

Microalgae growing in stressed environments and their antioxidant potential from production of  
secondary metabolites

By

Miranda Rose Gauthier

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**APPROVED/APPROUVÉ**

Thesis Examiners/Examineurs de thèse:

Dr. JA Scott  
(Supervisor/Directeur(trice) de thèse)

Dr. CA Laamanen  
(Committee member/Membre du comité)

Dr. Nathan Basiliko  
(Committee member/Membre du comité)

Dr. Geruso Senhorinho  
(Committee member/Membre du comité)

Dr. David J. Garbary  
(External Examiner/Examineur externe)

Approved for the Faculty of Graduate Studies  
Approuvé pour la Faculté des études supérieures  
Dr. Serge Demers  
Monsieur Serge Demers  
Acting Dean, Faculty of Graduate Studies  
Doyen intérimaire, Faculté des études supérieures

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# Abstract

Microalgae are photosynthetic microorganisms found in aquatic environments around the world. There is interest in using microalgae to capture carbon (CO<sub>2</sub>) from industrial off-gas, but sulphur dioxide often present in these gasses increases growing media acidity making it essential to find microalgal strains able to survive in pH 3.0-4.0. High metal concentration, acidity, solar irradiance, and nutrient limitations can instigate the production of protective secondary metabolites with antioxidant potential. Therefore, the antioxidant potential of novel microalgal isolates bioprospected from acidic mine-impacted water systems, identified as *Coccomyxa* sp. and *Chlamydomonas* sp., and a culture collection strain *Chlamydomonas reinhardtii* were tested using three antioxidant assays. Results showed that low pH conditions (pH 3.0) increased biomass production of *Coccomyxa* sp. but induced the death of *C. reinhardtii*. Under both pH 3.0 and uncontrolled pH conditions, the bioprospected strains had higher antioxidant potential than *C. reinhardtii*, with *Coccomyxa* sp. having the highest potential.

## *Keywords*

Antioxidants, Free radicals, Fresh-water microalgae, Green microalgae, Oxidative stress, Reactive oxygen species

# Co-Authorship Statement

Chapters 1 and 2 of this dissertation are co-authored by Dr. J.A. Scott and Dr. G.N.A. Senhorinho. Both co-authors contributed to developing the main conceptual ideas, supervising the project, providing critical feedback, and contributing to final manuscript. Chapter 3 of this dissertation is co-authored by Dr. J.A. Scott, Dr. G.N.A. Senhorinho, Dr. N. Basiliko, and Sabrina Desjardins. Dr. J.A. Scott and Dr. G.N.A. Senhorinho contributed to formulating the experimental hypothesis and supervising the project. Dr. G.N.A. Senhorinho and Sabrina Desjardins contributed to developing and executing experimental procedures. All authors contributed to providing critical feedback and editing for the final version of this chapter.

# Preface

There is an increasing urgency for industries to reduce their environmental emissions to curtail the ongoing climate crisis, which brings a corresponding urgency for creative and cost-effective solutions. Upon entering the Chemical Engineering program at Laurentian University, I discovered the exciting complexity of metallurgy and the intriguing necessity for the mining industry to modify its practices to continue to supply the many mineral products we use every day while maintaining the integrity of the environment. Being a steward for the environment with great interest in metallurgy I decided to pursue a graduate degree with Dr. Scott and Ongen to research novel ways to reuse resources in the mining industry.

Expanding on earlier research performed by Ongen, I focused my research on microalgae bioprospected from abandoned mine sites in Northern Ontario. Microalgae are incredibly resilient microorganisms that can survive in various harsh environments, including the acidic and metal-rich waters resulting from mining activities. The stress imparted on the microalgae by these harsh conditions, known as oxidative stress, instigates the microorganisms to produce compounds with antioxidant potential to protect themselves. Antioxidant compounds can be extracted from microalgal cells and used commercially in nutraceuticals for human consumption.

Humans are exposed to an array of pollutants and oxidative stressors that cause an accumulation of reactive oxygen species within our bodies. Research shows that reactive oxygen species contribute to the development of several degenerative diseases including cardiovascular disease, chronic inflammation, and cancer. A diet having plenty of fruits and vegetables rich in antioxidant compounds has been found to reduce the development of such diseases. When a balanced diet is

unattainable, modern society turns to nutraceutical supplements to fulfill nutritional deficiencies. However, many synthetically produced antioxidants are carcinogenic and unstable, making naturally produced antioxidants a desirable commodity.

Microalgae have exceptional carbon capturing abilities that can be applied to industrial off-gas pollution mitigation. Compounds in industrial off-gases, such as sulphur dioxide, can acidify the algal growing media making it unlivable for many strains of microalgae and cause oxidative stress. The purpose of my research was to determine if microalgae bioprospected from mine-impacted waters can survive in acidic conditions similar to those that would be caused by industrial off-gas while still producing economically beneficial antioxidant compounds.

The following dissertation is based on experiments designed and performed as a requirement for a Master of Applied Science in Natural Resource Engineering degree from Bharti School of Engineering at Laurentian University in Sudbury, Ontario, Canada. This project is funded by MITACS (project IT11703) and the Ontario Centre of Excellence (project 29434).

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# Table of Contents

<b>Abstract.....</b>	<b>iii</b>
Keywords .....	iii
<b>Co-Authorship Statement .....</b>	<b>iv</b>
<b>Preface.....</b>	<b>v</b>
<b>Acknowledgments .....</b>	<b>vii</b>
<b>Table of Contents .....</b>	<b>viii</b>
<b>List of Figures.....</b>	<b>xi</b>
<b>List of Tables .....</b>	<b>xiii</b>
<b>List of Abbreviations .....</b>	<b>xiv</b>
<b>1 Microalgae under environmental stress as a source of antioxidants .....</b>	<b>1</b>
1.1 <i>Microalgae: Oxidative Stress and Antioxidant Production.....</i>	2
1.2 <i>Abstract.....</i>	2
Key words .....	3
1.3 <i>Introduction.....</i>	3
1.4 <i>Reactive Oxygen Species and Antioxidants .....</i>	5
1.5 <i>Metals and Acidic Conditions.....</i>	16
1.5.1 <i>Metal Entry Into the Cell .....</i>	17
1.5.2 <i>Reactive Oxygen Species (ROS) Production and Cellular Response to Metal Stress</i> 18	
1.5.3 <i>Acidic Conditions and Metal Stress.....</i>	20
1.6 <i>High Solar Irradiance.....</i>	23
1.6.1 <i>Reactive Oxygen Species (ROS) Production and Cellular Response to High Solar</i> Irradiance .....	24
1.7 <i>Nutrient Limitations.....</i>	27

1.7.1	Reactive Oxygen Species (ROS) Production and Cellular Response to Nutrient Limitations .....	28
1.8	<i>Conclusions</i> .....	31
<b>2</b>	<b>Hypothesis and Objectives .....</b>	<b>33</b>
2.1	<i>Hypothesis</i> .....	33
2.2	<i>Objectives</i> .....	34
<b>3</b>	<b>Green photosynthetic microalgae from low pH environments as a potential source of antioxidants .....</b>	<b>35</b>
3.1	<i>Green Photosynthetic Microalgae From Low pH Environments as a Potential Source of Antioxidants</i> .....	36
3.2	<i>Introduction</i> .....	37
3.3	<i>Materials and Methods</i> .....	39
3.3.1	Chemicals and Materials.....	39
3.3.2	Microalgal Strains.....	39
3.3.3	DNA Extraction and Sequencing.....	40
3.3.4	Microalgal Culture Conditions and Extraction .....	41
3.3.5	Trolox Equivalence Antioxidant Assays .....	42
3.3.6	Chlorophyll Colourimetric Assay .....	45
3.3.7	Statistical Analysis.....	45
3.4	<i>Results</i> .....	46
3.4.1	Microalgal Identification .....	46
3.4.2	Effect of Controlled pH on Antioxidant Activity and Chlorophyll Content .....	47
3.5	<i>Discussion</i> .....	56
<b>4</b>	<b>Conclusions.....</b>	<b>62</b>
4.1	<i>Summary of Results</i> .....	62
4.2	<i>Future Work</i> .....	63

**References ..... 65**

# List of Figures

Figure 1.	Initiation, propagation, and termination reactions involved in lipid peroxidation [21,22]. Polyunsaturated fatty acid (LH) initiated by free radical ( $R \cdot$ ) and becomes a fatty acid radical ( $L \cdot$ ) and reduced radical (RH). Fatty acid radical reacts with oxygen to become a fatty acid peroxy radical ( $LOO \cdot$ ) which reacts with other fatty acids to become lipid hydroperoxide (LOOH). Lipid hydroperoxide breaks down to form reactive aldehyde compounds. Antioxidants reduce fatty acid radical back to polyunsaturated fatty acid. ....	7
Figure 2.	Pyrite ( $FeS_2$ ) oxidation causing acid mine drainage [39]. ....	16
Figure 3.	Iron driven Fenton and Haber-Weiss reactions producing reactive oxygen species in microalgal cells [14]. ....	20
Figure 4.	How irradiance ( $h\nu$ ) from the sun overstimulates photosynthesis, causing an excess of electrons ( $e^-$ ) and the production of reactive oxygen species (ROS). Mycosporine amino acids (MAA) are produced to shield the cell from irradiance. ....	26
Figure 5.	Nutrient deficiencies promote increased storage of lipids and starches, degradation of proteins, a production of reactive oxygen species (ROS) in microalgae. ....	30
Figure 6.	(A) Microalga M2 identified as <i>Coccomyxa</i> sp. (B) Microalga M23 identified as <i>Chlamydomonas</i> sp. ....	46
Figure 7:	Culture concentration of samples growing in controlled pH 3.0 medium and uncontrolled medium in grams of dry weight per litre of medium. Bar represents the mean $\pm$ SD (n = 3). Significance ( $p < 0.05$ ) between conditions with respect to each species expressed by lower case letter. ....	48
Figure 8:	Extraction yield of crude extracts from dry microalgal biomass of three species growing in unbuffered and pH 3.0 buffered conditions (% w/w). Bar represents the mean $\pm$ SD (n = 9). Significance ( $p < 0.05$ ) between conditions with respect to each species expressed as lower case letters. ....	50
Figure 9:	Significant ( $p < 0.05$ ) correlations between antioxidant activity in TEQ ( $\mu\text{mol trolox g}^{-1}$ dry weight biomass) and extraction yield of crude extract from dried biomass (% w/w) of <i>Coccomyxa</i> sp. samples growing at pH 3 and uncontrolled pH conditions. (n=15) ....	51

Figure 10: Trolox equivalent antioxidant activity (TEAC) ( $\mu\text{mol g}^{-1}$  dry weight biomass) according to ABTS, FRAP, and DPPH assays for extracts from strains grown in uncontrolled pH medium and buffered pH 3.0 medium. Bar represents the mean  $\pm$  SD (n = 9). ..... 53

Figure 11. Correlation between chlorophyll-a content in  $\text{mg g}^{-1}$  dry weight biomass and extraction yield of crude extract from dried biomass (% w/w) of *Coccomyxa* sp. samples growing at controlled pH 3.0 and uncontrolled pH conditions. (n = 15). ... 55

# List of Tables

Table 1. Antioxidant compounds with increased production by microalgae under oxidative stress. .... 10

Table 2. Ratio of chlorophyll-*a* to chlorophyll-*b* in three microalgal species growing in a buffered pH 3 medium and an unbuffered medium after 28 days. (NT = not tested as the cells died) (\*denotes significance across rows  $p < 0.05$ ). .... 54

## List of Abbreviations

<b>4-HNE</b>	4-hydroxynonenal	<b>GPx</b>	Glutathione peroxidase
<b>ABTS</b>	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid	<b>GR</b>	Glutathione reductase
<b>AMD</b>	Acid-mine drainage	<b>GSH</b>	Glutathione
<b>APX</b>	Ascorbate peroxidase	<b>HPLC</b>	High performance liquid chromatography
<b>BBM</b>	Bold's Basal Medium	<b>HSP</b>	Heat-shock protein
<b>BHA</b>	Butylated hydroxyanisole	<b>IR</b>	Infrared radiation
<b>BHT</b>	Butylated hydroxytoluene	<b>LC-MS</b>	Liquid-chromatography mass-spectrometry
<b>CAT</b>	Catalase	<b>MAA</b>	Mycosporine amino acid
<b>CPCC</b>	Canadian Phycological Culture Collection	<b>MDA</b>	Malondialdehyde
<b>DNA</b>	Deoxyribonucleic acid	<b>PAR</b>	Photosynthetically active radiation
<b>DPPH</b>	2,2-diphenyl-1-picrylhydrazyl	<b>PCR</b>	Polymerase chain reaction
<b>EDTA</b>	Ethylenediaminetetraacetic acid	<b>POD</b>	Peroxidase
<b>FRAP</b>	Ferric reducing antioxidant power	<b>PSII</b>	Photosystem II
<b>FTIR</b>	Fourier-transform infrared spectroscopy	<b>PUFA</b>	Polyunsaturated fatty acid
		<b>RNA</b>	Ribonucleic acid
		<b>ROS</b>	Reactive oxygen species
		<b>SOD</b>	Superoxide dismutase

**TPTZ** 2,4,6-tri(2-puridyl)-s-triazine

**UVR** Ultra-violet radiation

# Chapter 1

## **1 Microalgae under environmental stress as a source of antioxidants**

**(Review)**

Gauthier, M.R.,<sup>1,2</sup> Senhorinho, G.N.A.<sup>1,2</sup> and Scott, J.A.<sup>1,2,3</sup>

<sup>1</sup> Bharti School of Engineering, Laurentian University, Sudbury, Ontario, Canada

<sup>2</sup>Vale Living with Lakes Centre, Laurentian University, Sudbury, Ontario, Canada

<sup>3</sup>Department of Biology, Laurentian University, Sudbury, Ontario, Canada

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## 1.1 Microalgae: Oxidative Stress and Antioxidant Production

This chapter introduces background information about microalgae and how their environment influences their biochemical composition. The detriments of oxidative stress caused by reactive oxygen species and the antioxidants produced to alleviate this stress are discussed. Oxidative stress caused by acidity, metals, ultra-violet radiation, and nutrient limitations can induce production of desirable antioxidants in many species of photosynthetic green microalgae and blue-green algae. The mechanisms by which these stressors induce an accumulation of reactive oxygen species into the microalgal cell play a role in the cell's corresponding antioxidant response. These mechanisms and their application to commercial antioxidant production using microalgae are explored.

## 1.2 Abstract

The demand for naturally sourced compounds is rapidly growing as synthetically produced counterparts can be toxic and unsuitable for human consumption. Antioxidant compounds produced by microalgae under stress conditions are, therefore, becoming of commercial interest as potential natural health products. Oxidative stress caused by acidity, metals, ultra-violet radiation, and nutrient limitations can induce production of antioxidants in many species of photosynthetic green microalgae and blue-green algae. This review discusses the mechanisms of stress and the corresponding antioxidant response. Each microalgal species responds differently to each stress, yielding an increase in enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, and/or an increase in non-enzymatic antioxidants such as carotenes, xanthophylls, and flavonoids. There is potential to exploit these stress responses for commercial cultivation and the production of specific antioxidant compounds.

## *Key words*

Microalgae, antioxidants, oxidative stress, reactive oxygen species

### 1.3 Introduction

Microalgae are photosynthetic unicellular organisms that can be found in a wide range of aquatic and terrestrial environments [1]. These primitive eukaryotes have steadily evolved from prokaryotic cyanobacteria over the last 3.5 billion years and continue to prove their adaptability by acclimatizing to diverse strenuous environments [2]. It is speculated that there are 200,000 to 800,000 species of microalgae which can be found in fresh water, salt water, terrestrial, and marine ecosystems across the globe [3]. These microorganisms utilize solar energy to fix carbon dioxide at an efficiency that is up to 10-fold greater than terrestrial plants, making them essential for global carbon capture [4].

