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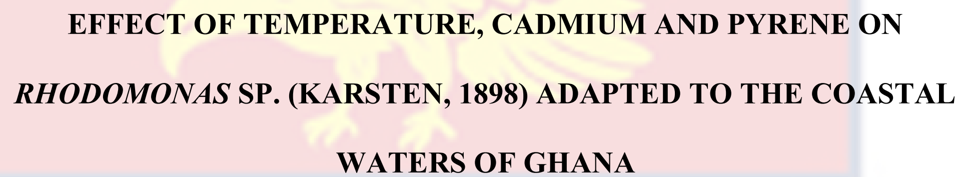

EFFECT OF TEMPERATURE, CADMIUM AND PYRENE ON *RHODOMONAS* SP. (KARSTEN, 1898) ADAPTED TO THE COASTAL WATERS OF GHANA

BENJAMIN DZIEDZORM GAWORNU

2023

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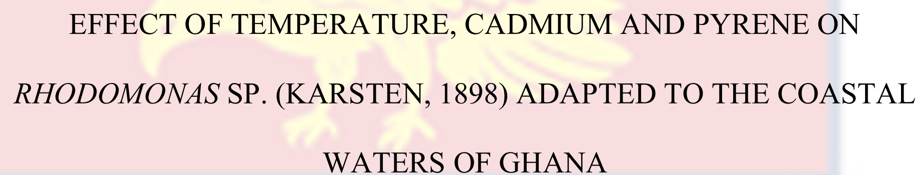

EFFECT OF TEMPERATURE, CADMIUM AND PYRENE ON *RHODOMONAS* SP. (KARSTEN, 1898) ADAPTED TO THE COASTAL WATERS OF GHANA

BY

BENJAMIN DZIEDZORM GAWORNU

Thesis submitted to the Department of Fisheries and Aquatic Sciences of the School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of a Master of Philosophy (M.Phil.) degree in Oceanography and Limnology

NOVEMBER 2023

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature …………………………… Date ……………………

Name: ………………………………………………………

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

Global projections suggest that human-induced factors such as climate change and pollution are altering the productivity of marine ecosystems with potential adverse consequences for nutrition and livelihood in many coastal communities. However, the combined effects of these stress factors remain largely unknown, particularly for tropical organisms. Using a microcosm experiment, this study investigated the response of the microalgae *Rhodomonas* sp., a major primary producer in many marine systems, to warming (2 – 6 °C above the average sea surface temperature of Gulf of Guinea), as well as pollution by cadmium (0.1, 1, 10 and 100 μgL^{-1}) and pyrene (0.2, 2, 20, 200 μgL^{-1}). Response of the species was assessed using its growth rate, dry weight, protein, lipid, carbohydrate contents and catalase activity. The results suggest that the combination of cadmium and pyrene pollution did not impact lipid content of the algae. In contrast, average dry weight (0.125 ± 0.003 $\mu gcell^{-1}$) of the cells increased (≈ 21 %) when pyrene and cadmium pollution exceeded 2 μgL^{-1} and 1 μgL^{-1} respectively. The increase in dry weight was related to increasing protein content of the cell. This was observed even when cells were exposed to warming, indicating that *Rhodomonas* sp. are able to buffer impact of environmental stress by producing heat shock proteins of relatively higher molecular weights. On the other hand, carbohydrate content of the cells decreased (≈ 86 %) when cadmium pollution exceeded 10 μgL^{-1} irrespective of pyrene pollution. Catalase activity, which indicates mechanism used by cells to neutralise impact of the stress conditions decreased (≈ 32 %) when pyrene and cadmium exceeded 0.2 μgL^{-1} and 0.1 μgL^{-1} respectively. These observations highlight the impact of multiple human-induced factors on marine organisms.

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KEY WORDS

Phytoplankton

Multiple stressor

Protein

Lipid

Carbohydrate

Enzyme

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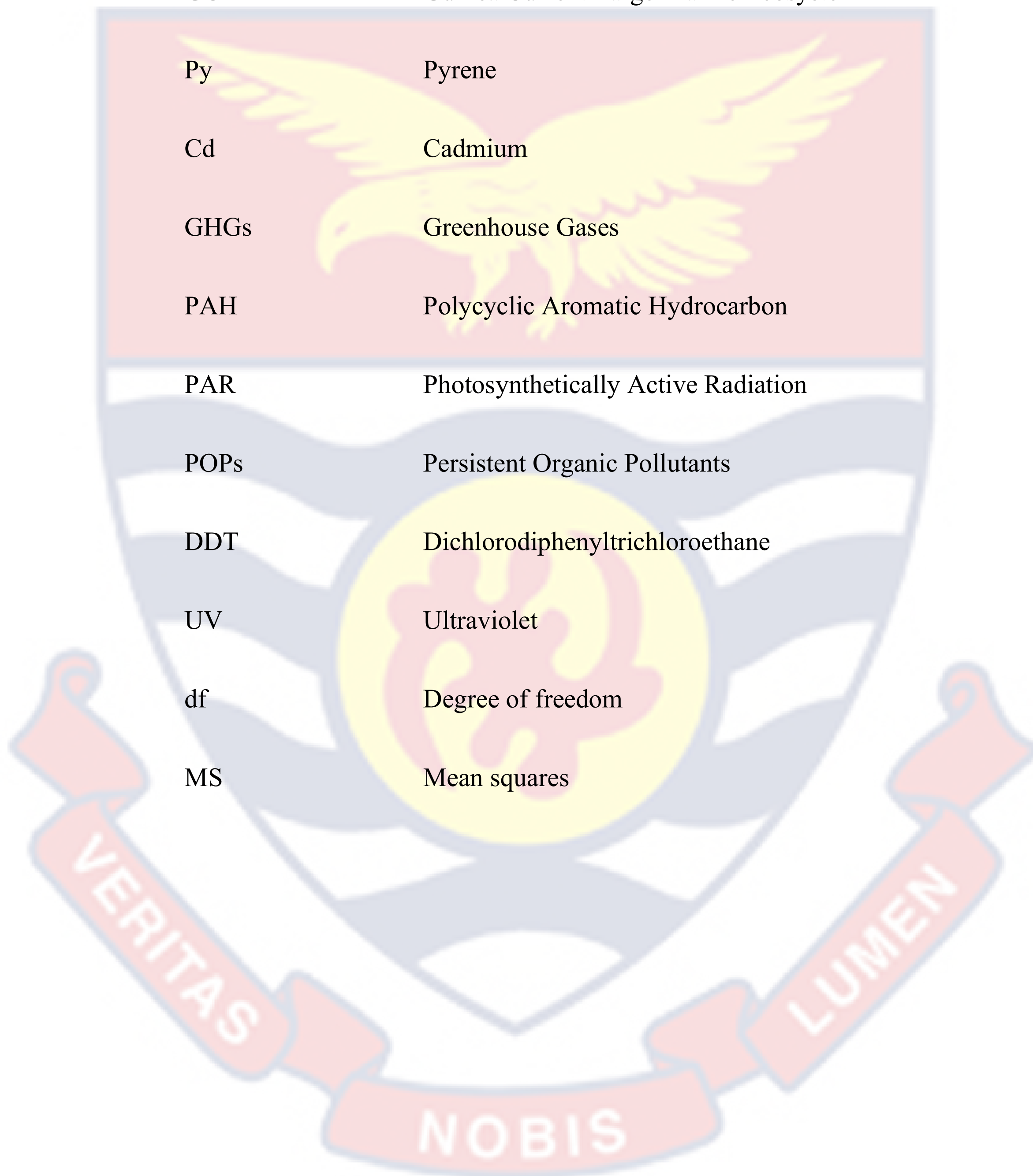

IPCC	Intergovernmental Panel on Climate Change
GCLME	Guinea Current Large Marine Ecosystem
Py	Pyrene
Cd	Cadmium
GHGs	Greenhouse Gases
PAH	Polycyclic Aromatic Hydrocarbon
PAR	Photosynthetically Active Radiation
POPs	Persistent Organic Pollutants
DDT	Dichlorodiphenyltrichloroethane
UV	Ultraviolet
df	Degree of freedom
MS	Mean squares

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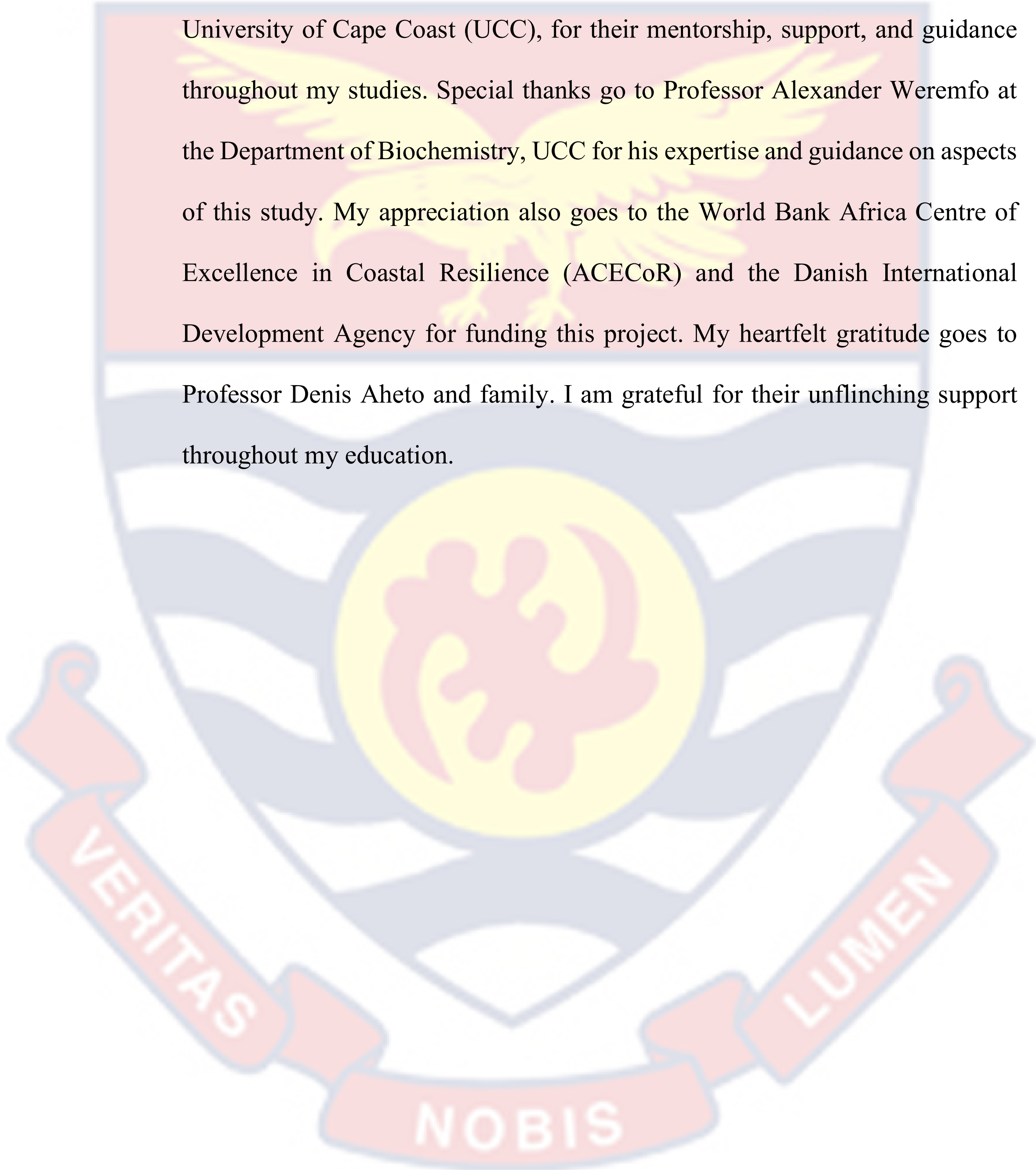
ACKNOWLEDGEMENTS

I express my profound gratitude to Dr. Emmanuel Acheampong and Dr. Paul Kojo Mensah of the Department of Fisheries and Aquatic Sciences (DFAS), University of Cape Coast (UCC), for their mentorship, support, and guidance throughout my studies. Special thanks go to Professor Alexander Weremfo at the Department of Biochemistry, UCC for his expertise and guidance on aspects of this study. My appreciation also goes to the World Bank Africa Centre of Excellence in Coastal Resilience (ACECoR) and the Danish International Development Agency for funding this project. My heartfelt gratitude goes to Professor Denis Aheto and family. I am grateful for their unflinching support throughout my education.



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DEDICATION

This research work is dedicated to my parents, who have shown me an abundance of love and support.

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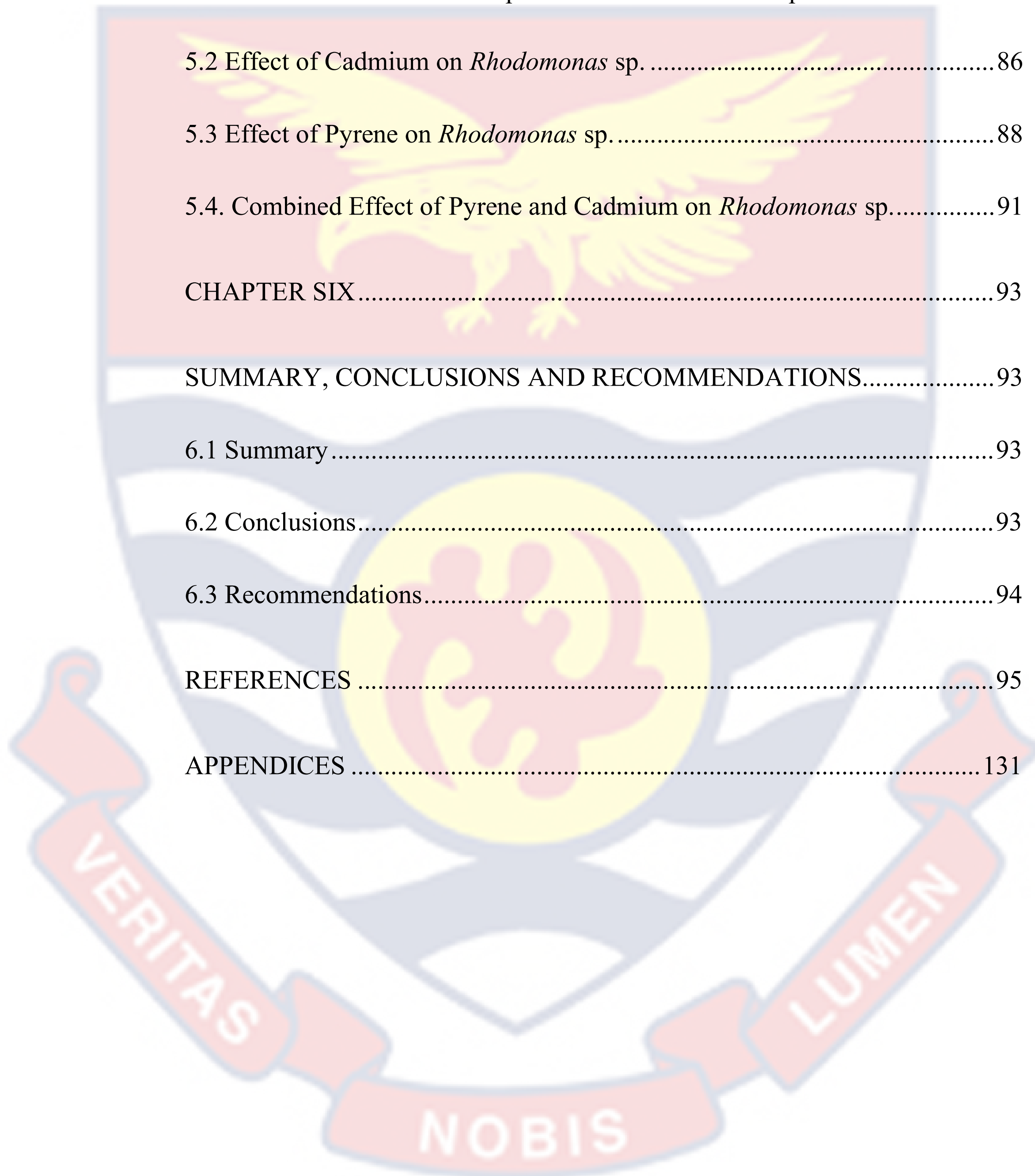

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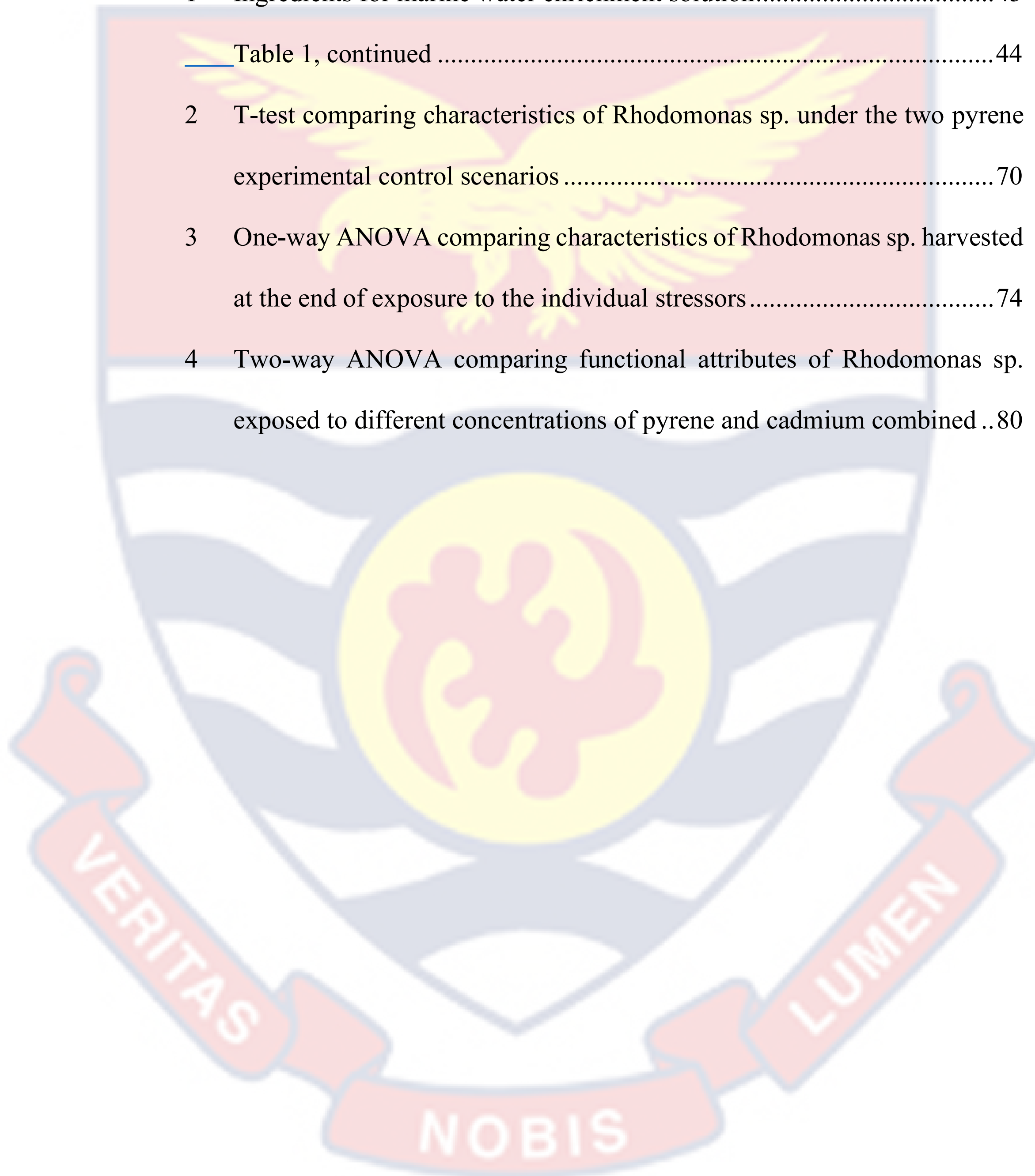

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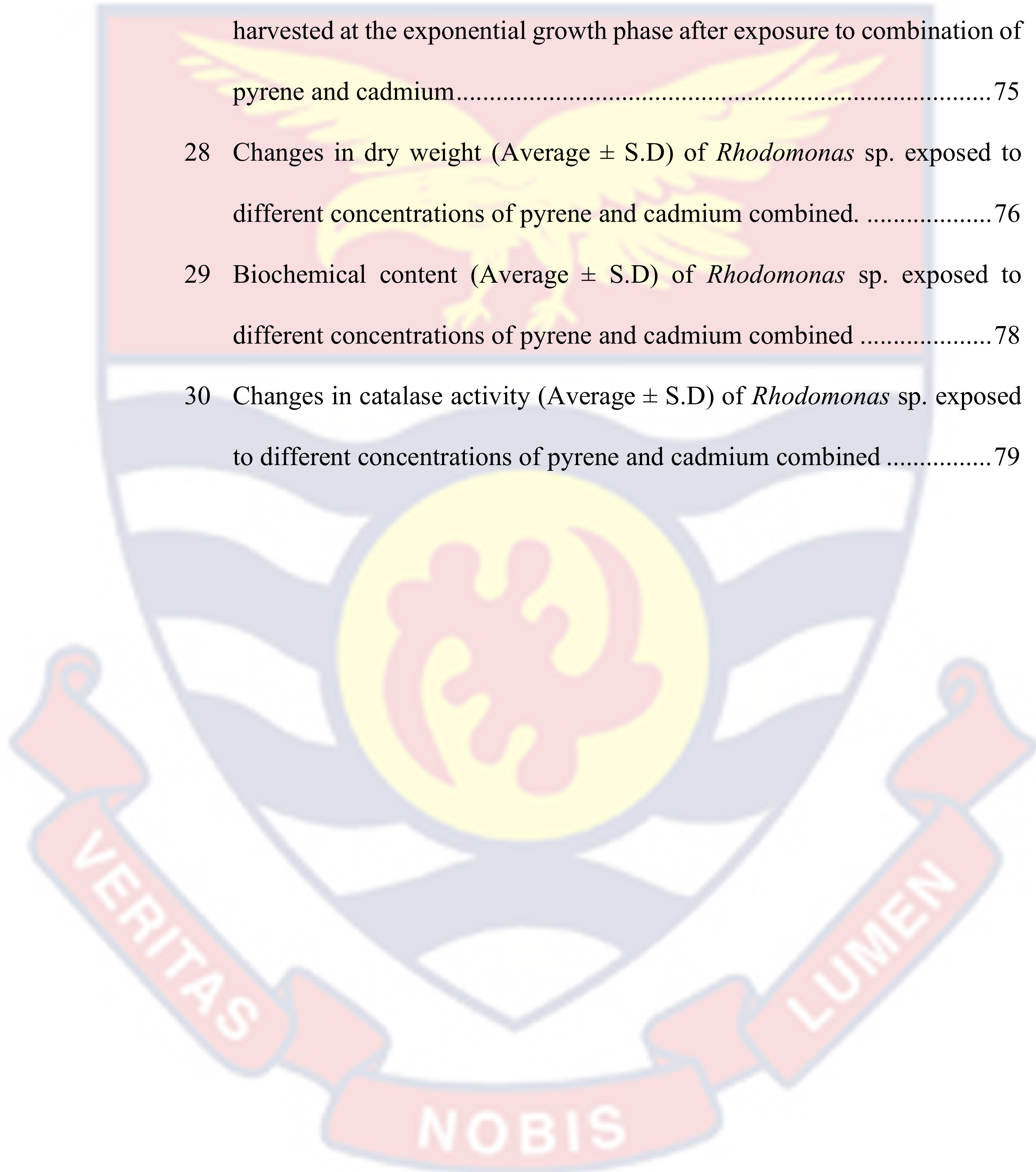

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CHAPTER ONE

INTRODUCTION

Coastal seas of Ghana form part of the Guinea Current Large Marine Ecosystem, rich in natural resources such as fish and oil (Chidi & Bradford 2020; Pretorius & Henwood, 2019). These resources provide a solid ground for numerous socioeconomic activities such as oil exploration and fishing. In spite of this, coastal areas of Ghana are being exposed to significant levels of pollution from various sources (Benson et al., 2018; Pappoe, Palm, Denutsui, Boateng, Danso-Abbeam & Serfor-Armah). In particular, reports suggest that pollution by heavy metals and petroleum is common along the coast of Ghana (Abdul et al., 2019; Ayamdoo, 2016; Okafor-Yarwood, 2018; Scheren, Ibe, Janssen & Lemmens, 2002).

