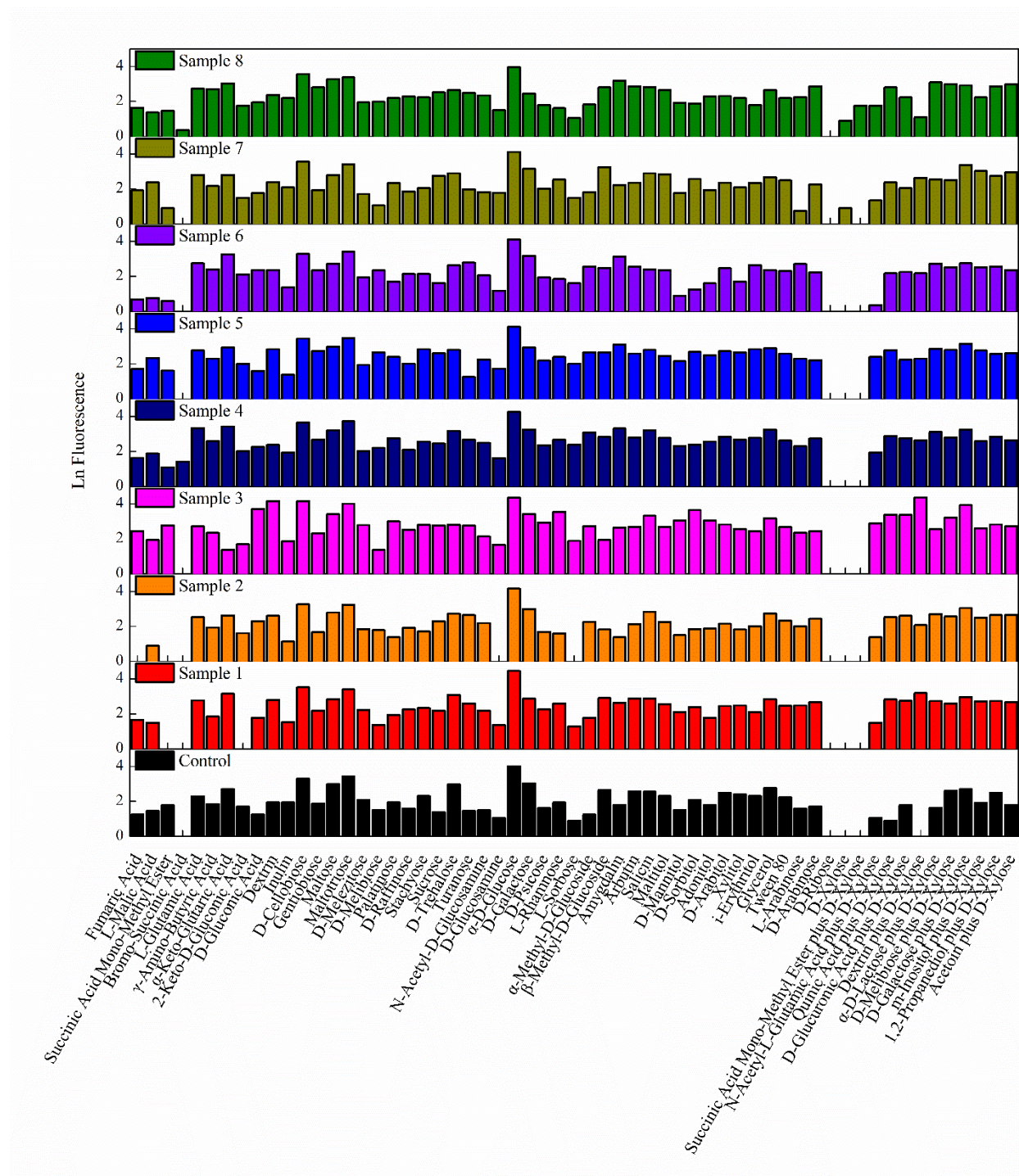
Figure 4.4 Fluorescence developments after 3 days for mixotrophic growth condition in the presence of different individual organic carbons. The average readings (n = 3) are used and standard deviations are within 10%. Description on samples can be referred to Table 4.1.

Table 4.3 Results of two way analysis of variance examining the effects of water source (WS), organic carbon (OC) and their interactions (WS X OC) on growth of algal community. Summary data used in the ANOVA are presented in Figure 4.3 and 4.4.