About 5,000 tons per year of dried microalgal biomass are produced commercially for use in biofuels, animal feed, and natural health products [4]. The accumulation of lipids by microalgae for use as biofuel has been investigated as a green energy source, with lipids containing 14-20 carbons commonly employed for biodiesel production [5]. The majority of the lipids obtained from microalgae are triacylglycerols, carotenoids, and long-chain polyunsaturated fatty acids [6–8].

Society's preference for naturally sourced nutraceuticals over synthetic alternatives is increasing, making the mass cultivation of microalgae for bioactive molecules a promising endeavour. Biochemicals of interest as potential health products, include long-chain polyunsaturated fatty acids with more than 20 carbons, as well as carotenoids, pigments, and some enzymes produced under stressing conditions that exhibit antioxidant properties [5].

Naturally sourced antioxidants are not only more stable, but synthetic antioxidants have been found to have potentially toxic effects and many are not suitable for human consumption [9]. Of the antioxidants widely produced by microalgae,  $\beta$ -carotene, astaxanthin, and lutein are those with the highest current market values [5].

It had been found that elevated levels of oxidative stress are correlated to many chronic and degenerative diseases in humans. A diet with plenty of antioxidant rich fruits and vegetables has been shown to reduce the prevalence of these diseases [10]. Natural source antioxidants, like the ones found in fruits and vegetables, are better for humans than synthetic antioxidants. Many mainstream synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been found to have a carcinogenic effect [10,11]. These pernicious health implications have turned antioxidant production trends towards developing safer biotechnological processes using microorganisms to produce antioxidants commercially [12].

There are many environmental influences that can impact the growth and composition of microalgae. These include metals, pH, UV stress, and nutrient availability [13]. The review by Paliwal *et al.* 2017 details how these and other abiotic stressors can influence valuable metabolite accumulation in microalgae [2]. It was noted that salinity stress and nutrient starvation are two of the most common methods used for upregulating the production of certain valuable metabolites for commercial production, especially lipids for biofuels and animal feed. They concluded that more research is needed to isolate genes responsible for enhancing pigment and valuable metabolite accumulation.

Manipulating environmental conditions during commercial cultivation can influence the production and accumulation of valuable bioproducts, such as antioxidant compounds (

Table 1). An applied stress can induce oxidative stress, which is a disproportion between prooxidant and antioxidant activity within a cell due to an increase of prooxidants [14]. Oxidative stress causes microalgae to produce valuable secondary defensive metabolites with antioxidant potential to compensate for this imbalance [10]. A review by Sathasivam *et al.* 2019 discussed some of the valuable metabolites produced by microalgae under regular and stress conditions and their commercial applications in food, aquaculture, farming, and pharmaceuticals [15]. The authors emphasized the importance of expanding opportunities for the commercial cultivation of microalgae. Commercial cultivation of microalgae is expensive and oxidative stress generally reduces the overall biomass production of the cultivated species [16]. Furthermore, Tang *et al.* 2020 discussed methods for cultivating and harvesting microalgae and the downstream processes of valuable metabolite extraction. The authors state improvements are needed on downstream extraction processes in order to make commercial cultivation of microalgae more economically feasible [17].

This review focuses on the exploitation of oxidative stress for enhancing commercial antioxidant production by microalgae. Specifically, high metal concentrations and acidic conditions, high solar irradiance, and nutrient limitations as mechanisms of oxidative stress and antioxidant responses in select species of microalgae are discussed.

## 1.4 Reactive Oxygen Species and Antioxidants

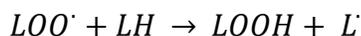
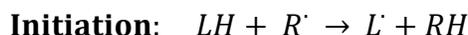
Reactive oxygen species (ROS) are the prooxidants known to cause oxidative stress in organisms and can accumulate in a cell through anthropogenic and natural abiotic sources as well as through *in vivo* processes. Cellular process that can produce ROS include photosynthesis, enzymatic reactions, and other reactions caused by cellular compounds and pollutants that enter the cell [14].

In aerobic organisms, ROS species include superoxide anions ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ), hydroxyl radicals ( $HO^{\cdot}$ ), and free radicals. Photosynthetic organisms are especially vulnerable to ROS since the reduction of molecular oxygen along the electron transport chain results in  $O_2^{\cdot-}$  [16,18–20].

Increased levels of ROS in microalgae can damage nucleic acids, proteins, lipids, and damage signal transduction [14]. This can lead to metabolic dysfunction within the cell and even cell death. ROS damages proteins and oxidizes amino acids, which potentially creates reactive hydroperoxides and carbonyls [14,18]. Lipids and membranes are vulnerable to lipid peroxidation caused by ROS, as can be seen in

Figure 1. Lipid peroxidation can produce highly reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), that can further damage proteins, amino acids, and DNA structure [19,21].

**Figure 1. Initiation, propagation, and termination reactions involved in lipid peroxidation [21,22]. Polyunsaturated fatty acid (LH) initiated by free radical ( $R^\cdot$ ) and becomes a fatty acid radical ( $L^\cdot$ ) and reduced radical (RH). Fatty acid radical reacts with oxygen to become a fatty acid peroxy radical ( $LOO^\cdot$ ) which reacts with other fatty acids to become lipid hydroperoxide (LOOH). Lipid hydroperoxide breaks down to form reactive aldehyde compounds. Antioxidants reduce fatty acid radical back to polyunsaturated fatty acid.**



Antioxidants are molecules with the ability to impede oxidation caused by ROS, thereby reducing cellular damages caused by oxidative stress [8,16]. Algae induce an antioxidant defence system consisting of enzymatic and non-enzymatic protection to mitigate organelle damage instigated by ROS, but the antioxidants produced will vary between algal species [10]. Enzymatic defences can include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxidase (POD), and ascorbate peroxidase (APX). Non-enzymatic defences can include glutathione (GSH), tocopherols, ascorbate (AsA),

ascorbic acid (AA), flavonoids, polyphenols, and carotenoids as well as the production of mycosporine amino acids (MAA) and proline [16,18,23–25].

Enzymatic antioxidants are a first-line of defence that breaks down and removes ROS causing oxidative stress by converting reactive oxygen products to  $H_2O_2$ , which is then converted to  $H_2O$  [26]. SOD is a powerful antioxidant found mainly in the cytosol, but is also present in chloroplasts, mitochondria, and peroxisomes. Its role is to convert harmful  $O_2^{\cdot-}$  to less harmful  $H_2O_2$  [8,18,26], which is then catalyzed by CAT, GPX, and APX enzymatic antioxidants. CAT is an efficient antioxidant typically found in peroxisomes, cell cytosol, and mitochondria. A single CAT molecule can convert millions of  $H_2O_2$  into  $H_2O$  and  $O_2$  in under a minute [8,18,22,26]. GPX reduces  $H_2O_2$  with GSH to form  $H_2O$  and glutathione disulfide (GSSG). GPX is mostly found in the cytosol of the cell, but can be also found in mitochondria, chloroplasts, and the endoplasmic reticulum [18,22,26]. APX can be found in chloroplasts and cell cytosol [8,18] and has a higher affinity for  $H_2O_2$  than CAT, making it highly important in scavenging of reactive  $H_2O_2$  molecules. APX reduces  $H_2O_2$  with AsA yielding  $H_2O$  and monodehydroascorbate (MDHA). These antioxidants work collectively to prevent lipid peroxidation and membrane damage [22,26].

Small molecule non-enzymatic antioxidants, such as AA and carotenoids, act as radical scavengers that attach to ROS and remove them from the cell [22]. Flavonoids have a high antioxidant activity and radical scavenging effect by chelating metal ions and protecting DNA against  $HO^{\cdot}$  damage [22]. Carotenoids and flavonoids make up the majority of the non-enzymatic antioxidants produced by microalgae [22].

Carotenoids are pigments responsible for broadening the spectrum of usable light for photosynthesis as well as protection against oxidative damage to light-harvesting pigments [20].

In microalgae, zeaxanthin, lutein, astaxanthin, and  $\beta$ -carotene are among the most common [20,27]. Carotenoids are split into two sub-classes, carotenes and xanthophylls. Carotenes consist of only hydrogen and carbon atoms, whereas xanthophylls have one or more oxygen atoms. The most prevalent carotene is  $\beta$ -carotene and the most prevalent xanthophyll is lutein [28].

**Table 1. Antioxidant compounds with increased production by microalgae under oxidative stress.**

Antioxidant type	Antioxidant compound	Stress condition	Microalgal species	Stress parameters	Productivity/activity of control	Productivity/activity with stress	Reference
Enzymatic	Ascorbate Peroxidase (APX)	Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 $\mu\text{M}$ for 7 days	< 0.2 $\mu\text{mol NADPH mg}^{-1} \text{ protein min}^{-1} *$	< 0.8 $\mu\text{mol NADPH mg}^{-1} \text{ protein min}^{-1} *$	[23]
			<i>Scenedesmus acuminatus</i>	Copper - 50 $\mu\text{M}$ for 7 days	< 0.6 $\mu\text{mol NADPH mg}^{-1} \text{ protein min}^{-1} *$	> 1.0 $\mu\text{mol NADPH mg}^{-1} \text{ protein min}^{-1} *$	
		Salinity	<i>Scenedesmus</i> sp. CCNM 1077	200 mM for 15 days	100% *	233.1% *	[29]
				2-Stage 400mM after 9 days of 15	100% *	311.9% *	
Catalase (CAT)	Metal Exposure	<i>Pavlova viridis</i>	Copper - 3 mg L <sup>-1</sup> for 14 days	< 150 U 10 <sup>6</sup> cells <sup>-1</sup> *	> 500 U 10 <sup>6</sup> cells <sup>-1</sup> *	[30]	
			<i>Scenedesmus vacuolatus</i>	Copper - 414 $\mu\text{M}$ for 7 days	< 100 U mg <sup>-1</sup> protein *	> 150 U mg <sup>-1</sup> protein *	[31]
		Nutrient Limitation	<i>Acutodesmus dimorphus</i>	Nitrogen starvation for 3 days	24.7 U x 10 <sup>3</sup> mg <sup>-1</sup> protein *	54.1 U x 10 <sup>3</sup> mg <sup>-1</sup> protein *	[25]
		pH Changes	<i>Spirulina platensis</i>	pH 7.5 from 9.0 for 14 days	2.8 U mg <sup>-1</sup> protein *	4.1 U mg <sup>-1</sup> protein *	[32]
		UV-B Radiation	<i>Dunaliella salina</i>	13.2 kJ m <sup>-2</sup> d <sup>-1</sup> (3rd day of exposure)	< 30 U mg <sup>-1</sup> protein *	63 U mg <sup>-1</sup> protein *	[33]
Glutathione Peroxidase (GPX)	Metal Exposure	<i>Pavlova viridis</i>	Copper - 3 mg L <sup>-1</sup> for 14 days	< 1000 U 10 <sup>6</sup> cells <sup>-1</sup> *	> 14000 U 10 <sup>6</sup> cells <sup>-1</sup> *	[30]	

\* denotes activity; dry weight (DW); fresh weight (FW)

**Table 1. Continued.**

Antioxidant type	Antioxidant compound	Stress condition	Microalgal species	Stress parameters	Productivity/activity of control	Productivity/activity with stress	Reference
Peroxidase (POD)		Metal Exposure	<i>Scenedesmus acuminatus</i>	Copper - 50 $\mu$ M for 7 days	< 2.0 $\mu$ mol oxi. Pyrogallol $\text{mg}^{-1}$ protein $\text{min}^{-1}$ *	> 3.0 $\mu$ mol oxi. Pyrogallol $\text{mg}^{-1}$ protein $\text{min}^{-1}$ *	[23]
		pH Changes	<i>Spirulina platensis</i>	pH 7.5 from 9.0 for 14 days	21.0 U $\text{mg}^{-1}$ protein *	30.5 U $\text{mg}^{-1}$ protein *	[32]
		UV-B Radiation	<i>Dunaliella salina</i>	13.2 $\text{kJ m}^{-2} \text{d}^{-1}$ (3rd day of exposure)	< 30 U $\text{mg}^{-1}$ protein *	141 U $\text{mg}^{-1}$ protein *	[33]
Superoxide Dismutase (SOD)		Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 $\mu$ M for 7 days	< 75 U $\text{mg}^{-1}$ protein $\text{min}^{-1}$ *	> 125 U $\text{mg}^{-1}$ protein $\text{min}^{-1}$ *	[23]
			<i>Pavlova viridis</i>	Copper -3 $\text{mg L}^{-1}$ for 14 days	< 20000 U $10^6$ cells $^{-1}$ *	> 40000 U $10^6$ cells $^{-1}$ *	[30]
			<i>Chlorella vulgaris</i>	Copper - 3 $\text{mg L}^{-1}$ for 72 h	< 100 $\text{mmol nitrate mg}^{-1}$ protein $\text{min}^{-1}$ *	> 140 $\text{nmol nitrate mg}^{-1}$ protein $\text{min}^{-1}$ *	[34]
			<i>Scenedesmus vacuolatus</i>	Copper - 414 $\mu$ M for 7 days	< 0.15 U $\text{mg}^{-1}$ protein *	> 0.25 U $\text{mg}^{-1}$ protein *	[31]
		Nutrient Limitation	<i>Acutodesmus dimorphus</i>	Nitrogen starvation for 2 days	687.3 U $\text{mg}^{-1}$ protein*	3857.9 U $\text{mg}^{-1}$ protein *	[25]
		pH Changes	<i>Spirulina platensis</i>	pH 10.5 from 9.0 for 14 days	10.2 U $\text{mg}^{-1}$ protein *	12.5 U $\text{mg}^{-1}$ protein *	[32]
		UV-B Radiation	<i>Dunaliella salina</i>	13.2 $\text{kJ m}^{-2} \text{d}^{-1}$ (4th day of exposure)	< 50 U $\text{mg}^{-1}$ protein*	107 U $\text{mg}^{-1}$ protein*	[33]

\* denotes activity; dry weight (DW); fresh weight (FW)

**Table 1. Continued.**

Antioxidant type	Antioxidant compound	Stress condition	Microalgal species	Stress parameters	Productivity/activity of control	Productivity/activity with stress	Reference	
Non-enzymatic	Ascorbic Acid (AA)	Nutrient Limitation	<i>Chlorella vulgaris</i>	Phosphorus limitation 0.01 mM for 5 days	< 1.0 mg g <sup>-1</sup> DW	> 1.0 mg g <sup>-1</sup> DW	[35]	
			<i>Phaeodactylum tricorutum</i>	Phosphorus limitation 0.01 mM for 5 days	< 1.0 mg g <sup>-1</sup> DW	> 1.5 mg g <sup>-1</sup> DW		
				Nitrogen limitation 0.2 mM for 5 days	< 1.0 mg g <sup>-1</sup> DW	> 1.0 mg g <sup>-1</sup> DW		
			<i>Tetraselmis suecica</i>	Phosphorus limitation 0.01 mM for 5 days	< 2.0 mg g <sup>-1</sup> DW	> 5 mg g <sup>-1</sup> DW		
				Nitrogen limitation 0.2 mM for 5 days	< 2.0 mg g <sup>-1</sup> DW	> 3 mg g <sup>-1</sup> DW		
			UV-B Radiation	<i>Chlorella vulgaris</i>	15 W m <sup>-2</sup> for 4 hours	< 5 mM g <sup>-1</sup> FW	> 5 mM g <sup>-1</sup> FW	[36]
		<i>Chlorococcum humicola</i>		15 W m <sup>-2</sup> for 4 hours	< 10 mM g <sup>-1</sup> FW	> 15 mM g <sup>-1</sup> FW		
		Ascorbate (AsA)	Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 μM for 7 days	< 0.3 μmol g <sup>-1</sup> FW	> 0.3 μmol g <sup>-1</sup> FW	[23]
				<i>Dunaliella salina</i>	13.2 kJ m <sup>-2</sup> d <sup>-1</sup> (5th day of exposure)	< 60 μmol g <sup>-1</sup> w. wt.	20.3 μmol g <sup>-1</sup> w. wt.	[33]

\* denotes activity; dry weight (DW); fresh weight (FW)

