Quantitative Sequestration of Anthropogenic Pollutants by Marine Microalgae

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Quantitative Sequestration of Anthropogenic Pollutants by Marine Microalgae

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville.

Mohammed Fahad Hasan
May 2019
Dedication

To Ammu, Abbu, Sharmi, Lisa and Frank Vogt
Acknowledgement

First and foremost, I am deeply indebted to Dr. Frank Vogt for his remarkable contribution to my time in graduate school. Beside academic mentorship, I highly value the personal relationship we forged over the years. I cannot believe that you are not here anymore, but I will try to maintain your legacy in my future work. I would also like to thank Dr. Christopher Baker and my doctoral committee members for their time and valuable discussions on this research. I also want to thank my colleagues for providing me a wonderful time in the graduate school. I want to acknowledge the support and assistance from all the staff members of Chemistry department at University of Tennessee. I am very thankful to National Science Foundation for funding this research under CHE-1710175.

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Abstract

Due to increased industrialization, the release of anthropogenic pollutants in the environment is increasing and the fate of these pollutants has become a global concern. Marine phytoplankton act as a considerable sink of many anthropogenic pollutants. However, chemical analyses of the biosequestration process often encounter challenges due to many simultaneously interacting chemical and biological parameters. This dissertation is motivated by understanding phytoplankton’s sequestration of anthropogenic pollutants by linking all the interactions between microalgae and their environment.

The sequestration of greenhouse gas CO₂ and its utilization for biomass production by marine microalgae was investigated by developing novel modeling methodologies with required microscopic spatial resolution. The validation experiments indicated that the model can accurately describe the chemically complex relation between atmospheric CO₂ and resulting biomass. Moreover, few applications of the model were presented to predict the impact of future environmental changes on the ecosystem.

In order to assess microalgae’s CO₂ sequestration a straightforward indicator, intracellular carotene quantity, was proposed. To accomplish this, intracellular carotene was measured by a combination of fluorescence spectroscopy and flow cytometry. By relating cells’ internal response (carotenoid) to external chemical changes (atmospheric CO₂), a slowdown in microalgae’s CO₂ sequestration was indicated by an increase of intracellular carotene quantity.

The last research topic was motivated by understanding the effect of two heavy metal pollutants (copper and nickel), on the uptake and biomass production by marine microalgae. The uptake of heavy metal by the contaminated cultures was quantified by inductively coupled plasma optical emission spectroscopy. This investigation suggested that the uptake of heavy metals and algal growth was strongly influenced by heavy metal. Furthermore, contaminated cells suffered significant intracellular damage due to the presence of heavy metal.
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Chapter 1. Introduction

1.1. Overview and Objectives

Over the last century, Earth has experienced one of the greatest transitions of modern humans; the urbanization. Driven by industrial evolution, humans have remarkably transformed their social, economic and technological conditions in this period. However, the aggressive fossil fuel burning for urban development has detrimental repercussions on the environment. The continuous release of greenhouse gas carbon dioxide in the atmosphere has a major impact on climate change including, global warming, ocean acidification and rising sea levels. Moreover, the pollutants from agricultural and industrial runoff can imbalance the ecosystems triggering changes in biodiversity and even, extinction. Therefore, it is crucial to understand the fate of these pollutants in our environment.

More than 71% of Earth’s surface is covered by marine ecosystem. This ecosystem contains phytoplankton which is the basis of the global food chain and a primary or secondary source to higher trophic levels. The anthropogenic pollutants enter this food supplying ecosystem by different natural or artificial pathways (rivers, rain, urban runoff, industrial discharge etc.). Ubiquitous marine phytoplankton can counterbalance these compounds from their surrounding chemical environment either as nutrients for growth or as luxury uptake. Phytoplankton respond to the continually changing chemical environment by altering their intracellular chemical signature. Such dynamic adaptation can lead to harmful environmental consequences including algal bloom, toxin release and incapability to sequester anthropogenic compounds. Therefore, it is essential to realize the interaction between phytoplankton ↔ environment and the potential consequence of this relationship in long-term climate changes.

Marine ecosystems are driven by many complex physical, chemical and phycological parameters. These parameters simultaneously influence the sequestration of anthropogenic compounds within the intracellular region of phytoplankton. Therefore, assessment of the phytoplankton ↔ environment relationships will be incomplete if these interactions are neglected. However, current analytical practices can only evaluate a certain number of parameters rather than linking all the considerations. Furthermore, the
microscopic sized phytoplankton can only interact with their immediate chemical environment. Yet, experimentally probing phytoplankton’s surrounding environment with microscopic spatial and temporal resolution is extremely challenging due to the presence of large number of cells in the ecosystem. Therefore, in order to accurately quantify and understand phytoplankton’s compound sequestration process, there is a need for a comprehensive approach that relates all these influential factors.

The aim of this dissertation is to quantitatively understand the sequestration of anthropogenic pollutants by marine microalgae by integrating all the interactions between microalgae ↔ environment. In order to accomplish this goal, the sequestration of greenhouse gas carbon dioxide and its subsequent transformation into biomass were estimated by linking computational models and experimental analyses. The rationale for modeling is to quantify CO$_2$ sequestration process by probing the large-scale ecosystem with a microscopic spatial resolution that straightforward experiments cannot access. From an analytical standpoint, this approach provides an accurate and comprehensive illustration of CO$_2$ fixation by microalgae. Furthermore, the CO$_2$ sequestration capacity of marine microalga *Dunaliella salina* was predicted by monitoring their intracellular pigment quantity as a straightforward indicator.

The secondary goal of this dissertation is to understand the effect of heavy metal (copper and nickel) toxicity on their remediation capacity by marine microalgae *Nannochloropsis oculata*. By relating cells’ internal response (biomass) to external (heavy metal) environment, this integrated approach gains insight on microalgae’s dynamic adaptation in response to these pollutants. From an environmental perspective, this will enable assessment of long-term impact of heavy metal toxicity.

1.2. **Scientific Background**

1.2.1. **Anthropogenic Pollutants and Their Influence on Environment**

Pollutants are contaminants that enter the natural environment and has detrimental effect on the ecosystem and human health. Due to industrial, residential and agricultural reasons, the release of anthropogenic pollutants into the environment has been continuously increasing over the last few decades. These anthropogenic contaminants are responsible for pollution in different forms including air, soil and water pollution. The
primary sources of air pollution are gaseous products including CO₂, CO, SOₓ, NOₓ, O₃, NH₃ and volatile organic compounds which are released in atmosphere during the combustion process. Anthropogenic soil pollution occurs primarily from heavy metals and hydrocarbons that originates due to construction, mining and agricultural activities. These pollutants can enter the food cycle through vegetation which has toxic effect on animal and human body. The pollutants from all sources can enter the fresh water or marine environment by natural or anthropogenic pathways to cause water pollution.

These pollutants have major impact on the ecosystem and climate change. The emission of CO₂, CH₄, N₂O and fluorinated gases are responsible for greenhouse effects. The consequence of this includes global warming effect, sea-level rise, declining arctic sea ice and ocean acidification [1, 2]. Due to the severity of greenhouse gas CO₂, NASA has launched the Orbiting Carbon Observatory (OCO-2) to monitor CO₂ levels and their distribution in the atmosphere [3]. The release of SOₓ and NOₓ gases are accountable for acid rain. Because of these pollutants, the World is experiencing a declining air quality that results in incidents like Yokkaichi asthma, Delhi smog, London smog and Los Angeles smog [4-7]. Pollutions from organic contaminants also have a threatening impact on the ecosystem. The relentless use of plastic materials is culpable for damaging the aquatic lives. Except a few biodegradable polymers, the plastic materials require longer time to decompose in the environment and therefore, can destroy the aquatic lives [8, 9]. Moreover, the application of excessive fertilizers, pesticides and herbicides has detrimental effect on human and wildlife [10].

The heavy metal pollution also has a serious damage on plant, animal and human health. The heavy metals enter the trophic chain primarily by their uptake by planktons. Since, this microorganism drives the food chain, they are bioaccumulated at a higher concentration within the higher trophic levels. Exposure to these pollutants, either short or long-term, can cause respiratory, cardiovascular and skin disease [11-13]. The heavy metal pollution has caused several major toxic outbreaks over the last century. Mercury poisoning in form of methylmercury has affected more than ten thousand people (1484 death) in Minamata Bay, Japan (also known as Minamata Disease) [14, 15]. The poisoning due to cadmium was responsible for the bone softening and kidney failure in itai-itai Disease (it hurts-it hurts disease), Japan [16, 17]. The arsenic poisoning through groundwater, even at trace concentration, is a major public health concern in many countries around the world [18].
1.2.2. **Significance of Microalgae**

Microalgae are unicellular organisms which are capable of producing oxygen through photosynthesis. They are ubiquitous and can sustain in terrestrial, freshwater and marine environment as well as in extreme conditions beneath the arctic ice and hot springs [19-24]. The first documented algal fossil is debated to date back at least 750 million years ago [25]. The number of living algal species is estimated to be between 30000 to 1 million [26]. These different species can be divided into two primary categories: eukaryotic and prokaryotic. The key feature of eukaryotic cells is a membrane-bound organelle that controls the regular cell metabolism and reproduction. On the other hand, prokaryotic cells do not have any internal cell membrane and thus, often referred as cyanobacteria (blue-green algae).

This phototrophic organism can produce their own food by directly harvesting the energy from sunlight. In this process, they convert the inorganic carbon dioxide and water to produce energy storing carbohydrates. In presence of solar radiation, the chloroplast converts the carbon dioxide into oxygen and adenosine triphosphate (ATP) into required energy for growth. Other primary nutrients required for their survival includes nitrogen, phosphorous and potassium at different quantities [27, 28]. Being at the bottom of food web, microalgae (*primary producers*) play an indispensable role as the keystone of the food chain. The photosynthesis by microalgae is responsible for producing about half the oxygen in the atmosphere [29]. In this process, they utilize almost half of the global carbon production and other pollutants as nutrients for biomass production [30-34]. Therefore, microalgae play a crucial role as a bioremediation tool for balancing a sustainable environment [33-41].

Over the last few decades, microalgae have gained a lot of interest for their commercial application to produce many useful end products [42-44]. Due to their fast growth rate, lipid contents of algae cultures can proliferate within a very short time [45]. The large-scale microalgae culture is extensively used in fuel industry to commercially produce biodiesel, bioethanol and biobutanol [29, 45-50]. Biofuels are now available in retails as an alternative to transportation fossil fuel [51]. Beside economic profits, these sources of clean energy have great environmental benefits to build a sustainable world [45, 51].

Microalgae is also used in pharmaceutical industries as a source of many nutritional supplements [42, 43, 52-54]. *Dunaliella salina* (also studied in this dissertation)
is the primary source of commercial beta-carotene production which is a precursor to vitamin A [52]. The chemical constituents extracted from other algal biomass are sources of proteins and unsaturated fatty acids. Ongoing researches indicate that these proteins have higher nutritional values compared to other plant sources [43, 53, 54]. They are often used as active pharmaceuticals ingredients for the treatment of heart disease and cancer [54]. In addition to that, large-scale cultures are used to extract innovative, high-value food additives from microalgae [55]. *Spirulina* is considered as a light-weight yet, high-value dietary supplement and thereby, consumed by astronauts in space [56]. Many breweries also use *Spirulina* as an ingredient to produce beers [54]. Furthermore, many cosmetics and skin products contain algae derived products for their application as antioxidants, thickeners and topical nutrient replenishment [42, 57].

### 1.2.3. Compound Sequestration and Chemical Analyses of Microalgae

Due to their ubiquity in large quantities, marine microalgae are major contributors to sequestration of inorganic compounds which serve as algal nutrients. Furthermore, due to their higher growth rate, algal biomass can double within hours which intensifies the compound sequestration process [46, 58, 59]. Among the sequestered compounds, carbon in form of CO$_2$ is the primary nutrient which cells utilize during the photosynthetic process [60-63]. Previous studies indicate that approximately 1.83 kg CO$_2$ can be sequestered by 1.00 kg algal biomass [50, 64]. During the photosynthetic process, cells also uptake and utilize nitrogen, phosphorus and a small quantity of cations [27, 28, 65]. These nutrients play a key role in their metabolic activity including the formation of DNA, ATP and cell membrane [47, 66].

Due to their ability to sequester large quantity of inorganic compounds within a short time-scale, microalgae are used as a bioremediation tool to remove contaminants from industrial flue gases. Algal pond containing species tolerant to acidic pH condition (i.e. *Chlorella* and *Tetraselmis* sp.) can remove a large quantity of CO$_2$ and NO$_x$ from the flue gas [49, 64, 67]. The flue gases from fossil fuel plant act as CO$_2$ sources for these microalgae cultures [68]. The algal biomass results out from the biosequestration process is used for biofuel and food application [50, 64]. Microalgae is also used as a sustainable tool for removing pollutants from industrial wastewater [69]. Algae growing in wastewater ponds have been tested for their CO$_2$ abatement ability [70]. The photobioreactor treatment process can efficiently remove carbon, nitrogen, phosphorus and organic pollutants from
the wastewater [71, 72]. The bioremediation process can be improved by regulating species-specific growth parameters such as light intensity, nutrient concentration, temperature, pH, salinity, and the culturing process [47, 73-78].

In order to promote the natural sequestration process, distributing iron to ocean surface layers has been proposed [79]. However, this approach is highly controversial as harmful algal blooms (HAB) may result from over-fertilization in agricultural areas [80]. HAB is responsible for interfering with the marine biosphere in particular marine mammals, sea-turtles, and sea-birds [34]. Moreover, HAB is responsible for releasing toxins that can destroy the aquatic lifecycle. These examples underline the importance of understanding chemical interactions between live microalgae and their chemical ambience [81].

The chemical interaction between microalgae ↔ environment can be probed by many bioanalytical techniques. These analytical methods are primarily focused on gaining insights on microalgae’s internal response to surrounding chemical concentration. A common approach is to monitor the intracellular algal protein concentration and their quantitative response to external nutrient concentration. For such studies, different spectroscopic and chromatographic techniques have been employed to investigate microalgae cultures [82-84]. Fourier Transform Infrared Spectroscopy (FTIR) and Raman Spectroscopy are widely used for their high selectivity and sensitivity to microalgae cells [85-89]. Furthermore, microalgae’s adaptation of intracellular pigment quantity is often used to understand their response to surrounding environment [73, 76]. Fluorescence excitation emission spectroscopy is often used for this analysis due to excellent fluorescence properties of the pigments (i.e. chlorophyll, carotenoids, phycoerythrin) [90, 91]. Moreover, inductively coupled plasma optical emission spectroscopy (ICP-OES) and atomic absorption spectroscopy (AAS) can be used to monitor the trace quantity of heavy metals sequestered by algae cultures [92-94]. Furthermore, mass spectrometry is often used for qualitative and quantitative analysis of cells’ internal lipid concentration [93, 95, 96].

The spectroscopic method is often combined with liquid and gas chromatographic techniques for separating the intracellular compounds. Furthermore, microalgae biodiversity has been determined by analyzing sequences of nucleic acids extracted from the cells [97, 98]. While this technique can detect a specific organism, designing probes for numerous species is time and cost intensive. Microscopic image analyses of size, shape and quantity of the algal cells are often used to understand phytoplankton’s
response to chemical environment [85, 94, 99]. Transmission and fluorescence microscopy are often used for in-vivo analysis of algal cell exposed to inorganic compounds [99, 100]. Cytometric analysis can also provide information on the transformation of carbon and other inorganic compounds into biomass [101-103].

While these analytical tools can gain insight on phytoplankton’s interaction with their surrounding chemical environment within the entire culture, they do not shed a light on the microscopic chemical changes around the cell. This is particularly important because, algal cells are of microscopic size (1-20 µm) [104]. Therefore, their compound uptake and utilization are restricted within the microscopic locality around the cells. Due to the limited spatial resolution, experimental techniques cannot probe cells’ microscopic vicinity. On the other hand, modeling approach can investigate cells’ microscopic surrounding with required spatial resolution. For this reason, in-silico computation modeling approach has gained a lot of interest among researchers to explore compound sequestration process.

Modeling of CO₂ sequestration into geological reservoirs [105, 106] has been investigated to assess their capacity to store CO₂ produced in industrial processes. Furthermore, release of liquefied CO₂ into deep ocean and its stabilization by limestone particles has been modeled [107]. However, these pilot studies were limited to small quantities of CO₂ removal only by geosequestration process. Modeling of microalgae’s nutrient uptake and biomass production for wastewater treatment has been performed [70]. Moreover, Algal growth in a photobioreactor containing flue gas has been modeled for pilot plant [68]. Nonetheless, most of these modeling studies were driven by engineering and economical aspects rather than environmental concerns.