Figure 3 and 4	Source of variation	Df	F	P
Heterotrophic	WS	8	158.4	<0.001
	OC	58	578.9	<0.001
	WS X OC	464	20.5	<0.001
Mixotrophic	Water source	8	468.2	<0.001
	OC	58	667.9	<0.001
	WS X OC	464	21.8	<0.001

4.3.3 Characterizing CLPP using principal component analysis (PCA)

Differences in the response of the assay presented by different profiles were then quantified via principal component analysis (PCA) and visualized graphically in three-dimensional spaces (Figure 4.5). The analyses identified three principal components (PC). The first principal component accounted for 35 and 38%, the second accounted for 15 and 13%, and the third accounted for 9 and 11% variation in the data for heterotrophic and mixotrophic growth conditions, respectively (Figure 4.5). The effects of water samples on the change of algal community were more pronounced under mixotrophic growth condition than under heterotrophic ones as the locations of points for different water samples are more distinguished (Figure 4.5 b) rather than clustered together (Figure 4.5 a). The relative differences are provided by the assay and showed its ability to distinguish the different water chemistry in same groups such as wetland water samples, Walkers pond, Pond mills and Redmond's pond, and oil sand process waters, treated and untreated OSPW (Figure 4.5 b). The similarity between waters in different groups (circled) can be investigated further by isolating from the other samples (Figure 4.5 d). Moreover, the power of the assay to distinguish between water samples was less in heterotrophic growth condition, albeit, Redmond's pond, primary effluent, North Thames River, and treated and untreated OSPW showed more distinctive profiles differentiating from other waters clustered (Figure 4.5 a). Again, when PCA was conducted for control, Walkers pond, Pond mills and secondary effluent, the differences were apparent (Figure 4.5 c). In both growth conditions, the differences still existed but not to the degree of disparate waters which might be due to the lack of sensitivity with five algal strains in community and proposing for usage of larger algal community, which

will add more sensitivity to the assay in the future.
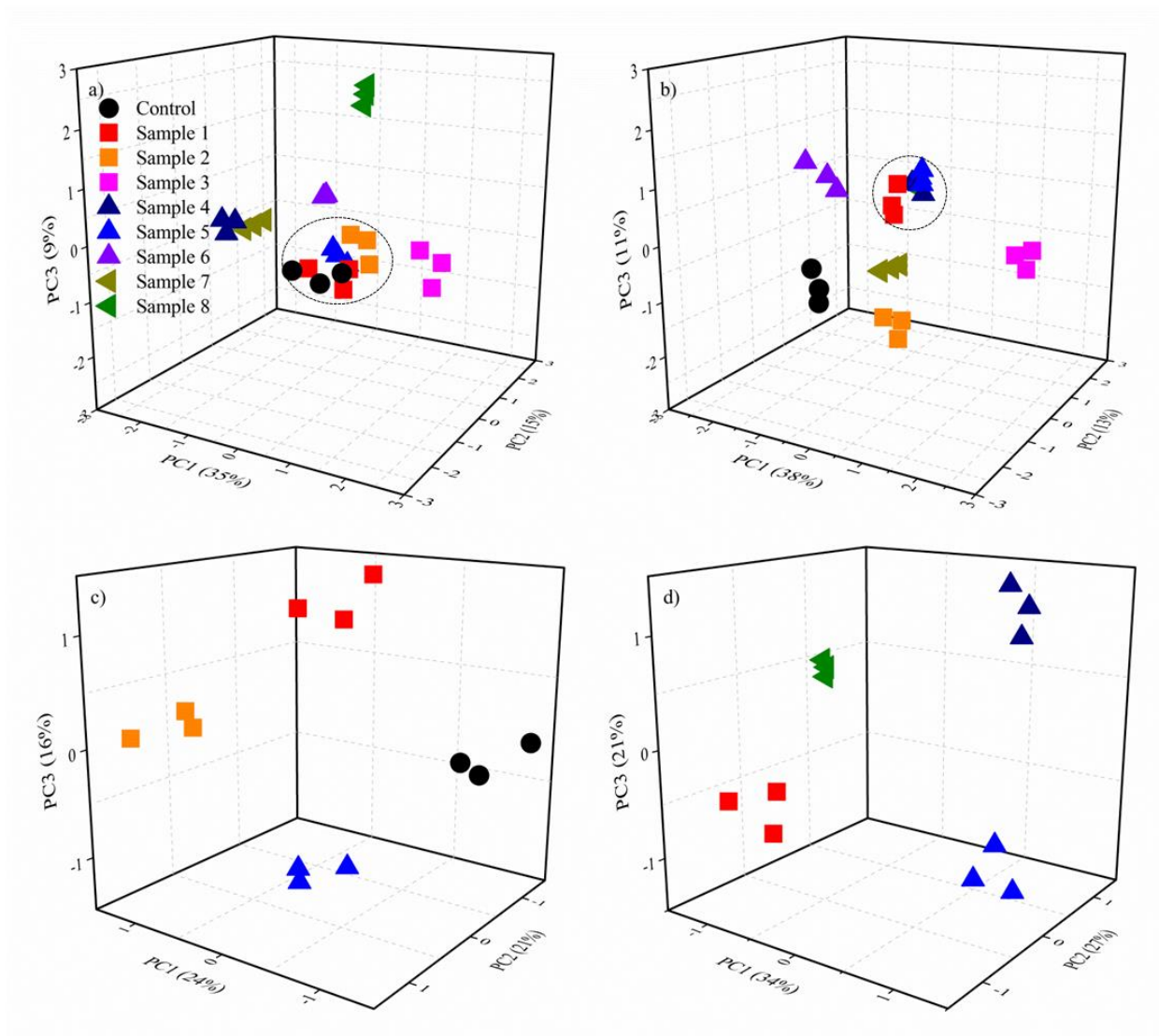
Figure 4.5 Water samples (see Table 4.1) under a) heterotrophic and b) mixotrophic conditions are shown using principal component analysis (PCA). Different profiles of natural logarithm (Ln) transformed fluorescence were used (refer to Figure 4.3 and 4.4). Points clustered are circled and again analyzed using PCA to resolve for c) heterotrophic and d) mixotrophic conditions.