**Table 1. Continued.**

Antioxidant type	Antioxidant compound	Stress condition	Microalgal species	Stress parameters	Productivity/activity of control	Productivity/activity with stress	Reference
Carotenoids		Light Intensity	<i>Chlamydomonas acidophila</i>	240 $\mu\text{E m}^{-2} \text{s}^{-1}$ for 20 days	< 40 mg L <sup>-1</sup> culture	> 50 mg L <sup>-1</sup> culture	[37]
		Temperature		40°C for 20 days	< 40 mg L <sup>-1</sup> culture	> 40 mg L <sup>-1</sup> culture	
		UV-A Radiation		10 $\mu\text{E m}^{-2} \text{s}^{-1}$ for 15 days	< 50 mg L <sup>-1</sup> culture	> 50 mg L <sup>-1</sup> culture	
		UV-B Radiation	<i>Chlorella vulgaris</i>	15 W m <sup>-2</sup> for 1 hour	0.98 mg g <sup>-1</sup> FW	1.18 mg g <sup>-1</sup> FW	[36]
		UV-B Radiation	<i>Chlorococcum humicola</i>	15 W m <sup>-2</sup> for 1 hour	1.02 mg g <sup>-1</sup> FW	1.36 mg g <sup>-1</sup> FW	
Glutathione (GSH)		Metal Exposure	<i>Pavlova viridis</i>	Copper - 3 mg L <sup>-1</sup> for 14 days	< 17.5 mg 10 <sup>6</sup> cells <sup>-1</sup>	> 35 mg 10 <sup>6</sup> cells <sup>-1</sup>	[30]
				Zinc - 6.5 mg L <sup>-1</sup> for 14 days	< 125 mg 10 <sup>3</sup> cells <sup>-1</sup>	> 175 mg 10 <sup>3</sup> cells <sup>-1</sup>	
			<i>Scenedesmus vacuolatus</i>	Copper - 414 $\mu\text{M}$ for 7 days	< 1.0 nmol 10 <sup>6</sup> cells <sup>-1</sup>	> 12.5 nmol 10 <sup>6</sup> cells <sup>-1</sup>	[31]
		UV-B Radiation	<i>Dunaliella salina</i>	13.2 kJ m <sup>-2</sup> d <sup>-1</sup> (6th day of exposure)	435 nmol g <sup>-1</sup> w. wt.	531 nmol g <sup>-1</sup> w. wt.	[33]
Mycosporine—like Amino Acids (MAA)	UV-B Radiation	<i>Dunaliella salina</i>	13.2 kJ m <sup>-2</sup> d <sup>-1</sup> (2nd day of exposure)	< 4 $\mu\text{mol g}^{-1}$ w. wt.	10.5 $\mu\text{mol g}^{-1}$ w. wt.	[33]	

\* denotes activity; dry weight (DW); fresh weight (FW)

**Table 1. Continued.**

Antioxidant type	Antioxidant compound	Stress condition	Microalgal species	Stress parameters	Productivity/activity of control	Productivity/activity with stress	Reference	
Polyphenols		Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 $\mu\text{M}$ for 7 days	< 6.0 gallic acid equivalent (mg g <sup>-1</sup> FW)	> 9.0 gallic acid equivalent (mg g <sup>-1</sup> FW)	[23]	
			<i>Scenedesmus acuminatus</i>	Copper - 50 $\mu\text{M}$ for 7 days	< 6.0 gallic acid equivalent (mg g <sup>-1</sup> FW)	> 9.0 gallic acid equivalent (mg g <sup>-1</sup> FW)		
		Nutrient Limitation	<i>Acutodesmus dimorphus</i>	Nitrogen starvation for 2 days	143.5 $\mu\text{g g}^{-1}$ FW	246.8 $\mu\text{g g}^{-1}$ FW	[25]	
		pH Changes	<i>Spirulina platensis</i>	pH 9.5 from pH 9.0 for 14 days	< 10 gallic acid equivalents (mg g <sup>-1</sup> DW)	> 12 gallic acid equivalents (mg g <sup>-1</sup> DW)	[32]	
Proline		Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 $\mu\text{M}$ for 7 days	< 10.0 $\mu\text{mol g}^{-1}$ FW	> 20.0 $\mu\text{mol g}^{-1}$ FW	[23]	
			<i>Scenedesmus acuminatus</i>	Copper - 50 $\mu\text{M}$ for 7 days	< 10.0 $\mu\text{mol g}^{-1}$ FW	> 60 $\mu\text{mol g}^{-1}$ FW		
			<i>Chlorella vulgaris</i>	Copper - 3 mg L <sup>-1</sup> for 72 h	2.1 nmol proline mg <sup>-1</sup> protein	12.1 nmol proline mg <sup>-1</sup> protein	[34]	
		Nutrient Limitation	<i>Acutodesmus dimorphus</i>	N-starvation for 2 days	15.1 $\mu\text{M g}^{-1}$ DW	71.5 $\mu\text{M g}^{-1}$ DW	[25]	
		Salinity	<i>Scenedesmus CCNM 1077</i>	sp.	400 mM for 15 days	29.2 $\mu\text{M g}^{-1}$ DW	131.5 $\mu\text{M g}^{-1}$ DW	[29]
					2-Stage 400mM after 9 days of 15	31.35 $\mu\text{M g}^{-1}$ DW	73.5 $\mu\text{M g}^{-1}$ DW	
UV-B Radiation			<i>Chlorella vulgaris</i>	15 W m <sup>-2</sup> for 4 hours	< 0.04 mM g <sup>-1</sup> FW	> 0.12 mM g <sup>-1</sup> FW	[36]	
			<i>Chlorococcum humicola</i>	15 W m <sup>-2</sup> for 4 hours	< 0.06 mM g <sup>-1</sup> FW	> 0.12 mM g <sup>-1</sup> FW		

\* denotes activity; dry weight (DW); fresh weight (FW)

**Table 1. Continued.**

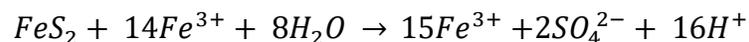
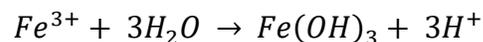
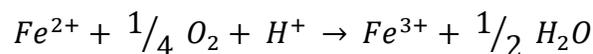
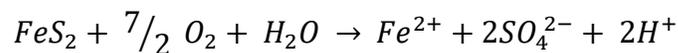
<b>Antioxidant type</b>	<b>Antioxidant compound</b>	<b>Stress condition</b>	<b>Microalgal species</b>	<b>Stress parameters</b>	<b>Productivity/activity of control</b>	<b>Productivity/activity with stress</b>	<b>Reference</b>
	Tocopherols	Metal Exposure	<i>Chlorella sorokiniana</i>	Copper - 25 $\mu\text{M}$ for 7 days	< 3.0 $\mu\text{g g}^{-1}$ FW	> 3.0 $\mu\text{g g}^{-1}$ FW	[23]
			<i>Scenedesmus acuminatus</i>	Copper - 50 $\mu\text{M}$ for 7 days	< 1.5 $\mu\text{g g}^{-1}$ FW	> 1.5 $\mu\text{g g}^{-1}$ FW	
		Nutrient Limitation	<i>Chlorella vulgaris</i>	Phosphorus limitation 0.01 mM for 5 days	< 0.4 $\text{mg g}^{-1}$ DW	> 0.6 $\text{mg g}^{-1}$ DW	[35]
			<i>Phaeodactylum tricorutum</i>	Phosphorus limitation 0.01 mM for 5 days	< 0.2 $\text{mg g}^{-1}$ DW	> 0.2 $\text{mg g}^{-1}$ DW	
			<i>Tetraselmis suecica</i>	Phosphorus limitation 0.01 mM for 5 days	< 0.2 $\text{mg g}^{-1}$ DW	> 0.2 $\text{mg g}^{-1}$ DW	

\* denotes activity; dry weight (DW); fresh weight (FW)

## 1.5 Metals and Acidic Conditions

Although metals are normally present in every water system in low concentrations, anthropogenic activities such as mining can drastically increase their levels. Commonly, mine-impacted water bodies consist of acidic streams and lakes with higher than natural concentrations of heavy metals, transition metals, and sulphates [38]. These conditions are introduced by acid mine drainage. Acid mine drainage is a result of the oxidation of pyrite to dissolved ferrous ions and aqueous sulphuric acid, which also makes effected water bodies become more acidic (Figure 2) [39]. As a result, the water body becomes further contaminated with sulphur, iron, copper, cadmium, zinc, and other metals [40]. These free radical contaminants are ROS that can impart oxidative stress amongst the organisms living within the water system [23]. The level of toxicity of metals to aquatic organisms depends largely on their oxidation numbers and reactivity [20], with the degree of the toxic effect on microalgae is species specific [8].

**Figure 2. Pyrite (FeS<sub>2</sub>) oxidation causing acid mine drainage [39].**



In general, acid tolerant and acidophilic algae are dominant in areas effected by acid mine drainage due to the high acidity of the environment. It can be speculated that some genera of

microalgae that are found in acid mine drainage areas can potentially be found in low pH areas as well. These genera include *Chlamydomonas*, *Dunaliella*, *Euglena*, and *Ochromonas* [38,40]. For example, large blooms of acidophilic algae have been found through the Iberian Pyrite Belt in South-Western Spain and the diversity coincides with the high metal contents of the site, the season, and acidity of the water [38]. In addition to the common genera of freshwater green microalgae, some diatoms and filamentous algae have been also found in extremely acidic areas [38].

Cellular metal toxicity is associated with oxidative stress since metal ions cause damage by driving ROS producing reactions, such as the Fenton reaction, within the cell [20]. The organisms that can successfully survive in metal polluted sites will have a genetic or physiological characteristic that aids their success [41]. Antioxidant production is dependent upon which metals are present, whether the metal stress is acute or chronic, and the concentration of each metal [20]. Under chronic stress, the organism has potential to acclimatize to the oxidative stress through a continual antioxidant response. Whereas, under acute stress, the oxidative stress will potentially supersede the antioxidant capacity of the organism [20].

### *1.5.1 Metal Entry Into the Cell*

Each species of microalgae has a contrasting toxic threshold for toxic metals as well as a different method for removing them from the cell, and different antioxidant responses [8,39]. Metal ions enter the cell by passing through the cell membrane and can compete with nutrients for binding to multivalent ion carriers or by binding to thiols to actively transport through the membrane [20]. Generally, when the concentration of a metal is too high, the microalgae detoxifies

itself extracellularly by active transport of metal ions through the cell wall or by binding metal ions to the cell wall [31,41].

Extracellular exclusion is a result of a decrease of cell membrane permeability, reducing the instance of metal ions entering the cytosol [42]. Microalgae can also detoxify intracellularly by storing metals into vacuoles, or by inducing an antioxidant defense system [31,41]. Storage of the ions into cell vacuoles is a form of intracellular detoxification that removes the metal ions that have passed through the membrane into the cell, but exacerbates the biomagnification of metals in the ecosystem [20].

### *1.5.2 Reactive Oxygen Species (ROS) Production and Cellular Response to Metal Stress*

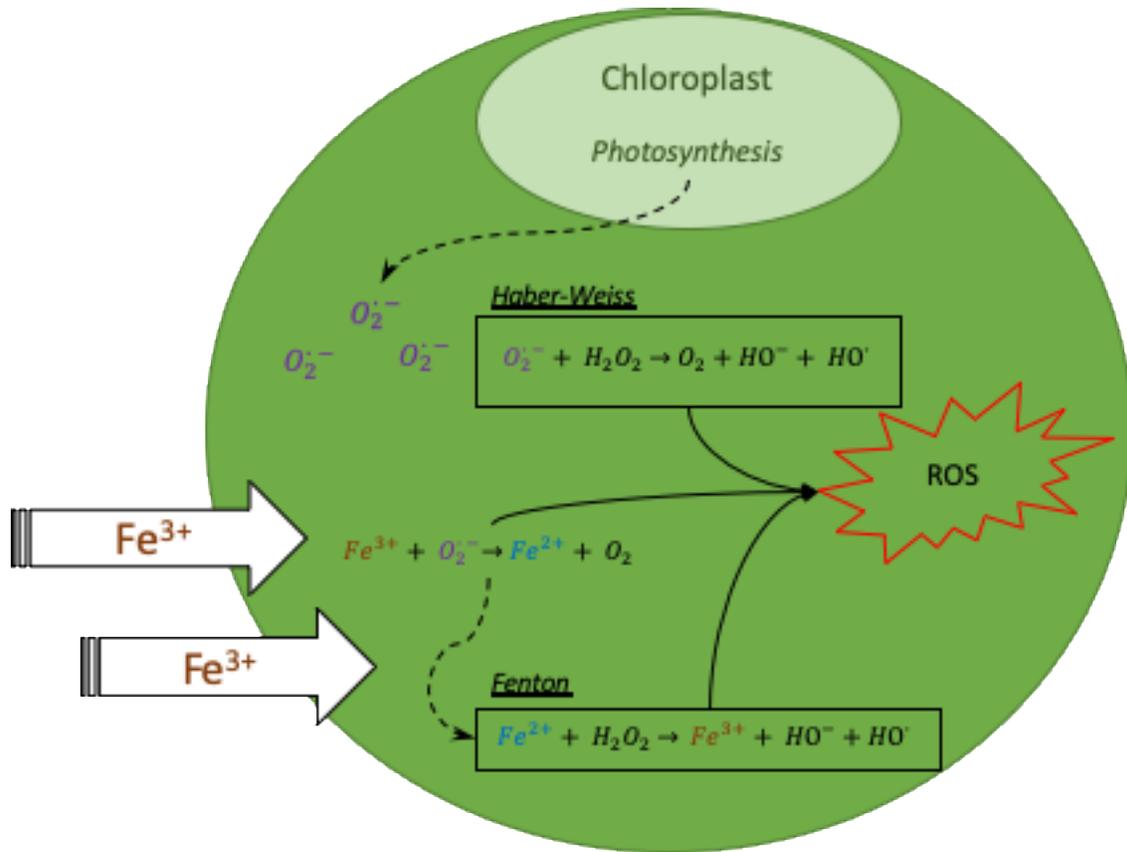
Copper, zinc, cadmium, iron, lead, mercury, and chromium have all been shown to play a role in ROS production through the Haber-Weiss reaction, Fenton process, and/or the electron transport chain. Copper and zinc are metals essential for enzyme production and photosynthesis within the cell. Whereas, others, such as lead, cadmium, and mercury, are not essential to cell functioning and can inhibit the cell's ability to perform photosynthesis, depolarize the cell membrane, acidify the cytoplasm, and/or disrupt overall cell homeostasis [20]. Green microalgae are photosynthetic organisms with chloroplasts that are a significant target for lipid peroxidation due to their polyunsaturated fatty acid rich membranes [31]. This makes defences against oxidative stress especially important for organisms that rely on photosynthesis.

The oxidative stress imposed on microalgae by high metal concentrations is primarily caused by the Fenton and Haber-Weiss reactions, and  $O_2^{\cdot-}$  production during photosynthesis and other cellular processes (

Figure 3) [8,14,18,41,43]. The Haber-Weiss reaction produces  $HO^\bullet$  and is catalyzed by iron or other metal ions in biological systems via the Fenton reaction [14]. The Fenton reaction can be described as the augmented oxidative potential of  $H_2O_2$  with iron, or another metal in a low oxidation state, being used as a catalyst in, generally, acidic conditions [44].

Even though other metals in low oxidation states can catalyze the Fenton process, iron is the primary contributor of the production of  $HO^\bullet$  in biological systems [14]. The Haber-Weiss reaction can produce more ROS from  $O_2^{\cdot-}$  and  $H_2O_2$  than produced enzymatically [14]. The antioxidant response invoked by the afflicted microalgae is likely to vary between species, type of metal contamination, and any associated oxidative stress that comes from the metals exposed to.

**Figure 3. Iron driven Fenton and Haber-Weiss reactions producing reactive oxygen species in microalgal cells [14].**



### 1.5.3 Acidic Conditions and Metal Stress

The optimal pH for growth of a microorganism is species specific and growth rates decrease as the pH deviates from it [45]. Eukaryotic microalgae surviving at low pH are impacted by high inflows of protons and develop defensive mechanisms to limit proton entry into the cell, and to preserve a neutral cytosol. Some acidophilic microalgae compensate for these influxes by developing a plasma membrane that is resistant to high levels of protons. This membrane overall reduces the

amount of energy required for the cell to pump against passive protons converging at the membrane to maintain the neutral pH of the cytosol [46]. This resistant membrane also attributes to the general tolerance acidophilic and acid tolerant algae have to heavy metals and toxic anions and deters them from entering the cell [46,47].