1.3.  Dissertation Outline

The goal of this dissertation is to understand phytoplankton’s sequestration of anthropogenic pollutants by linking all the interactions between microalgae and their environment. This enables an accurate understanding of the in-situ compound sequestration process as previous studies were restricted to a limited number of interactions. This dissertation is focused on two groups of anthropogenic pollutants: greenhouse gas CO₂ and heavy metals. The quantitative sequestration of the greenhouse gas and heavy metals by microalgae were analyzed by linking computational tools and
experimental analysis. In broader sense, understanding the sequestration of these pollutants can accurately predict the impact of future environmental changes on the ecosystem.

The second chapter of this dissertation outlines the development of a novel modeling framework to describe the transformation of inorganic carbon into microalgal biomass. This enables probing cells’ surrounding environment with microscopic spatial resolution, which conventional experiments cannot access by a straightforward method. For this reason, computational hard modeling approach was implemented first, to create an artificial ecosystem containing algal cells. Then, cells’ species-specific carbon nutrient uptake and their utilization for biomass production were predicted by ‘concentration field’ model. In order to assess the impact of future environmental scenarios, the simulations were performed as a function of atmospheric CO$_2$ concentration. Furthermore, the model was trained to investigate the impact of nutrient competition among multiple species.

Chapter 3 explains the validation of the model developed previous chapter. Since, phytoplankton’s surrounding carbon concentration cannot be experimentally measured for millions of cells, validation was performed through biomass quantification. For testing the model, two microalgae species Dunaliella salina and Nannochloropsis oculata were cultured individually and in binary mixtures at different atmospheric CO$_2$ concentrations. The biomass concentration was measured by flow cytometry technique over the course of several days. The experimentally measured and model predicted cell concentration were compared to assess the validity of the model. The comparison demonstrated an excellent agreement between the model and the experiments. Once validated, two applications of this robust model were presented to predict the effect of long-term CO$_2$ sequestration capacity and the impact of ocean acidification on biomass production.

Chapter 4 depicts an application of the ‘concentration field’ model. In this work package, the quantities of inorganic carbon consumed by different algal species were predicted and compared. The benefits of this modeling are bidirectional. First, by keeping the initial culturing conditions similar, the simulations provided a framework to compare carbon sequestration by different species. Secondly, it gained insights on the quantitative effect of nutrient competition on inorganic carbon capture. Interestingly, it was found that, some algal species may even invert their sequestration capacity when competition effect exists among multiple species.
The following Chapter 5 attempts to assess microalgae’s ability to sequester atmospheric CO$_2$. Microalgae’s carbon sequestration process is simultaneously influenced by many different physical, chemical and biological parameters. Therefore, in order to understand their ability to capture carbon, cumbersome experiments are required to analyze these parameters at the same time. This section of the dissertation proposed a novel indicator, namely intracellular β-carotene quantity, for assessing the CO$_2$ sequestration capacity of *Dunaliella salina*. The intracellular β-carotene quantity was measured by a combination of fluorescence spectroscopy and flow cytometry technique. By relating cells’ internal response (carotenoid) to external chemical changes (carbon concentration), this novel approach provides a comprehensive yet straightforward indicator to assess microalgae’s ability to sequester CO$_2$.

Chapter 6 discusses the quantitative uptake of heavy metals (copper and nickel) by marine phytoplankton *Nannochloropsis oculata*. For this purpose, algae cultures were exposed to different concentrations of copper and nickel. The uptake of heavy metal by algal cultures were measured over the course of several days by inductively coupled plasma optical emission spectroscopy (ICP-OES). At the same time, the impact of copper and nickel on microalgal growth were assessed by flow cytometry. Furthermore, scanning electronic microscopy (SEM) imaging were used to illustrate intracellular damages cells suffer due to heavy metals.

Ultimately, the aim of this dissertation is to understand the sequestration of anthropogenic pollutants by microalgae. This was accomplished by linking the dynamic adaptation of algal cells to the changing pollutant environment. Understanding microalgae’s sequestration performance will also open new opportunities to predict future scenarios and long-term impacts of climate change.
Chapter 2. Modeling the Transformation of Atmospheric CO$_2$ into Algal Biomass

This chapter is based on a publication by Mohammed F. Hasan and Frank Vogt:


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This author’s primary contribution to above publication includes: i) development of the problem into a work package, ii) development of the computational model, iii) designing and conducting the experiments, iv) process and analysis of the simulated and experimental data, v) most of the writing.

2.1. Introduction

Due to modern industrialization, the release of anthropogenic CO$_2$ has been considerably increased [108] and the fate of this inorganic pollutant has become a major concern [109]. On the other hand, ubiquitous marine phytoplankton counterbalance this greenhouse gas via its uptake as nutrient and is responsible for about half of the global primary carbon production [30-41]. Therefore, it is crucial to quantitatively understand the transformation of atmospheric CO$_2$ into phytoplankton biomass. It has been found that, phytoplankton cells’ surrounding chemical environment effects the biomass production and therefore, the amount of fixed CO$_2$ [60-63]. Therefore, in order to quantify the transformation process, the surrounding chemical environment and its interaction with the phytoplankton need to be investigated simultaneously.

Marine microalgae-based CO$_2$ sequestration occurs via the uptake of HCO$_3^-$ in following pathway [33]: $\text{CO}_2\, (g) \rightarrow \text{CO}_2\, (aq) + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$. Therefore, analysis of HCO$_3^-$ concentrations around the algal ecosystem would enable the quantitation of CO$_2$ sequestration. However, experimental measurement of HCO$_3^-$ concentration around algal cells cannot be studied straightforwardly because ecosystem often consists of $10^4$-$10^7$ cells per mL. Furthermore, cells (1-10 µm [104]) in ecosystem can only access to their immediate, microscopic surrounding for HCO$_3^-$ uptake and thereby create an inhomogeneous concentration gradient around them. Therefore, it is compulsory
to probe the $\text{HCO}_3^-$ concentrations around each cell with microscopic spatial resolution which is experimentally very complicated and expensive. Modeling method, on the other hand, can quantitatively describe the cells’ interaction with their chemical surrounding with microscopic spatial resolution. By linking all the chemical interactions involved in the sequestration process, computational models can quantitatively predict the quantity of fixed CO$_2$ and their transformation into algal biomass. From an analytical perspective, such an integrated approach can provide a novel framework to investigate this experimentally inaccessible environmental system.

This chapter presents the development of modeling methodologies for predicting microalgae-based CO$_2$ sequestration and its transformation into algal biomass. These innovative modeling approaches are based on describing all the steps in the sequestration process including, CO$_2$ dissolution, its transport to consumers as well as species specific nutrient uptake, nutrient competition among different species and utilization for biomass production [110, 111]. With the aim to understand the impact of future environmental changes on sequestration process, the model predicted biomass production is presented as a function of atmospheric CO$_2$ concentration. Since, the model links the chemical environment ($\text{HCO}_3^-$) of the macroscopic ecosystem to the produced biomass, this procedure also enables determining a $\text{HCO}_3^-$ distribution, which itself is difficult to probe, via a property that can easily be measured. Therefore, model validation has been accomplished by comparing model predicted cell concentrations with experimental flow cytometry measurements (explained in Chapter 3). Since the cells respond to their micro-environment as opposed to culture-average concentrations, the approach presented here gains a much more accurate assessment of the true conditions the cells experience. Therefore, quantitative statements derived by means of this modeling approach are more realistic and accurate. As an additional aspect, these microalgae species were cultured individually as well as together which then enabled investigating competition impacts on the biomass production.

2.2. **Theory of the Modeling**

In order to implement a modeling tool for describing the microalgae-based CO$_2$ sequestration, all the steps leading from atmospheric CO$_2$ to its accumulation into algal biomass need to be linked together. For this reason, the methodology 'concentration field'
$c(x,t)$ (unit: milli-molarity, mM) has been introduced. The concentration field is a space- and time- dependent term describing the distribution of $\text{HCO}_3^-$ concentration within an ecosystem. The motivation behind this approach is, the concentration of $\text{HCO}_3^-$ links the fate of atmospheric $\text{CO}_2$ and its uptake by algal cells to produce biomass. The concentration field is determined by four terms:

i) A ‘compound source’ $S(x,t)$ from the dissolution of atmospheric $\text{CO}_2$ into aqueous phase and its subsequent conversion into $\text{HCO}_3^-$ formation within an ecosystem

ii) A ‘compound transport’ $T(x,t)$ which comprises diffusion of $\text{HCO}_3^-$ within an aqueous ecosystem

iii) A ‘compound drain’ $D(x,t)$ describing microalgae’s uptake of $\text{HCO}_3^-$ out of the concentration field $c(x,t)$ to produce biomass. $D(x,t)$ needs to reflect that every microalgal species has specific compound uptake characteristics which may depend on $c(x,t)$ itself and potentially on the presence of competing species.

iv) A ‘nutrient utilization’ term describing how microalgae cells utilize the sequestered compound to produce new biomass. This is also a species- and concentration- dependent parameter which links the $\text{HCO}_3^-$ concentration with the produced biomass.

Among these terms, i) and ii) model the pathway by which atmospheric $\text{CO}_2$ is distributed within the ecosystem and communicate with the algal cells. Terms iii) and iv) represent the transformation of inorganic compound into algal biomass. The space- and time- dependent concentration field has been expressed by following 3-dimensional partial differential equation (PDE) which needs to be solved numerically:

$$\frac{\partial c(x,t)}{\partial t} = S(x,t) + T(x,t) + D(x,t)$$

(1)

2.2.1. Compound Source Term

The source function $S(x,t)$ quantitatively describes how atmospheric $\text{CO}_2$ is dissolved in the seawater medium to form $\text{HCO}_3^-$. In a first step, Henry’s law describes a thermodynamic equilibrium between gas and aqueous phase and therefore, determines
the quantity of CO$_2$(aq) that dissolves into seawater (CO$_2$(g) ↔ CO$_2$(aq)). From CO$_2$(aq), carbonic acid (H$_2$CO$_3$) forms which partially dissociates in water resulting in bicarbonate (HCO$_3^-$) which in turn produces a small quantity of carbonate ions (CO$_3^{2-}$). As will be discussed in the remainder of this section, the most relevant and stable product formed in this sequence of reactions is HCO$_3^-$.

$$CO_2(g) \leftrightarrow CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- \leftrightarrow H^+ \leftrightarrow CO_3^{2-} + 2 H^+$$

Quantitating the transformation of CO$_2$(g) into HCO$_3^-$ requires a detailed analysis. First, the partial pressure of CO$_2$ in the atmosphere (in atm) needs to be determined:

$$pCO_2 = xCO_2 \cdot (P - pH_2O) \cdot 10^{-6}$$

x CO$_2$ represents is the mole fraction of CO$_2$ in the atmosphere and P the total atmospheric pressure in atm (here: 1.0 atm). Since, CO$_2$ exchange occurs at the air-water interface, saturation vapor pressure of water, $pH_2O$ (atm) needs to be subtracted from the total pressure. Saturation water vapor pressure can be calculated by [112]:

$$\ln(pH_2O) = 24.4543 - \frac{6745.09}{T} - 4.8489 \cdot \ln\left(\frac{T}{100}\right) - 0.00054 \cdot S$$

T represents the temperature of the air-water interface in Kelvin and S denotes the seawater salinity in g/kg. Since, CO$_2$ is not an ideal gas, its fugacity $fCO_2$ (in atm) needs to be calculated from its partial pressure and ideal gas constant R [113]:

$$fCO_2 = pCO_2 \cdot \exp\left(P \cdot \frac{B + 2\delta}{R \cdot T}\right)$$

For calculating $fCO_2$, B and $\delta$ (both in m$^3$·mol$^{-1}$) are required which denote the virial coefficient and the cross-virial coefficient, respectively [114]:

$$B = (-1636.75 + 12.0408 \cdot T - 3.27957 \cdot 10^{-2} \cdot T^2 + 3.16528 \cdot 10^{-5} \cdot T^3) \cdot 10^{-6}$$

$$\delta = (57.7 - 0.118 \cdot T) \cdot 10^{-6}$$

After calculating fugacity, the CO$_2$ concentration (mol/kg) dissolved in seawater can be calculated from $[CO_2] = K_0 \cdot fCO_2$ with $K_0$ (mol/kg-atm) denoting CO$_2$ solubility coefficient which has been determined in [114] by:

$$K_0 = 10^{-6} \cdot \exp\left(\frac{9345.17}{T} - 60.2409 + 23.3585 \cdot \ln\left(\frac{T}{100}\right) + S \cdot (0.023517 - 2.3656 \cdot 10^{-4} \cdot T + 4.7036 \cdot 10^{-7} \cdot T^2)\right)$$
Dissolved CO$_2$(aq) is in equilibrium with HCO$_3^-$ and CO$_3^{2-}$ according to:

$$\text{CO}_2(aq) + H_2O \overset{k_1}{\rightleftharpoons} \text{HCO}_3^- + H^+$$

$$\text{HCO}_3^- \overset{k_2}{\rightleftharpoons} \text{CO}_3^{2-} + H^+$$

The equilibrium constants $k_1 = \frac{[\text{HCO}_3^-][H^+]}{[\text{CO}_2]}$ and $k_2 = \frac{[\text{CO}_3^{2-}][H^+]}{[\text{HCO}_3^-]}$ are temperature $T$ ($= 296K$) and salinity $S = \frac{g \text{ salt}}{kg \text{ seawater}}$ dependent functions (in mol/kg) and have been determined in [115] as:

$$\ln(k_1) = 2.83655 - \frac{2307.1266}{T} - 1.5529413 \cdot \ln(T) - \left(0.207608410 + \frac{4.0484}{T}\right) \cdot \sqrt{S} + 0.0846834 \cdot S - 0.00654208 \cdot S^{3/2} + \ln(1 - 0.001005 \cdot S)$$

$$\ln(k_2) = -9.226508 - \frac{3351.6106}{T} - 0.2005743 \cdot \ln(T) - \left(0.1069018 + \frac{23.9722}{T}\right) \cdot \sqrt{S} + 0.1130822 \cdot S - 0.00846934 \cdot S^{3/2} + \ln(1 - 0.001005 \cdot S)$$

$[H^+]$ (in mol/kg) can be determined from the pH (~8.2; buffered) and density of the seawater solution ($= 1.026 \frac{kg}{L}$). With [CO$_2$], [H$^+$], and $k_1$ known, $[\text{HCO}_3^-]$ (in mol/kg) can be calculated:

$$[\text{HCO}_3^-] = \frac{k_1 \cdot [\text{CO}_2]}{[H^+]}$$

Finally, gravimetric concentration units (mol/kg) are converted to millimolar (mM) through the density of seawater (kg/L). In this modeling application, this concentration of bicarbonate, $[\text{HCO}_3^-]$ (in mM) is used as the source function, $S(x, t)$. For completeness purposes, the carbonate ion concentration can be determined, too:

$$[\text{CO}_3^{2-}] = \frac{k_2 \cdot [\text{HCO}_3^-]}{[H^+]}$$

In order to simulate the real-world scenario, the source $S(x, t)$ is assumed to be spatially restricted to the interface between the atmosphere and the seawater surface. Therefore, $S(x, t)$ has been expressed as a Dirichlet boundary condition $S(x_{\text{top surface}}, t) = c(x_{\text{top surface}}, t)$. The atmospheric CO$_2$ is assumed to be continuously introduced in the aqueous medium only through this air-water interface. For the remaining
five walls of the ecosystem, \( S(x_{\text{walls except top}}, t) = 0 \) and thus Neumann boundary conditions \( \frac{\partial c(x,t)}{\partial x} \bigg|_{\text{walls except top}} = 0 \) were implemented.

### 2.2.2. Compound Transport Term

Compound transport within an ecosystem is governed by both diffusion and advection. The diffusion mechanism is defined by Fick’s second law, i.e. \( \frac{\partial c(x,t)}{\partial t} |_{\text{diffusion}} = \kappa \cdot \nabla^2 c(x,t) \). The diffusion coefficient \( \kappa \) of HCO\(_3\) as determined in [116] has been utilized in this study. In this modeling application, the diffusion coefficient \( \kappa \) is assumed to be independent of temporal and spatial factors. Mass transport due to advection is described by \( \frac{\partial c(x,t)}{\partial t} |_{\text{advection}} = \nabla \cdot (v(x,t) \cdot c(x,t)) \) where \( v(x,t) \) is the velocity of the medium. The net compound transport is a combination of these two mechanisms i.e. \( T(x,t) = \frac{\partial c(x,t)}{\partial t} |_{\text{diffusion}} + \frac{\partial c(x,t)}{\partial t} |_{\text{advection}} \). However, for this study, only diffusion has been considered since the cell cultures for model validation (in Chapter 3) were not mechanically moved and were maintained at a homogenous temperature. In conclusion:

\[
T(x,t) = \frac{\partial c(x,t)}{\partial t} |_{\text{diffusion}} = \kappa \cdot \nabla^2 c(x,t)
\]

(2)

### 2.2.3. Compound Drain Term

In order to produce another microalgae cell, a cell located at \( x_0 \) requires nutrients which it drains from the concentration field \( c(x_0, t) \) immediately surrounding it. Many quantitative models have been proposed for describing this phytoplankton based nutrient uptake for cell production [117-126]. For this modeling application, the classic Michaelis-Menten kinetics (MM) [120] has been chosen to characterize the nutrient uptake mechanism. MM describes a cell’s nutrient uptake rate as \( u_R = \frac{U_{\text{max}} \cdot c(x_0, t)}{\alpha + c(x_0, t)} \) with \( U_{\text{max}} \) denoting the maximum uptake rate and \( \alpha \) representing the cell’s nutrient affinity (or: ‘half-saturation constant’) [127]. When, over time, cells are being produced at \( x_0 \), a total of \( N(x_0, t) \) cells results which deplete the concentration field according to \( \frac{dc(x_0, t)}{dt} = -u_R \cdot \).
\[
N(x_0, t) = - \frac{U_{\text{max}} \cdot c(x_0, t)}{\alpha + c(x_0, t)} \cdot N(x_0, t) = D(x_0, t)
\]
and thereby establish the drain term for a single species culture which can be expanded to describe the drain performed by multiple species.