In addition, Ghana's marine waters as well the entire Gulf of Guinea is warming as a result of Global climate change (Asuquo & Oghenechovwen, 2019). The combination of the above stressors alter the functional attributes of organisms that form the basis the productivity of marine ecosystems (Brauko et al., 2020). These consequences are expected to be severe, particularly in tropical marine ecosystems where organisms are already living close to their tolerance limit (Nguyen et al., 2011). Therefore, the focus of this study was to assess the combined impact of sea surface warming and pollution on the functional and biochemical properties of phytoplankton adapted to Ghana's marine waters.

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1.1 Background to the Study

The term phytoplankton is derived from two Greek words (phyton), which means "plant" and (planktos), which means "drifter" (Cassidy, 2009). These autotrophic drifting organisms are the major producers in the pelagic environment. They produce sugars and other biochemical substances (proteins, lipids, etc.) from photosynthesis. These photosynthetic products are consumed by grazers (e.g., copepod) and the energy transferred through the food chain to higher trophic level organisms (Figure 1).

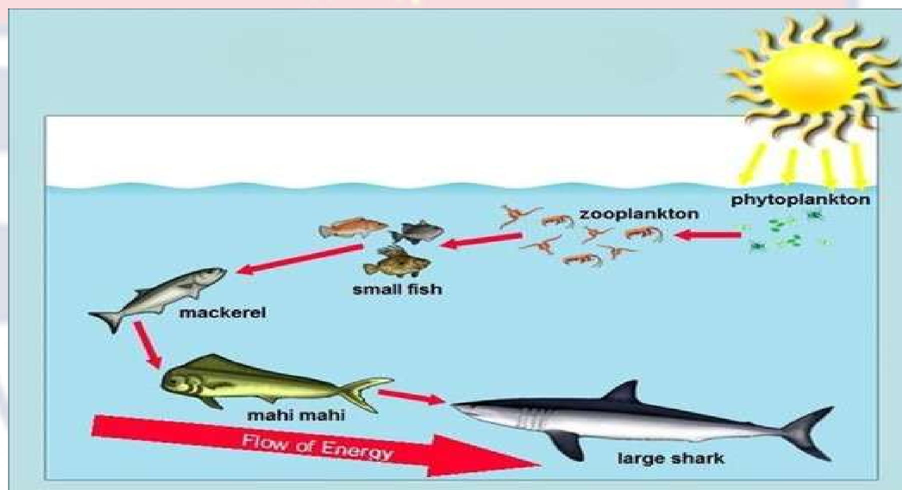

Figure 1: Energy transfer from phytoplankton to primary consumers (zooplankton) and then to higher trophic level organisms (Source: Cáceres, 2011).

The productivity of these photosynthetic cells is controlled daily changes in several environmental factors such as temperature, nutrient and light availability (Winder & Sommer, 2012). Global change factors such as climate and pollution can alter these environmental factors over a long term. In view of this, slight variations in environmental conditions of the ocean can significantly alter phytoplankton structure, taxonomic composition and their ability to synthesise essential biomolecules such as proteins.

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Research suggests that the warming of the ocean is one of the significant global change factors influencing growth and production of marine phytoplankton (Acheampong, Mantey & Weremfo, 2021; Bi, Ismar, Sommer & Zhao, 2017). According to Plumer (2019), more than 90% of the heat trapped by greenhouse gases is absorbed by the world's oceans due to their vast nature, leading to sea surface warming (Carrington, 2020). Since 1900, global sea surface temperatures have risen by an average of 0.7 °C (Jewett and Romanou 2017; Lea, Pak & Spero, 2000). This warming of the ocean is expected to be most noticeable in tropics and the Northern Hemisphere (Allen et al., 2019). By 2100, the ocean is expected to be two to four times warmer than the average reported in the 1970s; this warming is projected to be even worse, at five to seven times higher than the average in the 1970s under greenhouse gas emission scenarios. (Allen at al., 2019; Gattuso et al. 2015). This phenomenon of sea surface warming has been reported in the coastal seas of Ghana (Figure 2).

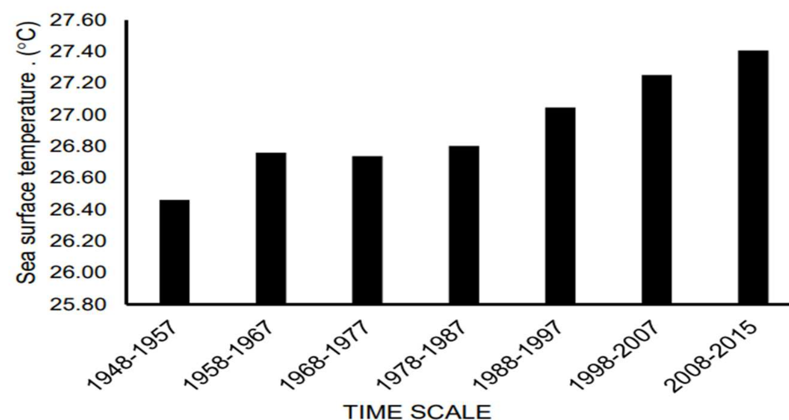

Figure 2: A decade-by-decade analysis of the changes in surface temperature of Ghana's sea during stable hydrographic period (Source: Ghana Meteorological Agency, 2016).

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In addition to sea surface warming, phytoplankton and other marine organisms are threatened by the pollution of the ocean (Landrigan et al., 2020; Sharma & Chatterjee, 2017). The types and sources of this pollution are diverse. This research is however focused on heavy metal and oil pollution. Heavy metals constitute pollutants that are of significant concern in recent years (Okereafor, Makhatha, Mekuto, Uche-Okereafor, Sebola & Mavumengwana, 2020). This is because they are generated by wide range of industrial and commercial activities and have potential to bioaccumulate and biomagnify in biological organisms. These metals enter the environment naturally and through human activities. Anthropogenic sources include mining, industrial waste and sewage disposal, application of insect and plant disease control agents as well as metallurgic works (Tamele & Vázquez Loureiro, 2020; Alava, Cheung, Ross & Sumaila, 2017). These activities are numerous along the coast of Ghana. Gbogbo & Otoo (2015) have reported high concentrations of cadmium in coastal water bodies and their associated resources in Ghana (Figure 3). Heavy metals persist in the marine environment and have the potential to bioaccumulate and biomagnify in biological organisms (Ensibi, Nejib & Yahia, 2017). According to Gallo, Morse, Hollnagel & Barros (2020), heavy metals cause oxidative stress in phytoplankton, thereby affecting the ability of various species to synthesise essential macromolecules such as carbohydrates.

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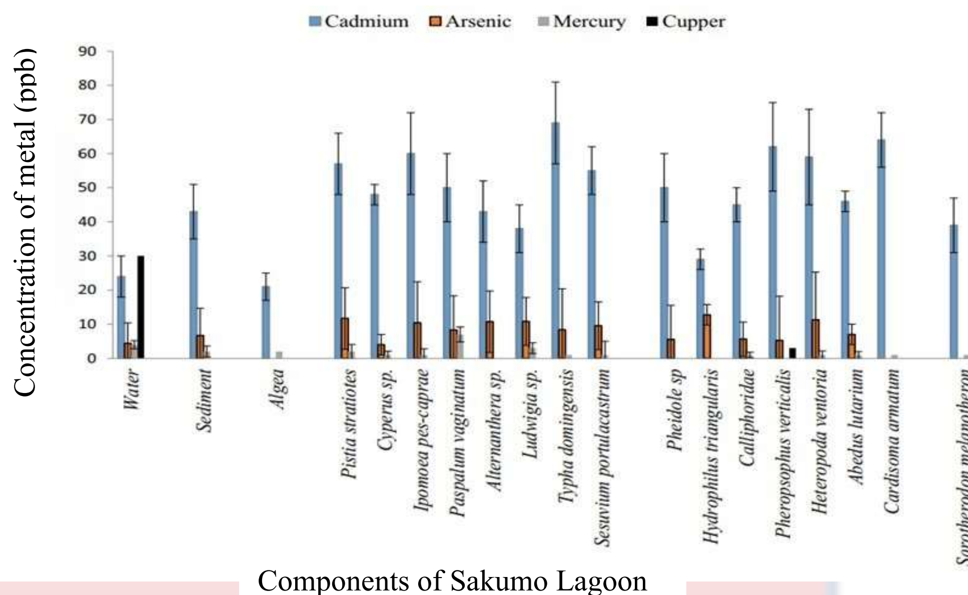

Figure 3: Concentration of dominant heavy metals in components of economically important coastal wetland in Ghana.

Petroleum is naturally formed in marine environments as a result of the decomposition of dead animals and plants that have been buried for millions of years on the seafloor (Hsu & Robinson, 2019). Occasionally, natural seepages do occur. These seepages are minor and have little impact on marine biota (Kennicutt, 2017). However, as the world's industries grow, so does the demand for petroleum products, driving up the exploration, production, refining, and transportation of petroleum at sea (Miller & Sorrell, 2013). This has increased the risk of petroleum pollution in aquatic environments (Kroon et al., 2020). Along the Gulf of Guinea, exploration and transportation of petroleum have been intensified over the recent years. These activities usually result in oil spills that are mostly unreported (Mukpo, 2021). According to Mukpo (2021), majority of oil slicks found in the Gulf of Guinea between 2002 and 2012 were related to shipping and offshore oil production. Particularly, in the coastal

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waters of Ghana, several Polycyclic Aromatic Hydrocarbons (PAHs) associated with petroleum have been found (Figure 4).

While numerous marine species are affected by petroleum pollution, planktonic communities present the greatest level of susceptibility (Almeda, Baca, Hyatt & Buskey, 2014). Oil films form shield on sea surface, thereby preventing light penetration for photosynthesis. This reduces carbon fixation by phytoplankton and subsequently reduce their growth (Tang, Sun, Zhou, Wang, Singh & Pan, 2019).

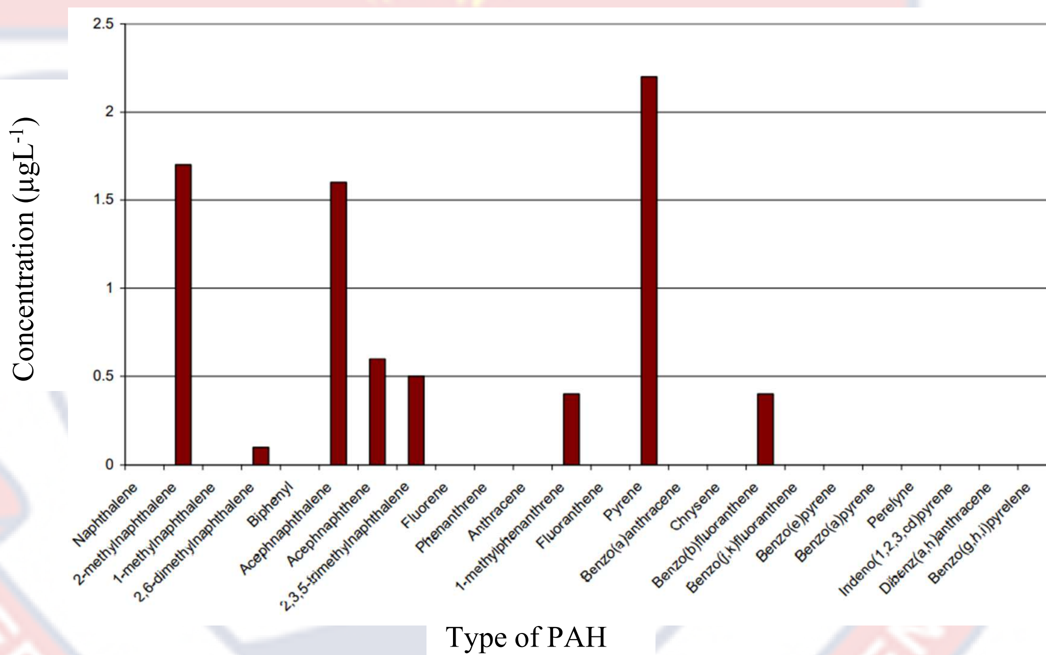

Figure 4: Levels of Polycyclic Aromatic Hydrocarbons found in coastal waters of Ghana (Source: Essumang, 2010).

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1.2 Statement of the Problem

Generally, sea surface warming and pollution do not occur in isolation in the marine environment (Crain, Kroeker, & Halpern, 2008). They act together to affect individual organisms as well as food web structure. However, efforts to address their combined impacts is hindered by several challenges. This is because a wide range of stressor pair can simultaneously occur in nature and it is difficult to draw general conclusions about the frequency with which various interaction types occur in nature (Griffen, Belgrad, Cannizzo, Knotts, & Hancock, 2016). Moreover, attempts to address this mostly involve studies conducted in temperate regions (Wernberg, Smale & Thomsen, 2012). There is little understanding on how tropical species are likely to respond to combination of these stressors. In particular, knowledge on how these stressors interact to affect nutritional quality (e.g., proteins, lipids, carbohydrates) of phytoplankton as well as the ability of the organism to mediate impact of the stressors using its enzyme system is lacking. Therefore, there is the need to investigate the combined impact of the above stressors on functional properties of tropical phytoplankton species.

1.3 Purpose of the Study

The purpose of the current study was to investigate the functional response of marine phytoplankton to combination of increasing sea surface temperature, and pollution by heavy metals and petroleum.

1.4 Research Objectives

The main objective of this study was to determine the combined impact of sea surface warming and pollution on the functional properties of phytoplankton.

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The specific objectives were to:

1. assess the individual impact of sea surface warming and pollution by cadmium (proxy for heavy metal) and pyrene (proxy for petroleum) on selected functional attributes (growth rate, dry weight, and biochemical composition) of *Rhodomonas* sp.
2. investigate the combined impact of cadmium and pyrene pollution on the functional attributes
3. assess the antioxidant response of *Rhodomonas* sp. to temperature, cadmium and pyrene and their combination.

1.5 Significance of the Study

Often, management decisions to counter the effect of environmental change involve the use of computer models and simulations (Menzie, MacDonell & Mumtaz, 2007; Shepard, 2005; Sutradhar et al., 2021). These tools allow managers to investigate the potential impact of different management actions before implementation and a crucial requirement for the development of this model is realistic descriptions indicating the behaviour of key organisms in the environment. These descriptions are usually derived from laboratory investigations simulating the different scenarios of environmental stress experienced by the target organism.

Whereas this is a common knowledge in literature, data for describing the behaviour of tropical organisms in general and those in Ghana's coastal waters in particular is lacking. This was one of the gaps the current research addressed. The design of the experiment was expected to produce results that can be useful

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for developing parameters to model marine food web dynamics in future climate scenarios.

1.6 Delimitations of the Study

In this study, only parameters under control were measured during the run of the experiment. This was done to avoid contamination of the cultures as much as possible. With regards to the experiment on petroleum pollution, a one-time spilling event was simulated to assess the effect of the PAH on functional properties of phytoplankton. This was done to avoid continuous addition of the pollutant to the cultures, which may have led to excess addition of the pollutant.

1.7 Limitations of the Study

A key component of this research was the determination of enzyme activity. Research suggest that the activity of these enzymes can be easily impacted by slight variation in temperature (Fahad et al., 2019). However, in this research, enzymes were harvested using a centrifuge (Eppendorf 5430) that did not have temperature regulator as recommended by standard analytical protocols. To overcome this, samples were cooled in refrigerator before centrifugation in order to keep the enzymes alive.

1.8 Organisation of the Study

The thesis is comprised of six chapters. The first chapter outlines the concept of the study, including its background information, problem statement, purpose, objectives, significance, and a summary. The second chapter provides a comprehensive review of relevant literature, including anthropogenic factors that affect phytoplankton growth, their causes and ecological effects. The third

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chapter details the materials, methods and statistical analysis utilized. The findings are presented in Chapter Four, followed by a discussion in Chapter Five. The final chapter, Chapter Six, offers conclusions and recommendations, along with reference list and appendices.

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CHAPTER TWO

LITERATURE REVIEW

This chapter reviews relevant literature on stressors in marine ecosystems, with focus on the factors impacting the growth and productivity of phytoplankton.

2.1 Classification and Ecological Importance of Marine Phytoplankton

In terms of groupings, phytoplankton are morphologically and physiologically diverse (Rengefors, Kremp, Reusch & Wood, 2017). Based on cell size, three main classes of phytoplankton exist (Brotas et al. 2022); these are picophytoplankton (size range: 0.2 - 2 µm), nanophytoplankton (size range: 2 - 20 µm) and microalgae (size range: 20 - 200 µm). According to Gaysina, Saraf & Singh (2019), the picoplankton constitutes major primary producers in many pelagic systems, including the ocean. *Prochlorococcus* and *Synechococcus* are examples of dominant picoplankton genus in oceanic waters. Indeed, Scanlan et al. (2009) have suggested that *Prochlorococcus* may be the most abundant photosynthetic organism on earth.

Aside size classification, phytoplankton can be grouped based on photosynthetic pigment they possess. These photosynthetic pigments absorb photons and transfer the energy into building their biomass. While all phytoplankton may contain chlorophyll a (Felip & Catalan, 2000), some photosynthetic pigments are peculiar to specific phytoplankton groups. Seoane, Laza, Urrutxurtu & Orive (2005), have previously associated chlorophyll b to bloom of a particular prasinophyte, *Cymbomonas tetramitiformis*. Report by Chai, Jiang, Cen, Ge & Lu (2016) suggests that diatoms can be identified by the presence of fucoxanthin (photosynthetic pigment in chloroplast of most brown

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algae). Dinoflagellates can be distinguished from haptophytes (flagellated algae with two golden-brown chloroplasts) because the former have dominant peridinin (pigment in chloroplast of most photosynthetic dinoflagellates) while haptophytes possess more chlorophyll c. In the marine ecosystem, diatoms and dinoflagellates constitute the major groups of phytoplankton (Kramer & Siegel, 2019; Roshith et al., 2018; Seymour, Amin, Raina & Stocker, 2017).

With the ocean covering more than 71 % of the earth, phytoplankton are responsible for producing up to 50% of oxygen as product of their photosynthetic activities (Lim, Phang, Abdul Rahman, Sturges & Malin, 2017; Pereira, 2021). In the marine environment, microbial communities utilise oxygen in decomposing organic matter. Other organisms such as fish depend on oxygen for cellular respiration. Phytoplankton are also responsible for regulating global climate. According to Cavicchioli et al. (2019), they are responsible for half of the global photosynthetic carbon fixation (net global primary production of approximately 50 Petagrams of carbon per year). This means they serve as a significant atmospheric carbon dioxide sink. Some unused carbon absorbed during photosynthesis is buried in sediments when phytoplankton die. Microbial decomposition of dead phytoplankton releases inorganic nutrients such as nitrates and phosphates back into surface waters for use by other organisms. Hence, phytoplankton play crucial role in carbon cycle.

2.2 Environmental Factors Regulating Growth of Marine Phytoplankton

The growth of phytoplankton is controlled by several environmental factors, including water temperature, nutrient availability and light intensity (Gao, Zhang & Häder, 2018; Reynolds, 2006). Nutrients have been long listed

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among the most important variables controlling phytoplankton community structure and biomass (Goericke, 2002; Mousing, Richardson & Ellegaard, 2018). Inorganic nutrients like phosphorus, nitrogen and silicon form part of the building blocks of phytoplankton cellular structure and are at particular times limiting factors for the phytoplankton growth (Geider & La Roche, 2002). Algal growth decreases when nitrogen and phosphorus are deficient (Kong, Zhu & Shen, 2010). In aquatic ecosystems, nitrogen exists in several dissolved forms. However, only reactive nitrogen (nitrate, nitrite and ammonium) is easily taken up by phytoplankton (Tyrrell, 1999). Phosphorus gets in the marine environment via river discharge and is present as phosphates for use by phytoplankton. Phytoplankton require these nutrients to perform metabolic functions and build cell structure (Kulk, van de Poll, Buma, 2018; Sommer, Peter, Genitsaris & Moustaka-Gouni, 2017). However, anthropogenic activities upland can alter availability of these nutrients in marine environment. The use of fertilisers on agricultural lands increases nitrate and phosphate loads in marine environment via river discharge and surface runoffs (Ngatia, Grace III, Moriasi & Taylor, 2019). This may cause bloom of phytoplankton in coastal marine waters (Wurtsbaugh, Paerl & Dodds, 2019; Zohdi & Abbaspour, 2019).

Light is another major environmental factor controlling growth of phytoplankton (Nissen, Vogt, Münnich, Gruber & Haumann, 2018). Phytoplankton trap energy from sunlight using chlorophylls to fix organic carbon through a process called photosynthesis (Bolin, 1970). This energy is considered Photosynthetically Active Radiation (PAR) and is within the visible light range – blue to red (Deo, Downs, Adamowski & Parisi, 2019; Wetzel, 2001). Availability of light for use by phytoplankton is affected by various

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anthropogenic factors. Notable among these is the presence of suspended particles in surface waters. Suspended particles disperse and absorb light, preventing its transmission to upper layers of the ocean (Matos, Faria, Martins, Henriques, Gomes & Goncalves, 2019). This reduces the amount of PAR available for phytoplankton for photosynthesis. The availability of these suspended particles is altered by anthropogenic factors such as sand mining on beaches and exploration of minerals on ocean floor (Ayyam, Palanivel & Chandrakasan, 2019; Yusuf, Pamungkas & Hudatwi, 2021).

Temperature is one of the primary drivers of most biological activities, influencing processes at all organisational levels, from subcellular to ecosystem (Thomas, 2013). Temperature affects phytoplankton growth dynamics directly and indirectly. Temperature directly controls enzymes responsible for carbon fixation (Moore et al., 2021). Phytoplankton grow and survive within certain thermal tolerance. In the tropics, most researches have reported water temperatures between 18 – 32 ºC as optimum growth temperature for most phytoplankton species (Fernández-González, Tarran, Schuback, Woodward, Arístegui & Marañón, 2022; Frieling et al., 2017; Mesquita, Prestes, Gomes & Marinho, 2020). According to Jin & Agusti (2018), there is a sharp decline in growth of phytoplankton when ambient temperature exceeds optimum than compared to when ambient temperature is below the optimum required. This makes species living close to their thermal tolerance limit more sensitive to warming than cooling (Jin et al., 2018).

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2.3 Drivers and Ecological Impact of Sea Surface Warming

Currently, the temperature of the sea is increasing at an alarming rate (Cheng et al., 2021). According to Swart, Gille, Fyfe & Gillett (2018) the warming is a result of increasing emission of greenhouse gases. Examples of these gases are carbon dioxide, hydrofluorocarbons and methane). Since the industrial revolution, greenhouse gas emissions into the earth's atmosphere have increased significantly. For example, atmospheric carbon dioxide levels are now 50% higher than before the industrial revolution (Ritchie, Roser & Rosado, 2020). Accumulation of these gases prevents the escape of heat energy into space, leading to global warming (Kweku et al., 2018). Energy, agricultural, transport, waste and industrial sectors account for major sources of these greenhouse gases (Ritchie et al., 2020).