4.4 Conclusions

This work demonstrates the successful application of a microalgal bioassay based on CLPP on various types of waters, including real environmental samples and differently treated municipal wastewater and OSPW. Therefore, this technology could be used for the monitoring and maintenance of the clean environmental water management and to check on different water treatments by characterizing their influences on the microalgal community.

4.5 Literature Cited

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Chapter 5. General Discussion and Recommendations

5.1 General Discussion

This research showed that focusing on a single carbon source or a single mode of cultivation was not enough to capture the potential toxicity effect of a micropollutant on a target algae species. This was shown by the variations in toxicity effects by two chemically similar QACs. The results provided here could be important for the further studies on toxicological mechanisms of QACs as the mechanisms of toxicity are still unknown. Moreover, the standardized microalgal toxicity test following the OECD guidelines should be modified in the future to include mixotrophic and heterotrophic growth conditions in addition to the autotrophic growth condition that has already been stipulated. Unlike other microbes, microalgae have both light and dark cycles and capability of catabolizing organic carbons in addition to inorganic carbon sources. These aspects should be considered in toxicity studies using microalgae.

This research showed for the first time, the application of community level physiological profiling (CLPP) technique on microalgae for the development of a microalgal bioassay. The developed bioassay was very sensitive and capable of detecting the toxicity effect of a tested micropollutant, hexadecyl trimethyl ammonium chloride (CTAC), at a relatively low concentration of 133 µg/L. Moreover, the results remained unchanged even with a very low inoculum density and a combination of two antibiotics, streptomycin and penicillin. With these features, this bioassay could be applied to the collected water from potential contaminated sites and fields for evaluating the water quality, or to various micropollutants listed in the environmental quality guidelines for re-evaluating the microalgal toxicity and incorporating the results in the modified version of guidelines in the future. Earlier, in case of a similar micropollutant tested here, didecyl dimethyl ammonium chloride (DDAC), the toxicity result to a single algal strain was exempted from the guideline setting calculation by the regulators because of its low sensitivity, however, with the developed bioassay, microalgae could take part in toxicology analysis of micropollutants setting new guidelines.

Furthermore, the application of the assay is not restricted to testing only toxic

compounds but also in distinguishing water of mixtures of various elements. The developed bioassay is expanded to differentiate various water sources of relevance to natural environment and could be adopted for field applications by the environmental engineers.

5.2 Recommendations for Future Work

A number of things needed to be improved and can be tried for future studies. The first improvement could be done by designing a microplate of organic carbons that are more specific and appropriate for microalgae utilizations. The commercially available Biolog plates with organic carbon substrates are more specific for bacteria, yeast, and fungi and are less efficient for microalgae. This is important as the power of the assay is directly correlated with carbon responses. Secondly, more of freshwater microalgae strains can be employed in the mixtures of community, which confers more sensitivity to the assay, as many microalgae have different responses for varying toxic compounds. Moreover, the assay can be explored using marine phytoplankton for assessing toxic compounds found in seawater and natural algal community for direct assessing the field conditions. Furthermore, the interpretation of the assay can be improved with various chemical analyzes.