The relationship between nutrient concentration and its transformation into algal biomass was proposed in Monod model [121]. In order to describe the cell production, i.e. \( \frac{dN(x_0, t)}{dt} \), Monod introduced a ‘nutrient efficiency’ \( \beta \) as the number of cells produced per unit of nutrient taken up. Thus, \( N(x_0, t) \) cells consume the resource at rate \( u_R \cdot N(x_0, t) \) and therefore, the cell count increases by \( \frac{dN(x_0, t)}{dt} = \beta \cdot u_R(t) \cdot N(x_0, t) \).

Expanding these two considerations to \( Q \) species located at \( x_0 \), all of which simultaneously drain \( c(x_0, t) \), leads to a system of \( Q + 1 \) ordinary differential equations (ODEs):

\[
D(x_0, t) = \frac{dc(x_0, t)}{dt} = - \sum_{q=1}^{Q} \frac{U_{\text{max}_q} \cdot c(x_0, t)}{\alpha_q + c(x_0, t)} \cdot N_q(x_0, t)
\]

\[
\frac{dN_q(x_0, t)}{dt} = \beta_q \cdot \frac{U_{\text{max}_q} \cdot c(x_0, t)}{\alpha_q + c(x_0, t)} \cdot N_q(x_0, t)
\]

(3)

Fusing the top equation of (3) and (2) into (1) together with the source term \( S(x, t) \) describes how the concentration field at \( x_0 \) changes over time. Since the considerations leading to (3) are valid for any \( x_0 \), they are valid at all \( x \). Thus, (4, top) describes how atmospheric \( \text{CO}_2 \) is sunk into an ecosystem. The equation (4, bottom) explains for all locations \( x \) the utilization of this nutrient to produce, over time, \( N_q=1, \ldots, Q \) cells of \( Q \) microalgal species. Integration of the concentration field and biomass produced after nutrient utilization together into (4) allows the quantitative analysis of the sequestration process.

\[
\frac{\partial c(x, t)}{\partial t} = S(x, t) + \kappa \cdot \nabla^2 c(x, t) - \sum_{q=1}^{Q} \frac{U_{\text{max}_q} \cdot c(x, t)}{\alpha_q + c(x, t)} \cdot N_q(x, t)
\]

\[
\frac{\partial N_q(x, t)}{\partial t} = \beta_q \cdot \frac{U_{\text{max}_q} \cdot c(x, t)}{\alpha_q + c(x, t)} \cdot N_q(x, t) \quad \text{with: } q = 1, \ldots, Q
\]

(4)
The initial conditions for (4) at $t = 0$ are:

$$\frac{\partial c_0(x,0)}{\partial t} = S(x, 0) + \kappa \cdot \nabla^2 c_0(x, 0)$$

$$\frac{\partial N_q(x,0)}{\partial t} = \text{experimental cell concentration at } t = 0$$

In conclusion, solving the system of $Q + 1$ partial differential equations (4) with initial conditions models the transformation of atmospheric $CO_2$ into microalgal biomass.

2.3. Programming and Implementation of Concentration Field

The concentration field model in (4) represents a dynamic system consisting of physical, chemical and phycological parameters influencing the $CO_2$ sequestration process. In order to approximate the solution of the system of PDEs in (4), numerical methods have been employed. For this modeling application, the simplest numerical approximation method i.e. Euler’s method has been chosen which can approximate (4) with known initial conditions. The principle of the Euler’s method is highlighted in following section.

In order to construct the numerical approximation using Euler method, a time-dependent ordinary differential equation of $y$ variable and known initial condition can be considered in the following equation.

$$\frac{dy}{dt} = f(y, t)$$

$$y_0 = y(t_0)$$

The forward Euler method (also, known as an explicit Euler method) for above equations can be derived from the forward finite difference approximation at $n$-th time point:

$$\frac{dy_n}{dt} = f(y_n, t_n) \approx \frac{y_{n+1} - y_n}{h}$$

$$\rightarrow y_{n+1} = y_n + h \times f(y_n, t_n)$$

Here, $h$ represents the uniform step size of $t$, i.e. $t_{n+1} - t_n$. The step size $h$ is very crucial for the proximity of the true solution. The approximation of the Euler method is
more accurate as the step size becomes smaller. The smaller step size reduces the difference between the numerical solution and the exact solution which is also known as local truncation error (LTE). The local truncation error is approximately proportional to \( h^2 \). However, the large number of steps of smaller size encompasses a higher computational cost.

From the aforementioned equation, as time \( t \) advances from its known initial condition \( t_0 \), the approximation of \( y \) at any \( n \)-th time point propagates as following:

\[
y_1 = y_0 + h \times f(y_0, t_0)
\]
\[
y_2 = y_1 + h \times f(y_1, t_1)
\]
\[
y_3 = y_2 + h \times f(y_2, t_2)
\]
\[
\vdots
\]
\[
y_n = y_{n-1} + h \times f(y_{n-1}, t_{n-1})
\]

From the first equation, \( y_1 \) can be calculated as the right-hand side consists of all known parameters. Recursively, the following \( y \)-values can be calculated from immediate previous \( y \)-iteration and the numerical solutions \( (y_n, t_n) \) up to \( n \)-th point can be approximated. A similar approach, i.e. Backward Euler or implicit Euler method is based on backward finite difference approximation which follows a similar scheme with final form:

\[
y_n = y_{n-1} + h \times f(y_n, t_n)
\]

Note that, unlike explicit Euler, the solution for \( y_n \) in implicit Euler depends on the function \( f(y_n, t_n) \). For this reason, implicit methods require one additional computation step and thereby, numerically more intensive compared to explicit Euler. However, both methods are numerically stable for approximating the numerical solution of a differential equation.

In this modeling application, two \( y \)-terms represent the concentration of \( \text{HCO}_3^- \) (top in (4) ) and biomass produced (bottom in (4) ) over time and space. Due to the complex and non-linear features of (4), a strong and powerful differential equation solver is required which enables the modeling of a large-scale macroscopic ecosystem with microscopic spatial resolution. For this reason, ‘Portable, Extensible Toolkit for Scientific Computation’ (PETSc) has been chosen to model concentration field [128, 129]. PETSc can solve data intensive scientific problems governed by PDEs on high performance computers. PETSc offers parallelization by means of message-passing communication
which enables the use of multi-core CPU machines. This is particularly effective for large-scale ecosystem modeling in order to maintain a reasonable computation time. Furthermore, the PDE solver library of PETSc is capable of using both implicit and explicit methods to handle stiff and non-stiff differential equations. PETSc handles large scale linear and nonlinear problems through the ‘Krylov Space method’ (KSP) and the ‘simplified nonlinear equation solver’ (SNES), respectively.

The application codes for PETSc can be written in many programming languages including, Fortran, C, C++ and Python. For this modeling application, C/C++ has been chosen for two reasons: First, compilers and math libraries are all available for C free of cost. Secondly, the standardize communication protocol ‘Message Passing Interface’ (MPI) which is a crucial component for parallel processing in PETSc has been written in C and can seamlessly be incorporated into C-based code [130-132].

In order to implement the concentration field first, an ‘artificial ecosystem’ representative of the macroscopic ecosystem needs to be modeled. The artificial ecosystem as shown in Figure 1 consists of cuboid ‘blocks’ with a uniform, microscopic spatial resolution along each direction which is referred as grid.

Prior to solving the system of PDEs, the artificial ecosystem needs to be initialized by the concentration field \( c(x, t) \) and biomass quantity. For initiating concentration field \( c(x, t) \), first the artificial ecosystem is assumed to be void of any \( \text{HCO}_3^- \). Then the atmospheric \( \text{CO}_2^{(g)} \) concentration above the ecosystem was set to a user selected level and ‘turned on’. For this modeling application, following range of \( \text{CO}_2 \) concentration was chosen: 300 ppm, 350 ppm, 400 ppm, 450 ppm and 500 ppm. These concentrations will respectively simulate the preindustrial, current and potential future \( \text{CO}_2 \) concentration in the atmosphere. After ‘turning on’, the atmospheric \( \text{CO}_2 \) partitions into the aqueous phase through the gas-liquid interface (top boundary) and enters within the artificial ecosystem. The top boundary, therefore, acts as the source of \( \text{HCO}_3^- \), namely \( S(x, t) \), for the entire concentration field. The aforementioned KSP solver is then applied to determine a steady-state solution of the diffusion equation \( \frac{\partial c(x, t)}{\partial t} = \kappa \cdot \nabla^2 c(x, t) \). This steady-state solution serves as the initialization of the concentration field, i.e. \( c(x, t = 0) \).
Figure 1: Cuboid ‘block’ representing the artificial ecosystem with equally spaced grid points along each direction.
Prior to solving the concentration field model in (4), a preliminary assessment of the model’s accuracy can be performed. For this reason, the model predictions were simulated at a certain atmospheric CO$_2$ concentration in an artificial ecosystem containing no algal cells, i.e. $D(x, t = 0)$. This simulation represents the steady-state HCO$_3^-$ concentration only due to diffusion at a certain physical and chemical conditions which was previously determined by well-known experiments [133]. The model predicted HCO$_3^-$ concentration at all atmospheric CO$_2$ conditions are compared against the corresponding theoretical values in Table 1. It has been found that, the HCO$_3^-$ concentration predicted by this model are ~98% accurate compared to the theoretical values.

For testing the concentration field model, first an artificial ecosystem with a grid size of $x \times y \times z = 128 \times 128 \times 32$ was assumed. The spatial resolution of the ecosystem was set 100µm along each direction. Then, a fixed number of algal cells ($10^9$) were inoculated only at a certain point ($x \times y \times z = 56 \times 64 \times 16$), and bicarbonate concentration as well as biomass population were predicted by the concentration field model (4). Figure 2(A) represents the colored map of bicarbonate concentration after 15 days at $z = 16$ plane where cells were inoculated within the 3D ecosystem. Figure 2(B) illustrates the concentration depletion around the cell location in 3D view with a projection on the top.

In order to ensure a reliable comparison between model predicted and experimentally measured cell concentration, the initial biomass quantity in both approaches needs to be distributed in the same way. Otherwise, any difference between them would amplify over time and thereby, model validation step will fail. For this reason, the first experimental cell concentration at $t = 0$ is chosen to be the ‘inoculation cell concentration’ for the modeling. In simulations, these cell number is homogeneously distributed among all grid points. However, compared to an ‘inner’ grid point, grid points on a container wall only got half that number assigned, grid points on a container edge a quarter, and corner grid points one eighth. Experimentally, a homogeneous distribution of $N_{q=1,...,Q}(x, t = 0)$ has been ensured by swirling the Erlenmeyer right after inoculation.
Table 1: Comparison between model predicted $\text{HCO}_3^-$ concentrations and corresponding theoretical values at different atmospheric $\text{CO}_2$ conditions.

<table>
<thead>
<tr>
<th>Atmospheric $\text{CO}_2$ concentration (ppm)</th>
<th>Concentration Field Model Predicted $\text{HCO}_3^-$ Concentration (mM)</th>
<th>Theoretical $\text{HCO}_3^-$ Concentration (mM)</th>
<th>Accuracy of the model (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>1.88831</td>
<td>1.92813</td>
<td>97.93</td>
</tr>
<tr>
<td>350</td>
<td>2.20303</td>
<td>2.24949</td>
<td>97.93</td>
</tr>
<tr>
<td>400</td>
<td>2.51774</td>
<td>2.57084</td>
<td>97.94</td>
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<td>450</td>
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<td>2.89220</td>
<td>97.92</td>
</tr>
<tr>
<td>500</td>
<td>3.14718</td>
<td>3.21355</td>
<td>97.77</td>
</tr>
</tbody>
</table>
Figure 2: Model predicted $\text{HCO}_3^-$ concentration (mM) within an artificial ecosystem. (A) represents a $(x \times y \times z) = (128 \times 128 \times 32)$ rectangular 3D block with algae cells inoculated at $(x \times y \times z) = (56 \times 64 \times 16)$. (B) depicts the $\text{HCO}_3^-$ concentration depletion around the inoculated cells.
Once the boundary conditions are implemented and model parameters are initialized, solutions of PDE system in (4) are calculated using PETSc’s time-stepper (TS) library augmented by SUNDIALS. The model has been considered as computationally more expensive ‘stiff’ problem for which the step size (here, space and time) needs to be extremely small for a stable numerical solution. For this reason, the model predicted $\text{HCO}_3^-$ concentration and the produced biomass were calculated with a time step of 1.8 seconds. Furthermore, backward Euler method (implicit) has been utilized for solving the PDE system. The Jacobian matrices for both differential equations were appropriately calculated and provided to TS library.

The software package was written into two layers: i) a generic core for the developers; and, ii) an application layer for the end-users. The core layer develops a generic modeling setup and explicitly describes all the hardcore functions for solving the concentration field model. Dividing the software package into the core and application layers would simplify and optimize the implementation process, because, the core only needs to be developed once. The application layer on the other hand, contains a number of variables including boundary conditions, physical and chemical parameters, etc. This software philosophy in conjunction with examples/tutorials for the application layer will also support dissemination.

The simulations were performed on a computer operating under CentOS 7 with two Intel Xeon processors E5-2650 (2x10 cores) and 128 GB RAM (Figure 3). For core-to-core and/or processor-to-processor communication, PETSc relies on the Message Passing Interface (MPI). For this reason, the openMPI version 1.10.0 were utilized in conjunction with the g++ compiler version 5.2.0.
Figure 3: Intel Xeon E5-2650 processors (20 cores) with 128 GB RAM used for the computation of concentration field model.
The concentration field has been solved on a grid size of \( x \times y \times z = 1024 \times 1024 \times 32 \) with a spatial resolution of 100\( \mu \)m in either direction. The volume of this artificial ecosystem is assumed to be equivalent of the experimentally realized culture volume for model validation. At a certain atmospheric \( \text{CO}_2 \) concentration, the simulations were performed to predict the \( \text{HCO}_3^- \) concentration and the biomass quantity for \( t = 15 \) culture days\(^\ddagger\). The model predicted results were then compared against the experimentally measured data for the validation purpose.

2.4. **Conclusion**

This work package has developed a novel modeling framework to describe phytoplankton based \( \text{CO}_2 \) sequestration and predict the transformation of this greenhouse gas into algal biomass. The modeling comprises of several steps, i.e. \( \text{CO}_2 \) partitioning from the atmosphere into marine ecosystem, its conversion into \( \text{HCO}_3^- \), bicarbonate's transport from source to consumers, and species-specific compound uptake for biomass production. The dynamics of the sequestered \( \text{CO}_2 \) and produced biomass were predicted by creating a representative 3-dimensional 'artificial ecosystem' containing algal cells. The simulations were implemented at preindustrial, current and future atmospheric \( \text{CO}_2 \) concentrations by means of a numerical differential equation solver (PETSc). Since, this integrated *in-silico* approach investigates phytoplankton's dynamic adaptation to chemical environments with a microscopic spatial resolution, the model predictions are expected to be more comprehensive and accurate than cumbersome experimental approach.

\(^\ddagger\) This only indicates the time duration of the simulated culture within the artificial ecosystems; not the computation time required for simulation in real-world time scale.
Chapter 3. Culturing Microalgae and Experiments for Model Validation

This chapter is based on a publication by Mohammed F. Hasan and Frank Vogt:


DOI: 10.1039/C7AN01054K.

This author's primary contribution to above publication includes: i) development of the problem into a work package, ii) development of the computational model, iii) designing and conducting the experiments, iv) process and analysis of the simulated and experimental data, v) most of the writing.

