BIOREMEDIATION OF WASTEWATER USING MICROALGAE

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ABSTRACT

BIOREMEDICATION OF WASTEWATER USING MICROALGAE

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Population expansion and industrial development has deteriorated the quality of freshwater reservoirs around the world and has caused freshwater shortages in certain areas. Discharge of industrial effluents containing toxic heavy metals such as Cd and Cr into the environment have serious impact on human, animal and aquatic life. In order to solve these problems, the present study was focused on evaluating and demonstrating potential of microalgae for bioremediation of wastewater laden with nitrogen (N) in the form of nitrates, phosphorous (P) in the form of phosphates, chromium (Cr (VI)) and cadmium (Cd (II)). After screening several microalgae, Chlorella vulgaris and algae taken from Pleasant Hill Lake were chosen as candidate species for this study. The viability of the process was demonstrated in laboratory bioreactors and various experimental parameters such as contact time, initial metal concentration, algae concentration, pH and temperature that would affect remediation rates were studied. Based on the experimental results, correlations were developed to enable customizing and designing a commercial Algae based Wastewater Treatment System (AWTS).
commercial AWTS system that can be easily customized and is suitable for integration into existing wastewater treatment facilities was developed, and capital cost estimates for system including installation and annual operating costs were determined.

The work concludes that algal bioremediation is a viable alternate technology for treating wastewater in an economical and sustainable way when compared to conventional treatment processes. The annual wastewater treatment cost to remove N,P is ~26x lower and to remove Cr, Cd is 7x lower than conventional treatment processes. The cost benefit analysis performed shows that if this technology is implemented at industrial complexes, Air Force freight and other Department of Defense installations with wastewater treatment plants, it could lead to millions of dollars in savings that could be repurposed for meeting other needs.
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TABLE OF CONTENTS

ABSTRACT.................................................................................................................. iv
ACKNOWLEDGEMENTS.............................................................................................. vi
LIST OF FIGURES......................................................................................................... xi
LIST OF TABLES............................................................................................................. xviii
LIST OF SYMBOLS, NOTATIONS, AND DEFINITIONS........................................... xx
CHAPTER 1 INTRODUCTION..................................................................................... 1
  1.1 Conventional Wastewater Treatment ................................................................. 2
  1.2 Nitrogen, Phosphorus and Heavy Metal Contamination ...................................... 4
     1.2.1 Nitrogen and Phosphorus Contamination ...................................................... 4
     1.2.2 Heavy Metal Contamination ..................................................................... 5
  1.3 Regulations .......................................................................................................... 7
  1.4 Conventional Removal Technologies ................................................................. 8
     1.4.1 Nitrogen Removal Technology ................................................................. 9
     1.4.2 Phosphorus Removal Technology ............................................................. 9
     1.4.3 Heavy Metal Removal Technology ............................................................ 9
  1.5 Need for Novel Technology ............................................................................... 10
  1.6 Microalgae ......................................................................................................... 11
CHAPTER 2 LITERATURE......................................................................................... 12
  2.1 Wastewater Treatment with Microalgae ............................................................ 12
  2.2 Mechanisms of Nutrient and Heavy Metal Removal .......................................... 13
     2.2.1 Nitrogen ................................................................................................. 14
     2.2.2 Phosphorus ........................................................................................... 15
     2.2.3 Heavy Metals ......................................................................................... 15
  2.3 Logic of Strain Selection ................................................................................... 16
  2.4 Growth Conditions of Chlorella Vulgaris ........................................................... 17
  2.5 Growth Controlling Factors of Microalgae ......................................................... 18
4.8.2 Algae Cell Growth ........................................................................................................ 57
4.8.3 Nitrate Analysis ............................................................................................................ 58
4.8.4 Phosphorus Analysis ................................................................................................... 58
4.8.5 Total Nitrogen Analysis ............................................................................................... 59
4.8.6 Metal Analysis ............................................................................................................ 59
4.8.7 Lipid Extraction ........................................................................................................ 60