This has serious implications for phytoplankton and their role in the marine food web. As temperature of the environment rises, unsaturated fat of algae decreases (Nalley et al., 2018; Svenning et al., 2019). This is because double bonds linking the carbon atoms of unsaturated fats loosen quickly (melt) under warmer conditions (Neidleman, 1987). Rate of enzyme activity responsible for carbon fixation rises with temperature. An optimum activity is reached at the enzyme's optimum temperature. A continuous increase in environmental temperature causes a sharp decrease in enzyme activity (Daniel, Danson, Eisenthal, Lee & Peterson, 2008). This is because increasing temperature alters activity of the enzyme by changing the shape of active site (Gardner, Biler, Risso, Sanchez-Ruiz & Kamerlin, 2020). These changes are likely to impact on the growth of the grazers, with cascading effect on the transfer of energy to the top of the marine food web (Murphy, Romanuk & Worm, 2020). This effect will impact

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on marine fish production and other ecosystem services (e.g., nutrient cycling) crucial for the health of marine ecosystems.

2.4 Drivers and Ecological Impact of Ocean Acidification

Increasing carbon dioxide levels in the atmosphere as a result of fossil fuel combustion and other human related activities such as unsustainable land use have a direct impact on ocean carbonate chemistry. This effect results in a phenomenon called ocean acidification. Ocean acidification refers to reduction in pH of the ocean over a long period of time (Feely, Doney, Cooley & Greeley, 2010; Orr et., al, 2005). This usually occurs when the ocean absorbs carbon dioxide from the atmosphere. When this happens, the ocean reacts with carbon dioxide to form carbonic acid, which dissociates to hydrogen and bicarbonate ions causing the ocean to become acidic.

Ocean acidification is also closely linked with impacts of climate change (Jewett & Romanou; Kump, Bralower & Ridgewll, 2009). Intensification of rainfall results in surface runoffs and freshwater input. During this process, nutrient runoff can contribute to coastal acidification by enhancing biological respiration. Nutrient loading generally enhances algal bloom, which are consumed by bacteria when they die. Bacteria respire carbon dioxide, and thus, bacterial blooms can result in acidification events (Waldbusser & Salisbury, 2014). Coastal waters can episodically experience riverine and glacial melt plumes that create conditions in which seawater can dissolve calcium carbonate structures (Evans, Mathis & Cross, 2014; Salisbury & Green, 2008). While these processes have existed in the past, increases in glacial melt and high-intensity precipitation events caused by climate change have the potential to

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produce larger freshwater plumes than in the past. Changes in precipitation, ice melt, and nutrient loading that have occurred over time may influence the magnitude of coastal acidification over time.

According to Feely et al. (2004), ocean surface waters have become 30 % more acidic over the last 150 years as they have absorbed large amounts of carbon dioxide from the atmosphere. Since the preindustrial era, the oceans have absorbed approximately 29% of all carbon dioxide emitted into the atmosphere (Le Quéré et al., 2016). Oceans currently absorb approximately 26% of the carbon dioxide emitted into the atmosphere by humans (Cheng, Trenberth, Fasullo, Boyer, Abraham & Zhu, 2017). By the end of the 21st century, ocean surface pH is projected to decrease from current average level of 8.1 to a possible average of 7.8 (Bopp et al., 2013). This has consequences for marine organisms. For most calcifying zooplankton, increasing acidity of the ocean affect shells. Davis, Rivest, Hill, Gaylord, Russell & Sanford (2017) have reported a reduced ability of the foraminifer (*Globigerina bulloides*) to precipitate shell material. Ability of the foraminifer to repair spine vital for buoyancy and feeding is also reduced. This could result in decline in zooplankton biomass, thereby affecting food web structure.

Major phytoplankton calcitic calcifier groups such as coccolithophores suffered reduction in their rate of calcification under simulated ocean acidification scenarios (Müller, Trull & Hallegraeff, 2017). Ocean acidification has also been reported to cause bloom of toxic dinoflagellates. This is because they utilise carbonates, which is a by-product of dissociation of carbonic acid (Barker & Ridgwell, 2012). They increase the rate of calcium carbonate formation, thereby causing bloom (Ouyang, Chen, Liu, He, Cai & Yin, 2018).

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According to Zhang et al. (2022), a more extensive bloom of such toxic phytoplankton groups may threaten marine food webs through trophic interactions.

2.5 Chemical Pollution

Chemical, plastic, pharmaceutical and industrial productions have steadily increased in recent years (Zhao, Ma & Hong, 2010). Every year, thousands of industrial chemicals find their way into the marine environment as a result of these productions (Laane, Slijkerman, Vethaak & Schobben, 2012). Over the last four decades, there has been growing concern about the potential harm that organic pollutants may cause to ecosystems and humans (Dachs & Méjanelle, 2010).

2.5.1 Persistent Organic Pollutants

Chemical compounds that are most dangerous in the marine environment are those that are persistent, toxic, and bioaccumulate (Hale, Arp, Schliebner & Neumann, 2020). These chemicals, known as persistent organic pollutants (POPs), have a long half-life in water, sediments and organisms (Jones & De Voogt, 1999). POPs are known for their chemical stability, which means that they do not break down easily in the environment or within organisms (Gaur, Narasimhulu & PydiSetty, 2018). They dissolve in fatty tissues and accumulate in the food chains. Important classes of POPs are organo-chlorine pesticides, Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated biphenyls (PCBs). PCBs are chemically stable at room temperature (Everaert, 2015).

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The Persistent Organic Pollutants are hazardous to the environment because of their extreme resistance to biological and chemical degradation by natural processes. They consist of a biphenyl ring with various chlorine substitutions (Everaert, 2015). PCBs were widely used as an additive to lubricating oils and greases, as well as in electrical installations, in the 1970s (Pascall, Zabik, Zabik & Hernandez, 2005). Organo-chlorine pesticides are chemically stable and hydrophobic synthetic compounds (Gaur et al., 2018). DDT (dichloro-diphenyl-trichloroethane), a well-known example of such a pesticide, is used as an insecticide in agriculture.

2.5.1.1 Emission of Persistent Organic Pollutants into the Environment

Organic pollutants get into the environment through a variety of pathways: some are purposefully used in most industrial processes, while others are accidentally released into the environment. (Šimůnek & van Genuchten, 2016). Chemicals can be released into freshwater, sea, the atmosphere, or the soil (Prevedouros, Cousins, Buck & Korzeniowski, 2006). The physical and chemical properties of chemicals, as well as the nature of the environment, influence their behavior and fate between these environmental compartments. Indeed, ocean and atmospheric currents can disperse pollutants all over the world. For example, volatile contaminants can be carried over large area by the wind and deposited in the aquatic environment far from the source area (Burkow & Kallenborn, 2000). In general, the ocean is the primary recipient for all organic contaminants (Law et al., 2010). Figure 5 depicts the sources and pathways through which pollutants can enter the aquatic environment.

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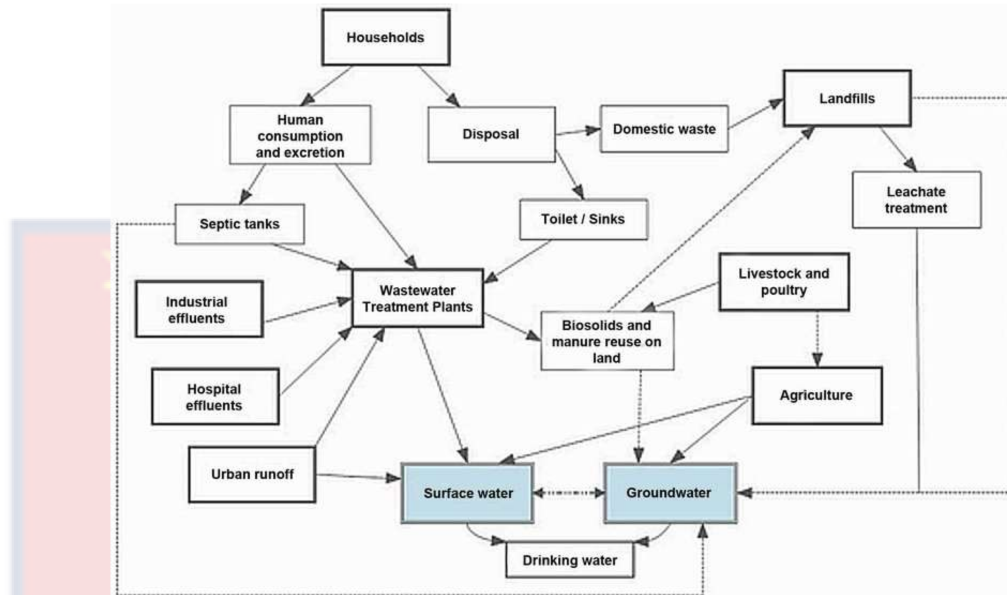

Figure 5: Diagram showing sources and pathways through which emerging contaminants enter aquatic ecosystems (Source: Stefanakis & Becker, 2020).

2.5.1.2 Physicochemical Properties of Persistent Organic Pollutants

The physical and chemical properties of organic pollutants affect their distribution, settlement, and remobilization in the marine environment. Aqueous solubility, vapour pressure, partition coefficients between the liquid, solid, and air phases, and half-lives in air, soil, and water are among these properties (Jones et al., 1999). When chemicals are dissolved in water, they go through a variety of processes and are distributed among the various phases (i.e., air, water, sediment and biota; Burns, 2000). POPs are typically hydrophobic and lipophilic, so they preferentially partition to solids (organic matter) and avoid the aqueous phase (Clarke & Cummins, 2015). Both dissolved and particulate organic matter in the water may act as 'sponges' to mop up organic pollutants (Hylland & Vethaak, 2011). As a result, sedimentation can remove them from the water column.

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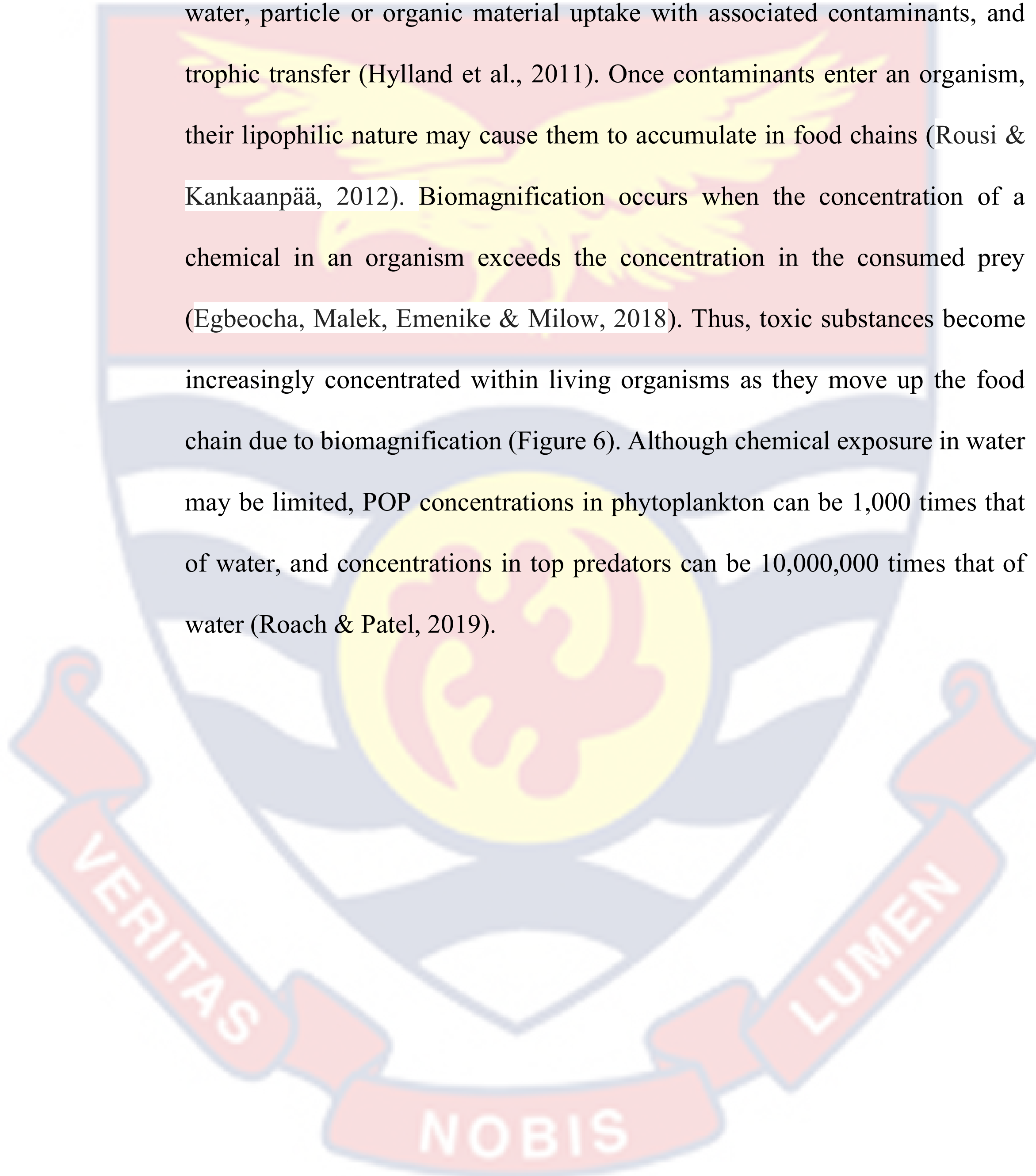
A polar organic pollutant tends to accumulate in biological tissues of organisms as well (Perelo, 2010). Pelagic organisms can be exposed to contaminants in different ways, including dissolved chemical pollutants in water, particle or organic material uptake with associated contaminants, and trophic transfer (Hylland et al., 2011). Once contaminants enter an organism, their lipophilic nature may cause them to accumulate in food chains (Rousi & Kankaanpää, 2012). Biomagnification occurs when the concentration of a chemical in an organism exceeds the concentration in the consumed prey (Egbeocha, Malek, Emenike & Milow, 2018). Thus, toxic substances become increasingly concentrated within living organisms as they move up the food chain due to biomagnification (Figure 6). Although chemical exposure in water may be limited, POP concentrations in phytoplankton can be 1,000 times that of water, and concentrations in top predators can be 10,000,000 times that of water (Roach & Patel, 2019).



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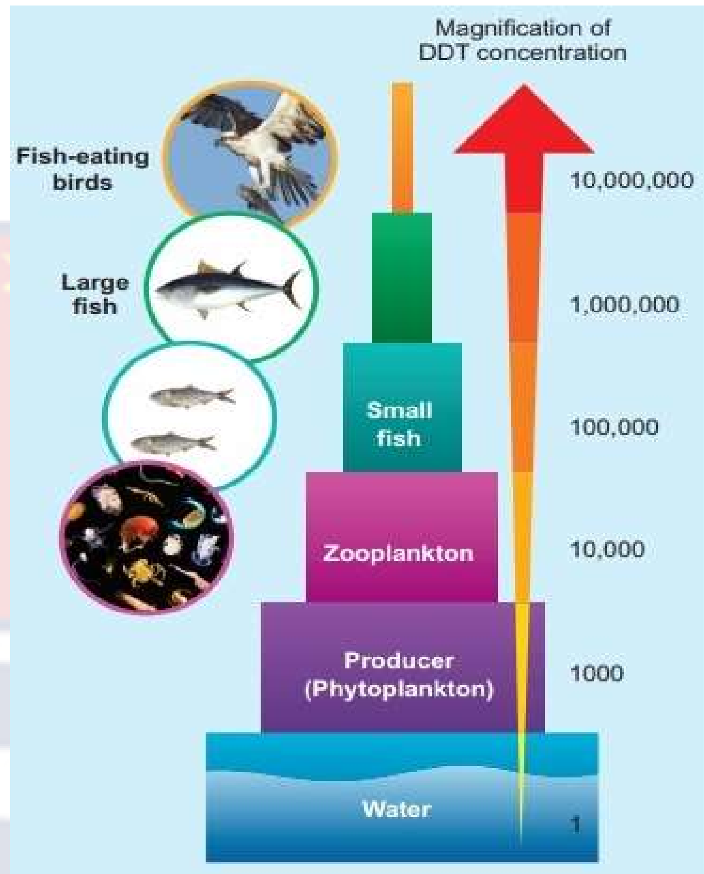

Figure 6: Magnification of persistent organic pollutants within the marine food chain (Adopted from Roach & Patel, 2019).

2.5.1.3 Ecological Impacts of Persistent Organic Pollutants

The effects of POPs are frequently seen in top predators such as marine mammals and predatory birds because they bioaccumulate and biomagnify in nature (Jones et al., 1999). The measurement of contaminant concentrations in selected marine organisms has a long history, and such data has frequently served as the foundation for environmental assessments (Law et al., 2010). According to Franke (1996), bioaccumulation can have long-term effects on individual species, communities, and ecosystems. The bioconcentration factor, which is the ratio between the concentration of a substance in an organism and

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the concentration in its environment, precisely determines the level of risk and concern for the environment. One of the most significant effects of POPs is their disruption of endocrine system of marine organisms (Katsiadaki, 2019). Exposure to POPs during developmental stages may induce abnormal thyroid function, decrease fertility rates and may also cause disruptions in the sex characteristics, thereby altering the sex ratios of the population (Tanabe, 2004). Certain POPs are also thought to harm the immune systems of marine species, leading to an increase in disease outbreaks (Law et al., 2010). Additionally, a broad spectrum of chemical contaminants can have an immediate effect on plankton communities by disrupting photosynthesis and other energy utilization and absorption processes (Booij et al., 2013). Among the most toxic biocides to marine phytoplankton are those with a herbicidal mode of action (Booij et al., 2013). Effects can range from slower population growth rates to shifts in species composition (i.e., toward species that are more tolerant of a particular pollution) (Hylland et al., 2011). Furthermore, many POPs have been established to have cancerous effects (Jones et al., 1999).

In addition to field surveys, toxicity tests conducted in the laboratory provide information about the ecotoxicological effects of POPs on marine species (Walker, Hopkin & Sibly, 2001). During laboratory tests, organisms are exposed to a chemical concentration range in order to obtain a concentration-response relationship, where response refers to the effect of the chemical on the organism. This allows for the estimation of the effective concentration (EC), which is the concentration at which a percentage effect is observed. This effective concentration reflects the tested species' sensitivity to the considered chemical, whereby a lower EC suggests greater sensitivity of the species to the

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tested chemical (De Laender, De Schamphelaere, Van Rolleghem & Janssen, 2007).

2.6 Chapter Summary

This chapter provided information on ecological importance of marine phytoplankton. Relevant information was also provided on stress factors related to global change and their effect on phytoplankton. The review broadened knowledge base in the research area and brought clarity to the research problem established.

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CHAPTER THREE

MATERIALS AND METHODS

This chapter outlines the materials and methods employed in the study. The experimental organism and its cultivation environment are thoroughly described. The microcosm experiments and laboratory analyses performed are also explained in this section. In addition, the statistical techniques used to analyse the results of the experiments are specified.

3.1 Experimental organism

In marine pelagic systems, diatoms and dinoflagellates constitute the dominant group of phytoplankton at the base of the marine food web (Cheung et al., 2021, Sherr & Sherr, 2007; Stoecker, Hansen, Caron & Mitra, 2017). However, research suggest that the zooplankton that feed on the phytoplankton prefer to ingest dinoflagellates as food (Turner, Levinsen, Nielsen & Hansen, 2001). This is because, unlike most diatoms that have silicious cell (Kale & Karthick, 2015), dinoflagellate cells are relatively easy to digest as they are almost "animal like" (Calbet, Broglio, Saiz & Alcaraz, 2002). Also, dinoflagellate cells are rich in essential molecules such as proteins and lipids required by planktonic grazers (Peltomaa, Aalto, Vuorio & Taipale, 2017). It was, therefore, expected that any environmental change on the cellular properties of dinoflagellates may significantly impact on marine food web (Barton, Irwin, Finkel & Stock, 2016; Lynam, Llope, Möllmann, Helaouët, Bayliss-Brown, & Stenseth, 2017). Based on this observation, the current experiment was conducted using a dinoflagellate species as test organism.

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The species used was *Rhodomonas* sp. (Figure 7). It belongs to Class Cryptophyceae. Its size ranges from 9 - 40 μm and has a pair of flagella extending from the cell's anterior, which is used for limited movement. It is typically marine in nature, although some studies have reported presence in freshwater habitats (Ballen-Segura, Felip & Catalan, 2017; Hill & Wetherbee, 1989). Its cells may appear red, brown or golden brown as it contains the pigment phycoerythrin (Derbel et al., 2022; Kugrens & Clay, 2003). It has been demonstrated that *Rhodomonas* sp. can achieve higher biomass concentrations in optimum environmental conditions in few days (Derbel et al., 2022). As a result, it served as the ideal test organism for this study, which sought to investigate their response to global change factors simulated in a short period of time.

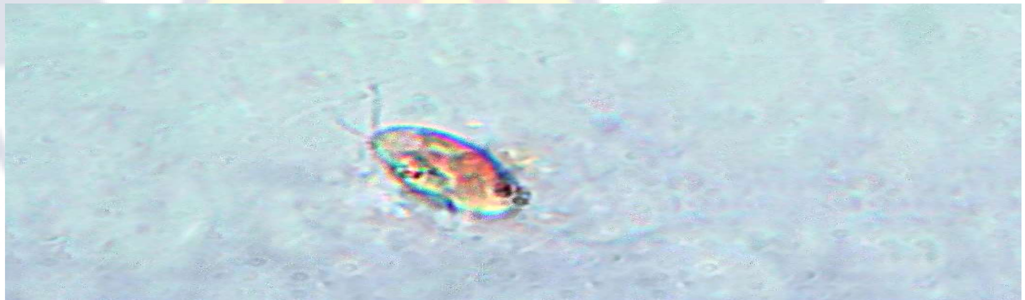

Figure 7: *Rhodomonas* sp. captured using Olympus CK-2 microscope (magnification 40x)

3.2 Preparation of Artificial Seawater

Artificial seawater was used in this experiment to overcome seawater quality problems and prevent complex interactions between pollutants and organic matter that may affect toxicity (His, Beiras & Seaman, 1999). Artificial seawater was prepared by dissolving laboratory formulated salt (brand: Instant Ocean) in UV sterilised deionised freshwater. For this experiment, all exposure

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media were created using artificial sea water at a salinity of 35. Thirty-five (35) grams of the artificial sea salt was weighed on analytical balance (Ohaus AX124/E, precision 0.001 g) and dissolved in 1 L of UV sterilised deionised water. Salinity was measured using refractometer (brand: Aichose).

This salinity was chosen based on salinity measurement of collected seawater at Elmina during the dry and wet seasons. According to Latsos, Bakratsas, Moerdijk, Houcke & Timmermans (2021), *Rodomonas* sp. have salinity tolerance ranging from 20 to 40. To confirm this, the salinities were replicated in a short experiment to determine cells produced after 3 days of culture. Artificial seawater salinity that produced growth of cells similar to the natural seawater (NS) salinity was selected for this experiment. The justification for this is that sufficient healthy cells were needed for biochemical analysis. Test results are presented in Figure 8.