3.1. Introduction

With an increase in atmospheric CO$_2$ level, phytoplankton-based CO$_2$ sequestration has gained attention among the ecology research community, environmental engineers, and governmental leadership. Microalgae are expected to play an important role in CO$_2$ mitigation since half of the global primary carbon production is due to algal photosynthesis [30-41]. While Chapter 2 of this dissertation develops simulation methodologies for predicting future environments, it is crucial to demonstrate and assess these models' accuracy and precision. To this end, Chapter 3 is focused on experimental validation of the modeling methods developed in Chapter 2.

Prior to modeling microalgae's compound uptake (drain $D(x,t)$), values for physiological parameters $\alpha$, $\beta$, and $U_{\text{max}}$ are required. However, these model-required parameters are unknown and hypothesized to depend on the cells' chemical environment i.e. HCO$_3^-$ concentration. In order to bridge this gap of knowledge, these values were determined experimentally by means of 'growth curves' [134]. For this reason, separate cultures were grown in a closed system under a series of fixed initial HCO$_3^-$ concentration. After obtaining cell counts over the course of several days, only the bottom equation of (4) were "fitted" by means of nonlinear least-squares regression to these data points and thereby derive values for $\alpha$, $\beta$, and $U_{\text{max}}$. Comparing these values as obtained from different chemical environments also derives insights in the ambience driving microalgae's
nutrient assimilation. Moreover, the concentration dependency of these parameters, i.e. $\alpha([\text{HCO}_3^-])$, $\beta([\text{HCO}_3^-])$, and $U_{\text{max}}([\text{HCO}_3^-])$ were estimated and hardwired into the concentration field model rather than constants.

Experimental validation of the concentration field $c(\mathbf{x}, t)$ (4) within a microscopic vicinity of a large number of phytoplankton cells ($10^5 - 10^7$ cells/mL) is very challenging. Therefore, simulations were validated indirectly by comparing model predicted biomass concentration, $N_q(t)$, and measured cell counts $n_q(t)$. If the model properly predicts experimental $n_q(t)$, the correctness of $c(\mathbf{x}, t)$ is implied as $N$ and $c$ are ‘entangled’ in the model comprising of (4). This work package has developed experiments to grow microalgae cultures under well-defined conditions and then applied flow cytometry technique to determine cell concentration, $n_q(t)$, derived under different chemical environments. These experimental findings were then compared to $N_q(t)$ reflecting the same ambient conditions. Comparing such experimental results to predicted data not only assesses the model’s validity but in turn also provides explanation of the experimental findings.

Once validated, two applications of the model have been presented. First, the model was used to realize the long-term trends in biomass production. This study will reveal whether or not a culture’s CO$_2$ sequestration capacity to produce biomass may slow down in the long-term. In addition, the model was utilized to assess the impact of ocean acidification on phytoplankton’s ability to sequester atmospheric CO$_2$.

### 3.2. Microalgae Culturing Techniques

For the validation of the concentration field model, two marine microalgae species were chosen, i.e. *Dunaliella salina* and *Nannochloropsis oculata*. These stock cultures were obtained from the UTEX Culture Collection of Algae at the University of Texas, Austin. *D. salina* is a well-characterized species ([135]) and *N. oculata* is known for its considerable chemical response to changing HCO$_3^-$ levels ([136], Figure 4). These two different microalgae species have been included into this study to demonstrate the model’s general validity as well as to investigate competition impacts on the biomass production.
Figure 4: Scanning Electron Microscopy (SEM) image of cells of *Nannochloropsis oculata.*
These algal species were cultured within Erlenmeyer flasks in ‘Enriched Seawater, Artificial Water’ (ESAW) growth medium. The ESAW growth medium recipe was prepared according to standard protocol [137, 138]. The reagents and their concentrations used for preparing 1-liter ESAW growth medium is listed in Table 2. These microalgae cultures were provided with different levels of $\text{HCO}_3^-$ in two approaches: i) by dissolving NaHCO$_3$ into the growth medium of cultures for estimating $\alpha, \beta, \text{ and } U_{\text{max}}$; ii) by flushing a certain CO$_2$ concentration into the headspace of cultures for model validation purpose. In order to avoid precipitation during the autoclave stage, the ESAW growth medium was buffered by Tris-HCl (~pH 8.2). The glassware, utensils and growth mediums used in the cell culturing step, requires sterilization prior exercise by using an autoclave. For this reason, they were wrapped in aluminum foils and labeled with autoclave tape to ensure the sterilization temperature. The autoclave cycle was performed at 121°C and 2.0 atm steam pressure with an exposure time of 40 minutes. After sterilization, the growth medium was cooled overnight followed by the addition of vitamin stock reagents (thiamin, biotin and vitamin B$_{12}$). Subsequently, the pH of the medium was confirmed (~pH 8.2) by using a Mettler Toledo Seven Compact pH meter. At this point, the ESAW medium was ready for culturing algae cells. The microalgae culturing setup for this application is shown in Figure 5.

For both model validation purpose and for estimating $\alpha, \beta, \text{ and } U_{\text{max}}$, the dynamics of biomass concentration of these cultures needs to be quantified. The cell concentrations in the resulting cultures were measured by means of flow cytometry (Guava easyCyte equipped with 488 nm laser). Both microalgae species show a unique red fluorescence which facilitated an accurate cell count. The clearly different sizes of $D. \text{salina}$ and $N. \text{oculata}$ ensured a reliable quantitation for both species even in mixture. Furthermore, six replicate flow cytometry data were extracted from the same culture and analyzed in order to assess the reproducibility of these cell counts. From these replicated analyses, the error bars shown in subsequent graphs comparing measured versus predicted cell counts were derived.
Figure 5: The microalgae culturing setup to ensure well-defined ambient conditions. (A) Opened growth cabinet where cultures are grown at different atmospheres. (B) Mass flow controllers for mixing synthetic air and CO₂ which create different atmospheres in the cultures’ headspace. (C) represents algae cultures growing under well-defined conditions. The violet and pink arrows indicate the nutrient replenishment via peristaltic pump and continuous supply of atmospheric CO₂ respectively. Not shown in the figure are departmental autoclaving facilities.
Table 2: List of reagents and their concentrations in the ESAW growth medium

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration in ESAW</th>
<th>Reagents</th>
<th>Concentration in ESAW</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (Fisher Scientific)</td>
<td>$6.04 \times 10^{-1}$ M</td>
<td>Na$_2$EDTA·2H$_2$O (Sigma Aldrich)</td>
<td>$6.56 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Na$_2$SO$_4$ (Fisher Scientific)</td>
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<td>FeCl$_3$·6H$_2$O (Fisher Scientific)</td>
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<td>KCl (Fisher Scientific)</td>
<td>$1.34 \times 10^{-2}$ M</td>
<td>ZnSO$_4$·7H$_2$O (Fisher Scientific)</td>
<td>$2.54 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>KBr (Fisher Scientific)</td>
<td>$1.21 \times 10^{-3}$ M</td>
<td>CoSO$_4$·7H$_2$O (Fisher Scientific)</td>
<td>$5.69 \times 10^{-8}$ M</td>
</tr>
<tr>
<td>H$_3$BO$_3$ (Fisher Scientific)</td>
<td>$6.19 \times 10^{-4}$ M</td>
<td>MnSO$_4$·4H$_2$O (Alfa Aesar)</td>
<td>$2.42 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>NaF (Fisher Scientific)</td>
<td>$1.11 \times 10^{-5}$ M</td>
<td>Na$_2$MoO$_4$·2H$_2$O (Acros Organics)</td>
<td>$6.12 \times 10^{-9}$ M</td>
</tr>
<tr>
<td>MgCl$_2$·6H$_2$O (Fisher Scientific)</td>
<td>$1.57 \times 10^{-1}$ M</td>
<td>Na$_2$SeO$_3$ (Sigma Aldrich)</td>
<td>$1.00 \times 10^{-9}$ M</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O (Fisher Scientific)</td>
<td>$3.05 \times 10^{-2}$ M</td>
<td>NiCl$_2$·6H$_2$O (Sigma Aldrich)</td>
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<td>SrCl$_2$·6H$_2$O (Acros Organics)</td>
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</tr>
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<td>Biotin (Fisher Scientific)</td>
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<tr>
<td>Na$_2$SiO$_3$·9H$_2$O (Sigma Aldrich)</td>
<td>$1.06 \times 10^{-4}$ M</td>
<td>Vitamin B$_{12}$ (Fisher Scientific)</td>
<td>$1.48 \times 10^{-9}$ M</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Varying (Fisher Scientific)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.1. Cultures for Determining Nutrient Uptake Characteristics:

Prior to modeling (4), values for the species-dependent nutrient uptake characteristics $\alpha$, $\beta$, and $U_{\text{max}}$ need to be determined experimentally. It has been hypothesized that these parameters depend on the species, on the $\text{HCO}_3^-$ concentration, and on the presence of nutrient competitors. For each species in individual and mixed cultures, these parameters’ values have been determined for a series of fixed initial $\text{HCO}_3^-$ concentration (0.518mM, 1.035mM, 1.553mM, 2.071mM, 2.919mM, 3.443mM) by means of growth curves (Figure 1 in [134]). For extracting $\alpha$, $\beta$, and $U_{\text{max}}$ from growth curves, a method has been employed which combines solving the ODE system (MM [120] and Monod [121] respectively):

$$\frac{dc(t)}{dt} = -\frac{U_{\text{max}} \cdot c(t)}{\alpha + c(t)} \cdot N(t)$$

$$\frac{dN(t)}{dt} = \beta \cdot \frac{U_{\text{max}} \cdot c(t)}{\alpha + c(t)} \cdot N(t)$$

This approach is based on nonlinear least-squares regression step that estimates values for $\alpha$, $\beta$, and $U_{\text{max}}$ such that the numerical solution of the ODE system is optimum in a least-squares sense. Nonlinear least-squares regression enables computing fit parameters that carry chemical information. In the aforementioned system of ODEs, the only accessible information is the cell concentration $N(t)$ from growth curves and all other parameters are unknown model parameters ($\Theta_{\text{unknown}} = \alpha \beta U_{\text{max}} c$). Based on least-square regression, unknown parameters ($\Theta_{\text{unknown}} = \alpha \beta U_{\text{max}} c$) are estimated so that the residual sum of squares (RSS) between regression model predicted cell concentration $n(t)$ and experimental cell concentration $N(t)$ are minimum.

$$\text{RSS}(\Theta) = \sum_m \frac{1}{\sigma_m^2} \cdot [n_q(t_m) - N_q(t_m)]^2$$

(5)

The initial values of $\Theta_{\text{init}}$ for solving the system of ODEs were chosen by a method called ‘guided random search’ (described in [139]). The ‘chosen’ initial solutions for $\Theta_{\text{init}}$ are utilized to solve the ODE system which yields some values for $n(t_m)$. These $n(t_m)$ are then used to calculate a (weighted) Residual Sum of Squares RSS($\Theta$). In each of the subsequent least-squares iterations, the parameters $\Theta$ are modified such that RSS($\Theta$) moves closer to a minimum. Using the updated $\Theta$ to again solve (5) results in new model
values $n(t_m)$ which are then utilized to re-calculate $\text{RSS}(\theta)$. The iterations continue until the final solution of $\theta$ explains the experimentally measured growth curve best in the least-square sense.

However, as opposed to linear regression, nonlinear regression is hampered by the 'local-minimum problem' [140] which is caused by the repeated runs of minimizing an RSS. In such case, the derived model fit parameters do not reflect the global minimum of the RSS, instead represent the local minimum. While this is not an ideal situation, model parameters that are sub-optimum but acceptable in terms of RSS and/or correlation can be utilized. It is recommendable though to perform repeated minimizations of the RSS utilizing the same experimental data and in order to gain insights into the spread of these model parameters’ values.

Once $\alpha$, $\beta$, and $U_{\text{max}}$ had been determined for all $\text{HCO}_3^-$ concentration and for all three culture types ($D. \text{salina}$, $N. \text{oculata}$, and $D. \text{salina} + N. \text{oculata}$), first and second order polynomials in $[\text{HCO}_3^-]$ were fitted to these data points (Figure 6). Resulting polynomials such as $\alpha([\text{HCO}_3^-]) = \theta_0 + \theta_1 \cdot [\text{HCO}_3^-] + \theta_2 \cdot [\text{HCO}_3^-]^2$ were then incorporated into the modeling software evaluating (4) to describe nutrient uptake characteristics as a continuous function of $[\text{HCO}_3^-] = c(x,t)$. Whether or not a zeroth, first, or second order polynomial is best, has been tested by means of an Analysis of Variance (ANOVA, confidence level 95% [141]). First, Figure 6 reveals that for all three culture types, all three parameters exhibit a strong dependency on the $\text{HCO}_3^-$-concentration which clearly justifies incorporating $\alpha([\text{HCO}_3^-])$, $\beta([\text{HCO}_3^-])$, and $U_{\text{max}}([\text{HCO}_3^-])$ into (4) as $\text{HCO}_3^-$-dependent functions rather than constants.
Figure 6: Concentration dependency of parameters $\alpha$, $\beta$, and $U_{\text{max}}$, which describe the cells' nutrient uptake, for individual *D. salina* (A-C) and individual *N. oculata* (G-I) cultures as well as their counterparts determined in binary mixtures of *D. salina* (D-F) plus *N. oculata* (J-L). Since nonlinear regression cannot determine unique solutions, nonlinear regression calculations have been repeated five time leading to the error bars.
Impacts of nutrient competition on the growth parameters $\alpha$, $\beta$, and $U_{\text{max}}$ can be deduced by comparing the following panels in Figure 6: A-C ($D. \text{salina}$ individual) to D-F ($D. \text{salina}$ in mixture) as well as G-I ($N. \text{oculata}$ individual) to J-L ($N. \text{oculata}$ in mixture). Values for the half saturation constant, $\alpha$, for both species in single and mixture were in the same order of magnitude and with exception of pure $D. \text{salina}$ at high concentration, $\alpha$-values were found to be increasing with the $\text{HCO}_3^-$-concentration. ANOVA implied a linear relationship for $D. \text{salina}$ in mixed culture, while nonlinearity was significant for the other three situations. The nutrient efficiency, $\beta$, for $D. \text{salina}$ was one order of magnitude lower than for $N. \text{oculata}$ in individual cultures. This is assumed to be the reason why a higher cell concentration of $N. \text{oculata}$ has been observed compared to $D. \text{salina}$. Moreover, competition for nutrients influenced both the shape and the magnitude of concentration dependent $\beta$-values for $D. \text{salina}$. On the other hand, $N. \text{oculata}$ exhibited similar trend in $\beta$-values with a slight increase in magnitude. Concentration dependency of the maximum uptake rate, $U_{\text{max}}$, for both single-species cultures was in comparable order of magnitude. For both species in mixed cultures, there was a significant decrease in the $U_{\text{max}}$ values compared to the corresponding single-species cultures. Moreover, the functional relation between $U_{\text{max}}$ and $[\text{HCO}_3^-]$ was found to be very similar between both single-species cultures as well as for both species in mixture but very different for single- and mixed-species cultures.

3.2.2. Cultures for Model Validation

For model validation purpose, experimental data were required which have been acquired from microalgae that were exposed to a known chemical steady-state environment. In particular, the concentration of atmospheric $\text{CO}_2$ in the cultures’ headspace needed to be well-defined and stable over the course of several days because only then the atmospheric conditions can be related to the microalgal biomass produced. By means of two mass flow controllers (Sierra Instruments), $\text{CO}_2$ (Airgas) had been mixed with synthetic air (Airgas) resulting in gas mixtures containing 300ppm, 350ppm, 400ppm, 450ppm, and 500ppm $\text{CO}_2$, respectively, which were then continuously flushed through five different cultures’ headspaces. These concentrations were chosen to simulate pre-industrial, current, and potential future atmospheric conditions. All other nutrients the cells require were provided via the culturing medium (ESAW). In order to replenish consumed, aqueous phase nutrients, fresh ESAW was slowly but continuously dripped into the
Erlenmeyers which were equipped with an overflow through which consumed medium was released. Over the course of several days, small aliquots of these cultures were extracted for determination of a culture’s current cell concentrations by means of flow cytometry. These experimental data were then compared to cell concentrations predicted by concentration field model (4).

3.3. **Results and Discussion — Model Validation**

The concentration field \( c(x, t) \) and species-specific cell counts \( N_q=1,\ldots,Q(x,t) \) have been computed from (4) for the same environmental conditions under which the real-world microalgae were grown. PETSc’s time-stepper advanced \( c(x, t) \) to the same points of time at which the cultures were submitted flow cytometry experiments †. During the computation, the species-specific nutrient uptake characteristics \( \alpha_q, \beta_q, \) and \( U_{\text{max}}q \) for single species cultures and their multi-species counterparts for species mixtures were utilized by the model. The model predicted absolute cell numbers which were converted into cell concentration utilizing the known culture volume. This procedure enabled a direct comparison between predicted and experimentally obtained cell concentration.