The natural acidity of a water body is influenced by proximity to sulphate rich geological deposits, biological processes, changing weather conditions, water temperature, salinity and industrial activity. Mining, afforestation and land development, industrial off-gas and effluents, and fossil fuel emissions are common anthropogenic factors causing an increase in water-system acidity [48]. Naturally, atmospheric  $CO_2$  dissolves into water and dissociates into inorganic carbon species used by the microalgal cell [49]. When water-systems are highly acidic (pH < 4.0) dissolved inorganic carbon becomes limited [46]. In areas afflicted by acid mine drainage and in geothermal hot-springs a decrease in pH is caused by dissolution and oxidation of sulphur compounds and production of sulphuric acid [43]. An increase in acidity expedites the dissolution of metals into the water and impedes the availability of nutrients [43,50].

A regularly investigated acid mine drainage impacted environment is the Rio Tinto River in Spain, which has a consistently acidic pH of 1.7-3.1, as well as high levels of dissolved metals and sulphate ions [50,51]. Additionally, the location of the river offers summers of extreme sun irradiance which favours species adapted to high levels of sunlight [50]. Overall, low pH, high metal contents, and high irradiance are major contributors to oxidative stress imparted upon the algae [37]. The level of oxidative stress was found to be strongly correlated to an increase in the production of the antioxidant compounds  $\beta$ -carotene, zeaxanthin, astaxanthin, and lutein by several isolated *Coccomyxa* sp., and *Chlamydomonas acidophila* from this area [37,51].

Carotenoids are valuable for their antioxidant and anti-inflammatory properties, pro-vitamin A activity, and their role in retina protection [9,37]. Astaxanthin is of particular interest as it has an antioxidant activity that is 10 fold that of  $\beta$ -carotene and more than 500 fold that of  $\alpha$ -tocopherol [27]. Synthetic astaxanthin is derived from petrochemicals and is considered to be unsafe for human consumption, driving the market value for naturally sourced astaxanthin to \$1,900 USD/kg in 2014 [9,52].

When microalgae are subjected to the additional oxidative stress that accompanies an acidic environment, such as increased exposure to dissolved metals, they respond by accumulating carotenoids and compounds with antioxidant potential [51]. These supplementary stressors are considered the primary cause of oxidative stress and cellular damage in an acidic environment [53]. In the Rio Tinto case of acid mine drainage, the bioprospected microalgae studied exhibited a higher accumulation of zeaxanthin, astaxanthin, and lutein under acidic and metal rich conditions than they would have without the imparted oxidative stress [51]. More research is, however, still needed to evaluate the impact of pH as a sole stressor and the culminating antioxidant cellular response of microalgae [32]. In particular, industrial processes whose off-gas may be used as a carbon dioxide for algal cultivation can drastically reduce the pH of the growing medium [54]. The extreme pH levels associated with these conditions need to be investigated on species that can survive in this stress.

In an experiment performed on the antioxidant potential of the blue-green algae *Arthrospira platensis* at a pH above and below the optimal of pH 9 was investigated [32]. These blue-green microalgae were found to produce higher levels of the antioxidant enzyme SOD at an increased, more alkaline pH. An increase in CAT and POD enzymes was correlated with a more

neutral pH [32]. SOD is a potent antioxidant that has been shown to protect against ROS induced diseases in humans [55]. In a similar experiment performed on the antioxidant activity of the freshwater green microalgae *Scenedesmus obliquus* SOD was found to be dependent on pH [56]. The microalgae were tested over a pH range of 6-8 and temperature range of 20-30°C. Biomass production increased at pH 6 and 30°C, but antioxidant production decreased at the low pH at every applied temperature. The experiment determined the best growing conditions for antioxidant production in *S. obliquus* to be pH 8 and 30°C. At these conditions, *S. obliquus* exhibited high antioxidant potential during the exponential phase of growth and had notably higher levels of  $\beta$ -carotene and lutein than under other tested growing conditions [56].

## 1.6 High Solar Irradiance

Solar irradiance that reaches the Earth's surface includes ultraviolet radiation (UVR, 280-400 nm), photosynthetically active radiation or visible light (PAR, 400–700 nm), and infrared radiation (IR, 700–1000 nm) [57,58]. Photosynthetic organisms, including green microalgae, rely on solar irradiance, particularly PAR, to drive carbon fixation via photosynthesis and biomass production [59]. It is well known that UVR can cause damage to plants and animals by contributing to the development of photochemical smog, photobleaching of vegetation, and photodamage to cells [36]. Cells can adapt to this damage by employing several defensive mechanisms, including an antioxidant defence system [60].

### *1.6.1 Reactive Oxygen Species (ROS) Production and Cellular Response to High Solar Irradiance*

Exposure to elevated levels of UVR over-stimulates photosynthesis and initiates the production of ROS (

Figure 4) [58]. An experiment including 15 different species of microalgae exposed to excessive irradiance showed a wide variety of species specific antioxidant responses [58]. Under the stress of excess irradiance some microalgae have been shown to accumulate carotenoids, POD, GSH, and AsA [58,60–62], and additionally initiate manufacturing of MAAs with antioxidant potential [60].

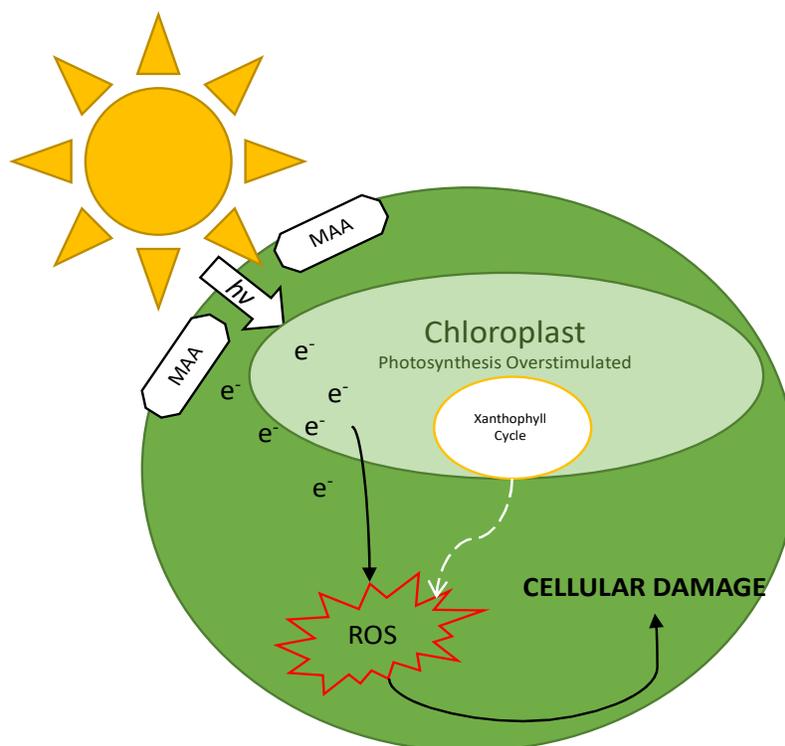
Carotenoids serve as photoprotectants, protecting the cell from photoinhibition, ROS, and damage to photosynthetic units [61]. Studies have found that the concentration of carotenoids acting as antioxidants outside of the thylakoid membrane increases in some microalgae after they were subjected to UVR [60–62]. Some pigments, such as violaxanthin and zeaxanthin, accumulate as a result of the xanthophyll cycle [58].

Many cyanobacteria and green microalgal species exposed to high irradiance have been found to produce UVR absorbing MAAs [60,63]. These small (<400 Da) molecules have an absorbance range of 307–362 nm [58,60]. The MAAs essentially act as a “sunscreen” for the cell by absorbing harmful UVR within this range before it can reach the chloroplast by converting it into heat [33]. They additionally exhibit antioxidant activity and have a high tolerance to other oxidative stress conditions, making them an ideal first-line antioxidant defence for the cell [60]. Commercially, they have been used in skincare products such as sunscreen and anti-aging products [24,64].

Suh *et al.*, in 2014 tested the MAA production of marine green microalgae *Chlamydomonas hedleyi*. The MAAs were isolated from algal extracts and identified using HPLC. It was found that the MAA mycosporine-Gly when tested in human keratinocytes exposed to UV irradiation had significant antioxidant activity and decreased biomarkers for skin inflammation. Furthermore, mycosporine-Gly exhibited anti-aging potential as it increased the expression of

elastin and procollagen promoting genes after they were suppressed due to UV exposure [24]. Shang *et al.*, 2017 used UV-B to induce MAA synthesis in cyanobacterium *Nostoc flagelliforme* [65]. They discovered a previously unidentified MAA, mycosporine-2-(4-deoxygadusolyl-ornithine) (M-2-DO). This unique compound has a higher molecular weight, at 756 Da, than most known MAAs and a large UV absorption spectrum. Genes responsible for the biosynthesis of MAAs in cyanobacteria were found giving opportunity for future research to exploit this oxidative stress response.

**Figure 4.** How irradiance ( $h\nu$ ) from the sun overstimulates photosynthesis, causing an excess of electrons ( $e^-$ ) and the production of reactive oxygen species (ROS). Mycosporine amino acids (MAA) are produced to shield the cell from irradiance.



A study investigated the antioxidant response and structural alterations under increased UV-B radiation of the green microalgae *Dunaliella salina* collected from seawater [33]. *D. salina* samples were exposed to  $8.8 \text{ kJ m}^{-2} \text{ d}^{-1}$  and  $13.2 \text{ kJ m}^{-2} \text{ d}^{-1}$  for 12-hours for six days. It was found that applying enhanced UV-B radiation significantly damaged organelles, the thylakoid membranes and the mitochondria swelled, lipid globules accumulated, and vacuoles increased in size. Excessive amounts of ROS were produced in the microalgae exposed to the higher UV-B dose while the amount of ROS in the control group did not vary.

The presence of MAAs increased initially with high UV-B, but after three days total MAAs decreased to below the levels found in the control samples. This suggests that the production of MAAs is stimulated by UV-B stress but is limited under excess irradiance. The quantity of carotenoids was found to be unaffected by the radiation, but the chlorophyll-*a* concentration initially increased before finally decreasing to levels lower than at time zero. The researchers speculated that carotenoids were involved more in antioxidant processes than in light-harvesting mechanisms. Levels of GSH and AsA increased with the enhanced UV-B. Enzymatic antioxidants SOD, POD, and CAT all had differing outcomes to the stress. Quantities of POD increased throughout the stress, SOD decreased, and CAT remained approximately the same. The overall enzymatic antioxidant activity of the stressed algae was originally greater than the control, but then reached similar levels after 4 to 5 days. This implies that the antioxidant defense of these microalgae against increased UV-B irradiance is short-term [33].

## 1.7 Nutrient Limitations

Green microalgae require roughly 30 different elements as micronutrients (Fe, Zn, Mn, Br, Si, B, Mo, V, Sr, Al, Rb, Li, Cu, Co, I, Se) and macronutrients (C, O, H, N, Na, K, Ca, P, S, Mg, Cl) to survive [66]. As the concentrations of these nutrients are changed, the biochemical profile of the algae is also affected [35]. Green microalgae dry weight can be composed of 1-14% nitrogen, 0.15-1.6% sulphur, and 0.05-3.3% phosphorus, depending on the nutrient availability of the growing medium [66]. Starving microalgae of nutrients, particularly the macronutrients N, S, and P, can result in several different cellular responses. These include: lipid and starch accumulation, ROS accumulation, antioxidant production, impairment of proteosynthesis, degradation of amino acids and pigments, and downregulation of photosynthesis (

Figure 5) [35,66]. Increased lipid accumulation during nutrient starvation is especially attractive for producing commercial bioproducts, as most of these lipids are triacylglycerols, polyunsaturated fatty acids, and carotenoids [67]. The application of nutrient stress for commercial lipid production purposes has been thoroughly investigated in many *Dunaliella* sp., *Haemotococcus* sp., and *Scenedesmus* sp. [35].

### *1.7.1 Reactive Oxygen Species (ROS) Production and Cellular Response to Nutrient Limitations*

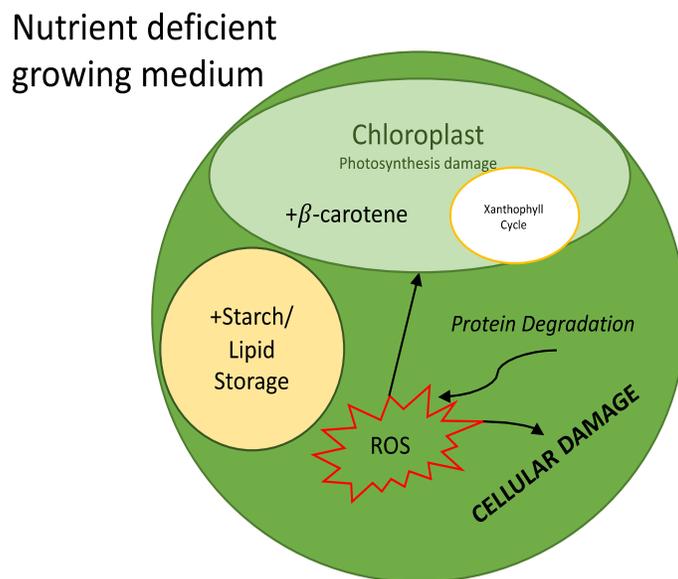
Changes in nutrient contents of growing media can invoke the production of ROS within the cell and a corresponding antioxidant response to alleviate the imbalance. Nitrogen deficiency induces ROS cellular toxicity and lipid peroxidation, and cells produce enzymatic and non-enzymatic antioxidants as a defence mechanism. Chokshi *et al.* demonstrated *Acutodesmus dimorphus* exposed to nitrogen starvation for 1 to 3 days increased SOD and CAT activity when compared to a population growing in a controlled (non-starvation) environment. The cellular concentration of proline and the total polyphenols also increased under nitrogen limitation [25]. Proline is an amino acid with antioxidant activity that plays a role in metal chelation and as a radical scavenger. Additionally, proline can help in the prevention of cardiovascular diseases [68]. Nitrogen starvation has been also associated with changes in pigment ratios as with *Coccomyxa* sp., Ruiz-Dominguez *et al.* found that  $\beta$ -carotene and lutein concentration slightly increased after two days of deprivation. The activities of the enzymatic antioxidants GR, APX, and CAT also increased [66,67].

Sulphur is necessary for the synthesis of compounds that play a role in scavenging ROS and sulphur starvation will impede the ability of the cell to produce these to relieve oxidative stress.

GSH is a sulphur containing non-enzymatic antioxidant impacted by deprivation as well as some metal chelating compounds [69]. In *Coccomyxa* sp.,  $\beta$ -carotene and lutein concentrations increased more after two days of sulphur starvation than phosphorus starvation, but less than with nitrogen starvation. Overall, there was no significant accumulation of carotenoids with any method of nutrient starvation in this study [67].

Deprivation of phosphorus has been shown to impose oxidative stress, although Yao *et al.* found it does not produce as much of an antioxidant response as nitrogen or sulphur starvation with the green microalgae *Tetraselmis subcordiformis* [70]. Carotenoid content increased, which can be associated with the initiation of the xanthophyll cycle [70]. *Coccomyxa* sp. expressed an increase in enzymatic antioxidants. GR and APX activities both increased during phosphorus starvation. Unlike nitrogen starvation, *Coccomyxa* sp. showed minimal change in the concentrations of  $\beta$ -carotene and lutein with phosphorus deprivation [67].