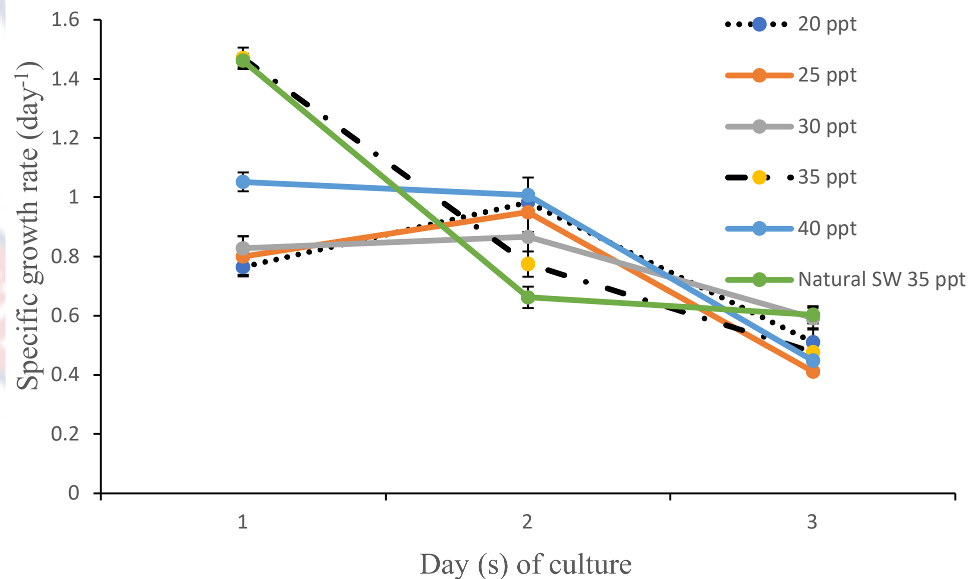

Figure 8: Specific growth rate (average ± S.D) of *Rhodomonas* sp. exposed to different salinities for 72 hours

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3.3 Preparation of Algal Growth Media

The algal growth media was prepared based on the nutrients provided by Hansen, 1989 (Table 1). The quantities indicated in Table 1 were weighed on analytical balance (Ohaus AX124/E, precision 0.001 g) and dissolved in separate Duran bottles containing 1 L of deionised water. The solutions were autoclaved at 15 psi for 15 minutes. To obtain B1 media, 1000 ml of solution 2 was added to 1 ml of solution 3. The B1 media was stored at ≈ 4 ºC. Vitamins was stored at – 20 ºC.

Table 1: *Ingredients for Marine Water Enrichment Solution*

Solution	Chemical formula	Name of ingredient	Quantity needed
1	$Na_2Si_3.9H_20$	Sodium metasilicate nonahydrate	30 mg
2	Na_2EDTA	Disodium ethylenediaminetetraacetate dehydrate	40 g
	$NaNO_3$	Sodium nitrate	100 g
	H_3BO_3	Boric acid	33.6 g
	NaH_2PO_4	Sodium dihydrogen phosphate	20 g
	$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate	0.36 g
	$FeCl_3.6H_2O$	Iron (III) chloride hexahydrate	1.3 g
3	$ZnCl_2$	Zinc chloride	2.1 g
	$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate	2.0 g
	$(NH_4)_6Mo_7O_{24}.4H_2O$	Ammonium heptamolybdate tetrahydrate	0.9 g

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Table 1, continued

	$CuSO_4.5H_2O$	Copper (II) Sulfate Pentahydrate	2.0 g
	Add drops of 0.1 N HCl until clear solution		
Vitamins	Thiamin		200 mg
	Biotin		1 mg
	Cyanocobalamin		1 mg

3.4 Maintenance of Algal Stock Culture

Stock culture of *Rhodomonas* sp. (strain: NIVA-15/12) was obtained from the Technical University of Denmark Aqua laboratory. They were adapted over six months to temperature and light conditions similar to those prevailing on the coast of Ghana before the beginning of the experiment. The cells were grown in transparent polyethylene bag (35 x 80 cm) containing artificial sea water (4.5 L) enriched with B1 algal growth media and vitamins. In following with the recipe by Hansen (1989), 1.1 mL of B1 media and 1.0 mL of vitamins was added to each litre of artificial seawater used for the experiment.

The stock of *Rhodomonas* sp. was maintained at 26 ℃, which falls within the range of long-term average sea surface temperature on Ghana's shelf (Ghana Meteorological Agency, 2016; Figure 9). They were managed using the procedure described in Acheampong et al. (2021). Each day, 50 % of the culture was diluted with freshly prepared media to keep culture at exponential phase as recommended by Solís-Salinas, Patlán-Juárez, Okoye, Guillén-Garcés, Sebastian & Arias (2021). Light source with a total irradiance of approximately 147 µmol m^{-2} s^{-1} was provided from above by eight short white fluorescent

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lamps on a 12-hour light to 12-hour dark cycle. Total irradiance was estimated based on the assumption that 1 W per unit surface area equals 4.6 µmol m^{-2} s^{-1}. According to Oostlander, van Houcke, Wijffels & Barbosa (2020), *Rhodomonas* sp. attain optimal growth at irradiance of 150 $m^{-2}.s^{-1}$. Therefore, the total irradiance used for this study was sufficient to support optimum growth of the species. Continuous aeration was provided using aquarium air pump (Danner pump AP-8) to ensure mixing and prevent self-shading of microalgae.

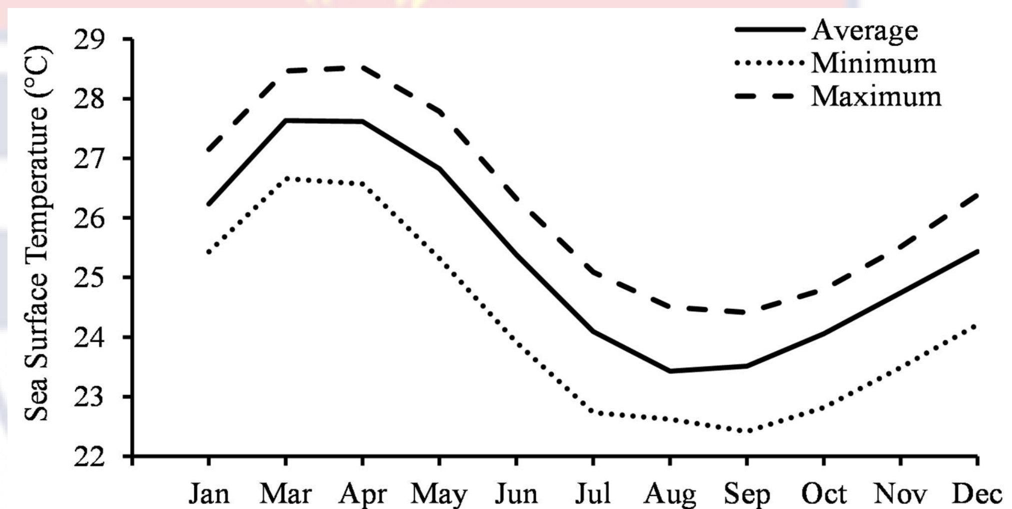

Figure 9: Observed Sea surface temperature on continental shelf of Ghana from 1948 – 2015 (Data source: Ghana Meteorological Agency 2016).

3.5 Effect of Stressors on *Rhodomonas* sp.

Experiments involving exposure of the species to the stressors were started using *Rhodomonas* sp. at a concentration of 664 cellmL^{-1}. This was to ensure that the cultures did not collapse during run of experiments due to high growth rate of the species investigated (Thoisen, Vu, Carron-Cabaret, Jepsen, Nielsen & Hansen, 2018). Salinity (35) and light supply (147 µmol m^{-2} s^{-1}) were kept

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constant at levels similar to those used for maintaining stock culture of the cells. The experiments were also run in water baths set to the desired temperatures as described in section 3.2. The cultures were shaken at least three times daily to keep cells in suspension during each exposure experiment.

3.5.1 Exposure of *Rhodomonas* sp. to Sea Surface Warming Temperatures

Impact of sea surface warming on microalgae was investigated by exposing *Rhodomonas* sp. to increasing sea surface temperatures. Four temperatures, including 26, 28, 30, and 32 °C were investigated. According to GMA (2016), the average SST of Ghana's coast during a stable hydrographic period is 26 °C. Therefore, 26 °C was used as control temperature. Experimental temperatures (28, 30 and 32 °C) represented + 2 and + 4 and + 6 °C above the average temperature. Each of the different experimental treatment was run in triplicate. The temperature treatments were set up in an 80-Liter water bath with a temperature regulated heater (EHEIM 200) (Fig 10). Water baths were continuously aerated with pump (Danner pump AP-8) to ensure even temperature distribution. Water temperatures were recorded every 15 minutes (Appendix 1) using Onset HOBO loggers (Pendant MX2201) during the culture period.

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Figure 10: Experimental setup showing suspended culture bags, water bath and light supply.

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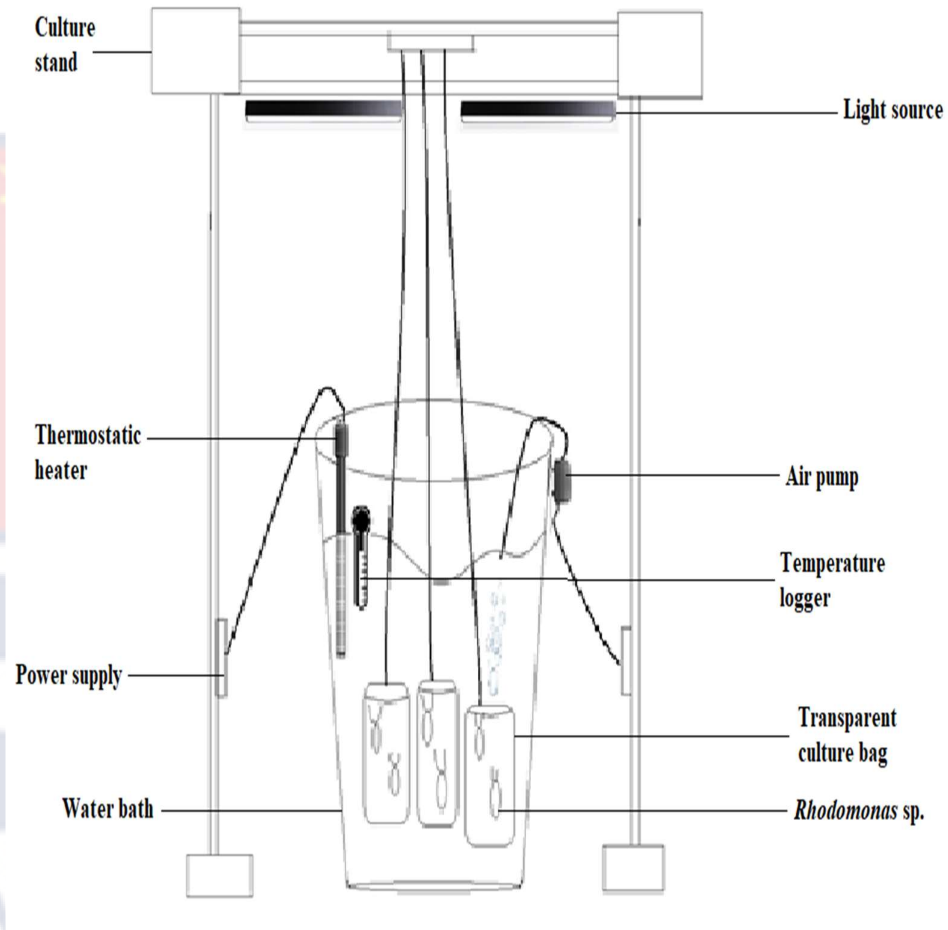

Figure 11: Schematic of experimental setup showing thermostatic heater, temperature logger, air pump, light source and culture bags containing *Rhodomonas* sp.

3.5.2 Exposure of *Rhodomonas* sp. to Cadmium

Along the coast of Ghana, cadmium (Cd) has been identified as the heavy metal with the highest concentration (Figure 3) (Gbogbo & Otoo, 2015). As a result, cadmium was used as proxy for heavy metal pollution and its effect was quantified. A stock solution (concentration: 100 mgL^{-1}) of this pollutant was prepared by dissolving 50 mg of Cadmium Chloride ($CdCl_2$) (Sigma-

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Aldrich, purity: 99.9 %) in 0.5 L of deionised water. The stock solution was kept in amber glass bottle at 4 °C for use later in the experiment.

3.5.2.1 Range-finding Test

Prior to the main experiment, a range-finding test was conducted in order to acertain actual concentration of the pollutant that could be tolerated by the species tested in this experiment. This procedure is consistent with previous experiments investigating the response of marine plankton to pollution (Asiedu, 2020; Ruiz et al., 2021). The range-finding test involved the determination of cadmium concentration that reduced growth of *Rhodomonas* sp by 50 %. This concentration is referred to as Lethal Concentration 50 (LC_{50}) (Martínez, Lumaret, Zayas & Kadiri).

It was determined after exposing the *Rhodomonas* sp. to the pollutant at the following concentrations 0, 0.1, 10, 100, 1000, 10,000 μgL^{-1}. The duration of this test was 72 hours based on the criteria suggested by the International Standardisation Organisation (ISO/TR 11044:2008). Concentrations below LC_{50} (Appendix 2) were selected and used in this experiment in line with previous studies (Asiedu, 2020).

3.5.2.2 Definitive Test

Based on the LC_{50} calculation, four different concentrations (0.1, 1 10, 100 μgL^{-1}) of the pollutant were used for the definitive test. The lowest concentration (0.1 μgL^{-1}) was selected as the nominal concentration of cadmium in line with previous study (Asiedu, 2020). Test concentrations were prepared by diluting portion of the stock solution with deionised water. This was done by pipetting a known volume of the stock solution into a volumetric flask and

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topped up to the 5 L mark using deionised water. The required volume of the stock solution used was calculated using Equation 1.

$$C_1*V_1 = C_2*V_2 \quad (1)$$

where C_1 = concentration of stock solution

V_2 = volume of stock solution to be taken

C_2 = concentration of diluted solution needed

V_2 = volume of the diluted solution needed

The tests were carried out in triplicates. A separate treatment was established as a control, in which no $CdCl_2$ was added.

3.5.3 Exposure of *Rhodomonas* sp. to Pyrene

Pyrene ($C_{16}H_{10}$) was selected as a representative of petroleum due to its widespread occurrence in areas with high oil and shipping traffic in marine environments (Almeda, Wambaugh, Wang, Hyatt, Liu & Buskey, 2013; Tiselius & Magnusson, 2017). Effect of pyrene was quantified. A stock solution (concentration: 606.75 mgL^{-1}) of this pyrene was prepared by dissolving 60.675 mg of granulated pyrene (Sigma-Aldrich, purity: 99 %) in 100 mL of acetone (Sigma-Aldrich, purity: 99.5 %). To avoid photodegradation, the stock solution was kept in amber glass bottle at 4 °C for use later in the experiment.

3.5.3.1 Range-finding Test

Prior to the main experiment, a preliminary test (LC_{50}) was conducted out in order to ascertain actual concentration of the pollutant that could be tolerated by the test organism. It was determined after exposing the

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dinoflagellate to the pollutant at concentrations of 0, 0.2, 20, 200 and 2000 μgL^{-1} (Appendix 3). Treatment with no pyrene pollution was set as control. Additionally, a control was set using acetone only to test its effect on the species investigated.

Pyrene has been shown to significantly degrade at higher temperature (37 ºC) over time (Grenvald, Nielsen & Hjorth, 2013). As a consequence, the level of the pollutant in the water can be expected to reduce over time under warming situations. These effects are usually avoided through the continuous addition of pollutant to culture setup (Ruiz et al., 2021). However, this study sought to investigate impact of pyrene in the case of a one-time oil spill event. It was expected that the concentration of the pollutant will remain the same for scenario. Hence, pyrene was not added continuously to the culture throughout the run of the experiment in this study.

3.5.3.2 Definitive Test

Based on the LC_{50} calculation, four different concentrations (0.2, 2, 20, 200 μgL^{-1}) of the pollutant were used for the definitive test. The lowest concentration (0.2 μgL^{-1}) was selected as the nominal concentration of cadmium. Test concentrations were prepared by diluting portion of the stock solution with deionised water. This was done by pipetting a known volume of the stock solution into a volumetric flask and topped up to the 5 L mark using deionised water. The required volume of the stock solution used was calculated using Equation 1. The tests were carried out in triplicates. A separate treatment was established as a control, in which no pyrene was added.

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3.5.4 Exposure of *Rhodomonas* sp. to Combination of Cadmium and Pyrene

For the experiment involving exposure of *Rhodomonas* sp to combination of heavy metal and petroleum, individual concentrations of the two chemicals (cadmium and pyrene) were combined with respect to their relative toxic fraction criterion as proposed by Mensah, Mgaba, Griffin, Odume and Palmer (2017). This was to ensure that the correct proportions of individual pollutants in the mixture was administered in order to obtain the desired concentrations. The relative toxic fractions (RTFs) were calculated using the results of the experiment involving the individual stress factors (cadmium or pyrene pollution) in line with the recommendation of Mensah et al. (2017). In this experiment, the 72 h LC_{50} values obtained from the previous experiments were used for the calculation of RTF for cadmium ($_{RTF}$ Cd; Equation 2) and pyrene ($_{RTF}$ Py; Equation 3).

$$_{RTF}\,Cd = \frac{LC_{50}Cd}{LC_{50}Cd + LC_{50}Py} \quad (2)$$

$$_{RTF}\,Py = \frac{LC_{50}Py}{LC_{50}Py + LC_{50}Cd} \quad (3)$$

where $LC_{50}Cd$ and $LC_{50}Py$ represent LC_{50} for cadmium and pyrene respectively. The amount of each individual pollutant needed for any concentration in the mixture of the two pollutants was estimated by multiplying the desired concentration with the relative toxic fraction of that particular pollutant.

The phytoplankton was incubated in a clear polyethylene bag (35 x 80 cm) mounted in water baths and exposed to combination of the stressors described above (Figure 3.4 b). Exposure bags were filled with deionised water

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enriched with sufficient (triple the required amount) B1 media and vitamins that would last through the run of each experiment. There were three replicates for each treatment.

3.6 Determination of Growth Rate of *Rhodomonas* sp.

Sampling procedure to determine growth rate was adopted from Acheampong et al. (2021). Daily cell counts were made to determine growth rate of the cells cultured in this experiment. Cultures in the transparent polyethylene bag were shaken before sampling to ensure uniform mixture of cells. Each day, 5 ml of the culture was collected into a 10 ml clear glass vial and fixed with one to two drops of Lugol's iodine solution (2 %). Then, 1 ml of the fixed sample was pipetted on Sedgewick – Rafter chamber and counted under microscope (magnification 10x, AmScope T390B).

Specific growth rate, μ, was calculated from the cell counts using the formular below (Renaud, Thinh, Lambrinidis, & Parry, 2002).

$$\mu = \frac{\ln (Y - Y_0)}{t - t_0} \qquad (4)$$

where Y = cell count (cells.ml^{-1}) on the day of sampling, t

Y_0 = cell count prior to the day of sampling t_0

3.6.1 Determination of Dry Weight of *Rhodomonas* sp.

For the determination of dry weight, 10 mL of each culture of the algae was used. This volume was filtered onto Whatman GF/F glass microfibre filter (mesh size: 0.7 μm) and dried in oven for 24 hours at 100 °C based on previous study (Teoh, Chu, Marchant & Phang, 2004). Blank filter papers without algae

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were also dried at the same time and temperature. After drying, the filters were weighed using analytical balance (Ohaus Adventurer® AX124/E, precision 0.001 g). The dry weights (D_w) of the algae were then calculated using the Equation below (Acheampong et al., 2021).

$$D_w = \frac{(w_2 - w_1)}{v_{algae} * \rho_{algae}} \quad (5)$$

where w_2 = dry weight of filter paper containing algae

w_1 = weight of filter paper without algae

v_{algae} = volume of algae filtered

ρ_{algae} = density of algae (cells.ml^{-1}) filtered

3.6.2 Determination of Biochemical Content of *Rhodomonas* sp.

For determination of biochemical content of *Rhodomonas* sp., the total volume (≥ 4 L) of the culture remaining after sampling for the determination of growth rate and dry weight in each treatment was used. They were collected into 50 mL falcon tubes for centrifugation in order to harvest the cells. Centrifugation was done at 3000 *g* for 10 minutes using Eppendorf 5430 centrifuge. After centrifugation, the supernatant was discarded and the remaining cells were washed three times using deionised water, and then collected into 50 mL sterile falcon tubes and stored at – 25 °C for biochemical analysis.

For the biochemical analysis, the simultaneous extraction method proposed by Chen & Vaidyanathan (2013) was followed. The cells were resuspended in 50 mL deionised water. After that, 10 mL of Sodium Hydroxide

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(1 N) in 25 % Methanol was added. The mixture was then vortexed (Joanlab VM-300S mixer) at 3000 rmp for 1 minute to ensure uniform mixing of the cells. The mixture was then heated for 30 minutes at 100 °C in order to break apart the individual cells. The mixture was allowed to cool and again centrifuged at 3000 *g* for 10 minutes. The supernatant obtained from this centrifugation was collected for protein, carbohydrate and lipid analysis as described below.

3.6.3 Protein Analysis

Protein content of the algae was determined using Bradford method (He, 2011). An aliquot of 100 µL of the supernatant was taken and mixed with 5 mL of Bradford reagent (composition of Bradford reagent: 10 mg Coomassie brilliant blue, 5 ml of 95 % ethanol, 10 ml phosphoric acid topped up to 100 ml using distilled water). The mixture was vortexed at 3000 rpm for 1 minute and absorbance of the solution (Figure 12) was read at 595 nm using InsMark UV100-star2 spectrophotometer. A blank was made by adding equal volume of the supernatant to a 5 mL solution containing ethanol, phosphoric acid and deionised water (without Coomassie brilliant blue). A calibration curve (Appendix 4) was prepared with different concentrations (100 – 1500 μgmL^{-1}) of bovine serum albumin (BSA) standard solution (2 $mgmL^{-1}$). Thereafter the standard solutions were subjected to the same procedure as the extracts. Protein content was quantified as a percentage of dry weight of each cell.

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Figure 12: Colour formed from reaction of sample protein with Bradford reagent.

3.6.4 Carbohydrate Analysis

Carbohydrate content of the algae was determined according to the anthrone-sulfuric acid method (Leng et al., 2016). A volume of 1 mL of the sample (supernatant) was mixed with 5 mL of the anthrone reagent (composition: 0.2 g anthrone dissolved in 100 ml of concentrated sulfuric acid) and the mixture vortexed at 3000 rpm for 1 minute. After cooling, the absorbance of the solution (Figure 13) was read at 625 nm using UV100-star2 spectrophotometer. Blank absorbance of the sample was read by reacting 1 mL sample with 5 mL H_2SO_4 without the anthrone reagent. Carbohydrate concentration was estimated using a calibration curve (Appendix 5) prepared from different concentration (10 – 250 μgmL^{-1}) of d-glucose standard solution (1 $mgmL^{-1}$). Total carbohydrate was expressed as a percentage of dry weight of each cell.

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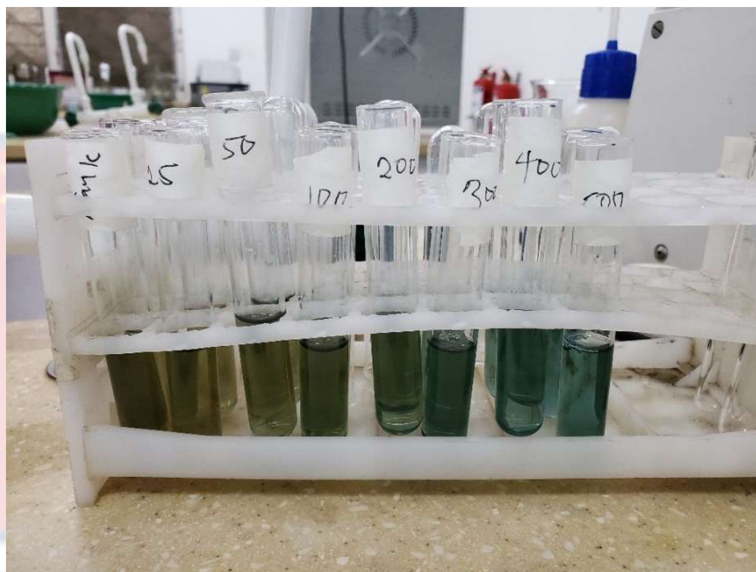

Figure 13: Colour formed from reaction of sample carbohydrate with anthrone reagent.