3.3.1. **Modeling of *Dunaliella Salina***

The left column of Figure 7 compares experimental and predicted cell concentrations obtained for single-species cultures grown under different atmospheric CO\(_2\) concentrations. For all CO\(_2\) situations a convincing agreement has been found which is indicative that the PDE (4) is a good description of the transformation of atmospheric CO\(_2\) into D. salina biomass. This agreement between theory and experiment stretches over the course of ten days which demonstrated that (4) not only properly describes quantities but also explains the dynamics of *D. salina* production. The right column depicts equivalent information but, in these cultures, *D. salina* was competing with *N. oculata*. Again, the predictions are capable of explaining the experimental data well. For all

† Note that it is important to utilize the single-species version of the species-specific nutrient uptake characteristics \( \alpha_q, \beta_q, \) and \( U_{\text{max}}q \) for single species cultures and their multi-species counterparts for species mixtures (see Figure 6)
investigated atmospheric CO₂ concentrations, the cell concentration is approximately one order of magnitude lower in the binary species cultures compared to sole D. salina cultures. The decrease in maximum uptake rate values ($U_{\text{max}}$) in mixed cultures (Figure 6 C vs. F) is possibly responsible for these reduced cell concentrations. It is also noticeable that compared to pure D. salina cultures, in such competition situations, the CO₂ concentration does have a pronounced impact on the resulting cell concentration which increases with atmospheric CO₂.

### 3.3.2. Modeling of Nannochloropsis Oculata

Figure 8 depicts information equivalent to Figure 7 but for N. oculata. First, it is notable that the model (4) describes the production of N. oculata cells well, too, and that the dynamics of cell production is again in good agreement with the experiment. This was found for single-species N. oculata cultures (Figure 8 left column) as well as for N. oculata in mixtures with D. salina (Figure 8 right column). For all investigated atmospheric CO₂ concentrations, the quantity of N. oculata biomass is considerably higher than for D. salina (cp. left columns of Figure 8 and Figure 7 respectively). This can be explained by N. oculata's higher nutrient efficiency values, $\beta$, compared to D. salina's (Figure 6 H vs. B) which implies that N. oculata produces more cells per nutrient unit. The cell concentration of N. oculata in binary mixture (Figure 8 right column) is approximately one order of magnitude lower compared to single-species culture. This can be explained by the reduced values of the maximum uptake rate, $U_{\text{max}}$, for N. oculata in binary mixture with D. salina compared to cultures containing exclusively N. oculata cells (compare Figure 6 L vs. I). It is also evident that, in contrast to single D. salina cultures, the atmospheric CO₂ concentration has a considerable impact on the N. oculata biomass production in both single-species and binary mixed cultures.

An overall comparison between model predicted and experimental result was summarized in Figure 9. This comparison indicates that, whether D. salina or N. oculata, the model predicted results are in a very good agreement with experimentally measured cell concentration for all atmospheric CO₂ concentrations. Therefore, it can be concluded that for the chosen species the concentration field method properly describes the main driving forces behind the transformation of atmospheric CO₂ into microalgal biomass. This assessment holds for individually cultured as well as for competing species.
Figure 7: Model validation with experimental data for *D. salina* in individual species cultures (left column) and binary species mixtures (right column) at different atmospheric CO$_2$-concentration (300-500ppm).
Figure 8: Model validation with experimental data for *N. oculata* in individual species cultures (left column) and binary species mixtures (right column) at different atmospheric CO₂-concentration (300-500ppm).
Figure 9: An overall comparison between model predicted and experimental cell concentration. Each point represents a certain species type (red - *D. salina* or blue - *N. oculata*), culture type (binary or mixed) and atmospheric CO$_2$ concentration.
3.3.3. **Model Application – Long Term Capability for CO₂ Sequestration**

For assessing phytoplankton’s CO₂ sink capacities, it has been investigated whether a microalgae culture reaches an upper limit in cell numbers or whether it continues growing unrestrainedly. If, over time, the cell production slows down, so does the increase of sequestered CO₂. If a culture’s growth is unrestricted, its CO₂ sinking capacity increases unimpededly. Model (4) has been employed for long-term predictions of produced cell numbers. These simulations have been performed for a period of 100 days under different atmospheric CO₂ concentrations ranging from 300-500 ppm.

Figure 10 displays predicted cell concentrations over time as a function of atmospheric CO₂ concentration. Four scenarios were considered, i.e. *D. salina* and *N. oculata* growing individually as well as both species competing with each other. From Figure 10 (A-B), it can be concluded that both species in individual cultures exhibit a qualitatively similar behavior with *N. oculata* having a faster growth and a stronger dependency on the CO₂ concentration. Most importantly though, it is noticeable that both species’ growth slows down as indicated by the surfaces becoming much flatter shape at later times. Based on the flatness of these plateaus, it can be concluded that *D. salina* slows down more than *N. oculata*. Moreover, *N. oculata* cultured at high CO₂ concentrations seem to slow down more and faster compared to low CO₂ concentrations. *D. salina*’s slowdown on the other hand features a much lower dependency on the CO₂ concentration. In conclusion, the main finding of this analysis is that overall the production of new cells slows down with time. This fact means that an increase in anthropogenic CO₂ releases may outweigh an increase in phytoplankton production.

In a competition situation (Figure 10 C-D), the cell production by both species generally shows a much stronger dependency on the CO₂ concentration. It also is considerably lower than in the corresponding single-species cultures (Figure 10 A-B). Apparently, competition suppresses cell growth with *D. salina* being the more impacted species. Moreover, the growth rates are lower in a competition scenario as indicated by the shallower slopes of the surfaces in time-direction. It is also obvious, that over time cell production tends to slow down but much less than in the single species cases. Nonetheless, it is reasonable to assume, that these surfaces reach a plateau at times >100 days. From these findings, it may be concluded that mixed species cultures sequester less CO₂ than a single-species culture. This is another aspect to consider when assessing phytoplankton’s CO₂ sequestration capacity.
Figure 10: Predicted cell concentrations increasing over time produced by different levels of atmospheric CO$_2$ (A) *D. salina* grown individually; (B) *N. oculata* grown individually; (C) *D. salina* in competition with *N. oculata*; (D) *N. oculata* in competition with *D. salina*; note the log$_{10}$-scale on the z-axis.
3.3.4. **Model Application – Effect of Ocean Acidification**

Another evident consequence of global climate change is the ocean acidification. Since the industrial revolution, atmospheric $\text{CO}_2$ is steadily dissolving in the seawater and increasing the acidity the ocean. Being at the bottom of the food web, phytoplankton’s response to ocean acidification has gained an increasing attention to marine researchers. To this end, concentration field model was applied to understand the impact of ocean acidification to phytoplankton growth. First, an artificial ecosystem was assumed to contain cells of *Dunaliella salina* or *Nannochloropsis oculata*. The $\text{CO}_2$ concentration at the air-water interface was chosen to be current level (400 ppm). Then, simulations of (4) were performed to predict the biomass quantity for 10 days at five different pH levels ranging from 7.82-8.22 with 0.1 interval. These ranges were chosen to simulate the pH of the seawater in *future, current* and *pre-industrial era* respectively. The model predictions were validated by the experimental flow cytometry measurements. For this reason, replicates of *D. salina* or *N. oculata* were cultured in growth medium at the chosen pH conditions. The pH of the growth medium was buffered initially and adjusted twice/day (Mettler Toledo S220 SevenCompact pH/Ion meter) to ensure a homogeneous growth condition. The cell concentration of these cultures was measured for the next 10 days using flow cytometry.

Three replicate simulations for *D. salina* cell concentration were plotted as a function of pH and time in Figure 11 (A-C). It was evident from the replicate *D. salina* cultures that, cell growth is pH dependent. The cell concentrations over the course of 10 days were decreased in relatively acidic growth medium i.e. low pH. Figure 11 (D) depicts a comparison between model prediction and experimental cell concentration. For all replicate *D. salina* cultures, the model predicted cell concentration can accurately describe the experimental measurements.

Figure 12 represents the equivalent information in Figure 11 but for cultures of *N. oculata*. Unlike the behavior observed for *D. salina* species, cell concentration of *N. oculata* were increased under acidic growth medium (A-C). This perhaps indicates that, some phytoplankton species may have the potential to endure the rapidly changing ocean climate. The comparison against the experimental cell concentration in Figure 12 (D) shows that, the model can satisfactorily explain the experiments.
Figure 11: (A – C) shows model predicted *D. salina* cell concentrations for three replicates over time as a function of pH in the growth medium. (D) shows the comparison between simulated and experimental data.
Figure 12: (A – C) shows model predicted N. oculata cell concentrations for three replicates over time as a function of pH in the growth medium. (D) shows the comparison between simulated and experimental data.
3.4. Conclusion

For modeling purposes, three parameters that describe the cells’ nutrient uptake characteristics had to be determined experimentally. It was found that these parameters depend on the species, the nutrient availability, and the presence of nutrient competitors. With these concentration dependent parameters describing the situation specific dynamics of nutrient uptake, species-specific biomass production could be expressed as a system of partial differential equations (PDEs). Numerically solving this PDE system then predicted the quantities of produced microalgae cells over time.

For experimental validation, two microalgae species (*Dunaliella salina* and *Nannochloropsis oculata*) had been chosen and were cultured individually and in a competition situation. Flow cytometry was used to measure experimental cell concentration over the course of ten days for both single-species and binary species cultures. For all three culture types (single *D. salina*, single *N. oculata*, and *D. salina* + *N. oculata*) and for all analyzed atmospheric CO$_2$-concentrations (300ppm - 500ppm) a very good agreement between experiment and model has been found.

After confirming that the novel modeling approach can with high accuracy predict the species-dependent production of microalgae cells, this model has been applied to study long-term trends in cell production. Motivation for this is to determine whether the cell production continues unimpededly or slows down as a culture grows. In the former case, the increase in phytoplankton-based CO$_2$-sequestration; in the latter, the CO$_2$-storage capacity of microalgae cells would diminish in increase. Simulations determined that the latter is the case for all considered atmospheric CO$_2$ concentrations and all three culture types. The alarming consequence of this is, the future increases of anthropogenic CO$_2$ releases may not be counterbalanced by growing phytoplankton cultures.

Another application of the model was presented to realize the impact of ocean acidification on microalgal growth. The model predictions in combination with experimental validation indicate that, the impact of acidification on algal growth is highly species specific. While some species are able to sustain life under low pH conditions, others experience a slower growth rate. This is a strong indication that the continual climate change can alter the life of the marine ecosystem.

Moreover, it was found that nutrient competition among different species considerably reduces the number of cells produced. This means that a real-world, mixed
species culture has a lower CO₂-sinking capability than the individual species. There are indications that the cell production in mixed-species cultures also slows down over time but that this slowdown is delayed. Again, a potential consequence is that mixed species cultures need to be considered when assessing phytoplankton’s CO₂ sequestration capacity.

Ultimately, the experimental investigations of dynamic adaptation of phytoplankton to a chemically changing environment and the modeling software determining quantities of sequestered inorganic compounds need to be fused together. This will then result in a comprehensive, quantitative description and prediction of future responses of marine environments to anthropogenic pollution.
Chapter 4. Quantification of CO₂ Sequestration by Microalgae

This section is based on a publication by Mohammed F. Hasan and Frank Vogt:

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This author’s primary contribution to above publication includes: i) development of the problem, ii) development and simulation of the computational model, iii) analysis of the simulated data, iv) most of the writing.

4.1. Introduction

Since the industrial evolution started, anthropogenic CO₂ releases have considerably increased over the last few decades [108]. This fact causes considerable environmental concerns and therefore, studying the fate of this greenhouse gas has become a major effort [109]. Since algal photosynthesis is producing about half of the global primary carbon generation [30-32], phytoplankton based CO₂ sequestration has a major impact on climate change [33-41]. Marine microalgae sequester CO₂ by the uptake of HCO₃⁻, a nutrient, which is produced via [33]: CO₂(g) → CO₂(aq) + H₂O ⇌ H₂CO₃ ⇌ HCO₃⁻ + H⁺. Therefore, knowing CO₂(g) and measuring HCO₃⁻ concentrations in marine ecosystem would enable quantitation of carbon sequestration by phytoplankton. However, microalgal cells (typical diameter 5-20µm [104]) produce a concentration gradient within a microscopic vicinity due to their very localized nutrient uptake. Therefore, in order to quantify the sequestration process, an in-situ measurement of HCO₃⁻ concentration with microscopic resolution would be required. But, such measurements are very challenging in particular since a macroscopic ecosystem contains a large number of microscopic cells [142]. On the other hand, modeling methods developed in Chapter 2 can accomplish a prediction of HCO₃⁻ concentrations with microscopic spatial resolution.

In this work, an assessment of microalgae based CO₂ sequestration is being reported with the goal to quantitatively describe the conversion of atmospheric CO₂ into microalgal biomass. This quantitative model is based on describing all physical and chemical processes namely dissolution of atmospheric CO₂, HCO₃⁻ formation and transport
within an aqueous ecosystem, microalgae species-specific \( \text{HCO}_3^- \) uptake, and \( \text{HCO}_3^- \) utilization for biomass production. Additionally, nutrient competition has been found to impact the \( \text{CO}_2 \) sequestration and therefore needs to be considered as well [61, 111, 134, 143].

### 4.2. Methods

In this study, the aforementioned steps describing the transformation of atmospheric \( \text{CO}_2 \) into microalgal biomass are linked by means of a novel ‘concentration field’ approach. This space- and time-dependent concentration field describes the \( \text{HCO}_3^- \) concentration distribution within an ecosystem. The dissolution of \( \text{CO}_2 \) from the atmosphere into an aqueous ecosystem and its subsequent conversion into \( \text{HCO}_3^- \) are described by chemical kinetics in the concentration field model. Additionally, a mass transport mechanism explains the distribution of \( \text{HCO}_3^- \) within an ecosystem. Two microalgae species, i.e. *Nannochloropsis oculata* and *Dunaliella salina*, have been considered here in two single-species as well as in a competition situation. To complete the model, several species-specific and environment dependent parameters that explain the cells’ nutrient uptake and utilization needed to be determined experimentally [120, 121, 127]. These parameters were determined in Chapter 3 and were utilized in this study.

In order to quantify the amount of \( \text{CO}_2 \) sequestered by a culture of microalgae, an entire marine ecosystem needs to be modelled. For this purpose, a macroscopic ecosystem is assumed to consist of microscopic, cuboid ‘blocks’ each of which represents a small volume surrounding a cell cluster. Each of these blocks was assumed to contain a single cell cluster of either *D. salina* or *N. oculata*, or for species mixtures two cell clusters for one of each, respectively. In the latter case, a competition between *N. oculata* and *D. salina* for the \( \text{HCO}_3^- \) supply establishes. From the overall \( \text{HCO}_3^- \) concentration, i.e. the sum contained in all blocks, a cell culture’s carbon sequestration has been derived.

An “artificial ecosystem” of \( 128\mu\text{m} \times 128\mu\text{m} \times 32\mu\text{m} \) (volume = 0.5nL) has been modeled with a spatial resolution of 1\( \mu \text{m} \) along each direction; this will be referred to as “grid”. Initially, the concentration field covering this artificial ecosystem has been assumed to be void of \( \text{HCO}_3^- \). Then, the \( \text{CO}_2(g) \)-concentration in the atmosphere above the artificial ecosystem was set to a user selected level and “switched on”. This has been done, in separate simulations, for 300 ppm, 350 ppm, 400 ppm, 450 ppm and 500 ppm. These
concentration ranges were chosen to simulate pre-industrial, present and potential future atmospheric CO$_2$(g) concentration. For each atmospheric CO$_2$(g) scenario, the top 1µm layer of the simulated culture represented the air-water interface where CO$_2$(g) partitions into the aqueous phase where it forms HCO$_3^−$. In this first step, this portion of the modeling was performed without cells being present in order to create, via diffusion, a realistic concentration field across the entire simulated area. In a second step, the cells’ HCO$_3^−$ uptake was “switched on” which modified the concentration field around them and initiated the production of additional cells. This turn-on defined $t = 0$. In the first simulation experiment, a fixed number ($10^9$) of either $D$. salina or $N$. oculata cells had been “inoculated” at a certain grid position of the artificial ecosystem. In case of species-competing mixtures, cell clusters ($10^9$) of both species were inoculated at two different locations. For single-species cultures, cell clusters of either $D$. salina or $N$. oculata were inoculated at grid point $x, y, z = 56, 64, 16$ or $x, y, z = 72, 64, 16$, respectively. For mixed-species cultures, one cluster of each species had been inoculated at the stated grid points. It has been assumed here that both cell clusters (and nutrients) are not moving due to advection.

The sequestration process causes local concentration depletions around cells which are then continuously replenished via diffusion of HCO$_3^−$ newly generated at the air-water interface. Thus, there is a continuous fixation of atmospheric CO$_2$ into phytoplankton biomass. In order to characterize the real-world ecosystem, all the simulations were performed at following physical and chemical conditions: temperature 20°C, atmospheric pressure 1.0 atm, pH 8.2 and salinity 35.0 g/kg of seawater.