Appendices
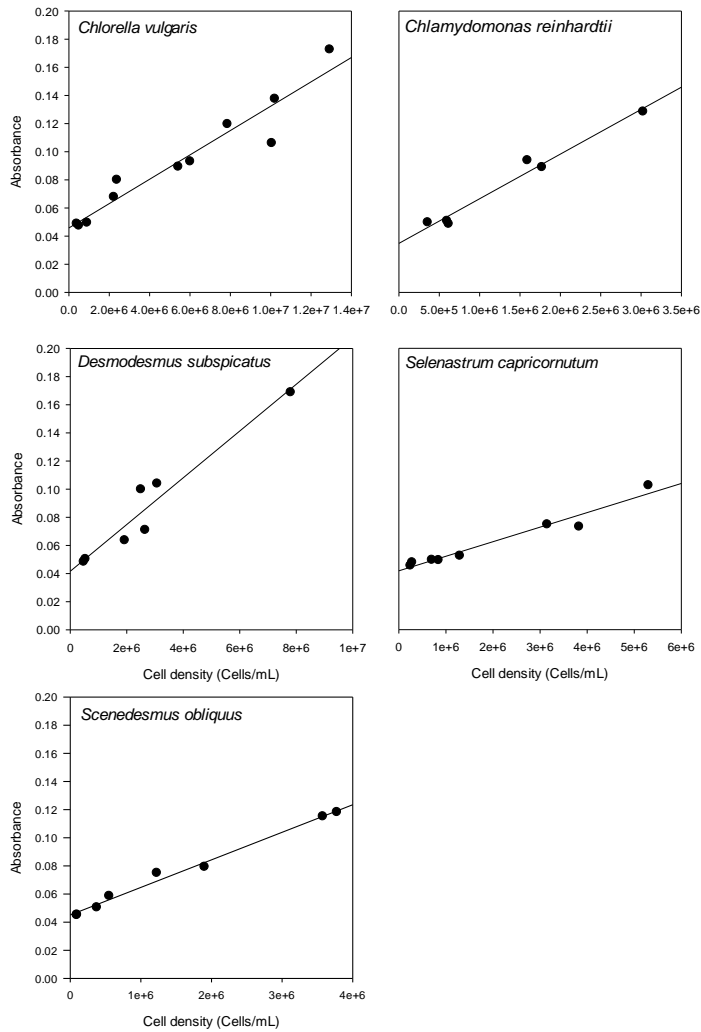
Appendix A Absorbance VS. Cell density