3.6.5 Lipid Analysis

Total lipid was determined by gravimetric method as suggested by Chen et al. (2013) and Folch, Lees & Sloane Stanley (1957). An aliquot of 0.5 mL of the supernatant was added to 1.5 mL of Chloroform/Methanol (2:1, V/V). The solution was vortexed at 3000 rpm for 2 minutes and then centrifuged at 12,000 *g* for another 2 minutes to separate it into aqueous and organic phases (Figure 14). The aqueous phase was discarded and 1 mL of the bottom organic phase was gently pipetted into pre-weighed glass vials (volume: 5 mL). The extract in the vial was evaporated at 60 °C in an oven for 24 hours after which it was placed in a desiccator for 3 hours at room temperature and weighed using Ohaus Adventurer® AX124/E (precision 0.001 g) until a constant weight was achieved. The weight of the lipid was obtained by subtracting the empty dish

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weight from that of the dish containing the lipid extract. The amount of lipid was expressed as percentage of the dry weight of each cell.

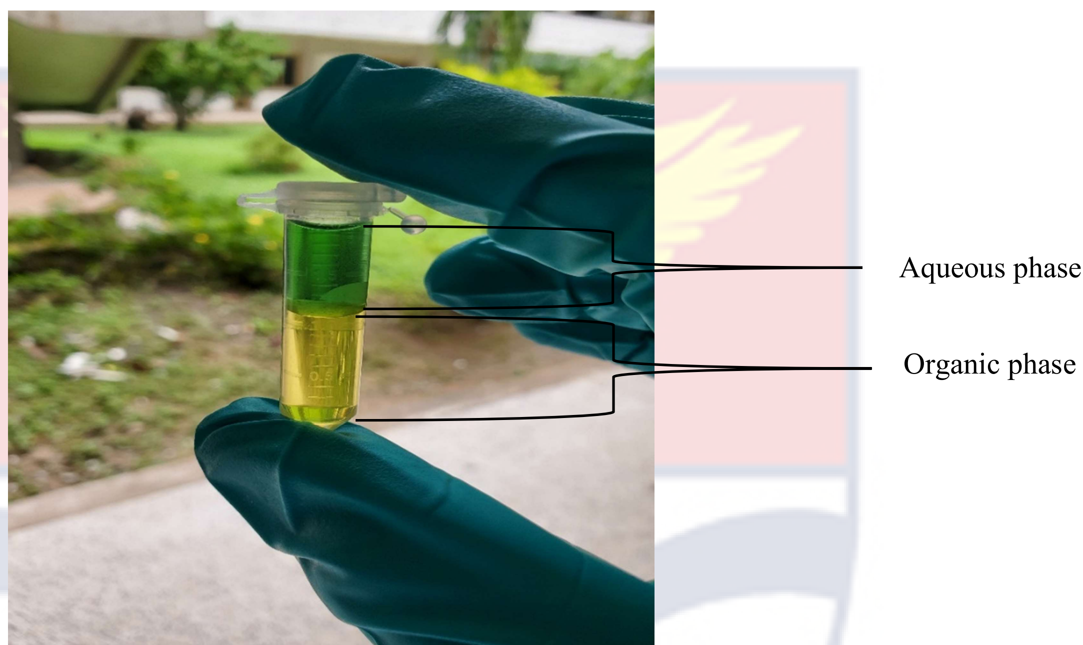

Figure 14: Separation of extract into aqueous and organic phases.

3.6.6 Measurement of Enzyme Activity

Catalase activity was used as a measure for quantifying the degradation of hydrogen peroxide generated by cells in response to the environmental stress factors (sea surface warming and pollution). This approach was based on previous report by Pinto et al. (2003). For the analysis, the supernatant obtained from centrifugation of the algal samples were pre-treated with Phosphate Buffer Saline (PBS) (0.01 M, pH 7.4). The enzyme activity was measured on a known amount of protein (1 mg) that was decomposed by dissolving in 1.0 µmol H_2O_2 for 1 minute at 37 °C. The activity was measured using an assay Kit (E-BC-K031-S; Elabscience, USA). For the analysis, 0.05 mL of the supernatant was reacted with reagents in the assay kit. Absorbance of the solution was read at

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405 nm using InsMark UV100-star2 spectrophotometer. The Catalase activity (CAT, ($Ucell^{-1}$) was estimated using the Equation 6 below extracted from Elabscience (2022).

$$\text{CAT activity} = \left[\frac{\Delta A * 32.5}{1 * V} * f \right] \div C_{pr} \qquad (6)$$

where $\Delta A = (OD_{control} - OD_{sample})$

V = volume of sample, ml

f = dilution factor (1) of sample before test

C_{pr} = number of cells analysed ($cellmL^{-1}$)

3.7 Data analysis

Testing for differences in growth rates between days was difficult because the cell counts used to calculate the rates were expected to be autocorrelated (Acheampong et al., 2021). Hence, warming effects were evaluated when the growth of the cells had reached exponential phase. Individual functional attributes (growth rate, dry weight protein, lipid, carbohydrate content and enzyme activity) were compared separately using one-way ANOVA with level of each stress factor as the source of variance. Regarding the pyrene experiment, a special control was set aside the "no pollution" control to test the effect of acetone alone on *Rhodomonas* sp. T-test was used to compare the means of the two controls for each functional attribute.

Individual biochemical content of each cell was expressed as the proportion of its dry weight. Therefore, values expressing the biochemical content of the algae were arcsine-transformed prior to the ANOVA. Whenever

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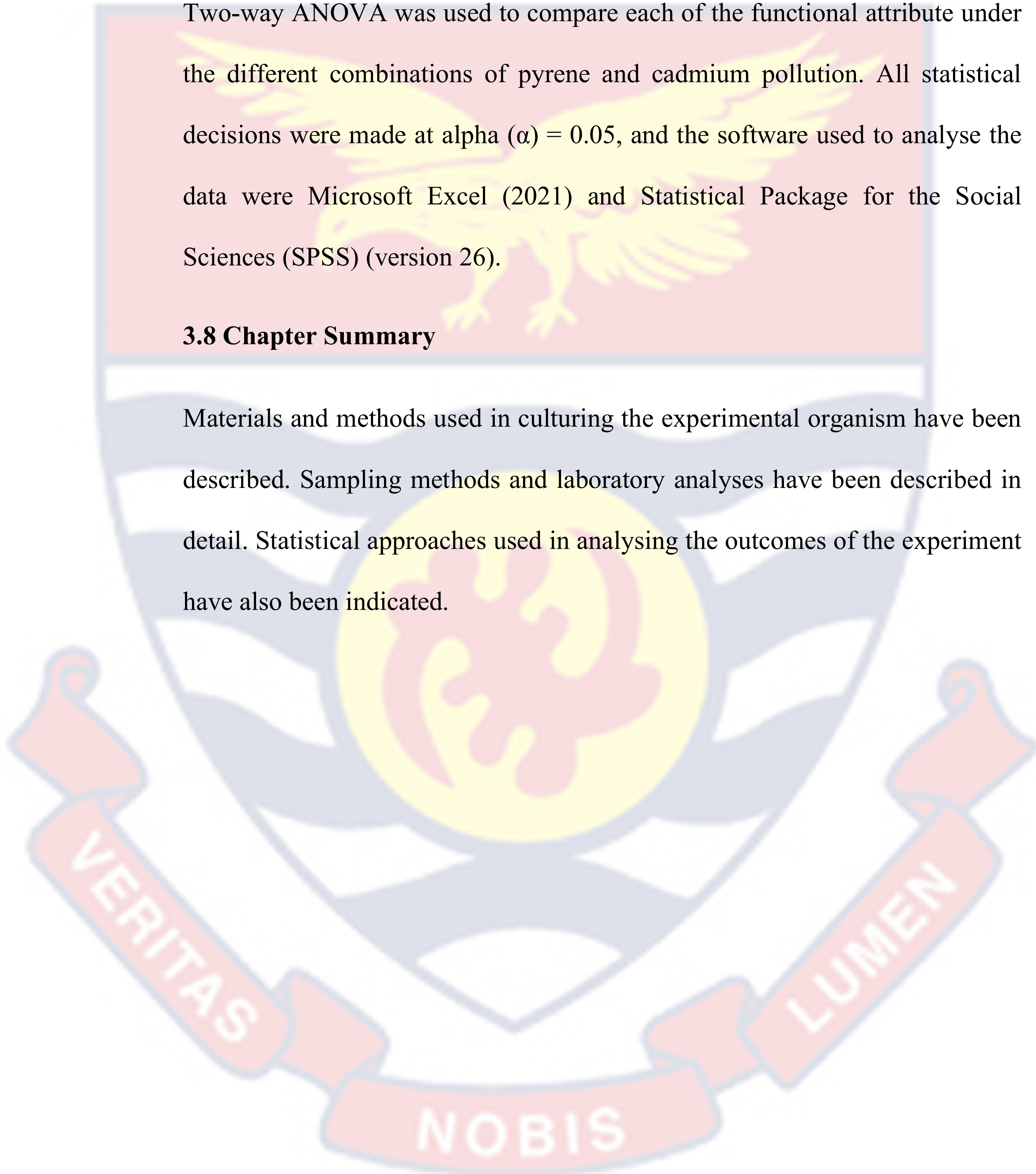

differences were identified between any sets of data, Tukey's HSD (Honestly Significant Difference) procedure was applied as a post-hoc test to identify which pairs of means were statistically significant (Sokal and Rohlf 1995). Two-way ANOVA was used to compare each of the functional attribute under the different combinations of pyrene and cadmium pollution. All statistical decisions were made at alpha (α) = 0.05, and the software used to analyse the data were Microsoft Excel (2021) and Statistical Package for the Social Sciences (SPSS) (version 26).

3.8 Chapter Summary

Materials and methods used in culturing the experimental organism have been described. Sampling methods and laboratory analyses have been described in detail. Statistical approaches used in analysing the outcomes of the experiment have also been indicated.

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CHAPTER FOUR

RESULTS

This chapter presents results obtained from laboratory experiments. The results are presented using appropriate graphs and tables. Standard deviation (S.D) around mean estimates have been indicated in graphs where necessary.

In this study, the effect of increasing sea surface temperature, cadmium and pyrene exposures and their interactions on growth rate, dry weight, contents of lipid, carbohydrate and protein, as well as catalase activity of *Rhodomonas* sp. were determined. Warming scenarios used were 28 °C, 30 °C and 32 °C above 26 °C, which is within the range of the long-term average sea surface temperature on Ghana's shelf. Cadmium was used as proxy for heavy metal pollution. Its effects were assessed at concentrations of 0.1, 1.0, 10.0 and 100.0 μgL^{-1}. Pyrene was used as proxy for petroleum pollution. Its effects were investigated at concentrations of 0.2, 2.0, 20.0 and 200 μgL^{-1}. The results of these laboratory experiments are outlined in the sections that follow.

4.1 Effect of Temperature on *Rhodomonas* sp.

The specific growth rate of *Rhodomonas* sp. when exposed to different sea surface temperatures is shown in Figure 15. Response of the dinoflagellate was immediate under all the warming scenarios, occurring just a day after exposure to the different levels of thermal stress. As shown in Figure 15, after 5 days of exposure, the growth rate had reduced by 4, 8 and 15 times at 28 °C, 30 °C, and 32 °C, respectively, as compared to the ambient temperature (26 °C). These reductions were significant (Table 3). Growth rate fell for two consecutive days when warming was 30 °C and 32 °C with 32 °C falling to the lowest (-0.5 ±

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0.1 day $^{-1}$) on the fifth day of exposure to thermal stress. The cells did not regain growth under all warming scenarios.

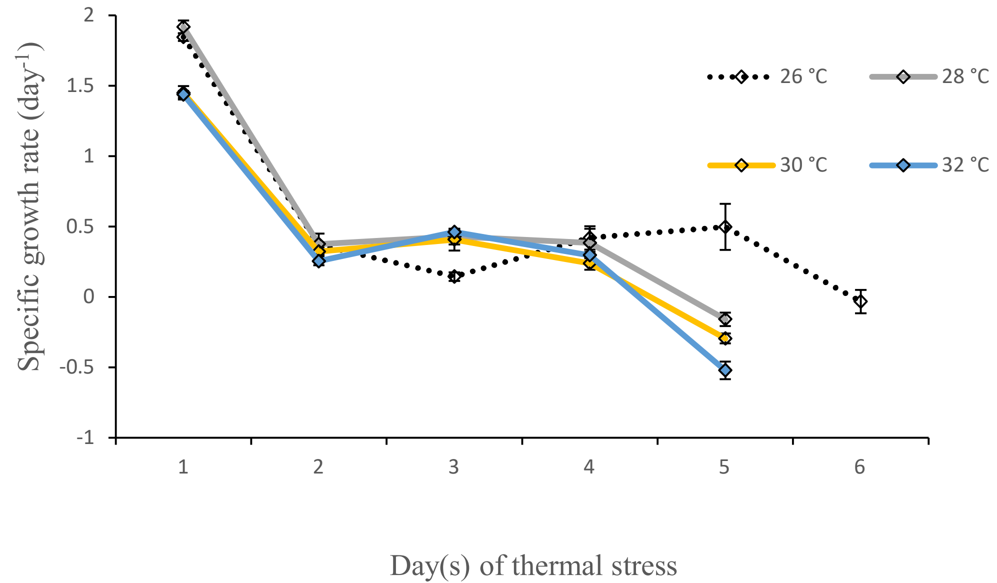

Figure 15: Changes in specific growth rate (Average ± S.D) of *Rhodomonas* sp. exposed to different sea surface temperatures

Figure 16 shows dry weights of the cells of Rhodomonas sp. exposed to different sea surface temperatures. The effect of the thermal stress was evaluated by comparing weights of the exposed cells to that of the average temparature of Ghana's coastal seas. After the experimental warming, weights of cells had significantly increased (Figure 16). Cells became heavier with increasing sea surface temperature (Figure 16). At the end of the thermal stress, cells exposed to 28 ° C were only about 0.13 times heavier than the parent stock. Cells exposed to 30 °C were about 0.96 times heavier whiles

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those exposed to 32 °C were about 1.3 times heavier than the control culture.

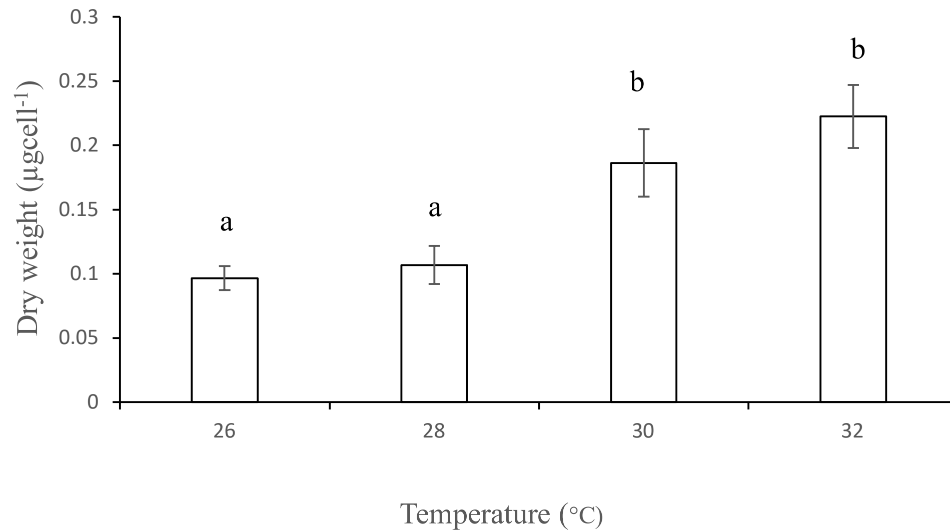

Figure 16: Changes in dry weight (Average ± S.D) of *Rhodomonas* sp. exposed to different sea surface temperatures. Mean values that are significantly different from another are represented by different letters (Tukey's post hoc at $p < 0.05$ after one-way ANOVA).

The effect of increasing sea surface temperature on the biochemical content of *Rhodomonas* sp. was evaluated by comparing the protein, lipid and carbohydrate content of cells exposed to the warming scenarios to that of their parent stock kept at the average temperature of Ghana's coastal seas (Figure 17). On average, protein content of the parental stock constituted ≈ 11% of dry weight. There was significant change ($p < 0.05$) in the protein content of *Rhodomonas* sp. cells exposed to the different sea surface temperatures (Table 3). In comparison with the stock culture, protein content reduced by 9 % when cells were exposed to 28 °C. However, the protein content increased by 17 and 21 % when the cells were exposed to 30 °C and 32 °C respectively. Although lipid content increased by 26, 35 and 38 % when cells were exposed to 28 °C, 30 °C and 32 °C respectively; this increase was not statistically significant

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(Table 3). However, there was significant effect of the thermal stress on carbohydrate content of *Rhodomonas* sp. Carbohydrate content increased by 99 % at 28 °C and then reduced at 30 °C and 32 °C by 66, 64 % respectively when compared to the stock.

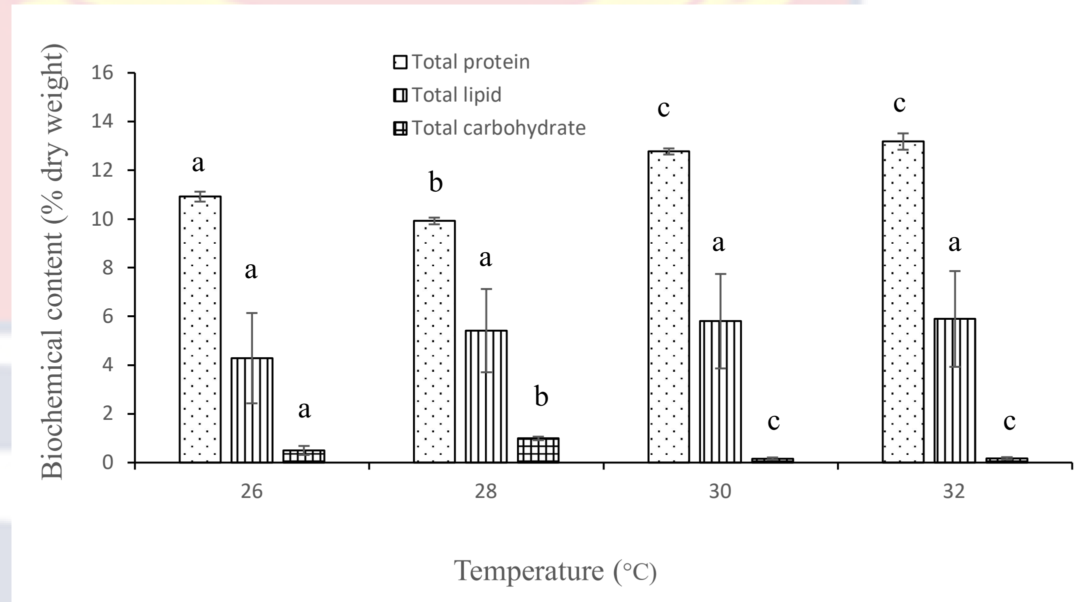

Figure 17: Biochemical content (Average ± S.D) of *Rhodomonas* sp. exposed to different sea surface temperatures. Alphabets on top of the bars indicate comparison between different temperature treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

The effect of warming on oxidative stress was measured using catalase activity in *Rhodomonas* sp. (Figure 18). The effect of the warming scenarios was evaluated by comparing catalase activity of the exposed cells to that of the long term average sea surface temparature of Ghana's coastal seas. After the exposure period, the catalase activity of *Rodomonas* sp. exposed to 28 °C, 30 °C, and 32 º C increased by 17, 72 and 100 % respectively. However, these

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increases were statistically not significant (P > 0.05) (Table 3).

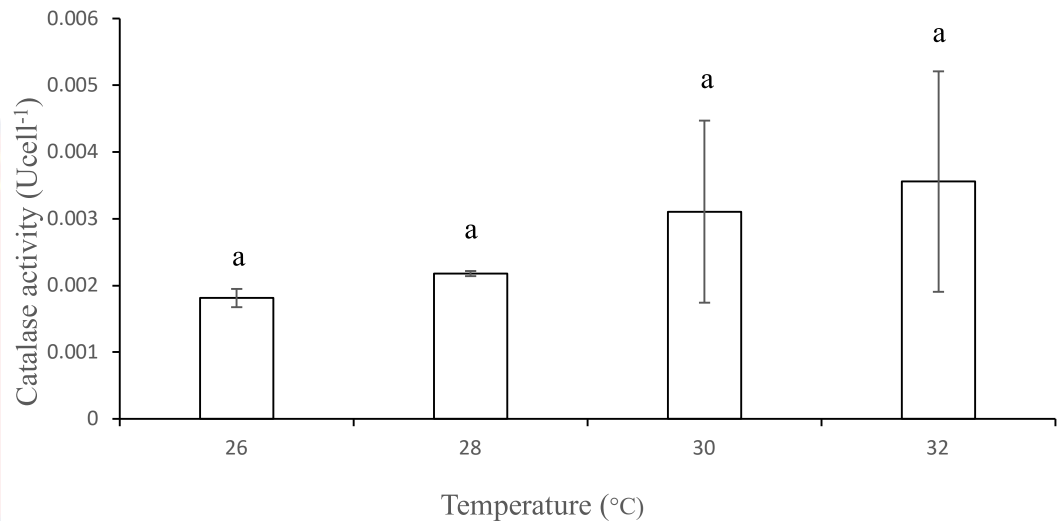

Figure 18: Changes in catalase activity (Average ± S.D) of *Rhodomonas* sp. exposed to different sea surface temperatures. Mean values that are not significantly different from another are represented by similar letters.

4.2 Effect of Cadmium on *Rhodomonas* sp.

The effect of cadmium on the growth rate of *Rhodomonas* sp. is shown in Figure 19. Response of *Rhodomonas* sp. was immediate, occurring on the second day of exposure to all concentrations of cadmium. The highest growth rate (2.2 ± 0.1 day^{-1}) was attained at day 1 in the pollution-free culture environment. At the end of exposure to 0.1, 1, 10 and 100 μgL^{-1} to cadmium, growth rate of the control cultures reduced by 4, 7, 12 and 31 % respectively. These reductions were statistically significant (Table 3). Cells reached their lowest growth

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rate on day 3 of exposure to 100 μgL^{-1} cadmium.

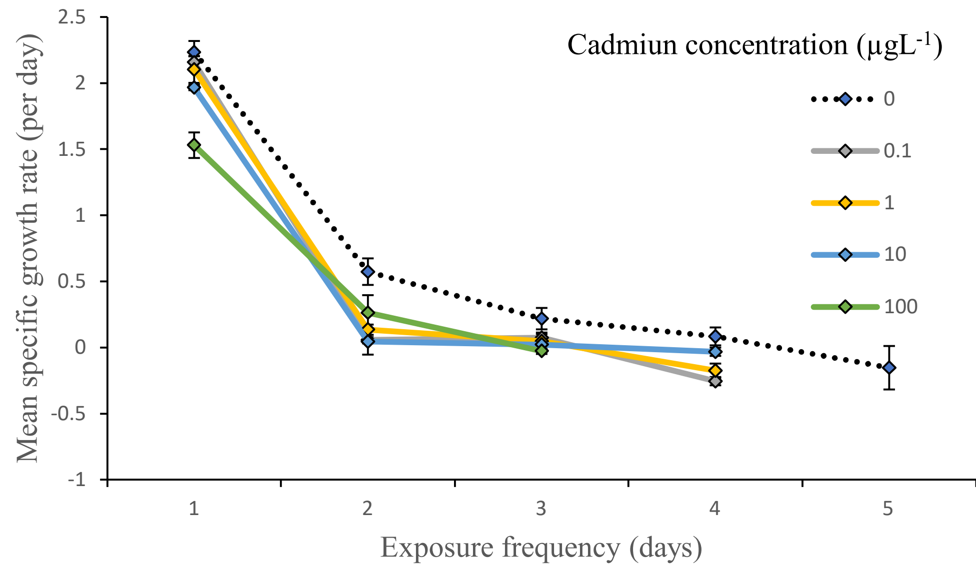

Figure 19: Changes in specific growth rate (Average ± S.D) of *Rhodomonas* sp. exposed to varying concentrations of cadmium.