4.3. **Results and Discussion**

The difference in HCO$_3^−$ concentrations between the initial concentration field, i.e. in absence of cells, and its counterpart containing an increasing number of cells were utilized to quantify the sequestration process over time. Based on the volume of the artificial ecosystem, concentration differences between absence and presence of cells could be translated into absolute quantities in nanograms of HCO$_3^−$ the cells had been sequestered. This had been done for several cultures and environmental conditions.
Figure 13 shows the heat map of $\text{HCO}_3^-$ concentration depletion around $D. \text{salina}$ (A-B) and $N. \text{oculata}$ (C-D) cells at the $z = 16$ plane where cells were inoculated. These figures indicate single-species cultures growing at 400 ppm atmospheric $\text{CO}_2$ concentration. Figure 13 (A) and (B) represents the $\text{HCO}_3^-$ concentration depletion by $D. \text{salina}$ cells after 10 and 15 days respectively. On the other hand, (C) and (D) represents equivalent information for $N. \text{oculata}$. Based on these heat maps, it can be concluded that single-species $D. \text{salina}$ cultures consume significantly higher quantity of $\text{HCO}_3^-$ than cells of $N. \text{oculata}$.

Figure 14 represents similar information as in Figure 13 but for binary mixed cultures. In this heat map, both $D. \text{salina}$ (A-B) and $N. \text{oculata}$ (C-D) cells were inoculated together at the same $z = 16$ plane. A comparison between Figure 14 and Figure 13 provides an insight on the impact of nutrient competition between two species. In these two figures it can be observed in (A-B) that, the presence of another species has considerably impacted the quantity of $\text{HCO}_3^-$ sequestered by $D. \text{salina}$ cells. However, the amount of $\text{HCO}_3^-$ sequestered by species $N. \text{oculata}$ in (C-D) were almost unaffected by the presence of another species. This initial assessment indicates that, perhaps some algal species can tolerate the impact of nutrient competition due to the low nutrient requirement for survival.

Figure 15 and Figure 16 depict the quantity of $\text{HCO}_3^-$ sequestered by $D. \text{salina}$ and $N. \text{oculata}$ cultures, respectively. The species-specific $\text{HCO}_3^-$ consumption (in ng) within the artificial ecosystem were plotted over the course of 20 days at different atmospheric $\text{CO}_2$ concentration (300-500 ppm). It has been found in Figure 15 and Figure 16 that the uptake behavior depends on the growth time and more dominantly, on the atmospheric $\text{CO}_2$ concentration. The flatness of these surfaces indicates that, $D. \text{salina}$ has a stronger dependency on the atmospheric $\text{CO}_2$ concentration than $N. \text{oculata}$ and has quantitatively higher $\text{HCO}_3^-$ uptake capacity (~1 order) at said environmental conditions. In fact, the difference in the nutrient uptake behaviour is not unexpected as in a previous study, the maximum bicarbonate uptake rate for species $D. \text{salina}$ were determined to be significantly higher than that of $N. \text{oculata}$ (Figure 6 in Chapter 3).
Figure 13: Heat map of the bicarbonate concentration distribution around the *D. salina* (A-B) or *N. oculata* (C-D) single-species culture growing at 400 ppm atmospheric CO$_2$ at $z = 16$ plane (cell location) within the artificial ecosystem. (A) and (B) represents the bicarbonate concentration depletion near *D. salina* cells after 10 and 15 days respectively. (C) and (D) represents equivalent information for *N. oculata* after 10 and 15 days respectively. Note the different scales of bicarbonate color map for (A-D).
Figure 14: Heat map of the bicarbonate concentration distribution around the *D. salina* (A-B) or *N. oculata* (C-D) in a binary mixed culture growing at 400 ppm atmospheric CO$_2$ at $z = 16$ plane (cell location) within the artificial ecosystem. (A) and (B) represents the bicarbonate concentration depletion near *D. salina* cells after 10 and 15 days respectively. (C) and (D) represents equivalent information for *N. oculata* after 10 and 15 days respectively. Note the different scales of bicarbonate color map for (A-D).
Figure 15: Quantification of $\text{HCO}_3^-$ sequestered (in ng; log-scale) over time at different levels of atmospheric $\text{CO}_2$ by $D. \text{salina}$ species within the artificial ecosystem.

Figure 16: Quantification of $\text{HCO}_3^-$ sequestered (in ng; log-scale) over time at different levels of atmospheric $\text{CO}_2$ by $N. \text{oculata}$ species within the artificial ecosystem.
Figure 17 shows the combined sequestration of each species in binary mixed cultures. In this case, two cell clusters of each species ($2 \times 10^9$ cells total) were inoculated at the two aforementioned locations and the quantity of $\text{HCO}_3^-$ sequestered were determined. In such a competition situation, the $\text{HCO}_3^-$ uptake by both species combined were found to be similar to the uptake behaviour of *N. oculata* species in Figure 16. It is also evident that *D. salina*'s sequestration performance is more impacted by the competition. This competition effect modifies the species-dependent bicarbonate uptake characteristics, more dominantly for *D. salina*. In a competing situation, the sequestration performance of species can apparently invert. This means that competition needs to be factored in when assessing phytoplankton-based CO$_2$-sequestration. This is a strong indication that multi-species sequestration models need to be species-specific.

4.4. **Conclusion**

The CO$_2$ concentration in the atmosphere is continuously rising which is considered a source of global warming. Since marine microalgae counterbalance this greenhouse gas through photosynthesis-based carbon fixation, it is crucial to understand mechanisms that control this sequestration. This study quantitatively investigates microalgae-based CO$_2$ sequestration by means of a novel modeling technique. This modeling approach is based on describing CO$_2$ dissolution out of the atmosphere into bodies of water where it forms $\text{HCO}_3^-$. This $\text{HCO}_3^-$ is transported to phytoplankton cells which utilize it as a nutrient for biomass production. It was found that this utilization is highly species-specific. Furthermore, real-world ecosystems contain multiple species which compete for this common nutrient source. Therefore, phytoplankton-based CO$_2$ sequestration should consider the species composition of an ecosystem. For example, when cultured by itself, *D. salina* sequesters more $\text{HCO}_3^-$ out of an environment than *N. oculata*. However, when both species compete for $\text{HCO}_3^-$, competition effects invert the sequestration capabilities of both species. Moreover, the overall quantities of fixed carbon drops considerably. This will be investigated in subsequent studies. It has also been found that an increasing CO$_2$ concentration in the atmosphere results in a higher quantity of resulting biomass. This is an indication that phytoplankton do counterbalance to a certain extent increased releases of this greenhouse gas.
Figure 17: Quantification of the combined $\text{HCO}_3^-$ sequestration (in ng; log- scale) by *D. salina* and *N. oculata* over time at different levels of atmospheric CO$_2$. 
Chapter 5. Predicting the CO$_2$ Sequestration Capacity of *Dunaliella salina*

This chapter is based on a submitted manuscript (Algal Research) by Mohammed F. Hasan and Frank Vogt entitled 'Intracellular β-carotene as an indicator for the CO$_2$ sequestration capacity of marine phytoplankton Dunaliella salina'.

This author’s primary contribution to above manuscript includes: i) development of the problem ii) proposition of the work package for the potential solution, iii) designing and conducting the experiments, iv) process and analysis of the experimental data, v) most of the writing.

5.1. **Introduction**

Due to increased industrialization, anthropogenic CO$_2$ emission have considerably increased, and the fate of this inorganic pollutant has become a global concern [108, 109]. Marine phytoplankton counterbalance this greenhouse gas via its uptake as nutrient, and is responsible for about half of the primary carbon production [30-34]. Therefore, phytoplankton’s ability to counterbalance CO$_2$ releases has a major impact on long-term climate changes [34, 37-41].

Marine microalgae sequester atmospheric CO$_2$ in form of HCO$_3^-$ which they utilize as a nutrient, which is produced via: CO$_2$(g) → CO$_2$(aq) + H$_2$O ⇌ H$_2$CO$_3$ ⇌ HCO$_3^-$ + H$^+$. This sequestration process is influenced by numerous chemical and physiological parameters, e.g. chemical environment, light, temperature, pH, salinity, nutrient concentration, nutrient uptake rate, specific growth rate etc. [47, 73-78]. To understand phytoplankton’s ability to sequester atmospheric CO$_2$, the impacts of these parameters on the transformation of atmospheric CO$_2$ to biomass need to be considered simultaneously. Due to the sheer number of continuously varying parameters, previous studies have been limited to only few selected factors [48, 59, 88, 144-146]. Therefore, there is a need for an overall indicator that describes phytoplankton’s ability to sequester atmospheric CO$_2$.

It has been found that, besides adapting biomass production to their surrounding environment, marine phytoplankton also respond by actively modifying its internal chemical composition [61, 63, 86, 110]. An example of such a dynamic adaptation process
is the accumulation of accessory pigments, namely carotenoids, in the lipid globules of the marine microalgae species *Dunaliella salina* under abiotic stressed conditions such as nutrient deprivation [52, 131, 132, 147, 148]. The presence of β-carotene in cells of *D. salina* can be qualitatively assessed from epifluorescence microscopy in Figure 18. Under stressed conditions during photosynthesis, the electronic spins of the intracellular chlorophyll can be altered through intersystem crossing to form triplet state chlorophyll: $\text{Chl} \rightarrow ^3\text{Chl}$ [149-152]. This triplet $^3\text{Chl}$ favors the conversion of triplet oxygen into its singlet form: $^3\text{Chl} + ^3\text{O}_2 \rightarrow ^1\text{O}_2 + \text{Chl}$ [149, 152, 153]. Singlet oxygen is highly reactive and responsible for photooxidative damage of the cells by reacting with proteins and pigments [151, 154, 155]. In order to protect the cells against the singlet oxygen damage, carotenoids are accumulated within the lipid globules because of their antioxidant properties [149, 155-157]. Carotenoids can directly quench singlet oxygen and the triplet chlorophyll which is responsible for the singlet oxygen production by physical and/or chemical quenching mechanisms [158-160]. On the other hand, it has also been found that the biomass production of *D. salina* and thus their sequestration of anthropogenic CO$_2$ slows down when nutrients are depleted over time [161, 162]. Therefore, an inverse relation is hypothesized between the carotenoid content of *D. salina* and the cells’ ability to produce more biomass. The aim of this research is to utilize the intracellular carotenoid content, namely β-carotene, as a sensitive indicator to predict the CO$_2$ sequestration capability of *D. salina*.

Cultures of *D. salina* were cultivated at different atmospheric CO$_2$ concentrations to introduce stress only due to carbon nutrient deficiency. Due to excellent fluorescence property of carotenoids, β-carotene concentrations of these cultures were measured by means of fluorescence spectroscopy. The resulting cell concentrations of *D. salina* cultures were measured by flow cytometry experiments. From these two pieces of information, an averaged intracellular β-carotene content per cell (pg/cell) has been derived by chemometric data analyses. This novel approach enables determining the time dependent β-carotene quantity of a cell and to relate it to the dynamics of biomass production. By relating a cell’s internal (carotenoids) response on a microscopic scale to external (CO$_2$ concentrations) changes on a macroscopic scale, the proposed research introduces a comprehensive approach to understand algae’s CO$_2$ fixation capacities.
Figure 18: Epifluorescence microscopy of stressed *D. salina* cells with arrows indicating β-carotene containing globules.
5.2. **Materials and Methods**

5.2.1. **Microalgae Culture Preparation**

*Dunaliella salina* obtained from the UTEX Culture Collection of Algae (https://utex.org) were cultured in Erlenmeyer flasks with ‘Enriched Seawater, Artificial Water’ (ESAW) growth medium. All nutrients except carbon for growth were provided in the ESAW medium at concentrations according to the standard protocol [137, 138]. In order to predict CO$_2$ sequestration capacity of *D. salina*, the cultures needed to be exposed to a known, well-defined and steady-state CO$_2$ concentrations. For this reason, two mass flow controllers (Sierra Instruments) were used for mixing 5% CO$_2$ and synthetic air to produce gas mixtures containing 300ppm, 350ppm, 400ppm, 450ppm, and 500ppm CO$_2$. These concentrations were chosen to simulate pre-industrial, current, and potential future atmospheric conditions, respectively. These gas mixtures were then continuously flushed through the headspace of different cultures. In order to replenish the consumed nutrients, fresh ESAW medium were continuously pumped in the culture by means of a peristaltic pump (Gilson, 8mL/day). This experimental setup incorporated a relative stress only due to carbon deficiency in cultures. These cultures were cultivated in a growth chamber at 20°C under continuous light with a photon intensity of 75± 10 µmol m$^{-2}$ s$^{-1}$.

Three replicate cultures of each CO$_2$ concentration were prepared to assess the reproducibility of the experiment. In order to maintain a consistent initial condition in all cultures, *D. salina* were inoculated with a fixed volume from the same stock culture. After inoculation, small aliquots (5mL) of these cultures were obtained over the course of 10 days for the quantitation of intracellular β-carotene.

5.2.2. **Quantification of β-carotene Concentration**

In order to quantify the intracellular β-carotene content of *D. salina*, β-carotene concentrations of the *D. salina* cultures were measured by a fluorescence spectrophotometer (Cary Eclipse, Agilent). For calibration purpose, pure β-carotene (EMD Millipore) was dissolved in methanol under low light to prepare six different standards: 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 mg/L with an additional blank. The fluorescence spectroscopy of these standards was performed in a 1 cm quartz cuvette. The excitation wavelength was set to 350 nm wavelength as it produced the most intense β-carotene peak (detail in
Appendix 2). The emission spectra were recorded between 400-600 nm with 0.5 nm interval. The slit widths of the excitation and emission monochromators were set to 5 nm.

In order to quantify β-carotene concentrations in algal cultures, prior to spectroscopic measurement, intracellular β-carotene needed to be extracted out of the cells of *D. salina*. For this reason, 4.50 mL cell suspension was centrifuged (Eppendorf 5702) at 2500 rpm for 12 minutes (Figure 19, A→B). The growth medium supernatant was discarded, and the pellet was resuspended in 4.50 mL methanol solution under a fume hood. The cell suspension was centrifuged again at 3000 rpm for 3 minutes. At this point, *D. salina* cells were broken down and lipid globules containing β-carotene and other lipophilic compounds were extracted by methanol (Figure 19, B→C). The supernatant was then separated from the residual cell debris for fluorescence spectroscopy. All these steps were performed daily for three replicate cultures growing at a certain atmospheric CO₂ concentration prior to fluorescence spectroscopy. The fluorescence emission spectra of the extracted β-carotene were recorded under the same instrumental settings used in standard calibration.

5.2.3. Quantification of Biomass Concentration

In order to assess the CO₂ sequestration capacity of *D. salina* cells, the intracellular β-carotene content must be related to the produced cell concentration. Flow cytometry (Guava easyCyte equipped with 488 nm laser) was used to measure the cell concentration of *D. salina* cultures growing at a certain atmospheric CO₂ concentration. For this purpose, 200 µL cell suspension of each replicate culture in all atmospheric CO₂ condition was added to a round-bottom 96 well plate. Based on the red fluorescence detector of the flow cytometer, the algal population was gated from the background signals. At least 5000 cells were analyzed to increase the accuracy of the cell population measurement. An autosampler was used to perform these experiments at the same time points of the spectroscopic measurements. The averaged cell concentration was determined from three replicate cultures and four replicate measurements of each culture.
Figure 19: Extraction process of intracellular β-carotene. (A) → (B): Small aliquot of the cell suspension is centrifuged. The supernatant is discarded, and the equal volume of methanol is added to extract β-carotene. (B) → (C): After centrifuge, the intracellular pigments are transferred in methanol with cell debris residing at the bottom.
5.3. **Results and Discussion**

5.3.1. **Quantification of Intracellular β-carotene Content**

In order to assess intracellular β-carotene as an indicator for the CO₂ sequestration capacity of *D. salina*, first, the time dependent β-carotene quantity per cell needed to be determined and then compared against the dynamics of biomass production. For this reason, the β-carotene concentration (mg/L) of the *D. salina* cultures were determined from fluorescence spectroscopy.

Figure 20 and Figure 21 demonstrate the similarity of the emission spectra of pure β-carotene in methanol standards and β-carotene extracted from *D. salina* culture grown at 300 ppm atmospheric CO₂ condition, respectively. Both figures share a prominent feature of the broad β-carotene peak with a peak maximum at 495±2 nm which validates the accuracy of β-carotene extraction technique. Minor deviations between the spectra of artificial and extracted β-carotene samples are due to the presence of other lipophilic compounds (predominantly, chlorophyll) in the supernatant. Furthermore, it was observed in Figure 21 that the β-carotene concentration in *D. salina* culture was increasing over time. Compared to the inoculation point (*t = 0*), the peak intensity after 10 days was increased by a factor of ~10. The emission spectra of the extracted β-carotene cultured at different CO₂ concentrations produced a similar behavior and therefore, were not included in graphics.