**Figure 5. Nutrient deficiencies promote increased storage of lipids and starches, degradation of proteins, a production of reactive oxygen species (ROS) in microalgae.**



A study in 2015 by Goiris *et al.* applied nitrogen and phosphorus stress on three species of microalgae, the marine diatom *Phaeodactylum tricornutum*, the marine green microalgae *Tetraselmis suecica*, and freshwater green microalgae *Chlorella vulgaris* [35]. Although several species of microalgae are known to increase carotenoid production under nutrient starvation [5,35], the species tested in this study displayed a reduction in carotenoid content. *P. tricornutum* exhibited the greatest decrease in carotenoids under nitrogen starvation, while *T. suecica* and *C. vulgaris* showed a decrease in carotenoids under phosphorus and nitrogen limiting conditions. Conversely, the concentration of tocopherols and AA increased for all species under nutrient stress. Overall, a decrease in antioxidant activity was found under nutrient stress conditions despite the increase of tocopherols and AA, which implies carotenoids and phenolics are major contributors to antioxidant activity. It was concluded that the antioxidant yield from these microalgae under these nutrient limitations was insufficient for commercial production of antioxidants. Though the

increase in AA and tocopherols is promising, the decrease in biomass indicates the nutrient limiting conditions need to be optimized for commercial cultivation.

## 1.8 Conclusions

The biochemical composition of microalgae adjusts to changes in the environment. Abiotic stress can instigate the production of harmful ROS as well as a unique cellular defensive antioxidant response. The enzymatic and non-enzymatic antioxidants produced by this response will vary between each algal species and stressor [58]. Metal exposure, acidic conditions, increased UV irradiation, and nutrient starvation have been all found to induce oxidative stress in a variety of green microalgal species and initiate accumulation of antioxidant compounds.

There are well in excess of 200,000 different species of microalgae, yet only a few species of *Spirulina* and *Chlorella*, as well as *Dunaliella salina*, and *Haematococcus pluvialis* have been deemed safe for human consumption and considered feasible for economic commercial cultivation [9,71,72]. With many species of microalgae still unexplored, further research is still needed to identify more algal species with high metabolite productivity suitable for large-scale commercial cultivation. There is also potential to exploit oxidative stress as a cultivation method to upregulate the production of specific desirable antioxidant compounds. Cultivating in a stressful environment that exhibit low pH, high metal concentrations, high solar irradiance, and/or nutrient limitations could be also beneficial for mitigating potential contamination by competing microorganisms [35,41]. For instance, the extremophilic nature of *Dunaliella salina* to saline conditions is also exploited to promote the production of  $\beta$ -carotene. The saline conditions of the growing medium mitigate contamination from non-halophilic organisms and when combined with increased solar irradiance and nitrogen limitation in a raceway pond carotenoid production can reach up to

200 mg/m<sup>2</sup> per day [33,73,74]. Research shows that bioprospected extremophiles have potential to be exceptional accumulators of bioactive metabolites [35,41,75]. However, investigation of extremophiles under oxidative stress for the commercial production of nutraceutical compounds is still needed [39,49,76,77].

With the global market for carotenoids predicted to reach around \$1.53 billion by 2021 [71] and although naturally sourced carotenoids are preferred for their greater suitability for human consumption and higher antioxidant capacity, less expensive synthetically produced carotenoids represent the majority of the carotenoid market [52,71]. However, as there is growing consumer desire for naturally produced antioxidants, further work looking for new microalgal species, and exploiting conditions that enhance antioxidant production are a route to satisfying this demand.

# Chapter 2

## 2 Hypothesis and Objectives

The demand for naturally sourced compounds is rapidly growing as synthetically produced counterparts can be toxic and unsuitable for human consumption. Antioxidant compounds produced by microalgae under stress conditions are, therefore, becoming of commercial interest as potential natural health products. An excess of ROS in microalgal cells can induce oxidative stress and a corresponding ancillary response. Each microalgal species responds differently to stress conditions, yielding an increase in enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, and/or an increase in non-enzymatic antioxidants such as carotenes, xanthophylls, and flavonoids. By investigating how specific stressors affect the antioxidant production of convenient microalgal species there is potential to exploit these stress responses for commercial cultivation and the production of specific antioxidant compounds.

### 2.1 Hypothesis

This dissertation explores how microalgae originating from acidic mine-impacted water systems respond to being cultivated in stressful acidic conditions. It was hypothesized that the oxidative stress caused by the acidic conditions would induce an antioxidant response in these microalgae and their previous acclimatization to an acidic environment would allow them to maintain biomass production.

## 2.2 Objectives

The objective was to investigate two previously isolated novel strains of green microalgae bioprospected from mine-impacted sites in Northern Ontario for their antioxidant potential and biomass production in acidic conditions. These two strains were compared to a culture collection acquired microalgae *Chlamydomonas reinhardtii*.

# Chapter 3

## **3 Green photosynthetic microalgae from low pH environments as a potential source of antioxidants**

**(Original Research)**

Miranda Gauthier<sup>1</sup>, G.N.A. Senhorinho<sup>1</sup>, Nathan Basiliko<sup>2,3</sup>, Sabrina Desjardins<sup>1</sup>, & John Ashley Scott<sup>1,3</sup>

<sup>1</sup> Bharti School of Engineering, Laurentian University, Sudbury, Ontario, Canada

<sup>2</sup>Vale Living with Lakes Centre, Laurentian University, Sudbury, Ontario, Canada

<sup>3</sup>Department of Biology, Laurentian University, Sudbury, Ontario, Canada

[SUBMITTED TO PHYCOLOGICAL RESEARCH]

### 3.1 Green Photosynthetic Microalgae From Low pH Environments as a Potential Source of Antioxidants

Utilizing microalgae for carbon (CO<sub>2</sub>) capture from industrial off-gas is environmentally beneficial and has the potential to produce valuable secondary metabolites. Due to an increase in acidity of the growing media caused by acid gasses, such as sulphur dioxide, also present in off-gasses, microalgae able to survive at pH 3.0 – 4.0 while still producing useful metabolites are needed. Two strains of microalgae bioprospected from mine-impacted water bodies in Northern Ontario and a culture collection strain *Chlamydomonas reinhardtii* were investigated for their biomass production, antioxidant capacity and chlorophyll content while growing under low pH conditions. The putatively novel microalgal isolates were identified as *Coccomyxa* sp. and *Chlamydomonas* sp. based on ITS sequences. Green microalgae were grown at pH 3.0 and uncontrolled pH media for 28 days. The antioxidant potential of the cultures was evaluated through three complimentary assays. The results showed that pH influenced the biomass production of *Coccomyxa* sp. and low pH induced the death of *C. reinhardtii*. Moreover, it was found that *Coccomyxa* sp. had significantly higher antioxidant potential than *Chlamydomonas* sp. at pH 3.0 in one of the antioxidant assays, and both bioprospected species showed higher antioxidant capacity than *C. reinhardtii*. A strong correlation between chlorophyll-*a* content and antioxidant activity of *Coccomyxa* sp. infers chlorophyll-*a* is a major contributor to the antioxidant activity of the algal extracts.

## 3.2 Introduction

The mining industry is rapidly expanding, causing many acute and chronic negative impacts on land and water-systems in proximity. Many mine-impacted water bodies are acidic and contain high concentrations of metals, transition metals, and sulphates as a result of acid mine drainage [38]. Some of these environments may exhibit extreme or harsh conditions, rendering them unlivable for many higher organisms [50]. The acidic and metal-rich water systems associated with mining operations and sulphide deposits have, however, been found to harbour several species of freshwater green microalgae [6]. High acidity in acid mine drainage affected waters has been shown to select for acid tolerant and acidophilic microalgae [38,50].

Factors that influence microalgal growth include pH, temperature, light intensity, UV exposure, presence of anthropogenic contaminants, and availability of essential nutrients [13]. Alterations to these conditions will either influence changes in the cellular biochemical composition as a response to stress, or result in cell death [2]. Manipulating microalgal growing conditions causes oxidative stress, which can potentially be used commercially to stimulate an increase in production of specific bioactive compounds [8]. Valuable antioxidant metabolites include carotenoids and other pigments and enzymes. Several carotenoids, specifically lutein,  $\beta$ -carotene, and astaxanthin, have the highest current market value of the antioxidant compounds produced [5]. The demand for naturally sourced nutraceuticals is expanding since several synthetic ones, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been found to have carcinogenic effects [10]. This drives the need to research new avenues to enhance biological expression of valuable natural compounds to make commercial production economically feasible.

Oxidative stress is caused by increase in prooxidants causing an imbalance with antioxidants. Reactive oxygen species (ROS) are prooxidants that accumulate in the cell through abiotic sources and certain *in vivo* processes. Under oxidative stress, ROS can damage nucleic acids and proteins, cause lipid peroxidation, instigate metabolic dysfunction within the cell, and even cause cell death [2,14]. When microalgal cells have overabundance of ROS, secondary defensive metabolites with antioxidant potential are produced as a way to compensate [10]. The metabolites that are produced vary between algal strains and stress conditions.

Cultivating microalgae commercially for their valuable bioactive compounds while taking advantage of their exceptional carbon-capturing abilities through carbon dioxide (CO<sub>2</sub>) uptake is becoming increasingly popular among many industries, including the mining sector. Sparging CO<sub>2</sub> containing off-gas into tanks of microalgae is a means to reduce industrial environmental impacts by mitigating greenhouse gas emissions [6]. It has been found, however, that using off-gas from industrial operations as a carbon source for microalgal cultivation often presents a challenge as the sulphur dioxide (SO<sub>2</sub>) also present in the off-gas lowers the pH of the growing medium to pH 3.0 - 4.0 [54]. Strains of microalgae that can survive in these acidic conditions while still producing viable quantities of bioactive compounds are needed to address this issue.

pH conditions ranging from pH 6 - 8 combined with varying growing temperatures between 20 °C to 30 °C have been shown to impact the antioxidant production in freshwater green microalgae *Scenedesmus obliquus* [56]; notable levels of  $\beta$ -carotene and lutein accumulated in *S. obliquus* have been observed when cultivated at pH 8 and 30 °C [56]. Research is, however, needed to determine the impact of acidic pH, as would be caused by industrial off-gasses, on the antioxidant capacity of green microalgae. In this study, two axenic strains of green microalgae isolated from

mine-impacted aquatic systems in Northern Ontario were investigated and compared to a type strain of *Chlamydomonas reinhardtii*. The extracts of these two novel isolates have previously been found to have antibacterial activity against Gram-positive bacteria [78]. Here the strains are sequenced for taxonomic identification and investigated for their ability to produce antioxidants under controlled acidic conditions similar to the low pH caused by sparging in industrial off-gas in industrial carbon capture bioreactor systems.

### 3.3 Materials and Methods

#### 3.3.1 Chemicals and Materials

All chemicals and reagents were of analytical grade and purchased from Fisher Scientific Mississauga, Ontario, Canada unless otherwise specified. Sterile Milli-Q water was used in all experiments. All components of the Bold's Basal Medium (BBM) solution [79] were purchased from Fisher Scientific Canada. Cultures were buffered using 1.0 M sulphuric acid and 1.0 M potassium hydroxide purchased from Fisher Scientific Canada. Centrifugation took place in a Sorvall ST8 Centrifuge (Thermo Scientific, Suzhou, China). The Oakton pH 150 Meter (WD-35614-30, Oakton Instruments, Vernon Hills, United States of America) was calibrated before each use and pH readings were taken according to ASTM D1293-18 [80].

#### 3.3.2 Microalgal Strains

Two putatively novel microalgal strains called M2 and M23 previously isolated were utilized for this study [78]. Isolates were obtained from abandoned mine-waste impacted sites exhibiting pH

of < 3.0 in Northeastern Ontario, Canada. *Chlamydomonas reinhardtii* CPCC11 was obtained from the Canadian Phycological Culture Centre (Waterloo, Canada).

### 3.3.3 DNA Extraction and Sequencing

The procedure for lysing microalgal cells was modified from Fawley and Karen (2004) [81]. A loopful of microalgal biomass was taken from a BBM agar plate and suspended in 200 µL of milliQ water in a 2 mL conical microcentrifuge tube. The suspension was centrifuged at 16000 g for 1 minute. The supernatant was removed, and the pellet was resuspended in 200 µL of an extraction buffer containing 70 mM Tris-hydrochloride (Tris-HCl), 30 mM ethylenediaminetetraacetic acid (EDTA), and 1 M sodium chloride (NaCl) at pH 8.6. The pellet and extraction buffer were vortexed until thoroughly mixed and acid washed glass beads (Sigma G8772-10G) were added to the conical portion of the microcentrifuge tube. The tubes were placed in a Mini-BeadBeater (Biospec, Bartlesville, United States of America) for 4 x 30 second cycles and were placed on ice between cycles. Then, the tubes were centrifuged at 4000 g for 10 minutes and the resulting supernatant was used for DNA extraction.

A DNeasy Plant Pro Kit (Qiagen Montréal, Québec, Canada) was used for the DNA extraction. The kit was followed according to the provided manufacturer's protocol. The ITS1-5.8S-ITS2 region in rDNA was amplified according to Aslam *et al.*, 2017 with ITS Forward1 (5'-ACCTAGAGGAAGGAGAAGTCGTAA-3') and ITS Reverse1 (5'-TTCCTCCGCTTATTGATATGC-3') primers (Thermo Fisher Scientific) [76]. Phire Green Hot Start II PCR Master Mix (Thermo Scientific) was used. The mixtures were placed in a Bioer LifePro Thermal Cycler TC-96/G/H(b)A (Hangzhou, China) with PCR conditions adapted

from Mahmoud *et al.*, (2016). Amplification was initiated by DNA denaturing at 94 °C for 5 minutes followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes before holding at 4 °C. Agarose gel (1.2 %) electrophoresis for 2.5 hours at 70 V confirmed PCR products to be 750 bp and 700bp for M2 and M23 respectively. The resulting PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Montréal, Québec, Canada). A Biotek Synergy HT spectrophotometer (Winooski, United States of America), with a Biotek Take3 Microvolume Adaptor and Biotek Gen5 V 2.04 software was used to verify DNA quality of the purified product prior to sending it to Génome Québec in Montréal, Canada for Sanger sequencing.

#### 3.3.4 *Microalgal Culture Conditions and Extraction*

The three microalgal strains, M2, M23, and *Chlamydomonas reinhardtii* CPCC11 were batch grown in biological triplicate in 500 mL of BBM for 28 days on a gyratory table orbiting at 125 r min<sup>-1</sup> at room temperature (20°C ± 2°C) and with natural sunlight (approximately 11 h light and 13 h dark, and 10.5 µW cm<sup>-2</sup>). Biological triplicates were grown in media regulated at pH 3.0 throughout the entirety of the experiment and grown in media without pH regulation. For the samples growing in controlled pH 3.0 medium, cultures were inoculated into BBM with a pH of 3.0. The pH was monitored and adjusted daily using 1 mol L<sup>-1</sup> sulphuric acid and 1 mol L<sup>-1</sup> potassium hydroxide for buffering. The media for the samples growing without pH control started at the pH of originating water body conditions. Therefore, the bioprospected samples were inoculated into BBM initially at pH 2.9 to replicate the acidity of the environment they were collected from. Type strain *C. reinhardtii* was inoculated into a neutral BBM (pH 7.0). The pH of each culture was monitored daily with no pH buffering. Earlier unpublished studies discovered

bioprospected strains M2 and M23 are in the exponential phase of growth after 28 days of cultivation. Therefore, after 28 days, each flask was harvested and centrifuged at 3250 g for 10 min. The resulting biomass was freeze-dried for 24-28 h and weighed prior to extract preparation.

Crude algal extracts were extracted using a method adapted from Goiris *et al.* (2012). Freeze-dried microalgal biomass was ground into a powder using a mortar and pestle and 2 mL of 95% reagent alcohol was added. The mixture was vortexed for 5 min and then agitated for 24 h with no exposure to light [82]. After 24 h extraction, the suspension was vortexed for 5 min and then centrifuged at 3250 g for 10 min. The supernatant was removed, and the process repeated three times. The extracts were finally combined and stored at -20°C until further analysis. Prior to analysis, the extracts were concentrated in a Savant Speed Vac Plus SC210A rotary vacuum (Fisher Scientific, Mississauga, Canada) for 2 to 4 h. The resulting solid extracts were weighed and resuspended in 2 mL of 95% reagent alcohol prior to antioxidant and chlorophyll assays.