Figure 20 shows the effects of different concentrations of cadmium exposure on the dry weights of the cells. The effect of cadmium was evaluated by comparing weights of the exposed cells to that of a no pollution scenario. Cells were about 1.3 times heavier than the control when they were exposed to the nominal concentration of cadmium (0.1 μgL^{-1}) found in Ghana's coastal seas. The cells became 1.2 times heavier when the nominal concentration was increased by 10 folds. The increase in cell dry weight was

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statistically significant (Table 3).

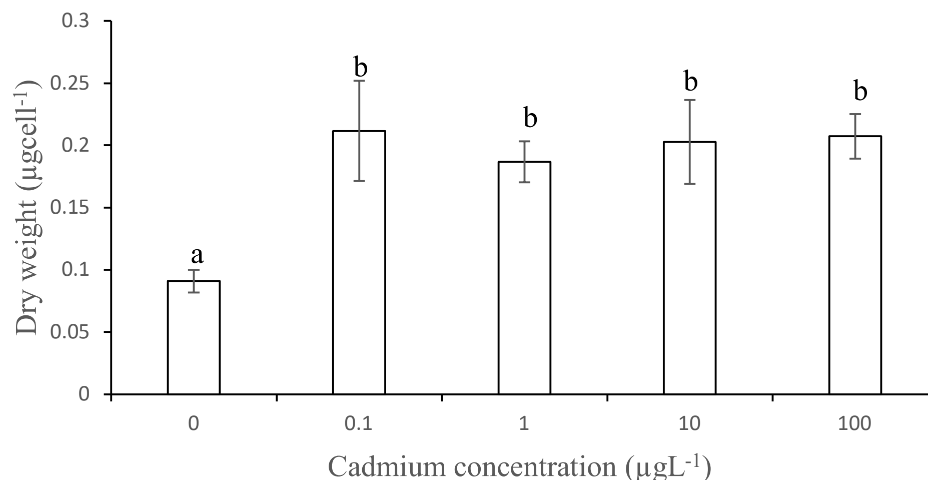

Figure 20: Changes in dry weight (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of cadmium. Mean values that are significantly different from another are represented by different letters (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

The effect of cadmium on the biochemical content of *Rhodomonas* sp. was evaluated by comparing the protein, lipid and carbohydrate content of cells exposed to different concentrations of cadmium (Figure 21). On average, protein constituted 8 % of dry weight of the control culture. There was significant increase ($p < 0.05$) in the protein content of *Rhodomonas* cells exposed to the different cadmium concentration (Table 3). Protein content increased by 2 % when exposed to 1 and 10 μgL^{-1} of culture medium. There was also a 24 % increase in cellular protein content at 100 μg.L^{-1}. Lipid content of *Rhodomonas* sp. reduced by 46, 53, 51 and 52 % after exposeure to 0.1, 1, 10 and 100 μgL^{-1} respectively (Figure 21). However, there was no significant increase in lipid content of *Rhodomonas* sp. exposed to the varying cadmium concentrations (Table 3). In contrast, there was a significant effect ($p <0.05$) of cadmium on carbohydrate content of *Rhodomonas* sp. exposed to different

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cadmium concentrations. Cellular carbohydrates had reduced by 94, 96, 96 and 98 % after exposure of *Rhodomonas* sp. to 0.1, 1, 10 and 100 μgL^{-1} respectively.

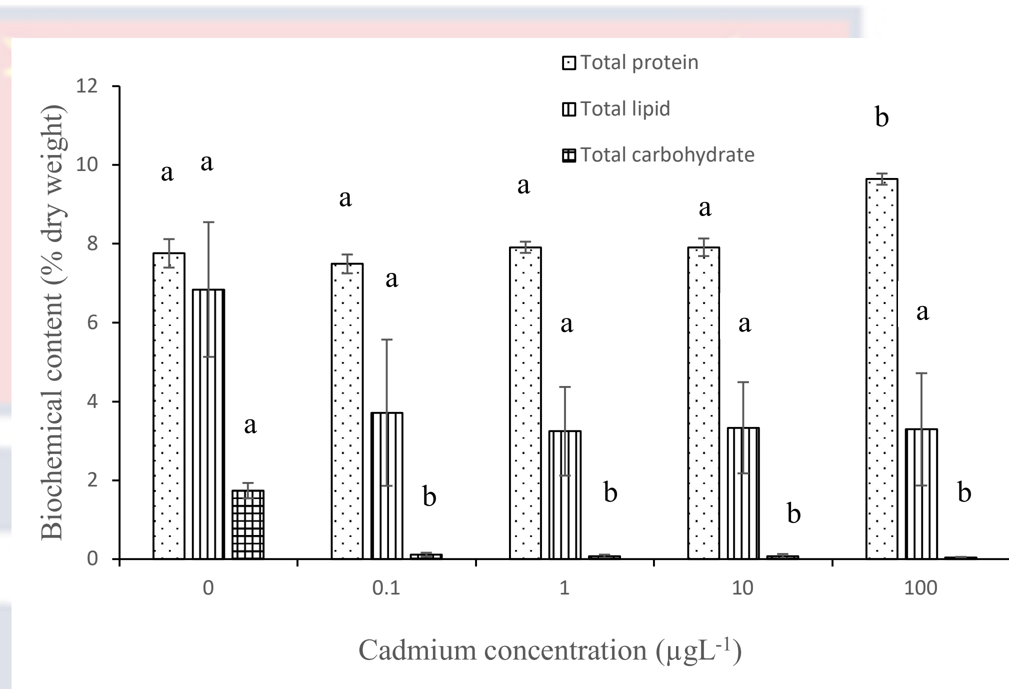

Figure 21: Biochemical content (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of cadmium. Alphabets on top of the bars indicate comparison between different cadmium treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

The ability of *Rhodomonas* sp. to neutralise the oxidative effect by the different concentrations of cadmium is presented in Figure 22. The effect of cadmium was evaluated by comparing activity of the exposed cells to that of no pollution scenario. Addition of cadmium had significant impact on the enzyme activity. After the exposure period, cells of *Rhodomonas* sp. exposed to 0.1, 1, 10 and 100 μgL^{-1} had significantly increased catalase activity by 100, 94, 71 and

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100 % respectively (Figure 22).

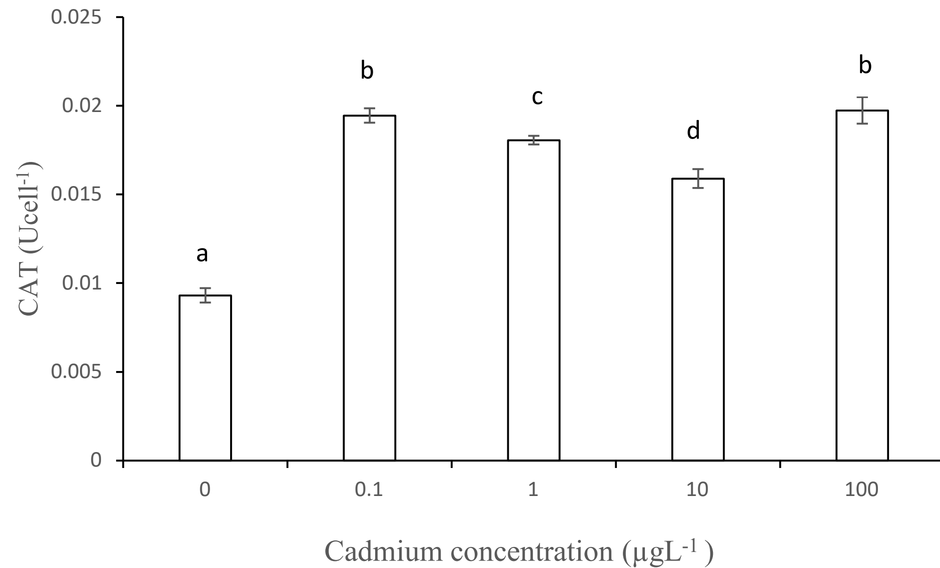

Figure 22: Changes in catalase activity (Average ± S.D) of *Rhodomonas* sp. exposed to different cadmium concentrations. Alphabets on top of the bars indicate comparison between different cadmium treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

4.3 Effect of Pyrene on *Rhodomonas* sp.

The effect of pyrene on the growth rate of *Rhodomonas* sp. is shown in Figure 23. The Response of *Rhodomonas* sp. was immediate in all concentrations, occurring just a day after exposure to pyrene. Growth rate fell by 18, 54, 66 and 37 % after exposure to 0.2, 2.0, 20.0 and 200.0 µgL^{-1} of pyrene. However, this reduction was not statistically significant (Table 3). Acetone used to dissolve pyrene in the pollutant mixture did not have significant ($p > 0.05$) impact on

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the growth rate of *Rhodomonas* sp. (Table 2).

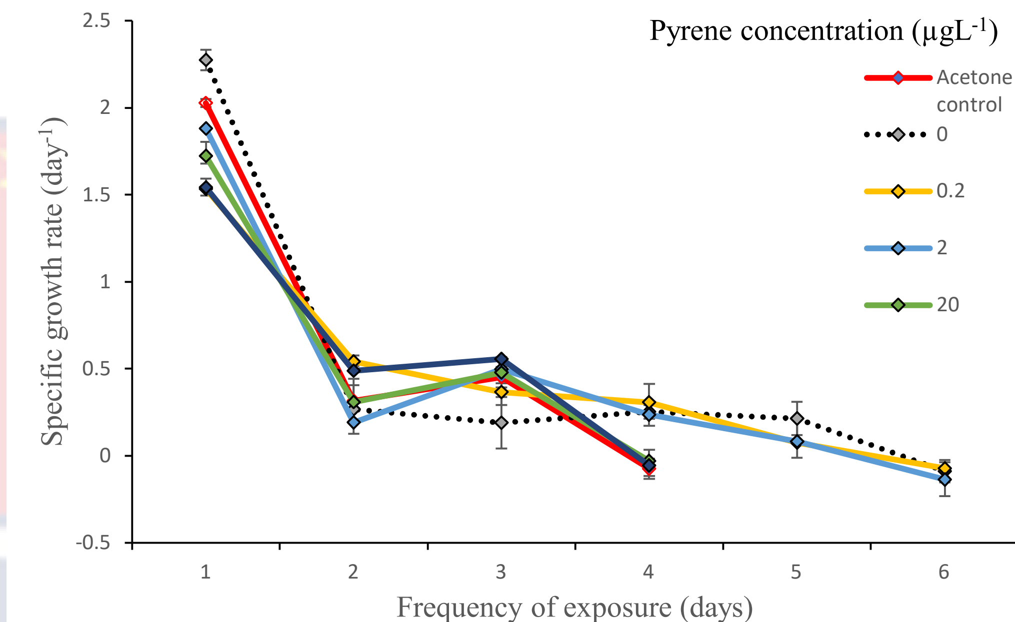

Figure 23: Changes in specific growth rate (Average ± S.D) of *Rhodomonas* sp. exposed to varying concentrations of pyrene.

Table 2: *T-test Comparing Characteristics of Rhodomonas sp. under the Two Pyrene Experimental Control Scenarios. df = 2*

Functional attribute	t Stat	P(T<=t) two-tail
Specific growth rate, μ (day^{-1})	2.679618003	0.115611259
Dry weight, dw ($\mu g cell^{-1}$)	4.112503576	0.054351612
Protein content (% dw)	1.842130342	0.20679168
Lipid content (% dw)	0.672405653	0.570602346
Carbohydrate content (% dw)	2.782930994	0.108506684
Catalase Activity ($U cell^{-1}$)	55.51785458	**0.000324282**

Figure 24 shows dry weights of the cells of *Rhodomonas* sp. exposed to diffirent concentrations of pyrene. The effect of the pyrene was evaluated by comparing weights of the exposed cells to that of a no pollution scenario. At the time of harvest, weights of the cells had not changed after exposure to 0.2 $\mu g L^{-1}$ of

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pyrene. The weights reduced by 4 % after exposure to 2.0 μgL^{-1} of pyrene and began to increase by 23 % after exposure to 20.0 and 200 μgL^{-1} of the pollutant. However, these changes in cell dry weights were statistically not significant (Table 3). Also, acetone alone did not have significant effect on weights of the species (Table 2).

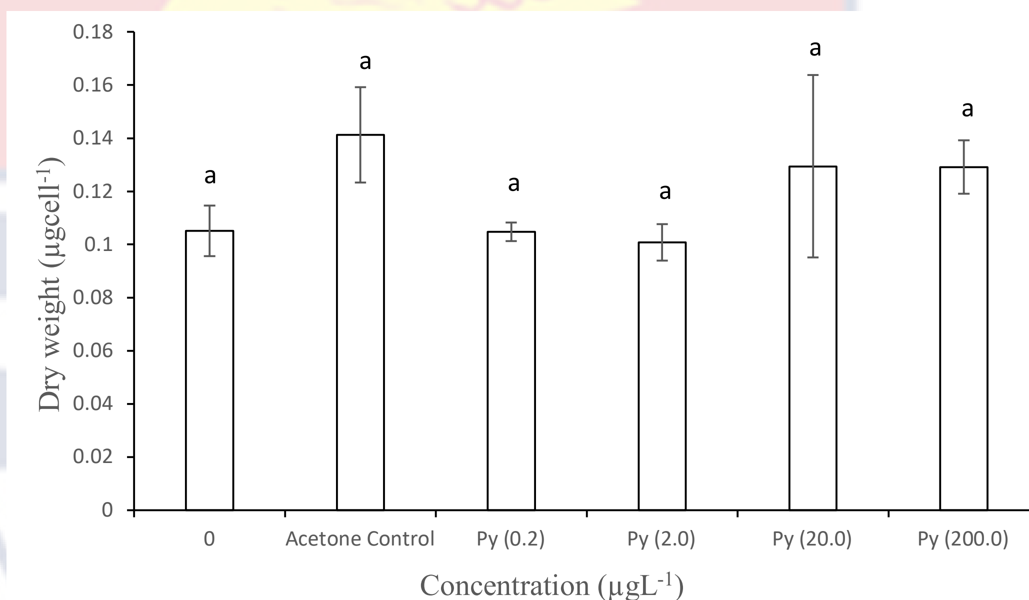

Figure 24: Changes in dry weight (Average ± S.D) of *Rhodomonas* sp. exposed to different pyrene concentrations (Py). Mean values that are not significantly different from another are represented by similar letters.

The effect of pyrene on the biochemical content of *Rhodomonas* sp. was evaluated by comparing the protein, lipid and carbohydrate content of cells exposed to different concentrations of pyrene (Figure 25). On average, protein constituted 6 % (SD: 0.1) of dry weight of the control culture. Protein content of the stock culture had significantly increased by 40, 41, 42 and 28 % after exposure to 0.2, 2.0, 20.0 and 200.0 μgL^{-1} respectively (Figure 25). There was

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no significant effect of pyrene on lipid content of *Rhodomonas* sp. exposed to varying pyrene concentrations (Table 3). However, carbohydrate content of *Rhodomonas* sp. exposed to different pyrene concentrations reduced significantly by 16, 81, 94 and 97 % after exposure to 0.2, 2.0, 20.0 and 200.0 μgL^{-1} of pyrene (Table 3). Acetone alone did not have significant impact on protein, lipid and carbohydrate content of *Rhodomonas* sp. (Table 2).

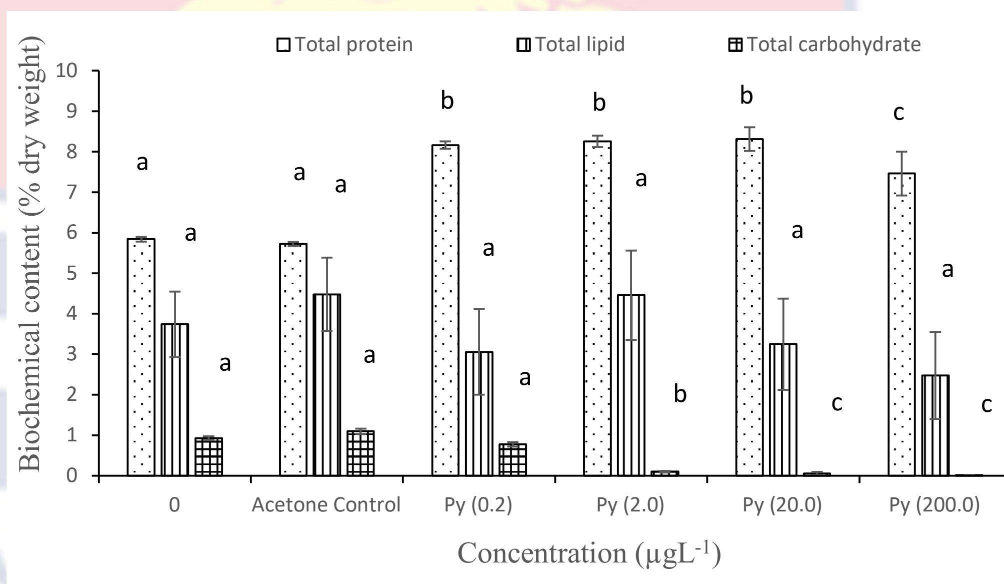

Figure 25: Biochemical content (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of pyrene (Py). Alphabets on top of the bars indicate comparison between different pyrene treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

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The ability of *Rhodomonas* sp. to neutralise the oxidative effect of pyrene is presented in Figure 26. The effect of the pyrene was evaluated by comparing catalase activity of the exposed cells to that of no pollution scenario. After the exposure period, cells of *Rhodomonas* sp. exposed to 0.2, 2.0, 20.0 and 200.0 μgL^{-1} had catalase activity significantly reduced by 22, 22, 11 and 33 % respectively (Figure 26).

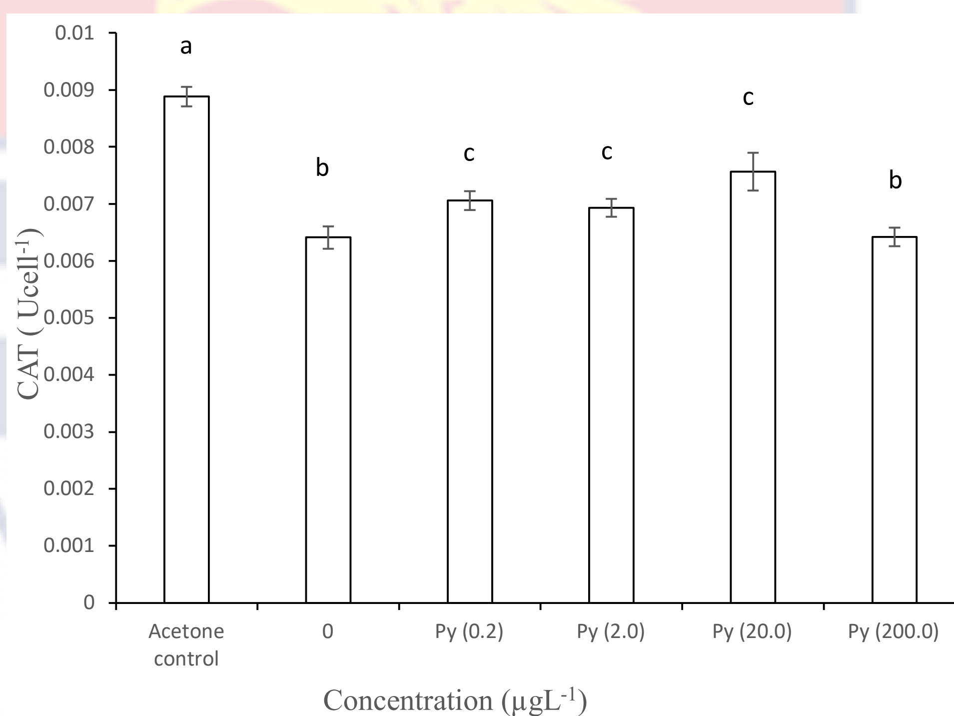

Figure 26: Changes in catalase activity (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of pyrene (Py). Alphabets on top of the bars indicate comparison between different pyrene treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after one-way ANOVA).

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Table 3: One-way ANOVA Comparing Characteristics of Rhodomonas sp. Harvested at the End of Exposure to the Individual Stressors. df: Thermal Stress = 3; Cadmium = 4; Pyrene = 5

Stress factor	Algal functional attribute	F value	*p*-value
Thermal stress	Specific growth rate, μ (day^{-1})	36.96181	**4.91E-05**
	Dry weight, dw ($\mu g cell^{-1}$)	27.98478	**0.000136**
	Protein content (% dw)	158.1634	**1.85E-07**
	Lipid content (% dw)	0.524095	0.677792
	Carbohydrate content (% dw)	36.25113	**5.27E-05**
	Catalase Activity ($U cell^{-1}$)	1.692285	0.245212
Cadmium	Specific growth rate, μ (day^{-1})	4.418132	**0.02584**
	Dry weight, dw ($\mu g cell^{-1}$)	11.15758	**0.001045**
	Protein content (% dw)	35.93209	**6.61E-06**
	Lipid content (% dw)	2.62047	0.098732
	Carbohydrate content (% dw)	120.057	**2.08E-08**
	Catalase Activity ($U cell^{-1}$)	222.8522	**9.99E-10**
Pyrene	Specific growth rate, μ (day^{-1})	0.499113	0.771383
	Dry weight, dw ($\mu g cell^{-1}$)	2.943881	0.058213
	Protein content (% dw)	69.29065	**1.98E-08**
	Lipid content (% dw)	1.611494	0.236463
	Carbohydrate content (% dw)	324.6836	**1.48E-11**
	Catalase Activity ($U cell^{-1}$)	60.35338	**4.36E-08**

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4.4 Combined Effect of Cadmium and Pyrene on *Rhodomonas* sp.

The combined effect of pyrene and cadmium on the growth rate of *Rhodomonas* sp. harvested at the exponential growth phase is shown in Figure 27. Growth reduced by ≈ 20 % when pyrene and cadmium exceeded 2.0 µgL^{-1} and 1.0 µgL^{-1} respectively. In general, there was significant interactive effect of pyrene and cadmium on growth of the *Rhodomonas* sp. (Table 4).