Based on the emission spectra of the β-carotene standards in Figure 20, the concentrations of the β-carotene extracted from *D. salina* cultures were determined by chemometric data analyses. Principle component regression (PCR) was used to build a calibration model between spectroscopic information and pure β-carotene concentration. Furthermore, it was observed in Figure 21 that the baseline of the spectra drifted over time due to the complexity of the algal samples. To overcome this, the background drift correction was performed simultaneously in the calibration and prediction step by 'pseudo principal component', described elsewhere [163, 164]. Based on the calibration model, β-carotene concentration on each time point were predicted from emission spectra as the response variable.
Figure 20: Emission spectra of β-carotene standards in methanol at 350 nm excitation wavelength

Figure 21: Emission spectra of β-carotene extracted from cells of *D. salina* cultured at 300 ppm atmospheric CO₂ over 10 days.
In order to translate a culture’s $\beta$-carotene concentration (mg/L) into intracellular $\beta$-carotene quantity (pg/cell), the biomass concentration (cells/mL) of the algal cultures was measured by flow cytometry. For cultures growing at all atmospheric $CO_2$ conditions with replicates, the biomass concentration over time was quantified and averaged based on replicate cytometric measurements. At the end, the time dependent averaged $\beta$-carotene quantity in each cell (pg/cell) has been determined from following relationship:

$$\text{Intracellular } \beta\text{-carotene quantity (pg/cell)} = \frac{\beta\text{-carotene concentration of culture (mg/L)}}{\text{biomass concentration (cells/mL)} \times 10^6}$$

Furthermore, *D. salina* cells’ ability to respond internally to external changes ($CO_2$ concentrations) needs to be assessed. For this reason, the dynamics of intracellular $\beta$-carotene quantity of cultures growing at different atmospheric $CO_2$ concentrations were compared. Figure 22 displays the averaged intracellular $\beta$-carotene quantity over time as a function of atmospheric $CO_2$ concentration. It has been found that, the atmospheric $CO_2$ condition of a culture certainly has a strong impact on the quantity of $\beta$-carotene produced in each cell. The intracellular $\beta$-carotene quantity was almost similar at inoculation time ($t = 0$) for all cultures growing at different $CO_2$ conditions. However, the $\beta$-carotene quantity accumulated within the cell after 10 days was found to be higher at lower atmospheric $CO_2$ conditions. For a relatively stressed 300 ppm culture, the $\beta$-carotene quantity can be nearly twice as high as cultures growing at nutrient richer 500 ppm $CO_2$ concentration. This validates the fact that, cells of *D. salina* can respond to stressed culture conditions only due to carbon deficiency by accumulating different quantity of $\beta$-carotene pigment within the cell.
Figure 22: The averaged intracellular β-carotene quantity over time produced at different atmospheric CO$_2$ concentration.
5.3.2. **Correlation of intracellular β-carotene and CO₂ sequestration capacity**

In order to validate the intracellular β-carotene quantity as an indicator for CO₂ sequestration capacity, the time dependent carotenoid quantity needs to be compared against the quantity of sequestered CO₂. However, quantitation of sequestered atmospheric CO₂ is experimentally very challenging. On the other hand, it has been found in previous work that the biomass concentration of a *D. salina* culture is related to the quantity of sequestered CO₂[161]. For this reason, the intracellular β-carotene quantity over time was compared against the dynamics of the biomass production to assess the validity of β-carotene as an indicator.

Figure 23 depicts the comparison between the averaged intracellular β-carotene quantity (right y-axis) against the dynamics of biomass concentration (left y-axis) for cultures grown at different atmospheric CO₂ concentration. The error bars originated from the replicate cultures as well as replicate measurements of the same culture. All cultures growing under different atmospheric CO₂ concentration were inoculated with a comparable initial cell concentration (~3.0 × 10⁵ cells/mL). It has been found that the dynamics of biomass production, i.e. the CO₂ sequestration capacity of *D. salina* increases at higher atmospheric CO₂ concentration. The cultures growing at lower CO₂ concentration experienced a relatively declining growth phase after a few days, while cultures at 500 ppm did not go through such lethargic growth phase. However, regardless of the atmospheric CO₂ concentration, the β-carotene quantity within each *D. salina* cell were evidently increased during the declining growth phase. In other words, the potential slowdown of the CO₂ sequestration by *D. salina* can be monitored by a rapid increase of the intracellular β-carotene quantity.
Figure 23: Comparison of intracellular β-carotene quantity (right y-axes) against biomass production (left y-axes) over time for cultures grown at different atmospheric CO₂ concentration. The error bars resulted from the replicate cultures as well as the replicate measurements of the same culture.
5.4. Conclusion

Anthropogenic CO₂ releases into the atmosphere is continuously increasing. Since, photosynthesis-based carbon fixation plays a crucial role in counterbalancing this greenhouse gas emissions, it is essential to understand phytoplankton’s CO₂ sequestration capacity. However, the microalgae based CO₂ sequestration process is influenced by numerous chemical, physical, and physiological parameters. In order to predict microalgae’s capacity for sequestering atmospheric CO₂ into biomass, the impact of these parameters need to be investigated concurrently which renders the experiments challenging.

In this study, a comprehensive yet straightforward indicator has been presented to estimate phytoplankton’s sequestration of atmospheric CO₂. Marine phytoplankton Dunaliella salina respond to external chemical environments by adapting their internal chemical composition. For example, when nutrient concentration is deficient for sustaining life, D. salina respond to such stressed culture condition by accumulating β-carotene pigments within their cells. At the same time, biomass producing capacity of D. salina and thereby, their ability to sequester atmospheric CO₂ slows down under stressed nutrient condition. Therefore, it has been hypothesized that an inverse relationship exists between D. salina’s intracellular carotenoid content and their ability to produce biomass. This has been validated by measuring both the effect (internal carotenoid and biomass quantity) and the cause (stress due to nutrient deficiency) of environmental changes. Under a series of atmospheric CO₂ condition, β-carotene content in each cell of D. salina cultures and number of produced cells, respectively, has been quantified by a combination of fluorescence spectroscopy and flow cytometry. The results from these experiments indicate that, D. salina cultures responds to the shifting chemical condition by adapting their intracellular β-carotene quantity. Algal cultures cultivated at a relatively stressed atmospheric CO₂ conditions accumulate larger quantity of β-carotene within each cell. Furthermore, it has also been found that, the intracellular β-carotene quantity is linked to cells’ ability to produce biomass and thereby, endorse its validity as an indicator for monitoring CO₂ sequestration. Therefore, this approach enables precise predictions of microalgal CO₂ sequestration based on intracellular carotenoid quantities. Understanding microalgae’s sequestration performance with a straightforward indicator can open new opportunities to predict future scenarios and long-term impacts on climate changes.
Chapter 6. Uptake of Heavy Metals by Marine Microalgae

*Nannochloropsis oculata*

6.1. **Introduction**

Due to industrial, agricultural and technological advancement, the release of heavy metals in the aquatic environment has been continuously increasing over the last few decades [13, 165]. The presence of heavy metals in aquatic ecosystem even at trace concentration (ppb) poses a serious concern for both ecology and human exposure [11, 65, 166-168]. Therefore, it is crucial to understand the fate of this toxic heavy metal ions in the aquatic ecosystem.

Marine phytoplankton are considered as the primary tool for the uptake and accumulation of heavy metals in large scale [65, 85, 169]. Some of these heavy metals play an essential role for the regular metabolic activity of microalgae cells while others can be toxic and suppress their natural growth [12, 85]. The bioaccumulation of heavy metal ions take place in two possible steps [12, 87, 170-173]: i) adsorption of the heavy metal ions on the surface of the cell wall and ii) transport of the metal ions through the membrane toward the cytoplasm where it binds with intracellular proteins through complex formation. In this way, marine microalgae respond to heavy metal pollutants in their surrounding environment by sequestering them within their intracellular level. However, the presence of excess heavy metals within the intracellular level of microalgae have detrimental effect on their ability to sustain growth and proliferation [12, 13, 93, 165]. In such case, intracellular heavy metals are responsible for producing reactive oxygen species (ROS) that cause photooxidative damage by reacting with cellular proteins and pigments [151, 154, 155]. Ultimately, cells are ruptured into debris and intracellular compounds are released back in the environment [149, 151, 154, 156]. This indicates that, when heavy metal concentration in the environment exceeds cells’ capacity, their growth rate and bioremoval capacity of heavy metals are inhibited. For this reason, it is crucial to quantitatively understand the effect of heavy metals both on their uptake capacity and on the growth of microalgae.

This goal of this research is to quantitatively investigate the uptake of copper and nickel by marine microalgae *Nannochloropsis oculata*. Copper and nickel were chosen
since they have extensive applications in many industries including building construction, electronics, batteries, transportation equipment, coatings and consumer products. From these sources, the heavy metals enter the trophic chain by phytoplankton’s uptake and eventually, bioaccumulate in animal and human body. In this study, cells of *N. oculata* were exposed to different concentrations of heavy metals. The uptake of trace amount of copper and nickel was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) over the course of several days. Furthermore, the toxic effect of these heavy metals on *N. oculata*’s growth was simultaneously measured by flow cytometry. Moreover, it is hypothesized that, intracellular copper and nickel can trigger rupture and fragmentation of *N. oculata* cells. To test this, the intracellular damage from excessive heavy metals on *N. oculata* was demonstrated by scanning electron microscopy (SEM). By linking cells’ internal response (biomass) to their surrounding heavy metal concentration, this integrated approach can accurately assess the effect of inorganic pollutants on the phytoremediation capacity.

6.2. **Materials and Methods**

6.2.1. **Microalgae Culture Preparation**

For this study, marine microalgae *Nannochloropsis oculata* was chosen for its well-known intracellular response to changing chemical environment [136]. *N. oculata* was obtained from the UTEX Culture Collection of Algae (https://utex.org). Cells of *N. oculata* were cultured in Erlenmeyer flasks with ‘Enriched Seawater, Artificial Water’ (ESAW) growth medium. All nutrients for growth were provided in the ESAW growth medium at concentrations according to the standard protocol in Table 2 [137, 138].

In order to understand the effect of copper and nickel on their uptake by *N. oculata*, the inorganic salts of heavy metals were added to the growth medium at different concentrations. Copper was supplied by anhydrous copper (II) chloride to produce following Cu concentrations in the growth medium: 0.25, 0.55, 1.00, 2.30, 5.33, 11.00 ppm. Similarly, the source of nickel was nickel (II) chloride hexahydrate to produce following Ni concentrations in the growth medium: 0.09, 0.32, 0.80, 1.90, 4.71, 9.59 ppm. This increasing order was chosen to represent the metal concentration from current levels to point sources concentration respectively. Since, all other nutrients in the growth medium were uniform, a comparison between these cultures would only outline the impact of
different copper and nickel concentrations on *N. oculata*. Furthermore, an uncontaminated growth medium was prepared according to the ESAW protocol⁶ which served as the controlled culture.

The cells of *N. oculata* were inoculated in the growth medium containing different concentrations of heavy metals. The cell inoculation was carefully performed to obtain a similar initial cell concentration (*t* = 0) among all *N. oculata* cultures. This would then enable a reliable comparison between different heavy metal exposed cultures. Both control and contaminated cultures were cultivated in a growth chamber for 14 days under continuous light (24 hour). Figure 24 shows the culturing setup in the growth chamber for different concentrated Ni cultures. From visual assessment, the impact of different Ni concentrations was evident in these cultures after 8 days. Small aliquots of these cultures were withdrawn each day for ICP-OES and flow cytometry analysis.

⁶ Note that, the ESAW growth medium contains 0.00145 ppm nickel which is required according to standard ESAW protocol. The lowest Ni concentration in the contaminated cultures were ~60 times higher than the recommended Ni standard in ESAW.
Figure 24: The culturing setup of Ni contaminated *N. oculata* cultures. The Ni concentration in the growth medium is increasing from left to right. From visual perspective, the effect of different Ni concentration in the culture was evident after 8 days.
6.2.2. ICP-OES for Heavy Metal Quantification

The biosorption of copper and nickel were quantitatively measured by ICP-OES (Agilent Technologies 5110 ICP-OES). For calibration purpose, Cu and Ni standards (100 ppm) were purchased (inorganic ventures) as multiple element standards in 1% HNO₃. By a series of dilution, following Cu and Ni standards were prepared from the purchased stock: 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 ppm. Prior to quantification of heavy metal uptake by *N. oculata* cells, these standards were analyzed by ICP-OES. The operating parameters of the instruments are listed in Table 3. After plasma generation, the standards are introduced in the plasma chamber by means of a nebulizer. The excited copper and nickel atoms are relaxed to their ground state by photon emission. Since, the energy of the photons corresponds to characteristics wavelength of an atom, this principle is utilized for detecting heavy metal concentration even at trace quantity. In this case, copper and nickel concentration were quantified based on 327.395 and 231.604 nm wavelength respectively. The instrument response (intensity, a.u.) was plotted against the standard metal concentration of metal to build a linear calibration curve (Figure 36 and Figure 37 in Appendix 3). The $R^2$ values for Cu and Ni calibration standard were found to be 0.99968 and 0.99976 respectively. Based on these two linear calibration curves, the uptake of heavy metals by *N. oculata* were predicted.

The best practice for quantifying phytoremediation of heavy metal would have been measuring intracellular Cu and Ni quantity of *N. oculata* cultures. However, this approach requires extraction of intracellular metal contents by an organic solvent. Although it is not impossible to analyze organic solvent using ICP-OES, it demands additional expensive instrumentation. For this reason, an alternative approach was adopted to avoid the use of volatile organic solvent. Instead of measuring intracellular metal quantity, the concentration of Cu and Ni in the aqueous growth medium was monitored over time. For this purpose, a small aliquot of cell suspension from each culture was withdrawn each day. The cell suspension was centrifuged (Eppendorf 5702) for 8 minutes at 2000 rpm. At this point, the cell pellet was separated from the supernatant growth medium. The supernatant was then carefully transferred for trace metal analysis by ICP-OES. Using the similar operating parameters of Cu and Ni standards, the heavy metal concentration of the growth medium was quantified from the calibration curve. Three replicate measurements of Cu and Ni concentration were determined which contributed to statistical error bars. This step was repeated for all contaminated cultures 14 days.
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</table>
6.2.3. Flow Cytometry for Biomass Quantification

In order to assess the effect of Cu and Ni on *N. oculata*’s biomass producing capacity, the cell concentration of both control and contaminated cultures was quantified by flow cytometry (Guava easyCyte). For this reason, 200 µL of cell suspension was transferred in a round-bottom 96 well plate. Based on the flow cytometer’s red fluorescence detector, the signals from live algal cells were separated from the background. The red fluorescence intensity was chosen because of the intracellular chlorophyll pigment in *N. oculata* cells. To improve the accuracy of the measurement, minimum 6000 live cells were analyzed for cell concentration quantification. In order to perform the ICP-OES and flow cytometry analysis at the same time, an autosampler was used. The averaged cell concentration was measured from four replicate measurements of each culture.

Furthermore, cell rupture and fragmentation caused by excessive Cu and Ni concentration were preliminary assessed by flow cytometry. This was performed by monitoring the smaller sized cell debris which are produced due to heavy metal toxicity. Both copper and nickel were found to produce a significant amount of cell debris after 7 days while the control culture did not indicate such fragmentation. This initial assessment urged for further investigation which was performed by microscopy imaging.