### 3.3.5 Trolox Equivalence Antioxidant Assays

#### 3.3.5.1 ABTS<sup>+</sup> Radical Scavenging

The antioxidant capacity of the extracts were evaluated using the Trolox equivalent antioxidant capacity with ABTS<sup>+</sup> radical scavenging adapted from Li *et al.* [10]. A 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) cation solution was produced through a reaction of 7 mM ABTS and 2.45 mM potassium persulphate in Milli-Q water. The reaction took place in a dark room for 16 hours at room temperature, and the resulting solution was utilized within 48 hours of its creation. Using a plate-reader spectrophotometer the ABTS<sup>+</sup>

solution was diluted with 95 % reagent alcohol until an optical absorbance of  $0.700 \pm 0.050$  at 734 nm was achieved. Extract samples were diluted with 95 % reagent alcohol to 20 – 80 % of this reading [10]. Trolox solutions (600  $\mu\text{M}$ , 450  $\mu\text{M}$ , 390  $\mu\text{M}$ , 300  $\mu\text{M}$ , 150  $\mu\text{M}$ , 120  $\mu\text{M}$ , 90  $\mu\text{M}$ , and 0  $\mu\text{M}$ ) were used to develop a standard curve for the absorbance readings. Linear regression using the standard curve ( $R^2 > 0.95$ ) was used to determine the antioxidant capacity of microalgal extracts in trolox equivalencies (TEQ). Trolox is a synthetically produced vitamin E derivative and TEQ is a unit of  $\mu\text{mol trolox g}^{-1}$  dry weight biomass. Exactly 20  $\mu\text{L}$  of sequentially decreasing concentrations of Trolox solution were added to a 96-well plate with 200  $\mu\text{L}$  of ABTS<sup>+</sup> solution and the absorbance reading was taken. Using a 96-well plate, 20  $\mu\text{L}$  of the sample was added with 200  $\mu\text{L}$  of ABTS<sup>+</sup> solution or reagent alcohol to each well. Wells containing sample and reagent alcohol were used as a blank reading. The plate was incubated at room temperature in the dark for 30 minutes prior to absorbance readings being taken using the Biotek Synergy HT spectrophotometer [83].

#### 3.3.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power assay was performed according to Benzie and Strain [84] with modifications. A 300 mM sodium acetate buffer solution was created by adding 0.16 g of sodium acetate into 100 mL of 0.28 M acetic acid. The pH of the resulting solution was adjusted to 3.6 using 1.0 M sodium hydroxide (NaOH) and 1.0 M hydrochloric acid (HCl). A 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (T1253-1G, Sigma-Aldrich, Oakville, Canada) solution was made by dissolving 0.31 g of TPTZ into 100 mL of 40 mM HCl. A 20 mM ferric chloride solution was made by dissolving 0.135 g of ferric chloride hexahydrate into 25 mL of Milli-Q water. Prior to performing the assay, a FRAP working solution was made by mixing acetate buffer, TPTZ

solution, and ferric chloride solution in a 10:1:1 ratio. This working solution was used immediately after creation. In a similar method to the ABTS<sup>+</sup> assay, a calibration curve was created using the same sequentially decreasing concentrations of Trolox in the presence of FRAP working solution. The results were measured according to this calibration curve as TEQ similar to the ABTS<sup>+</sup> assay. Using a 96-well plate, 20 µL of the sample was added with 200 µL of FRAP working solution or 95 % reagent alcohol. Wells containing sample with reagent alcohol were used as a blank reading. The optical absorbance was read at 593 nm using the same spectrophotometer after 30 minutes of incubation in the dark at 37 °C [84].

#### 3.3.5.3 DPPH assay

The DPPH antioxidant capacity assay adapted from Brand-Williams *et al.* [85] was used. A  $6 \times 10^{-5}$  M 2,2-diphenyl-1-picrylhydrazyl (DPPH) working solution was created in the dark by dissolving 2.4 mg of DPPH into 100 mL of 95 % reagent alcohol using gentle sonication for complete dissolution. The solution was used immediately after being created. Similar to the ABTS<sup>+</sup> and FRAP assays, a calibration curve using the same sequentially decreasing concentrations of Trolox solution with the freshly made DPPH working solution was developed. Results were determined using the calibration curve as TEQ. Algal extract samples were diluted with 95 % alcohol until they were within 20 – 80 % of the blank absorbance. In a 96-well plate, 20 µL of the sample was added along with 200 µL of DPPH working solution or 95 % alcohol. Wells containing sample and reagent alcohol were used as blank readings. Using the spectrophotometer, the optical absorbance was read at 515 nm after 30 minutes of incubation in the dark at room temperature [85].

### 3.3.6 Chlorophyll Colourimetric Assay

The colourimetric assay for chlorophyll content estimation was adapted from Lichtenthaler and Buschmann [86]. 2 mL of 95 % ethanol was added to each of the solid extracts from the rotary vacuum and vortexed until well mixed in the absence of light. 200  $\mu$ L of the freshly diluted extract was added to a 96-well plate for analysis. 200  $\mu$ L of 95 % ethanol was used as a blank reading. The plate was read using a spectrophotometer at 649 nm and 664 nm. The chlorophyll-*a* and chlorophyll-*b* contents of each extract was measured using the following formulas from Lichtenthaler and Buschmann:

$$C_a = 13.36A_{664} - 5.19A_{649}$$

$$C_b = 27.43A_{649} - 8.12A_{664}$$

Where  $C_a$  and  $C_b$  are the estimated quantities of chlorophyll-*a* and chlorophyll-*b* respectively in  $\mu$ g mL<sup>-1</sup>.  $A_{649}$  is the absorbance reading at 649 nm and  $A_{664}$  is the absorbance reading at 664 nm.

### 3.3.7 Statistical Analysis

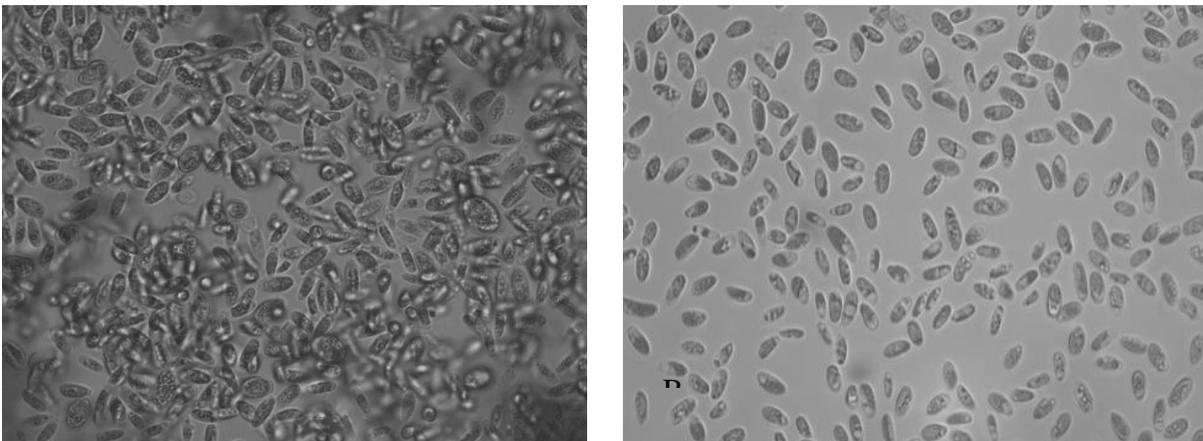
Statistical analysis was performed using IBM SPSS Statistics for Macintosh, version 25 (IBM Corp., Armonk, NY, USA). Significance was determined by conducting bivariate one-way ANOVA tests. Tukey's HSD post-hoc test was applied to all multiple comparison tests. Correlations were found using Pearson Correlation calculations. Significance was established by  $p < 0.05$ . Values are expressed as average of three independent replicates  $\pm$  the standard deviation of the mean unless otherwise specified.

## 3.4 Results

### 3.4.1 Microalgal Identification

Morphological identification of each axenic bioprospected strain identified strain M2 to be *Coccomyxa* sp., and M23 to be *Chlamydomonas* sp., (Figure 6) [78]. Sanger sequencing for the ITS rDNA sequence and analysis via BLAST using the NCBI GenBank confirmed these morphological identifications. The closest BLAST match for sample M2 was of the *Coccomyxa* genus and the closest match for sample M23 was of the *Chlamydomonas* genus. Henceforth, bioprospected strains M2 and M23 will be referred to as *Coccomyxa* sp. and *Chlamydomonas* sp. respectively.

**Figure 6.** (A) Microalga M2 identified as *Coccomyxa* sp. (B) Microalga M23 identified as *Chlamydomonas* sp.



### 3.4.2 Effect of Controlled pH on Antioxidant Activity and Chlorophyll Content

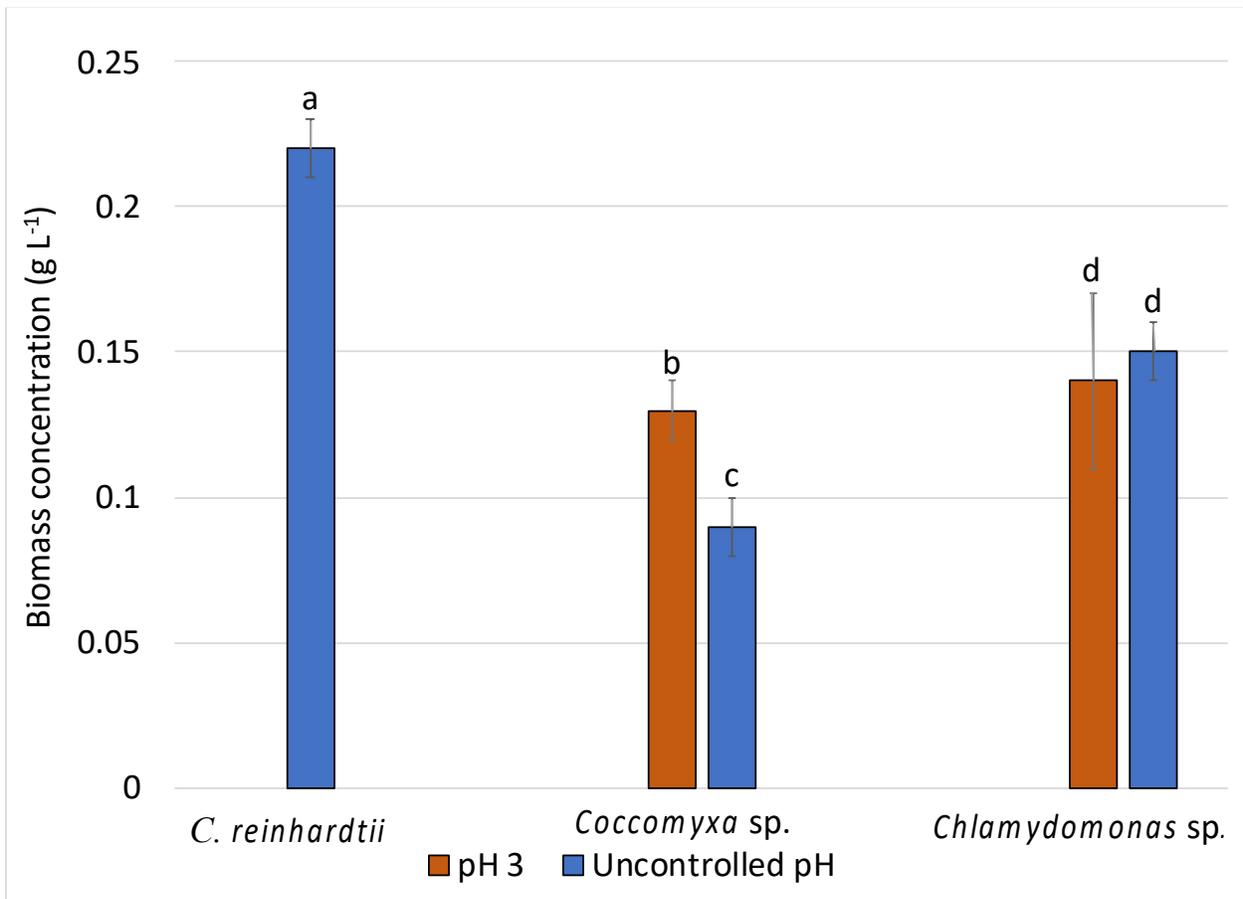
#### 3.4.2.1 Biomass and extraction

Cultures growing in uncontrolled pH medium were inoculated into a medium at the pH of the original growing conditions of the species. Cultures of *Chlamydomonas* sp. and *Coccomyxa* sp. were inoculated into a pH 2.9 medium and CPCC *C. reinhardtii* into a pH 7.0 medium. After 28 days of growth without pH alteration, the pH of the three cultures of *Coccomyxa* sp. were pH 3.5, 3.6, and 3.9 whereas the *Chlamydomonas* sp. cultures were pH 3.4, 3.4, and 3.5. Cultures growing in controlled pH 3.0 medium were inoculated into a pH 3.0 medium. After 3 - 4 days the *C. reinhardtii* cultures died in the pH 3.0 controlled medium. The pH of *C. reinhardtii* in the uncontrolled pH medium, however, did survive in all samples and were harvested after 28 days at pH 6.8, 7.1, and 7.2.

The biomass production of the microalgae is illustrated in Figure 7. The bioprospected *Coccomyxa* sp. showed an increase in biomass production under controlled pH 3.0 conditions ( $p < 0.01$ ). Controlled pH cultures had a concentration of  $0.13 \pm 0.01$  g L<sup>-1</sup> dry weight per medium after 28 days while the uncontrolled pH cultures had an average concentration of  $0.09 \pm 0.01$  g L<sup>-1</sup> dry weight per medium after 28 days. For *Chlamydomonas* sp., the controlled pH had no impact on biomass production. The strains growing at controlled pH 3.0 had a concentration of  $0.14 \pm 0.03$  g L<sup>-1</sup> dry weight per medium after 28 days and the uncontrolled pH strains had a concentration of  $0.15 \pm 0.01$  g L<sup>-1</sup> dry weight per medium after 28 days. The culture collection *C. reinhardtii* under uncontrolled conditions had the highest biomass production with a

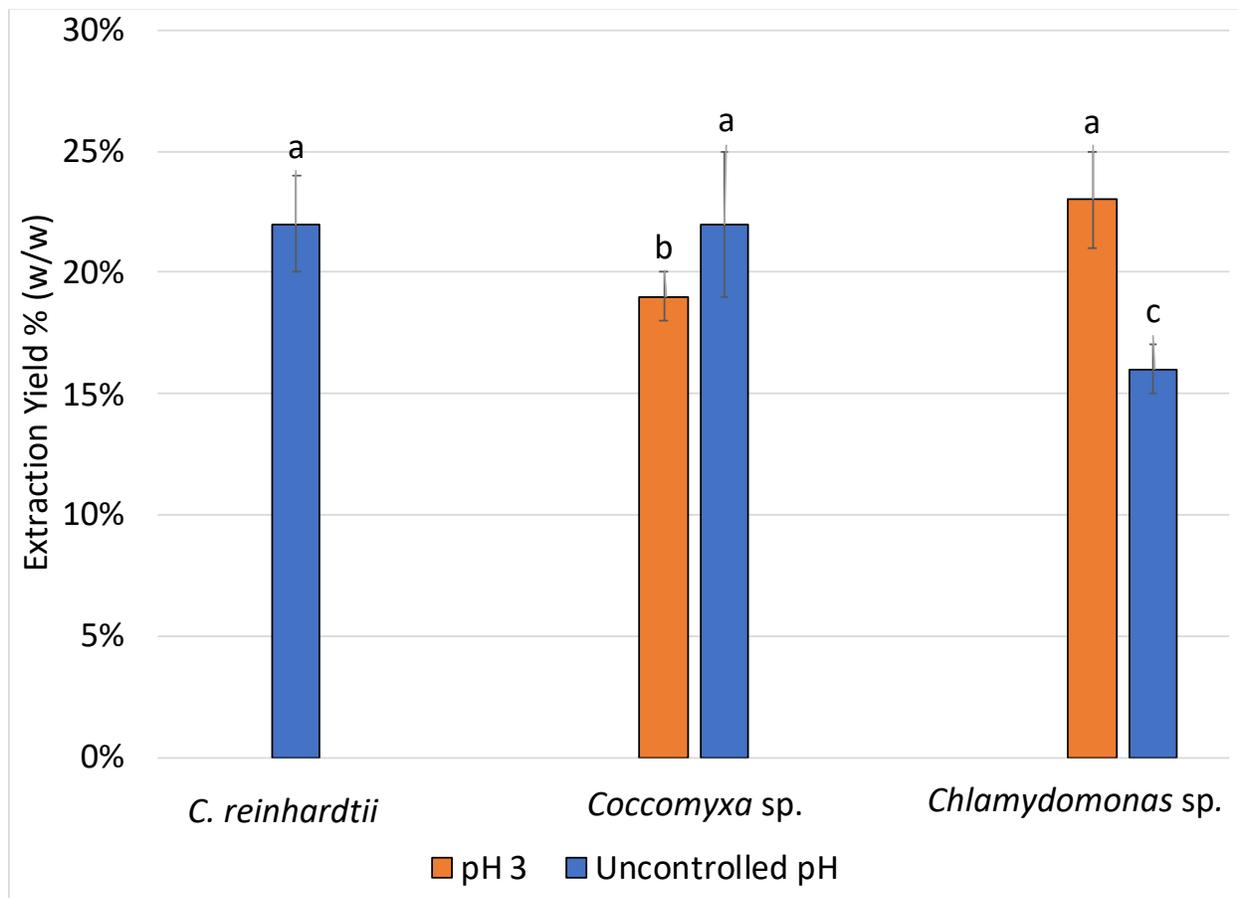
biomass concentration of  $0.22 \pm 0.01 \text{ g L}^{-1}$  dry weight per medium after 28 days but had no biomass production in controlled pH 3.0 medium.