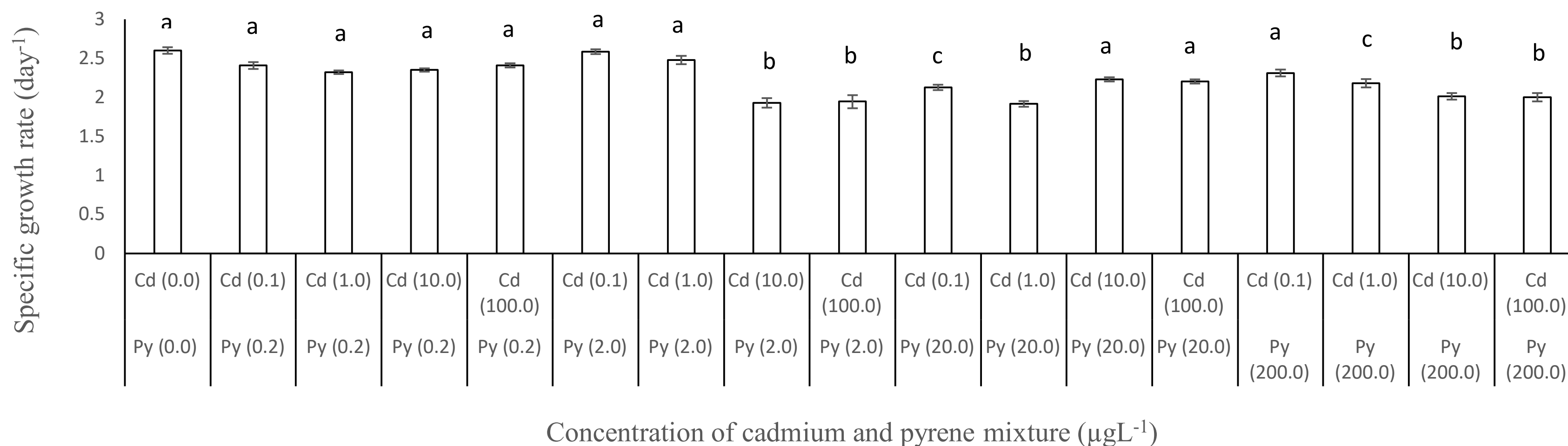

Figure 27: Changes in specific growth rate (Average ± S.D) of *Rhodomonas* sp. harvested at the exponential growth phase after exposure to combination of pyrene and cadmium. Alphabets on top of the bars indicate comparison between different pyrene and cadmium combination treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after two-way ANOVA).

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Figure 28 shows dry weights of *Rhodomonas* sp. cells exposed to combined concentrations of pyrene and cadmium. Dry weight of cells in control culture was 0.125 µgcell^{-1} ± 0.003. Weight of the cells increased by ≈ 15 % when pyrene and cadmium exceeded 0.2 µgL^{-1} and 100.0 µgL^{-1} respectively. There was significant interractive effect of cadmium and pyrene on dry weight of the organims (Table 4).

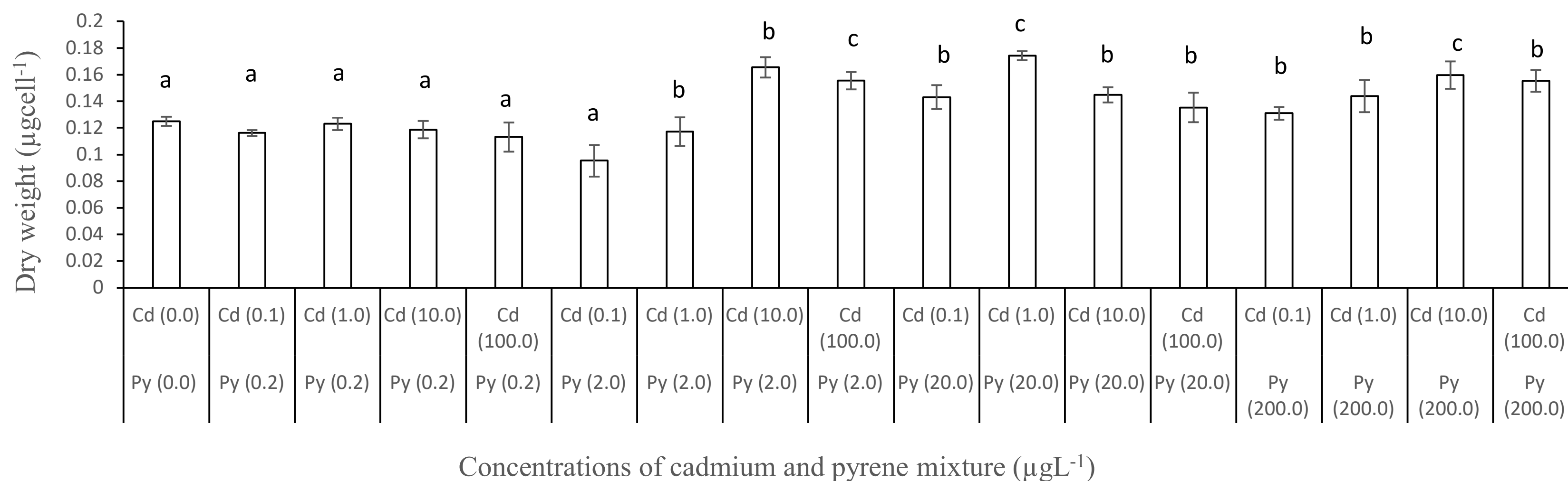

Figure 28: Changes in dry weight (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of pyrene and cadmium combined. Alphabets on top of the bars indicate comparison between different pyrene and cadmium combination treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after two-way ANOVA).

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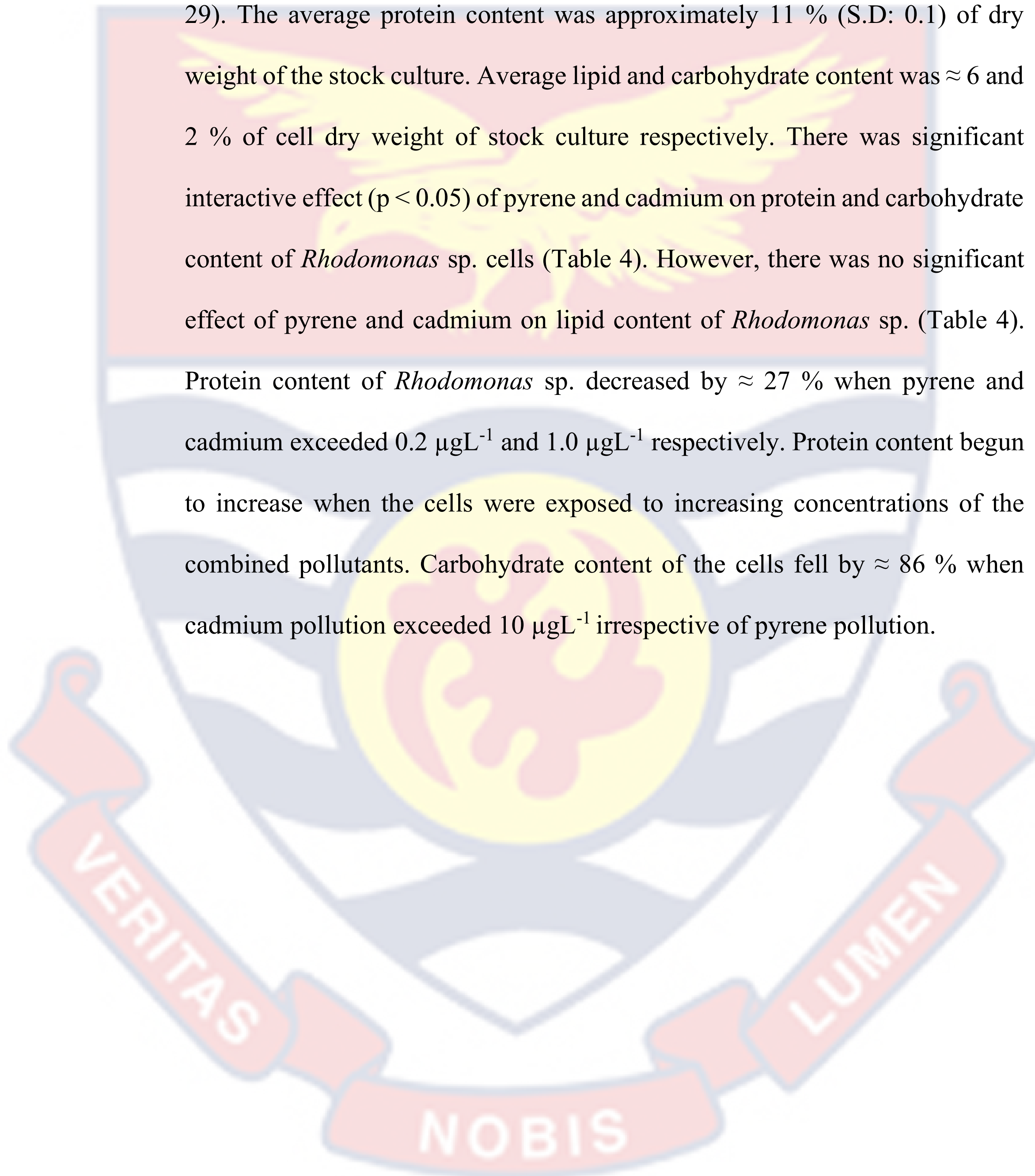

The combined impact of pyrene and cadmium on the biochemical content of *Rhodomonas* sp. was evaluated by comparing protein, lipid and carbohydrate content of cells exposed to the different combinations of the stressors (Figure 29). The average protein content was approximately 11 % (S.D: 0.1) of dry weight of the stock culture. Average lipid and carbohydrate content was ≈ 6 and 2 % of cell dry weight of stock culture respectively. There was significant interactive effect ($p < 0.05$) of pyrene and cadmium on protein and carbohydrate content of *Rhodomonas* sp. cells (Table 4). However, there was no significant effect of pyrene and cadmium on lipid content of *Rhodomonas* sp. (Table 4). Protein content of *Rhodomonas* sp. decreased by ≈ 27 % when pyrene and cadmium exceeded 0.2 μgL^{-1} and 1.0 μgL^{-1} respectively. Protein content begun to increase when the cells were exposed to increasing concentrations of the combined pollutants. Carbohydrate content of the cells fell by ≈ 86 % when cadmium pollution exceeded 10 μgL^{-1} irrespective of pyrene pollution.

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Figure 29: Biochemical content (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of pyrene and cadmium combined. Alphabets on top of the bars indicate comparison between different pyrene and cadmium combination treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after two-way ANOVA).

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The ability of *Rhodomonas* sp. to neutralise the oxidative effect of combined concentrations of pyrene and cadmiumis presented in Figure 30. The effect of the combined stressors was evaluated by comparing catalase activity of the exposed cells to that without pollution scenario. There was significant interractive effect of pyrene and cadmium on the enzyme activity (Table 4). Catalase activity decreased by ≈ 32 % when pyrene and cadmium exceeded 0.2 µgL^{-1} and 0.1 µgL^{-1} respectively.

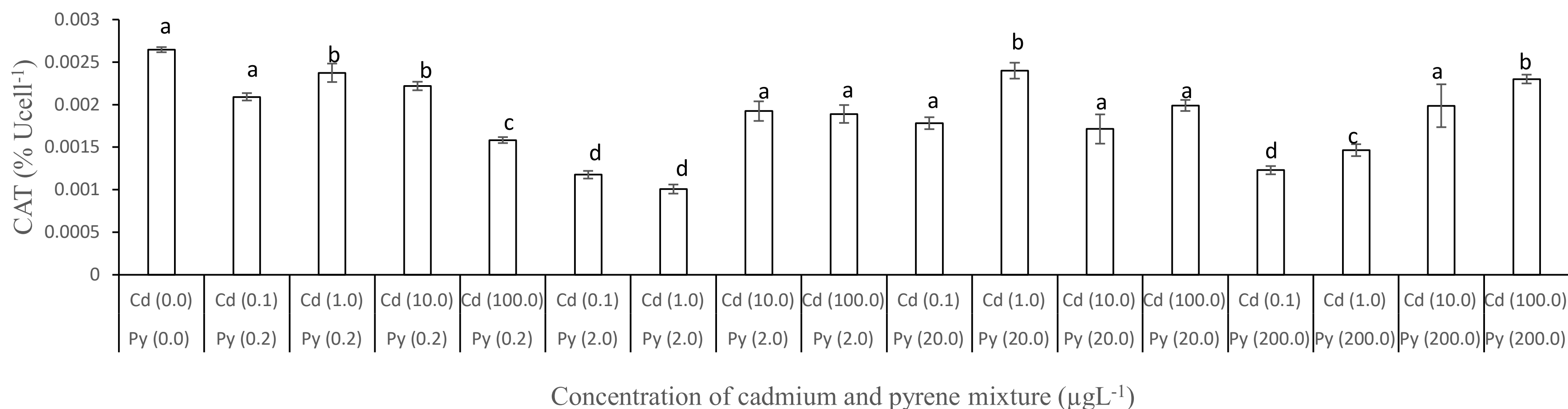

Figure 30: Changes in catalase activity (Average ± S.D) of *Rhodomonas* sp. exposed to different concentrations of pyrene and cadmium combined. Alphabets on top of the bars indicate comparison between different pyrene and cadmium combination treatments; average values that are significantly different are indicated by different alphabets (Tukey's *post hoc* at $p < 0.05$ after two-way ANOVA).

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Table 4: *Two-way ANOVA Comparing Functional Attributes of Rhodomonas sp. Exposed to Different Concentrations of Pyrene and Cadmium Combined*

Functional attribute	Source of variance	*df*	*MS*	*F-value*	*P-value*
Specific growth rate, μ (day^{-1})	Pyrene	3	0.169143	86.4456	**1.96E-15**
	Cadmium	3	0.13229	67.61103	**6.09E-14**
	Pyrene * Cadmium	9	0.11946	61.05387	**1.3E-17**
Dry weight, dw ($\mu g.cell^{-1}$)	Pyrene	3	0.002578	36.28654	**2.06E-10**
	Cadmium	3	0.00144	20.27645	**1.51E-07**
	Pyrene * Cadmium	9	0.001067	15.02278	**3.59E-09**
Protein content (% of dw)	Pyrene	3	12.80607	807.0489	**3.23E-30**
	Cadmium	3	1.764075	111.1735	**5.27E-17**
	Pyrene * Cadmium	9	2.974693	187.4676	**4.33E-25**
Lipid content (% of dw)	Pyrene	3	8.773406	0.386714	0.763303
	Cadmium	3	9.57706	0.422137	0.738393
	Pyrene * Cadmium	9	8.762035	0.386212	0.933004
Carbohydrate content (% of dw)	Pyrene	3	30.00655	904.8752	**5.29E-31**
	Cadmium	3	6.118828	184.5189	**2.85E-20**
	Pyrene * Cadmium	9	3.961059	119.4494	**4.79E-22**
Catalase Activity, CAT ($U.cell^{-1}$)	Pyrene	3	7.69E-07	73.29943	**1.99E-14**
	Cadmium	3	3.88E-07	36.99803	**1.62E-10**
	Pyrene * Cadmium	9	5.48E-07	52.20337	**1.34E-16**

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CHAPTER FIVE

DISCUSSION

The focus of this study was to assess the response of a common dinoflagellate in Ghana’s coast to the effect of increasing sea surface temperature, cadmium and pyrene pollution. To the best of my knowledge this is the first study to look at the combined effect of the aforementioned global change factors on the biochemical content of a phytoplankton species in Ghana's coastal waters. The effect of the stressors was assessed by measuring changes in growth rate, dry weight, protein, lipid, carbohydrate content and catalase activity of *Rhodomonas* sp. The outcomes are discussed in the following sections.

5.1 Effect of Sea Surface Temperature on *Rhodomonas* sp.

Temperature is a primary environmental factor that regulates the physiological processes of organisms, including phytoplankton. According to Abram, Boivin, Moiroux & Brodeur (2017), temperature drives metabolic activities of organisms. Specifically for phytoplankton, temperature regulates enzymes responsible for carbon fixation (Rillema, MacCready & Vecchiarelli, 2020; Young, Goldman, Kranz, Tortell & Morel, 2015). In the present study, *Rhodomonas* sp. was subjected to four levels of sea surface temperature. The findings showed specific growth rate of the *Rhodomonas* sp. was significantly reduced at the end of the exposure period (Figure 15). Under all levels of thermal stress, the response was immediate, occurring just a day after exposure. The response of *Rhodomonas* sp. was, however, different in terms of the degree of thermal stress suffered. Compared to the stock culture, the growth rate declined between 4 – 15 times under the different thermal stress. The

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temperature scenarios investigated in this study were significantly higher than the temperature (26 ± 0.2 °C) of the stock culture. In addition to temperature, nutrient and light which are the major limiting factors for growth of phytoplankton (Liu, Chen, Zhang, Zhang, Wu & Hu, 2021; Thomas et al., 2017) – were provided in levels that could sustain the cultures until the end of each experiment. Hence, it can be said that the different growth rates exhibited by *Rhodomonas* sp. is directly related to the simulated sea surface temperatures. Similar effect of temperature on two species of tropical microalgae have been reported by Acheampong et al. (2021).

It has also been shown that increase in temperature mostly increases enzyme activities responsible for photosynthesis (Schaum et al., 2017). As a result, organisms grow faster when ambient temperature is within tolerable limits; growth reduces when temperature increases above tolerable limits (Pörtner & Farrell, 2008). Specifically for phytoplankton, research has shown that growth rate peaks at an optimum temperature, then decreases as temperature exceeds the optimal level (Barten, Wijffels & Barbosa, 2020; Morales, Sánchez & Revah, 2018). This was observed in the present study, where the growth rate of *Rhodomonas* sp. decreased significantly with increased sea surface temperatures (Figure 15). The decline in growth rate observed at temperatures above 26 °C was in line with previous studies (Acheampong et al., 2021). The steep drop in growth rate at 30 °C and 32 °C may have been because the species was investing most of its energy in metabolic maintenance for survival at higher temperatures, rather than growth (Acheampong, Hense & John, 2014). This metabolic maintenance includes the requirement to meet energy demands for physiological processes such as thermoregulation (Bennett

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& Ruben, 1979) and protein or biomass turnover (Mente, Coutteau, Houlihan, Davidson, & Sorgeloos, 2002). The remaining substrate is reinvested into growth only after the cost of maintenance has been met (Acheampong, Nielsen, Mitra, & St. John, 2012). The effect of warming on the growth rate observed in this study (Figure 15) suggests that it is likely for the growth of the phytoplankton to be negatively impacted by the increase in sea surface temperatures projected under global climate change (IPCC, 2018). Such an effect would directly result in decline in the organism's productivity with consequence on their subsequent accumulation of macromolecules vital for energy transfer in marine food webs as phytoplankton form the base of the marine food web.

Primary producers, including phytoplankton synthesise biomolecules such as proteins and lipids. These biomolecules are important cellular components of marine organisms because they are involved in cell membrane function, energy storage, and trophic relations in food webs (Dalsgaard, John, Kattner, Müller-Navarra & Hagen, 2003; Jin, Hutchins & Gao; 2020). Temperature is important for synthesis of biomolecules in organisms. Previous studies have attributed changes in biochemical content of different types of phytoplankton to elevated temperatures (Nalley et al., 2018; Smith & Morris, 1980). On average, protein content of the stock culture of *Rodomonas* sp. as a percentage of cell dry weight was more than lipid followed by carbohydrates. This observed pattern in the biochemical composition agrees with the general pattern reported in most microalgae (Lavens & Sorgeloos, 1996; Militão, Fernandes, Bastos, Martins, Colepicolo & Machado, 2019). The exposure of the species to the different sea surface temperatures had a pronounced effect on two

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biomolecules – protein and carbohydrate content of the test organism (Table 3). Protein content reduced by 9 % when temperature was 28 C°. However, it began to increase when temperature was 30 °C and 32 °C. The results agree with observations made by Thompson, Guo & Harrison (1992), indicating significant increase in algal protein content at higher growth temperatures. Other studies have reported otherwise. A study conducted by Acheampong et al. (2021) on a tropical dinoflagellate found that protein content decreased with increasing sea surface warming scenarios. Similarly, Renaud et al. (2002) also observed significantly lower percentages of protein content in the dinoflagellates *Isochrysis* sp. and *Cryptomonas* sp., cultured above 33 °C. Previous reports demonstrate that marine microalgae including dinoflagellates (e.g., *Scrippsiella trochoidea*) and diatoms (e.g., *Ditylum brightwellii*) synthesise heat shock proteins to protect themselves against stress induced by warming and other environmental factors (Deng, Li, Hu, Yue & Tang, 2021; Döhler, 1994; to Uji, Gondaira, Fukuda, Mizuta & Saga, 2019). These are medium sized proteins weighing on average 40 – 71 Kilodalton generally produced by cells exposed to stressful conditions (Döhler, 1994). Therefore, a possible reason for the increase in protein content of *Rhodomonas* sp. exposed to different sea surface temperatures in the present study could be attributed to the synthesis of heat shock proteins. The accumulation of these medium sized proteins may also explain the increase in the dry weight of cells exposed to the different temperatures in the present experiment (Figure 16).

According to Ardila- Álvarez et al. (2017), microalgae under any form of stress produce bioactive substances which could alter lipid synthesis and favour production and accumulation of lipids. This corroborates the results

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obtained in this study. As the thermal stress was increasing, *Rhodomonas* sp. accumulated more lipids. However, the effect of temperature on lipid content was not significant. The effect of the different experimental temperatures was also significant on the carbohydrate content of *Rhodomonas* sp. Carbohydrate content of the cells reduced significantly when warming was 30 °C and 32 °C. This could be due to the fact that more of the fixed carbon was directed towards synthesis of proteins and lipids rather than carbohydrates. Catalase (CAT) is one of the principal antioxidant enzymes that removes hydrogen peroxide (H_2O_2) from algae, protecting the cells from oxidative damage caused by reactive oxygen species (ROS) (Lozano, Trombini, Crespo, Blasco & Moreno-Garrido, 2014). ROS have the ability to disrupt chlorophyll synthesis and inhibit cell growth (Chia, Chimdirim & Japhet, 2015). As a result, changes in CAT activity can be easily linked to the organism's level of stress. In the present experiment, catalase activity increased with increasing experimental temperature (Figure 18). The observed increase in catalase activity may be associated with the increase in protein content as temperature increases (Table 3). The increase in protein may have been directed towards increasing the biosynthesis of catalase enzyme needed to battle the stress conditions induced by temperature in the algae. Thus, the increased catalase activity implies an increase in ROS content and hence increasing the synthesis of catalase enzyme to reduce the effect of ROS and further reduce oxidative damage (Branco, Lima, Almeida & Figueira, 2010; Crespo, Lozano, Blasco & Moreno-Garrido, 2013; Lozano et al., 2014).

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5.2 Effect of Cadmium on *Rhodomonas* sp.

The distribution of cadmium in surface waters is comparable to that of major aquatic nutrients (Lane & Morel, 2000; Xu & Morel, 2013). This suggests that the pollutant may be taken by phytoplankton in surface waters. The metal when taken up by phytoplankton has potential to interfere with their cellular metabolism (Duque, Montoya & Botero, 2019). In the present study, *Rhodomonas* sp. was exposed to varying cadmium concentrations (0.1 – 100 μgL^{-1}). Research suggests that uptake of cadmium by phytoplankton can directly impact its growth (Hindarti & Larasati, 2019; Permana & Akbarsyah, 2021). The present study found that growth rate of *Rhodomonas* sp. reduced significantly (≈ 14 %) when exposed to cadmium above 0.0 μgL^{-1}. This could be attributed to interaction of the metal with cell membrane of the *Rhodomonas* sp. According to Permana et al. (2021), absorption of cadmium by phytoplankton occurs in two stages. The initial stage (fast passive absorption), occurring at the cellular level involves direct interaction of cadmium with cell cover. This process reduces photosynthetic activity of the cells thereby inhibiting cell division (Ferdian, Hindarti & Permana, 2020). This effect may have accounted for the immediate decline in growth of *Rhodomonas* sp. found in this study (Figure 19). Several research have reported a decline in growth of dinoflagellates with increasing cadmium concentration (Intwala, Patey, Polet & Twiss, 2008; Payne & Price, 1999). These findings agree with the present study. Other studies have reported otherwise, suggesting that addition of cadmium accelerate growth in some marine diatoms (Lee, Roberts & Morel, 1995; Morel, Reinfelder, Roberts, Chamberlain, Lee & Yee, 1994).