6.2.4. Scanning Electron Microscopy of *N. oculata*

The toxic effect of heavy metals on *N. oculata* to produce cell debris was investigated by scanning electron microscopy (SEM). For this reason, a small droplet of cell suspension was transferred on a silicon wafer. Once dried, the cells were imaged by the Zeiss Auriga Crossbeam FIB/SEM instrument located at Joint Institute of Advance Materials, University of Tennessee. In order to reduce the charging of cells in the electron beam, the samples were gold coated. The control and heavy metal treated cells were analyzed after 10 culturing days. A comparison between the control and Cu/Ni treated cultures would then provide a qualitative insight about the cell wall rupture and debris formation due to contamination.
6.3. Results and Discussion

6.3.1. Uptake of Heavy Metals by *N. oculata*

Figure 25 contains a stacked plot to depict the changes of Cu concentration in the growth medium over 14 days. The error bars originated from replicate measurement of the Cu concentration from ICP-OES analysis. The biosorption of Cu by *N. oculata* cells qualitatively exhibits a similar behavior for all contaminated cultures despite their varying initial concentrations. Within the first 6-8 days after inoculation, the biosorption of Cu was dominant by the *N. oculata* cells. For cultures growing at lower initial Cu concentration (0.25 and 0.55 ppm), the cells were tolerant to heavy metals for 8 days. On the other hand, cells growing at higher initial Cu concentration experienced a rapid uptake for 6 days. The biosorption of Cu within this phase were found to be significant by means of an Analysis of Variance (ANOVA, 95% confidence interval [141]). Another interesting finding is the quantity of Cu removed from cells’ surrounding. As the initial metal concentration increased from top to bottom, the Cu concentration in growth medium were reduced by 0.15–1.00 ppm. After the initial uptake phase, the Cu concentration in the growth medium were unexpectedly increased. This feature was prominent among the cultures exposed to high Cu concentration. It is assumed that, at this time, the cells were no longer tolerant to heavy metal exposure. During this phase, the intracellular protein bound heavy metals were released back into the growth medium when cells could not proliferate under this harsh culture conditions.

\[\text{**} \text{Due to the excellent reproducibility of ICP-OES technique, some error bars appear to be missing (100% reproducible) in Figure 25.}\]
Figure 25: Concentration of Cu (ppm) in the different growth medium over time. Each colored graph in the stack represents corresponding labeled initial Cu concentration (at $t = 0$, ppm) in the growth medium.
Figure 26 contains information equivalent to Figure 25, but for the biosorption of nickel by \textit{N. oculata}. In this stacked plot, the change of Ni concentration in the growth medium is plotted over time for all Ni contaminated cultures. Similar to the behaviors observed for Cu contaminated cultures, the biosorption of Ni from the growth medium was noticed within the first 7-8 days. This remediation process was initialized at a slower rate for the first 3-4 days which was followed by a rapid and shorter Ni uptake. For 0.091 and 0.32 ppm cultures, the Ni concentration reached minimum after 8 days while for other concentrated cultures it happened even earlier. Analysis of Variance (ANOVA, 95% confidence interval [141]) was performed to confirm the significance of the Ni uptake within this phase. The Ni concentration in the growth medium for different metal treated cultures were reduced by \(~0.03–0.80\) ppm. The heavy metal uptake phase was followed by an increase of Ni concentration in the growth medium. At this stage, the cells could not remediate any more Ni from their surrounding environment. The presence of excess heavy metals than required for sustaining life causes intracellular damages. As a result of this, cells of \textit{N. oculata} break apart and the remediated heavy metals are released back in the environment.

6.3.2. \textbf{Effect of Heavy Metals on Biomass Production}

Figure 27 demonstrates the effect of initial heavy metal concentrations on their dynamics of biomass production. The error bars in the figure derived from replicate flow cytometry measurement of the contaminated cultures. It was found that, all contaminated cultures experienced a slower growth rate compared to uncontaminated control culture. Similar to the uptake behavior observed in Figure 25, the biomass concentration of the Cu treated cultures was increasing for the first few days. However, after this timeline, the cell concentration of these cultures started to decline. This behavior was particularly dominant for the cultures exposed to higher Cu concentration. After the declining growth phase, the dynamics of biomass concentration appeared to reach a steady state. At this stage, the cell concentration of the Cu contaminated cultures was almost twice as low as the controlled culture.
Figure 26: Concentration of Ni (ppm) in the different growth medium over time. Each colored graph in the stack represents corresponding labeled initial Ni concentration (at $t = 0$, ppm) in the growth medium.
Figure 27: Effect of Cu concentration of the biomass concentration over time. Each color represents corresponding initial Cu concentration (ppm) in the culture.
Another interesting finding from the flow cytometry experiment was the amount of debris formed in the Cu treated cultures. The presence of debris was indicated by a relatively lower forward scattering intensity compared to healthy live cells. The red fluorescence intensity of these events confirmed that, these signals originated only from the cell fragments and not from growth medium salts. While the healthy control cultures had no debris for the chosen growth period, the contaminated cultures started to form considerable amount of cell debris after 7-8 days.

Figure 28 represents the effect of Ni metal concentration on *N. oculata*’s ability to produce biomass over time. The growth rate of the Ni exposed cultures was initially similar the control culture for the first ~5 days. However, at a later stage, the cultures experience a significantly slower growth rate. Furthermore, a comparison between Figure 27 and Figure 28 indicates that, *N. oculata*’s ability to produce biomass is more adversely influenced by nickel than copper. The cell concentration of the culture initially containing 9.59 ppm Ni was almost ~20 times lower than the uncontaminated culture.

In case of Ni treated cultures, the flow cytometry experiments detected the presence of fragmented cells in the growth medium. Compared to healthy living cells, the debris produced signals with low forward scattering intensity. Furthermore, the red fluorescence intensity due to intracellular chlorophyll confirmed that, this came from the cell debris. Since, the control culture did not indicate any debris formation, this was another effect of heavy metal on *N. oculata* cultures.

### 6.3.3. Scanning Electron Microscopy of *N. oculata*

Figure 29 and Figure 30 represent the SEM images of control and metal treated cultures respectively after 10 days. While the image from healthy control culture in Figure 29 did not indicate any cell debris formation, the cell fragmentation was evident in heavy metal treated images in Figure 30. Figure 30 (A) and (B) are images for Cu contaminated cultures growing at high and low concentration respectively. Although, it is difficult to draw a quantitative conclusion from these images, the cells exposed to higher Cu concentration produced more debris than cultures grown at lower Cu concentration. Figure 30 (C) and (D) shows the effect of high and low Ni concentration, respectively, on cell fragmentation. Again, the high concentrated Ni culture suffered more cell fragmentation than cells exposed to low Ni concentration. The rupture of the cell wall also caused a leakage of cell inclusions to form *algal mud* as evident in Figure 31.
Figure 28: Effect of Ni concentration of the biomass concentration over time. Each color represents corresponding initial Ni concentration (ppm) in the culture.
Figure 29: SEM image of a control *N. oculata* culture after 10 days.
Figure 30: SEM images of heavy metal contaminated cultures after 10 days. (A) and (B) represent images of Cu contaminated cultures growing at relatively higher and lower Cu concentration respectively. (C) and (D) represent images of Ni treated cultures growing at relatively higher and lower Ni concentration. The red arrows represent the fragmentation due to cell rupture while the yellow arrows represent the presence of cell debris.
Figure 31: SEM image of *N. oculata* growing under copper treated culture showing the evidence of *algal mud* and debris formation after 10 days.
6.4. **Conclusion**

The heavy metal pollutants can have detrimental effect on phytoplankton. The phytoremediation of heavy metals by marine microalgae as well as their ability to sustain life are adversely affected by heavy metal exposure. In this study, the effect of copper and nickel on *Nannochloropsis oculata* was assessed by monitoring their heavy metal uptake and growth simultaneously. The uptake of copper and nickel by *N. oculata* was quantified by inductively coupled plasma optical emission spectroscopy. It was found that, *N. oculata* cells exposed to copper and nickel concentration were intolerant to the contaminated environment. Although, the heavy metal uptake was unaffected for the first few days, later due to intracellular damage, the sequestered heavy metal was released back in the environment. This was more dominant for cultures growing at higher heavy metal concentration. This unorthodox uptake behavior was also supported by their dynamics of biomass production. For this reason, flow cytometry was used to measure the cell concentration of healthy and heavy metal contaminated *N. oculata* cultures. A comparison between their growth rate indicated that cells became highly intolerant to copper and nickel exposure. Even though, their growth rates were initially similar to that of control culture, at the end the number of live cells in contaminated cultures were significantly less than contaminated culture. Furthermore, the cell rupture and fragmentation suffering from the presence of heavy metals were imaged by scanning electron microscopy.

From environmental perspective, this is the most intimidating finding because it indicates that, phytoplankton’s may not be able to remediate the uprising heavy metal release in the environment. In the long run, the heavy metal pollution can trigger damages in biodiversity and even, extinction.
Chapter 7. Conclusions and Future Directions

Due to industrialization, the release of anthropogenic pollutants in the environment has been considerably increasing. The consequences of this pollutants possess serious environmental and health risks. Therefore, studying the fate of the anthropogenic pollutants has become a major concern for predicting future environmental scenarios. Marine phytoplankton act as a considerable sink for many of these pollutants. Therefore, it is crucial to quantitatively understand the sequestration of anthropogenic pollutants by marine phytoplankton. However, the chemical analyses of phytoplankton are challenging due to sheer number of simultaneously influencing chemical and biological parameters. Current analytical approaches are often limited to one independent parameter and thus, do not shed light on the overall picture. Therefore, there is a need for a holistic analytical approach to explore phytoplankton’s sequestration of anthropogenic pollutants. This dissertation quantitatively describes the microalgal sequestration of anthropogenic pollutants by linking all the interactions between microalgae and their chemical environment. Of particular interest are anthropogenic pollutants greenhouse gas CO\textsubscript{2} and heavy metals (copper and nickel).

In order to quantitatively predict CO\textsubscript{2} sequestration and microalgal biomass production, it is crucial to accurately describe the relation between nutrient concentration and resulting biomass. However, since cells can only chemically interact with their microscopic vicinity, CO\textsubscript{2} sequestration must be investigated on a micrometer spatial resolution. However, macroscopic ecosystems cannot straightforwardly be studied experimentally on microscopic scales especially when cultures contain large number of cells. To overcome this limitation, modeling methodologies were presented which describe sequestration processes within microscopic vicinities of a large number of cells. These innovative modeling approaches were based on describing CO\textsubscript{2} dissolution, its transport to consumers as well as species-specific compound uptake and utilization. Moreover, real-world ecosystems contain multiple microalgae species that compete for a common nutrient source. Hence, competition effects among species were incorporated into these novel models, too. Model validation was accomplished under series of chemical conditions by comparing the predicted cell concentration with flow cytometry experiments. For this reason, two marine microalgae species *Dunaliella salina* and *Nannochloropsis oculata* were chosen. The comparison between predicted and experimental cell concentration
demonstrated an excellent agreement between the model and the experiments. The validated model was then applied to predict microalgae’s long-term \( \text{CO}_2 \) sequestration capacity and the impact of ocean acidification on biomass production. Furthermore, the model was implemented to predict the quantities of inorganic carbon consumption by microalgae. Since, the initial culturing conditions can only be similar in the model, the simulations provided a novel framework to compare carbon sequestration by different species in individual and mixed cultures.

This modeling approach can make significant contribution to environmental community as it introduces a comprehensive approach to quantify \( \text{CO}_2 \) sequestration. This holistic approach has the potential to open new perspective for both engineers and ecologists to investigate biofuel production and ocean pollution respectively. From biologists’ perspective, this innovative approach can also be applied to other prokaryotic and eukaryotic organisms to understand their responses to surrounding environment. Furthermore, the model can be augmented to predict phytoplankton’s adaptation to multiple pollutants at the same time. This will then present an accurate description of future responses of marine ecosystem to anthropogenic pollutions.

Furthermore, an indicator, namely intracellular \( \beta \)-carotene quantity, was proposed to monitor atmospheric \( \text{CO}_2 \) sequestration capacity of marine microalgae \textit{Dunaliella salina}. Cultures of \textit{D. salina} respond to their surrounding atmospheric \( \text{CO}_2 \) environment by adapting their intracellular \( \beta \)-carotene quantity. Furthermore, \( \text{CO}_2 \) sequestration and biomass producing capacity of \textit{D. salina} is strongly influenced by their \( \text{CO}_2 \) concentration. For this reason, an inverse relationship was hypothesized between intracellular \( \beta \)-carotene quantity and sequestered \( \text{CO}_2 \). It was found that, the potential slowdown of \( \text{CO}_2 \) sequestration can be monitored by an increase of the intracellular \( \beta \)-carotene quantity of \textit{D. salina}. By linking the cause (\( \text{CO}_2 \) concentration) and the effect (intracellular \( \beta \)-carotene quantity) of the environmental change, this holistic approach can predict the future impact of greenhouse gas emission. Furthermore, this approach can broaden the scope beyond a laboratory sample to predict microalgal \( \text{CO}_2 \) sequestration performance in freshwater and marine ecosystems.

The uptake of anthropogenic heavy metal release by marine phytoplankton plays a crucial role for the environmental remediation. On the other hand, heavy metals have detrimental impact on marine biodiversity. In this research, the uptake of copper and nickel by marine microalgae \textit{Nannochloropsis oculata} was quantitatively investigated. Moreover,
the effect of copper and nickel on *N. oculata*'s ability to produce biomass was assessed. Furthermore, the intracellular damage due to the uptake of heavy metal was qualitatively investigated by imaging techniques. This study suggested that, the phytoremediation of copper and nickel were initially unaffected by the presence of heavy metals in their environment. However, after the initial uptake phase, *N. oculata* became intolerant to excessive heavy metal concentration and suffered intracellular damages i.e. cell wall rupture, cell fragmentation. Ultimately the initially remediated heavy metals were released back in the environment. This observation has the potential to open new research opportunities in this area. Since, intracellular protein molecules are responsible for binding with heavy metals, cell metabolic pathways for heavy metal binding need to be investigated. Furthermore, the impact of other industrial heavy metals on phytoplankton’s response in terms of growth and metal uptake need to be assessed.

The goal of this dissertation was to quantitatively understand the sequestration of anthropogenic pollutants by linking the interactions between microalgae and their environment. The quantitative sequestration of greenhouse gas and heavy metals were assessed by linking computational tools and experimental analyses. However, it is important to realize that, this work can only gain insight on the aftermath of anthropogenic pollution. In order to reduce the anthropogenic pollution, eventually humans need to shift to a sustainable and eco-friendly way of living. Only then humans can preserve a better world for future generations to come.
List of References


Appendices
Appendix 1

Figure 32: The impact of atmospheric CO$_2$ concentration on the dynamics of biomass production of individually grown cultures of *D salina* (left) and *N oculata* (right). In the right figure, although the initial cell concentration for 400 ppm culture was lower than 300 ppm CO$_2$ concentration, after a few days, the produced biomass in former was higher than the latter case.

Figure 33: The impact of atmospheric CO$_2$ concentration on the dynamics of biomass production of binary mixed cultures of *D salina* (left) and *N oculata* (right). Even though, the initial cell concentration for 400 ppm culture in the right figure was lower than 300 ppm CO$_2$ concentration, after a few days, the produced biomass in former was higher than the latter case.
Figure 34: Emission spectroscopy of pure β-carotene (50 mg/L) at different excitation wavelength. This validates the choice of excitation wavelength 350 nm as it produces the most intense β-carotene signal.
Figure 35: Schematic of the fate of heavy metals within microalgae.

**Step 1: Microalgae cells cultured at heavy metal treated growth medium**

**Step 2: Heavy metal transports across the cell membrane to be i) adsorbed on the cell wall (fast), and/or ii) accumulated within the chloroplast (slow)**

**Step 3: Intracellular heavy metal binds with the protein by forming metal-protein complex**

**Step 4: Excess heavy metals in the chloroplast disrupts the metabolic activity by breaking the metal-protein complex**

**Step 5: The toxic effect of heavy metals causes the cells to rupture into debris.**
Figure 36: Calibration curve of Cu standards from ICP-OES ($R^2=0.99968$).

Figure 37: Calibration curve of Ni standards from ICP-OES ($R^2=0.99976$).
Appendix 4

Summary of Graduate School Honors, Publications and Presentation

Honors

- Poster winner (3rd place) at the Eastman-NETSACS Student Research Symposium, 2018.
- Graduate Students Senate travel award, University of Tennessee, 2018.
- National Science and Technology Fellowship, Ministry of Science and Technology, Government of Bangladesh, 2012.
- Applied Chemistry and Chemical Technology Alumni Scholarship, University of Dhaka, 2009.

Publications

- M. Hasan*, F. Vogt, Modeling the transformation of atmospheric CO₂ into microalgal biomass, Analyst, 142, 4089-4098, 2017. DOI: 10.1039/C7AN01054K.

Presentations (3 oral and 4 poster)

- M. Hasan*, F. Vogt, 2018, Intracellular carotenoid quantity as an indicator for CO₂ sequestration capacity of Dunaliella salina, SciX, Atlanta, Georgia and Eastman-NETSACS Student Research Symposium, Kingsport, Tennessee
- M. Hasan*, F. Vogt, 2017, Modelling the transformation of inorganic environmental pollutants into microalgal biomass, University of Dhaka, Bangladesh.
Vita

Mohammed Fahad Hasan was born in Mymensingh, Bangladesh, on November 13, 1989. He grew up in Dhaka, Bangladesh and attended primary, middle and high school in Udayan Higher Secondary School. Fahad obtained a Bachelor of Science degree in Applied Chemistry & Chemical Engineering from University of Dhaka. After graduation, Fahad started his doctoral studies at University of Wyoming from Spring 2014. In Fall 2014, he moved to University of Tennessee to reunite with his family. He performed his doctoral research in Analytical Chemistry division under the direction of Dr. Frank Vogt (deceased on December 4, 2018) and Dr. Christopher Baker. Fahad will graduate in May 2019 with a Ph.D. in Analytical Chemistry.