**Figure 7: Culture concentration of samples growing in controlled pH 3.0 medium and uncontrolled medium in grams of dry weight per litre of medium. Bar represents the mean  $\pm$  SD (n = 3). Significance ( $p < 0.05$ ) between conditions with respect to each species expressed by lower case letter.**

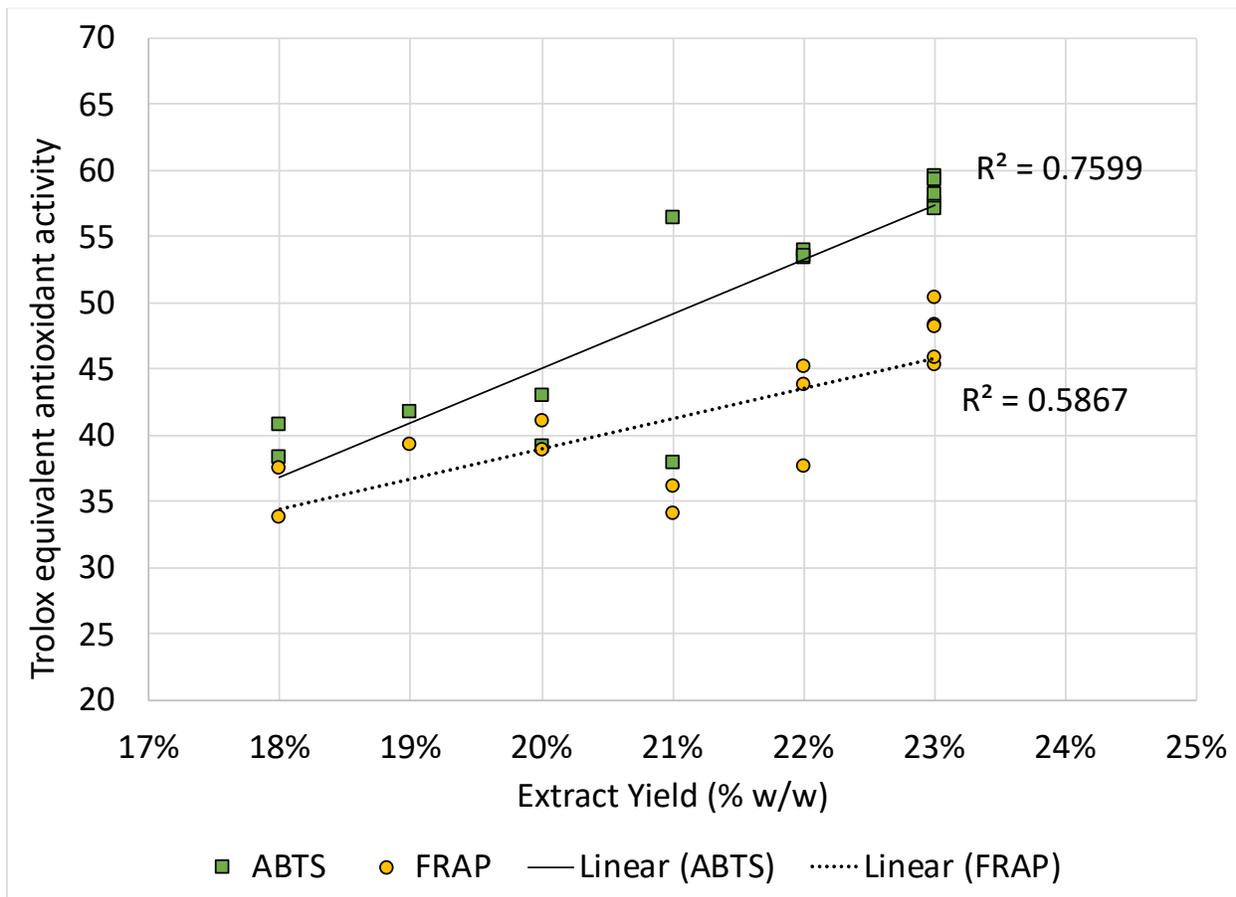


Of the bioprospected strains, *Coccomyxa* sp. showed a decrease in crude extract yield when grown under controlled pH medium regardless of having higher biomass production under the same conditions. Interestingly, the *Chlamydomonas* sp. biomass from controlled pH medium yielded higher quantities of crude extracts at  $23 \pm 2$  % (w/w) than the biomass harvested from uncontrolled pH conditions which resulted in  $16 \pm 1$  % (w/w) extraction as can be seen in Figure 8. In this study, *Coccomyxa* sp. expressed a strong correlation between crude extract recovery and antioxidant capacity per gram of dry biomass according to the ABTS<sup>+</sup> ( $r = 0.86$ ), DPPH ( $r = 0.59$ ), and FRAP ( $r = 0.76$ ) assays ( $p < 0.05$ ) (Figure 9).

**Figure 8: Extraction yield of crude extracts from dry microalgal biomass of three species growing in unbuffered and pH 3.0 buffered conditions (% w/w). Bar represents the mean  $\pm$  SD (n = 9). Significance ( $p < 0.05$ ) between conditions with respect to each species expressed as lower case letters.**



**Figure 9:** Significant ( $p < 0.05$ ) correlations between antioxidant activity in TEQ ( $\mu\text{mol trolox g}^{-1}$  dry weight biomass) and extraction yield of crude extract from dried biomass (% w/w) of *Coccomyxa* sp. samples growing at pH 3 and uncontrolled pH conditions. (n=15)

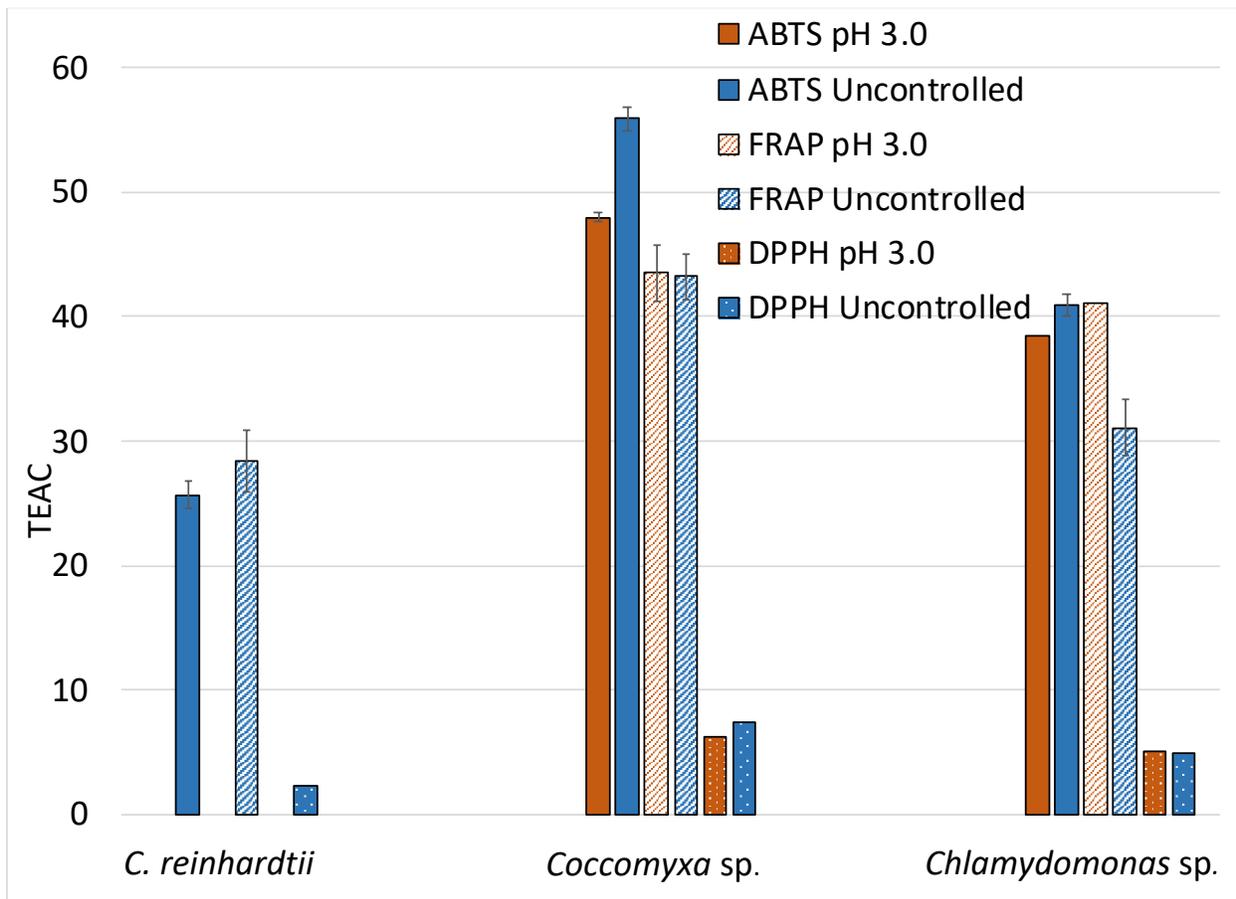


### 3.4.2.2 Antioxidant capacity assays

The extracts from each sample were tested for their antioxidant activity using three trolox equivalency assays: ABTS<sup>+</sup> radical scavenging, DPPH, and ferric reducing antioxidant power (FRAP). These colorimetric assays measure the change in optical density of an oxidant that is

reduced by the antioxidants in the algal extract or by trolox, the antioxidant control [87] and the results are shown in Figure 10. *Coccomyxa* sp. cultured under controlled pH showed no significant change in trolox equivalency when compared to uncontrolled pH condition. The antioxidant activity of *Chlamydomonas* sp. did increase under controlled pH conditions according to the FRAP assay ( $p < 0.01$ ) from  $31.1 \pm 2.3$  TEQ uncontrolled to  $41.1 \pm 2.2$  TEQ at pH 3, but slightly decreased according to the ABTS assay ( $p < 0.05$ ) from  $40.9 \pm 0.9$  TEQ uncontrolled to  $38.4 \pm 0.4$  TEQ at pH 3. When antioxidant activity of *Coccomyxa* sp. and *Chlamydomonas* sp. extracts were compared under controlled pH conditions, extracts of *Coccomyxa* sp. expressed a higher antioxidant activity according to the ABTS assay ( $48.0 \pm 4.3$  TEQ) while *Chlamydomonas* sp. had a lower activity ( $38.4 \pm 0.4$  TEQ). *Coccomyxa* sp. also had the highest activity when compared to *Chlamydomonas* sp. and the culture collection *C. reinhardtii* growing under uncontrolled pH conditions. Among the green microalgae strains tested, *C. reinhardtii* exhibited the lowest antioxidant activity and the FRAP assay did not show a significant difference between the antioxidant activity of *C. reinhardtii* and *Chlamydomonas* sp. under uncontrolled pH conditions.

**Figure 10: Trolox equivalent antioxidant activity (TEAC) ( $\mu\text{mol g}^{-1}$  dry weight biomass) according to ABTS, FRAP, and DPPH assays for extracts from strains grown in uncontrolled pH medium and buffered pH 3.0 medium. Bar represents the mean  $\pm$  SD (n = 9).**



### 3.4.2.3 Chlorophyll content

The chlorophyll-*a* and chlorophyll-*b* contents of ethanolic extracts from each sample were measured and the chlorophyll-*a*:chlorophyll-*b* ratios are given in Table 2. *Coccoomyxa sp.* showed no change in chlorophyll-*a* content ( $p > 0.05$ ) between controlled and uncontrolled pH conditions, but did express a significant accumulation of chlorophyll-*b* ( $p < 0.05$ ) under controlled pH

conditions, thereby reducing the ratio of chlorophyll-*a/b*. *Chlamydomonas* sp. showed no change in chlorophyll content under either condition ( $p > 0.05$ ).

**Table 2. Ratio of chlorophyll-*a* to chlorophyll-*b* in three microalgal species growing in a buffered pH 3 medium and an unbuffered medium after 28 days. (NT = not tested as the cells died) (\*denotes significance across rows  $p < 0.05$ )**

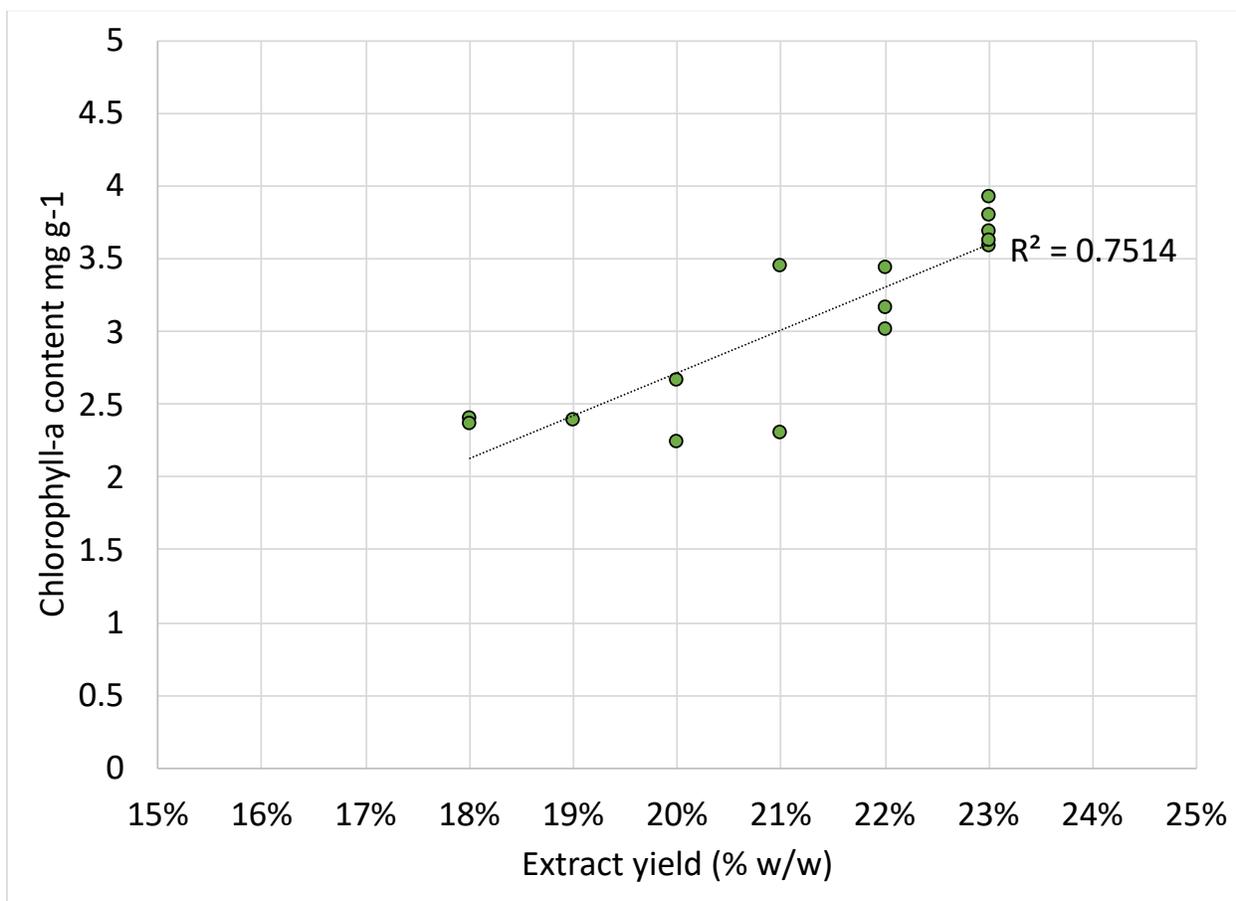
Species	Controlled (pH 3)	Uncontrolled pH
<i>C. reinhardtii</i>	NT	0.6 ± 0.1*
<i>Coccomyxa</i> sp.	1.2 ± 0.2	1.8 ± 0.1*
<i>Chlamydomonas</i> sp.	1.0 ± 0.1	1.2 ± 0.1