% Entering data *

DTAC_Mixotrophic=[0 28.47682   22.51656   50.33113   52.31788   58.27815   56.29139
0   22.51656   24.50331   55.62914   49.33775   46.35762   62.25166
0   41.22807   40.78947   57.89474   64.47368   89.47368   100
0   0   20   70   73.33333   100 90
0   12.8012 16.86747   23.19277   15.51205   12.8012 31.77711
0   0.83333 5.83333 20   30   45.83333   35.83333
0   18.07229   27.71084   27.71084   24.09639   26.50602   32.53012
0   15.56886   17.36527   44.31138   55.08982   51.49701   53.29341
0   24.6114 26.1658 36.26943   36.26943   46.37306   50.25907
0   40.76087   48.36957   47.28261   50.54348   49.63768   52.17391
0   21.01064   29.78723   43.61702   42.02128   48.40426   44.68085
0   0   4.45205 23.9726 13.69863   30.47945   76.36986
0   22.68041   31.95876   40.72165   37.62887   45.36082   47.42268
0   21.50776   28.82483   45.12195   55.43237   54.102   69.06874
0   28.60697   25.37313   27.98507   37.68657   38.0597 52.23881
0   28.77095   28.77095   41.34078   49.72067   53.07263   54.18994
0   22.15569   31.73653   48.50299   48.8024 49.7006 50.2994
0   14.94253   27.20307   35.05747   36.78161   36.39847   41.37931
0   22.0339 33.89831   56.77966   47.45763   50.84746   55.9322
0   24.59016   27.86885   37.70492   45.08197   52.45902   53.00546
0   23.18841   21.73913   43.47826   36.95652   47.10145   44.92754
0   24.64115   36.84211   46.88995   51.19617   47.84689   53.11005
0   20.19231   28.60577   46.63462   46.63462   52.40385   56.73077
0   13.82979   19.85816   29.07801   31.20567   41.48936   71.2766
0   19.62025   20.88608   32.27848   38.60759   42.61603   48.73418
0   13.33333   20   38.88889   40.83333   41.66667   48.33333
0   26.70807   36.02484   46.58385   36.02484   49.06832   47.82609
0   53.84615   42.30769   53.84615   53.84615   52.5641 50
0   25.75   41.5   52   53.5   53   51
0   18.42105   3.28947 34.86842   30.92105   27.96053   34.86842
0   12.9771 18.32061   53.05344   63.35878   76.33588   80.53435
0   56.28571   58.85714   71.42857   78.85714   87.42857   88.57143
0   10.6267 4.76839 46.04905   77.52044   88.55586   99.45504
0   18.3432 28.99408   44.97041   44.97041   52.07101   50.29586
0   19.88636   25.85227   41.47727   46.30682   47.72727   50
0   21.31148   26.22951   36.61202   41.80328   42.62295   48.08743
0   33.50515   32.47423   34.53608   34.53608   48.45361   45.36082
0   29.36047   40.69767   47.67442   58.13953   59.30233   59.88372
0   39.50276   44.47514   50.27624   49.72376   55.8011 47.51381
0   26.36364   4.54545 15.45455   4.54545 12.72727   18.18182
0   26.45161   30.80645   46.29032   48.70968   49.67742   51.93548
0   25.76531   39.54082   50   49.4898 57.14286   66.32653
0   29.87013   37.66234   53.24675   61.68831   68.83117   68.18182
0   16.66667   21.11111   43.33333   45.55556   60   70
0   50   75   87.06897   88.7931 98.27586   100
0   32.25806   32.25806   41.93548   51.6129 41.93548   37.09677
0   98.21429   100 100 100 100 100
0   16.30435   13.04348   9.78261 10.86957   18.47826   20.1087
0   27.14286   35.71429   38.57143   41.42857   47.85714   45.35714
0   21.15385   30.12821   36.53846   39.10256   52.5641 56.41026
0   34.84163   46.38009   45.70136   50.45249   55.20362   57.91855
0   43.39623   34.90566   34.19811   30.66038   41.98113   39.62264
0   16.4557 25   28.79747   34.49367   36.39241   44.93671
0   20.94595   21.4527 35.81081   32.60135   42.22973   45.27027

```
0     5.66038 29.24528      28.30189      30.18868      50    50.9434
0     28.7037 35.18519      35.18519      38.88889      45.37037      55.55556
0     27.35849      23.27044      35.84906      36.47799      47.16981      50];
```

```
c=[0     400 800 1200      1600      2000      2400];
```

% Regression analysis based on Hill Equation

```
Hilleq=fittype(@(EC50, m, x) 0+(100-0)./(1+(x./EC50).^(-m)));
```

```
p0=[200, 2];
for g=1:57

    options = fitoptions(Hilleq);
    options.Lower = [0 1];
    options.StartPoint = p0;
    curve = fit( c', DTAC_Mixotrophic(g,:)', Hilleq, options );
    newcoeffcients_DTAC_Mixotrophic(g,:)=coeffvalues(curve);

end
EC50_DTAC_Mixotrophic=newcoeffcients_DTAC_Mixotrophic(:,1);
```

%ploting

```
figure(1)
c_plot=linspace(min(c), max(c));
for g=1:57
    fitteddata_EC50_DTAC_Mixotrophic(g,:)= 0+(100-
0)./(1+(c_plot./newcoeffcients_DTAC_Mixotrophic(g,1)).^(-newcoeffcients_DTAC_Mixotrophic(g,2)));
    subplot(10,6,g)
    plot(c_plot, fitteddata_EC50_DTAC_Mixotrophic(g,:))
    hold on
    plot(c, DTAC_Mixotrophic(g,:), 'o')
end
```

Appendix B Matlab codes for EC_{50} estimation and graph for DTAC mixotrophic condition

Curriculum Vitae

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- Jun-Woo Kim, Lars Rehmann, Madhumita B. Ray (2017). Development of microalgal bioassay based on community level physiological profiling (CLPP). *Algal Research, 25, 47 – 53*
- Adnan Hossain Khan, Jun-Woo Kim, Mark Sumarah, Sheila M. Macfie, Madhumita B. Ray (2017). Toxicity reduction and improved biodegradability of benzalkonium chlorides by ozone/hydrogen peroxide advanced oxidation process. *Separation and Purification Technology, 185, 72 – 82*