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The second stage of cadmium absorption is known as slow active absorption. During this process, cadmium enters the cytoplasm of the cell. Series of reactions including synthesis of molecules occur within the cytoplasm. The pollutant targets enzymes and cytoplasm as the main destination. Synthesis of the macromolecules investigated in this study occur within organelles in the cytoplasm of *Rhodomonas* sp. (Villaruel-López, Ascencio & Nuño, 2017). Therefore, the changes in macromolecules observed in this study can be attributed to the impacts at the second stage of cadmium absorption by the microalgae. This study found that carbohydrate content had significantly reduced at all levels of cadmium exposure (Figure 21). Several studies have attributed similar changes to the effect of the metal on the light-harvesting organelles of phytoplankton (Hindarti et al., 2019; Bai, Jiang, Jiang, Zu, & Feng, 2022; Jai, Pan & Zhu, 2022). Phytoplankton harvest light for photosynthesis using chlorophylls and other accessory pigments contained in their chloroplast. Cadmium has been known to reduce the efficiency of phytoplankton to make glucose from carbon dioxide and water using the pigment contained in chloroplasts to trap sunlight. This could explain the significant decline of carbohydrate observed in this study when *Rhodomonas* sp. was exposed to all the concentrations of cadmium used in this study. Heavy metals can cause oxidative damage by directly increasing the concentration of reactive oxygen species (ROS) in the cell (Ali et al., 2019). A number of different ROS, including hydrogen peroxide and superoxide anion, occur transiently in aerobic organisms. ROS can oxidize proteins, lipids, and nucleic acids, causing alteration in cell structure (Ahmad et al., 2017). In this study, Cd exposure may have caused lipid peroxidation, as the macromolecule was found to decrease

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with increasing concentration of the metal (Figure 21). Lipid is known to be more susceptible to oxidative stress. This is because the polyunsaturated fatty acids (PUFA) in them serve as the most favorable substrate for peroxidation (Borza et al., 2013).

Tolerance of phytoplankton to heavy metals in the environment has long been linked to defence responses that prevent oxidative stress. Cells can also induce the synthesis of protective proteins as an alternative to keeping metals outside the cell. These protective proteins are referred to as antioxidant proteins. They do so by modulating their levels, which is an adaptive response to withstanding impact of the cadmium (Dat, Foyer & Scott, 1998; Okamoto, Pinto, Latorre, Bechara & Colepicolo, 2001). Indeed, the activity of catalase was found to significantly increase along the increasing concentrations of cadmium in this study. Increase in cellular protein content could also explain the increase in dry weights of cells exposed to the different concentrations of cadmium. This is because, proteins form the bulk biochemical content of most microalgae, including *Rhodomonas* sp. (Lavens et al., 1996). The findings suggest that the presence of cadmium in Ghana's coast is negatively affecting growth of phytoplankton. However, some species like *Rhodomonas* have developed a unique mechanism to preserve their essential biochemical content using their antioxidant system.

5.3 Effect of Pyrene on *Rhodomonas* sp.

Pyrene is likely to be found in most petroleum-polluted waters (Gustavon, Tairova, Wegeberg, & Mosbech, 2016; Vahabisani & An, 2021). The pollutant inhibits the natural development of most aquatic animals

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(Seemann et al., 2017; Tomar, Atre, Sharma, Rai-kalal & Jajoo, 2022). In this study, a one-time spilling event of pyrene was simulated to test its effect on *Rhodomonas* sp. Pyrene has been shown to have negative effects on the growth and productivity of marine phytoplankton (Ozhan & Bargu, 2014). Research suggests that pyrene concentrations above 0.1 µg/L can inhibit the growth and productivity of phytoplankton, potentially due to its toxic effects on photosynthesis and cellular respiration (Hjorth, Vester, Henriksen, Forbes & Dahllöf, 2007; Kim, Shin, Moon, Park & Chang, 2004). In the present study, growth of *Rhodomonas* sp. reduced by ≈ 44 % when the species was exposed to pyrene concentration above 0.0 μgL^{-1}. Several studies have attributed this decline to inhibition of the photosynthetic efficiency and electron transport rate of phytoplankton (Li et al., 2021; Marwood, Smith, Solomon, Charlton & Greenberg, 1999). Petroleum products such as pyrene are buoyant on water. According to Quigg et al. (2021), they inhibit light penetration vital for photosynthesis and growth of phytoplankton. In addition, pyrene can accumulate in the cells of phytoplankton and potentially interfere with their metabolism and reproductive capacity (Behera et al., 2018).

Moreover, pyrene has been long established to alter synthesis of biomolecules in planktonic organisms (Jaiswal, Kumar, Vlaskin & Nanda, 2021; Patel, Kumar, Kumar & Khan, 2018). In the present study, protein content of stock culture of *Rhodomonas* sp. increased significantly (≈ 38 %) at exposure concentrations above 0.0 μgL^{-1} (Figure 25). This significant increase in protein content with increasing pyrene concentrations could be attributed to the synthesis of protective proteins (Dat et al., 1998; Okamoto et al., 2001). These proteins are induced when organisms interact with chemicals (Hasegawa,

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Rahman, Kato, Maki & Rahman, 2013). Cells can also induce the synthesis of protective proteins as a mechanism of keeping polycyclic aromatic hydrocarbons (PAH) outside the cell. To the best of my knowledge, no such response pattern of *Rhodomonas* sp. to the specific PAH investigated (pyrene) has been previously reported. However, the current findings contradict the findings from previous study that documented decrease in cellular protein of *Chlorella* sp. with increasing concentrations of another PAH – fluoroethane (Tomar & Jagoo, 2021). This suggests that different species of phytoplankton respond differently to different petroleum pollutants (El-Sheekh, El-Naggar, Osm & Haieder, 2000). The increase in protein content could also explain why cells became heavier (≈ 23 %) at the higher pyrene concentrations. This is because proteins form the bulk of biomolecules of phytoplankton (Lavens et al., 1996).

Lipid content of the species were not significantly affected by pyrene exposure in this study (Table 3). However, carbohydrate content reduced by ≈ 72 % at exposure concentrations above 0.0 μgL^{-1} (Figure 25). Report by Tomar & Jojoo (2021) has proven that pyrene reduces the efficiency of phytoplankton to synthesise glucose during photosynthesis. This could explain the significant reduction in carbohydrate content observed in this study when *Rhodomonas* sp. was exposed to pyrene concentrations above 0.0 μgL^{-1}. The activity of catalase was found to significantly decrease (≈ 22 %) along the increasing concentrations of pyrene investigated in this study. Similar report has been made by Sun et al. (2021). The authors made known that the decrease in catalase activity could be due to inhibition of the enzyme's synthesis by pyrene. This suggests that, in

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pyrene polluted environment, catalase may not protect species such as *Rhodomonas* from oxidative stress.

5.4. Combined Effect of Pyrene and Cadmium on *Rhodomonas* sp.

Previous research has shown that organisms can be subjected to multiple environmental stressors at the same time (Crain et al., 2008; Griffen et al., 2016). The impact of these combined factors is not fully understood, particularly in tropical systems (Griffen et al., 2016). The impact of pyrene and cadmium interaction on *Rhodomonas* sp. was assessed based on growth and biochemical attributes of the organism at the exponential growth phase. Results of this study showed that pyrene and cadmium when presented simultaneously alter growth and biochemical properties of *Rhodomonas* sp. (Figure 27 – 30). According to Fleeger, Gust, Marlborough & Tita (2007), pyrene can form complexes with heavy metals in aquatic systems, thereby increasing heavy metal uptake by aquatic organisms from water. This effect has the potential to alter the ability of cell membrane of most aquatic organisms to adapt to changing environment (Gauthier, Norwood, Prepas & Pyle, 2014). Thus, exposing vital organs to increasing bioaccumulation of chemical pollutants and causing oxidative stress. In the present study, pyrene and cadmium mixture significantly (Table 4) inhibited the growth of *Rhodomonas* sp. (≈ 20 % reduction in growth) when pyrene and cadmium exceeded 2.0 $\mu g.L^{-1}$ and 1.0 μgL^{-1} respectively. This result corroborates the results from previous laboratory investigations. Wang, Luo, Ke, Luan & Tam (2013) reported a significant reduction in growth in a unicellular marine microalgal species (*Selenastrum capricornutum*) after exposure to a combination of different PAHs and heavy

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metals. Baścik-Remisiewicz, Aksmann, Żak, Kowalska & Tukaj (2011) also found that the combination of cadmium and a three-ring PAH (anthracene) inhibited the growth of *Desmodesmus subspicatus* more significantly than when the toxicants were administered individually.

Results from the current study could be attributed to inhibition of photosynthetic activity and the ability of microalgae to synthesise molecules relevant for their life processes (Subashchandrabose, Wang, Venkateswarlu, Naidu & Megharaj, 2017; Wang, Wufuer, Duo, Li & Pan, 2022). This is evidenced by results on the biochemical composition *Rhodomonas* sp. obtained in this study. Protein and carbohydrate contents of the species reduced significantly, suggesting an interactive effect of the two pollutants. However, pyrene and cadmium did not interact to significantly reduce the lipid content of *Rhodomonas* sp. (Table 4). Heavy metals and PAHs induce generation of reactive oxygen species (ROS) like hydrogen peroxide when the stressors are presented individually as well as combined (Li, Hu, Zhu, Chen, Kong & Liu, 2006; Mallick, 2004). Catalase plays a vital role in the degradation of hydrogen peroxide (Pinto et al., 2003). In the present study, the pollutants interacted significantly to reduce the activity of catalase when pyrene and cadmium exceeded 0.2 μgL^{-1} and 10 μgL^{-1} respectively (Figure 30). This may be related to decrease in cellular protein content (Figure 29). According to Kebeish, El-Ayouty & Husain (2014), reduction in protein content of microalgae affects the expression of catalase and hence its activity.

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CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The main aim of the study was to evaluate the combined impact of sea surface warming and pollution on the functional properties of *Rhodomonas* sp. The study provides preliminary but crucial information on the aforementioned global change factors on the biochemical content of a dinoflagellate associated with Ghana's coastal waters. This information can aid in developing parameters for modelling dynamics of marine food webs under global climate change. The study showed that the stressors investigated, when presented individually or combined has significant impact on nutritional quality of tropical marine dinoflagellate.

6.2 Conclusions

Based on the discussions of the experimental outcomes, the following conclusions were made in the present study:

Organisms at the base of the marine food web are simultaneously exposed to impacts of increasing sea surface temperature and pollution. It is crucial to understand how these organisms respond to the individual stressors as well as their combination. By exposing *Rhodomonas* sp. to the stress factors under laboratory simulations, this study established that the combination of cadmium and pyrene pollution, as well as the stressors presented individually does not impact lipid content of the species. Under all stress conditions investigated, carbohydrates were the macromolecules impacted most suggesting a significant

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impact of the factors on photosynthetic efficiency of the species. In contrast, cells became heavier when exposed to a combination of cadmium and pyrene. The increase in dry weight was related to increasing protein content of the cell. This was observed even when cells were exposed to warming alone. This suggests that under stress, dinoflagellates species like *Rhodomonas* are able to overcome oxidative stress from environmental stress by producing heat shock proteins of relatively higher molecular weights as well as increasing the activity of catalase. Given that the majority of tropical systems are anticipated to experience severe consequences of climate change and pollution, the combined effects of these stressors could have significant impact on the base of marine food webs. This, in turn, could have serious repercussions for human health and livelihoods.

6.3 Recommendations

Further studies are recommended to:

1. determine how effect of the combined stress factors manifest at higher trophic levels within the marine food web.
2. investigate the types of heat shock proteins synthesised by phytoplankton as a result of combination of the stress factors investigated.
3. Works should be extended to other functional groups such as diatoms and green algae.

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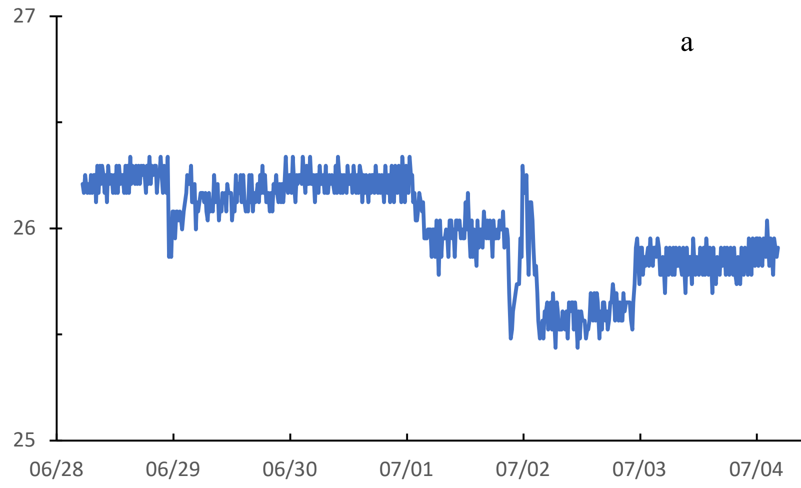
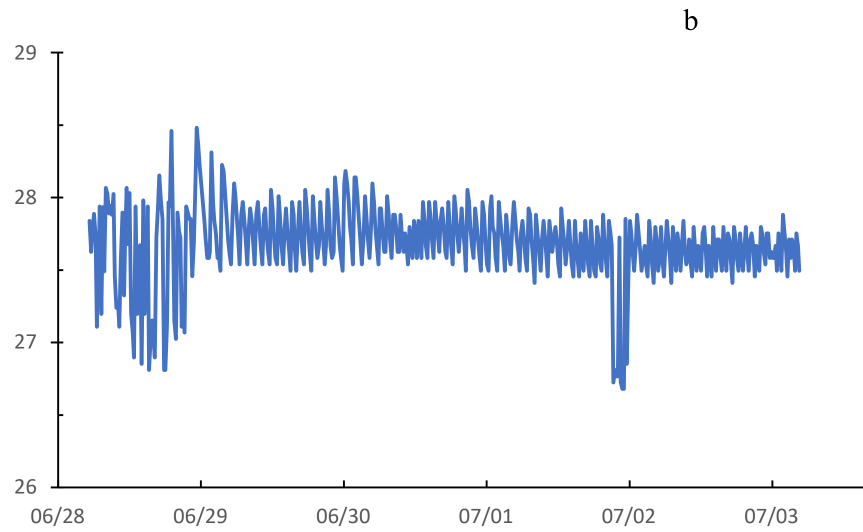
APPENDICES





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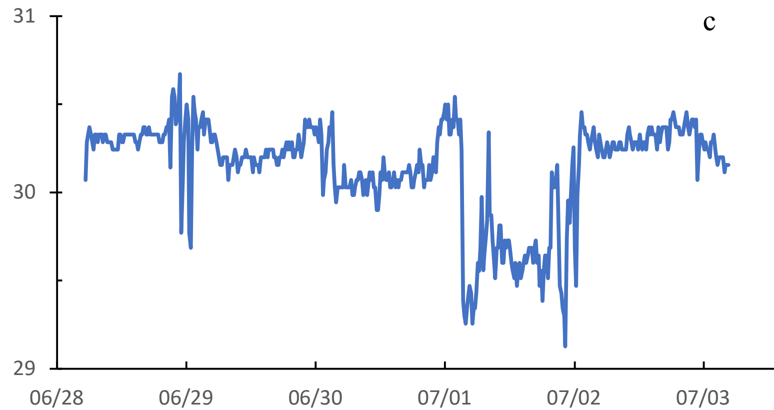

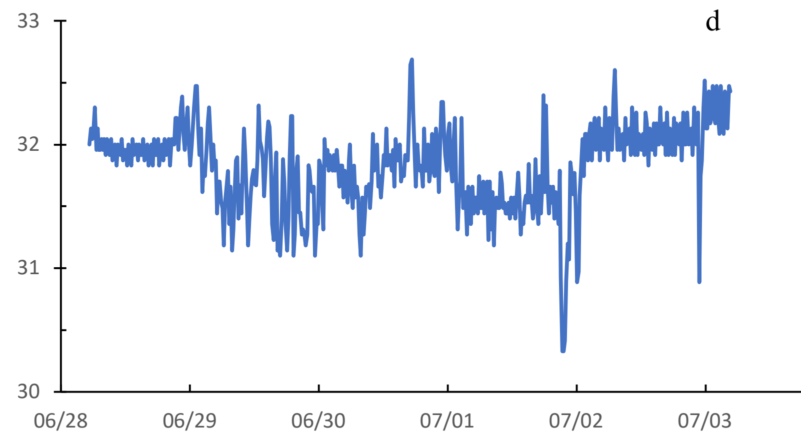

Appendix 1: Experimental temperatures recorded using temperature loggers (Onset HOBO Pendant MX2201) fixed in the water baths used for culture of the microalgae. The warming scenarios were the ambient temperature, 26 °C (a), 28 °C (b), 30 °C (c) 32 °C (d). Warming of the cultures began on 28 June 2022 for a maximum of 6 days.

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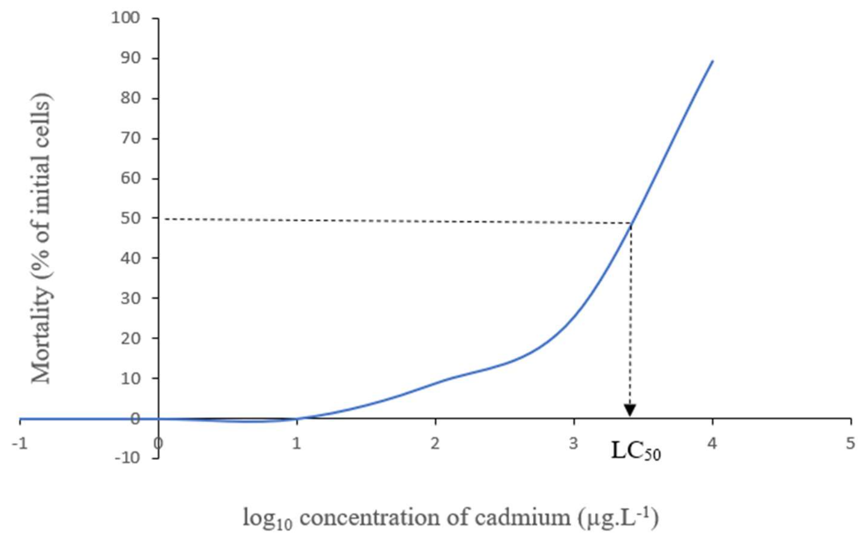
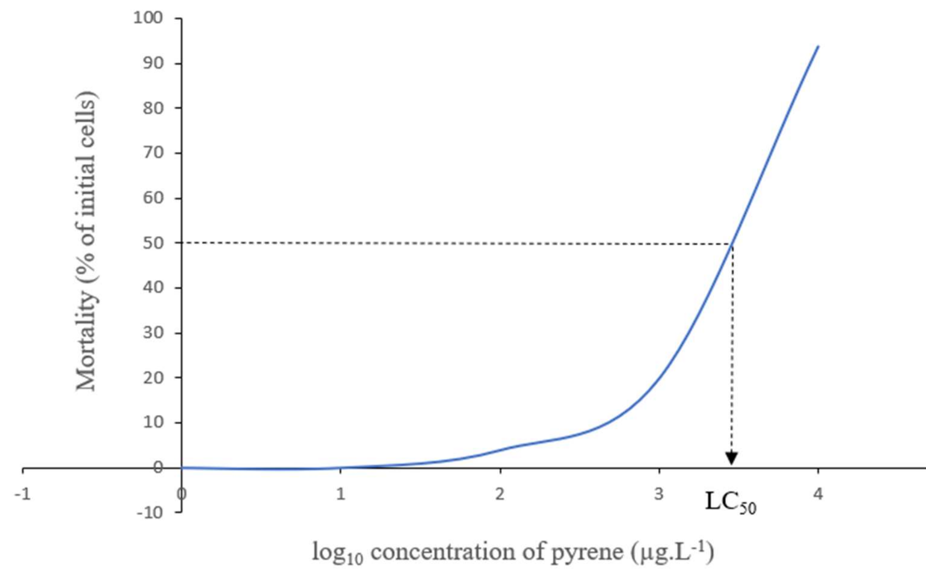


Appendix 2: Concentration – response curve of Rhodomonas sp. exposure to cadmium, showing LC_{50} of cadmium.



Appendix 3: Concentration – response curve of Rhodomonas sp. showing LC_{50} of pyrene.

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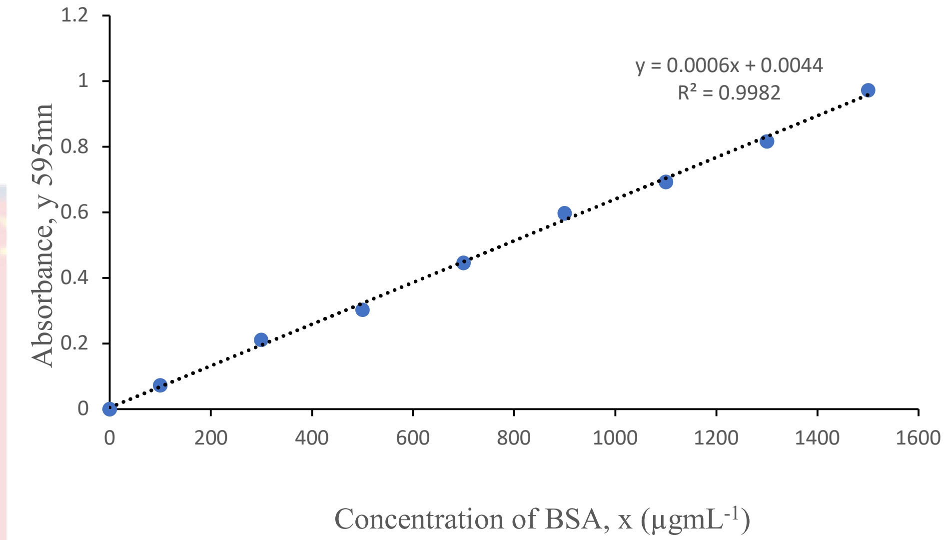
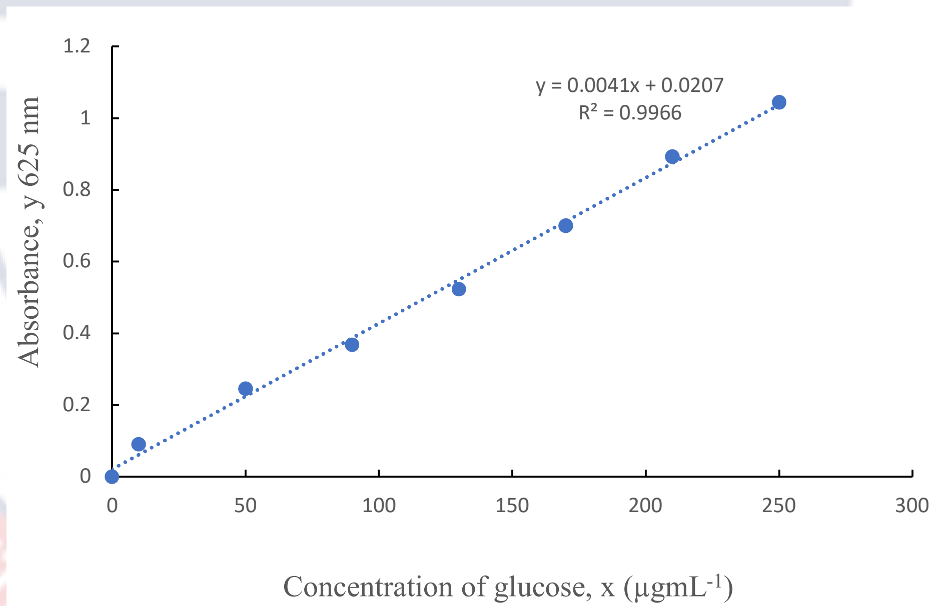


Appendix 4: Calibration curve for estimation of total protein content



Appendix 5: Calibration curve for estimation of total carbohydrate content

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