*Coccomyxa* sp. had the highest levels of chlorophyll-*a* when growing under pH 3.0 and under uncontrolled pH conditions ( $2.9 \pm 0.3 \text{ mg g}^{-1}$  dry weight and  $3.4 \text{ mg g}^{-1}$  dry weight respectively). Among the three species growing under uncontrolled pH conditions, *C. reinhardtii* had the lowest levels of chlorophyll-*a* ( $1.6 \pm 0.1 \text{ mg g}^{-1}$  dry weight) and the highest chlorophyll-*b* content ( $3.1 \pm 0.2 \text{ mg g}^{-1}$  dry weight). *Coccomyxa* sp. exhibited the lowest chlorophyll-*b* content ( $1.9 \pm 0.1 \text{ mg g}^{-1}$  dry weight).

A strong correlation ( $p < 0.01$ ) was found between chlorophyll-*a* content and the antioxidant capacity assays associated with extracts of *Coccomyxa* sp. and *Chlamydomonas* sp. For *Coccomyxa* sp., chlorophyll-*a* content was correlated, according to Pearson correlations, with the

antioxidant capacity of the extracts according to the ABTS<sup>+</sup> ( $r = 0.97$ ), DPPH ( $r = 0.63$ ), and FRAP ( $r = 0.80$ ) assays. Similarly, *Chlamydomonas* sp. extracts exhibited correlation between chlorophyll-*a* content and antioxidant capacity measured by ABTS<sup>+</sup> ( $r = 0.74$ ), and DPPH ( $r = 0.45$ ) assays. As can be seen in Figure 11, there was a correlation between crude extract recovery and chlorophyll-*a* ( $r = 0.86$ ) content ( $p < 0.05$ ) for the *Coccomyxa* sp..

**Figure 11.** Correlation between chlorophyll-*a* content in mg g<sup>-1</sup> dry weight biomass and extraction yield of crude extract from dried biomass (% w/w) of *Coccomyxa* sp. samples growing at controlled pH 3.0 and uncontrolled pH conditions. (n = 15).



### 3.5 Discussion

The results of the BLAST database search and morphological microalgal identification are in agreement with previous identifications of microalgae bioprospected from other mine-impacted sites; species of *Chlamydomonas* and *Coccomyxa* have been isolated from the acidic, metal-rich water of the Rio Tinto [38,51,88], and *Chlamydomonas acidophila* was isolated from acid mine drainage ponds outside of a copper mine in North Wales [40]. The genera of green freshwater microalgae that are predominant in acidic mine-impacted water systems are *Chlamydomonas*, *Dunaliella*, *Euglena*, *Ochromonas*, and *Coccomyxa* [38,40].

Oxidative stress can induce an antioxidant response in several species of freshwater green microalgae [78]. The stressors that coincide with acidic conditions, such as a high concentration of metals, are considered the main cause of oxidative stress in microalgae in acidic environments [51,53]. A significant obstacle in commercial cultivation of microalgae is that oxidative stress increases metabolite accumulation, but generally reduces overall biomass production [13]. This experiment investigated the impact of pH control on biomass, antioxidant, and chlorophyll production of two microalgal species, *Chlamydomonas* sp. and *Coccomyxa* sp., with demonstrated antibacterial activity [78] originating from acidic mine-impacted areas and a culture collection acquired *Chlamydomonas reinhardtii*. The three strains were grown in a controlled pH 3.0 medium and an uncontrolled pH medium for 28 days. Extracts were examined for antioxidant activity and chlorophyll production.

The bioprospected samples were likely able to survive in controlled pH 3.0 medium as they were bioprospected from water bodies that were pH 2.9. On the other hand, the culture collection

*C. reinhardtii* had no prior exposure to a low pH environment and could not acclimatize to the sudden change. With contamination of microalgal cultures by other microorganisms a concern during commercial production, the survivability of *Coccomyxa* sp. and *Chlamydomonas* sp. in acidic conditions may be beneficial for reducing the risk of contamination by neutrophilic microalgae, as well as bacteria and fungi [88] .

The solvents used for the extraction process can have a large influence on crude algal extract yield and composition [10], as well as extraction technique, extraction time, and culture growth conditions [89]. In this study, ethanolic extraction was chosen as many antioxidants, such as phenols, flavonoids, and chlorophylls, are polar compounds [90]. Ethanolic extracts have higher antioxidant potential when compared to water extracts despite ethanolic extracts having a significantly lower crude extract yield [89]. It was speculated this was due to ethanol extracting more hydrophilic antioxidants than water.

The growth conditions in this study resulted in significant ( $p < 0.01$ ) changes in the extraction yields from each species of microalgae. The increase of biomass production of *Coccomyxa* sp. under controlled acidity combined with the decrease in extraction yield may have resulted from an accumulation of lipophilic compounds and antioxidants that were not well extracted by the polar ethanol. These results are consistent with previous work in which antioxidant activity of green microalgae was suggested to be strain- specific [10].

Both the bioprospected microalgal samples expressed higher antioxidant activity per gram of dry weight biomass than the culture collection *C. reinhardtii*. *Coccomyxa* sp. showed the highest antioxidant activity between all three species in both tested conditions, with the highest activity recorded by the ABTS<sup>+</sup> assay with microalgae grown under uncontrolled pH conditions

( $55.9 \pm 1.0$  TEQ). In comparison, the antioxidant capacity of 23 marine microalgae using ABTS<sup>+</sup> assays found that highest antioxidant capacities were by the blue-green microalgae *Synechococcus* sp. *FACHB283* at  $29.6 \pm 1.24$  TEQ, green microalgae *Chlamydomonas nivalis* at  $24.3 \pm 0.47$  TEQ, and blue-green microalgae *Nostoc ellipsosporum* *CCAP 1453/17* at  $21.1 \pm 1.83$  TEQ [10]. These were similar to the culture collection species, but considerably lower than observed with the two bioprospected species.

The consistently lower values resulting from the DPPH compared to the ABTS assay may be attributed to pigmented, hydrophilic, and lipophilic antioxidants being better expressed through the ABTS assay [87]. This shows that although the assays follow a similar mechanism of changing colour when reduced in the presence of antioxidants, the level of response may differ depending upon the types of antioxidant compounds in the extract. That is, the antioxidants produced may vary between stress conditions and also microalgal species [10]. Stronger antioxidants, like astaxanthin, will reduce oxidants more readily than weaker ones [87]. For instance, ABTS<sup>+</sup> assays are best for quantifying antioxidant activity from carotenoids and polyphenols and FRAP assays are best for quantifying antioxidant activity from polyphenols and some carotenes and xanthophylls [82]. Additionally, in an experiment performed on methanolic guava fruit extracts it was found that FRAP and ABTS<sup>+</sup> assay reactions required 30 minutes to fully react with extracts, but DPPH a full 24 hours [91]. In a similar manner, the composite mixture of antioxidants present in crude algal extracts and their interaction with one another may cause a more prolonged antioxidative response that necessitates longer reaction times for antioxidant capacity assays [82]. Strains of *Coccomyxa* spp. and *Chlamydomonas* spp. isolated from samples collected from the

acidic metal-rich waters of the Rio Tinto in Huelva, Spain have been shown to accumulate high levels of carotenoids, particularly lutein and  $\beta$ -carotene[37,51,88].

The chlorophyll contents of each microalgal extract were also measured as chlorophyll and its derivatives have been shown to have antioxidant activity, making them a product of interest [92]. While some pigments, like carotenoids, tend to accumulate under oxidative stress, a decrease in chlorophyll-*a* content is an indicator of cell stress and toxicity [8,93]. For example, a decrease in chlorophyll content has been seen under salinity stress [94] and also nutrient stress [35]. Changes in the chlorophyll-*a* to chlorophyll-*b* ratio has been recorded under copper stress [31] as well as in the presence of excess irradiance [95]. The cell may convert between chlorophyll-*a* and chlorophyll-*b* as a means to mitigate the production of ROS and to maintain photosynthetic efficiency [95]. Previous research shows that divergence from ideal growing pH can restrict the cell's ability to synthesize chlorophyll and carotenoid pigments [32]. Although the levels of chlorophyll-*a* did not decrease to indicate cellular toxicity, the increase in chlorophyll-*b* exhibited by *Coccomyxa* sp. under controlled pH conditions may imply cells were compensating for the diminished photosynthetic capacity regularly associated with oxidative stress [96]. The antioxidant activity of chlorophyll may explain the correlation between chlorophyll-*a* content and antioxidant activities of *Coccomyxa* sp. and *Chlamydomonas* sp. This infers that chlorophyll is a major contributor to the antioxidant potential of these algal extracts.

Utilizing industrial off-gas emissions as a means to commercially cultivate microalgae while capturing and mitigating CO<sub>2</sub> has received a lot of attention. However, successful application could be aided by using strains of microalgae that can survive the acidic conditions created by acid off-gasses. This would, therefore, avoid costs associated with addition of alkali agents to neutralize

the impact of the acid gasses. Furthermore, microalgae that can tolerate acidic environments may have the advantage of growing in an environment that inhibits other organisms, such as predators and potentially competing microalgae.

We have investigated whether microalgae when growing at low pH also produce viable quantities of valuable antioxidant compounds. Two microalgae bioprospected from water bodies impacted by acid mine drainage (pH 2.9) known as *Coccomyxa* sp. and *Chlamydomonas* sp., that exhibited antibacterial activity [78] also displayed high antioxidant capacity. As a comparison, *Chlamydomonas reinhardtii* from a culture collection was used, and although it had a higher overall biomass production under uncontrolled pH conditions, it was unable to survive in the acidic growing medium.

In pH 3.0 conditions, the *Coccomyxa* sp. had a significantly higher antioxidant potential and chlorophyll-*a* content than the *Chlamydomonas* sp. A strong correlation between chlorophyll-*a* content and the antioxidant capacity of *Coccomyxa* sp. indicates that chlorophyll-*a* may be a major contributor to the antioxidant activity of the extracts. Furthermore, the controlled acidity did not change the biomass concentration of *Chlamydomonas* sp., but did instigate higher biomass production by *Coccomyxa* sp.

Generally, biomass production under stressful conditions is a major challenge for commercial cultivation, but the stress triggers an accumulation of certain valuable bioactive compounds, but reduces the cell's ability to produce biomass [13]. This further suggests *Coccomyxa* sp. as a good contender for commercial cultivation of antioxidants. Further testing such as total phenolic compounds and carotenoid content and type using FTIR [97], LC-MS [27] and/or HPLC [98]

should be carried out in order to determine whether these compounds are major contributors to the high antioxidant capacity exhibited by the bioprospected microalgae, particularly *Coccomyxa* sp.

# Chapter 4

## 4 Conclusions

This chapter summarizes the findings of the research and experiment performed in part of this dissertation. Future work regarding the nutraceutical potential of the tested bioprospected novel strains of microalgae is considered.

### 4.1 Summary of Results

Microalgae are photosynthetic microorganisms ubiquitous in aquatic environments around the world. The biochemical composition of these microorganisms is dependent on the environment in which it grows. Stressful environments, characterized by acidity, high metal concentrations, high solar irradiance, and nutrient limitations contribute to the oxidative stress that induces the cellular production of antioxidants. Antioxidants are valuable compounds that can be extracted and sold for commercial nutraceuticals. The demand for naturally sourced antioxidants is increasing, driving a need to find cost-effective methods for growing microorganisms like microalgae for the purpose of harvesting their bioactive compounds.

The two novel strains of microalgae, *Coccomyxa* sp., and *Chlamydomonas* sp., bioprospected from abandoned mine sites in Northern Ontario were examined for their antioxidant capacity and biomass production in acidic conditions. Experiments showed growing *Coccomyxa* sp. at a steady pH 3.0 did not affect the antioxidant production when compared to being grown in an uncontrolled

pH environment and interestingly increased biomass production. Furthermore, *Coccomyxa* sp. had a higher antioxidant potential than *Chlamydomonas* sp. and the culture collection strain *Chlamydomonas reinhardtii*. It was suggested that *Coccomyxa* sp. is a promising contender for being cultivated using industrial off-gas that acidifies growing media.

## 4.2 Future Work

Work is still needed to confirm the suitability of the novel strains, specifically *Coccomyxa* sp., for commercial antioxidant production using industrial off-gas as a main carbon source. Future investigation could include growing *Coccomyxa* sp. in media mimicking the acidity, carbon dioxide concentrations, and potential contaminants (i.e., metals) in industrial off gas and analyzing the impact on the antioxidant potential of *Coccomyxa* sp.

Primarily, carotenoids, phenols, and chlorophylls could be accurately quantified, which would help determining which compounds are the greatest contributors to the extracts' high antioxidant potential. Once the main antioxidants are identified, the crude extracts of *Coccomyxa* sp. and *Chlamydomonas* sp. could be fractionated and analyzed for the specific compounds through available analytical technologies such as high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and Fourier-transform infrared spectroscopy (FTIR). Further, different extraction solvents have been found to extract distinct types of antioxidant compounds. More extraction solvents could be tested, and their crude extracts analyzed to determine the best solvent for extracting certain compounds from the novel strains.

Future work is needed to draw more solid conclusions from the experiment conducted for this dissertation. Given the skewed results produced by the DPPH antioxidant assay, this assay should

be modified to account for the pH of the algal extracts. The assay should be redone and compared to the results found by the ABTS and FRAP assays. This research involved extracting DNA from two novel algal strains for Sanger Sequencing for identification. The identification of these strains in this research needs to be expanded on using a phylogenetic tree to determine a more accurate genus and species. The Ongen lab group is currently working on developing a phylogenetic tree for better identification.

Additionally, the experiment performed for this dissertation was a lab-scale experiment. A scale-up experiment conducted in bioreactors sparged with industrial off-gas should be used to determine if the antioxidant potential and biomass production of these strains is reproducible in for commercial cultivation. The cost associated with large-scale microalgal cultivation is a major challenge in commercial production and should be analyzed through a scale-up cost analysis.

With another major draw-back of commercial algal cultivation being biomass production under stress conditions, a two-stage cultivation of *Coccomyxa* sp. and *Chlamydomonas* sp. should be tested. This involves growing the microalgae at optimal growing pH then suddenly dropping/raising the pH prior to harvesting. A two-stage cultivation is used to preserve a high biomass yield and stimulate antioxidant production. It should be investigated as to whether these novel strains produce more antioxidants through this method than they produce when grown at a constant acidic pH.

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