CHAPTER 5 RESULTS AND DISCUSSION ............................................................................. 62
5.1 Nitrate and Phosphorus Removal .................................................................................... 63
  5.1.1 Preliminary Experiments (selection of microalgae) .................................................. 63
  5.1.2 Nitrate and Phosphorus Removal Experiments .......................................................... 67
    5.1.2.1 Effect of Nitrate and Phosphorus on Growth ..................................................... 68
    5.1.2.2 Effect of Initial Phosphorus Concentration on Nitrate Removal ..................... 72
    5.1.2.3 Effect of Initial Nitrate Concentration on Phosphorus Removal ..................... 75
    5.1.2.4 Effect of Nitrate Concentration on Lipid Content ........................................... 78
    5.1.2.5 Nitrogen Uptake Validation .............................................................................. 81
    5.1.2.6 Bio Kinetic Coefficients of Nitrate and Phosphorus Removal ......................... 86
  5.1.3 Effect of Temperature on Nitrate and Phosphorus Removal ..................................... 88
  5.1.4 Nitrogen Removal from Different Nitrogen Sources ............................................... 92
  5.1.5 Phosphorus Removal by Species Acclimated to High P Environments ............... 96
5.2 Heavy Metal (Cr, Cd) Removal Experiments ................................................................. 101
  5.2.1 Preliminary Studies .................................................................................................. 101
  5.2.2 Biosorption of Chromium and Cadmium from Synthetic Wastewater by
        Chlorella vulgaris ........................................................................................................ 108
  5.2.3 Kinetics Studies ....................................................................................................... 119
  5.2.5 Heavy Metal Removal Under Mixotrophic and Heterotrophic Conditions
        ..................................................................................................................................... 127
    5.2.5.1 Mixotrophic and Heterotrophic Growth ......................................................... 127
    5.2.5.2 Heavy Metal Removal ................................................................................. 133
5.3 Application of Algal Bioremediation for Wastewater Treatment at Industrial
    Complexes .......................................................................................................................... 135
5.4 Metal Recovery from Algae Surface ............................................................................ 138
5.5 Design of Algae-based Wastewater Treatment Technology (AWTS) ...................... 140
  5.5.1 Technology Assumptions ....................................................................................... 141
  5.5.2 Site Location and Climatic Conditions .................................................................. 142
  5.5.3 Design Theory ....................................................................................................... 143
5.5.4 Design Principle................................................................. 149
5.5.5 Cost Estimation................................................................. 153
5.5.6 Conventional Process vs AWTS for Treating Metal Laden Water........ 157

CHAPTER 6 SUMMARY AND FUTURE WORK .................................. 161
6.1 Summary .............................................................................. 161
6.2 Future Work ....................................................................... 162

BIBLIOGRAPHY ........................................................................ 163
APPENDIX A Cost Estimation .................................................. 188
LIST OF FIGURES

Figure 1.1 Schematic representation of conventional wastewater treatment plant........... 3

Figure 2.1. Process involved in a high rate algal pond. .................................................. 13

Figure 2.2 Conversion of inorganic nitrogen to organic nitrogen. ................................. 14

Figure 2.3. Metal binding sites of a typical algal cell. .................................................... 16

Figure 4.1. Microscopic photos of algae species .............................................................. 35

Figure 4.2. Bioremediation experimental set up. ............................................................... 42

Figure 5.1. Nitrate removal from wastewater. ................................................................. 63

Figure 5.2. Nitrate uptake per unit biomass. ................................................................. 64

Figure 5.3. Phosphorus removal from wastewater. ......................................................... 65

Figure 5.4. Phosphate uptake per unit biomass. .............................................................. 66

Figure 5.5. Growth of species in wastewater. ................................................................. 67

Figure 5.6. Growth curves of C.vulgaris in synthetic wastewater at different P loading, \( (\text{PO}_4^3) \) experiments [\( \text{PO}_4^3 \)-P (mg/L) (♦)13.1; (■) 26.21; (▲)52.42 ]; [\( \text{NO}_3^- \) ~183 mg/L]................................................................. 69

Figure 5.7. Growth curves of C.vulgaris in synthetic wastewater at different nitrate loading, \( (\text{NO}_3^-) \) experiments.[\( \text{NO}_3^- \) (mg/L) (♦) 90.12; (■) 182.68; (▲) 362.69; (●)741.56]; [\( \text{PO}_4^3 \) ~52 mg/L]. ........................................................................................................ 69

Figure 5.8. General pattern of microalgal growth in batch cultures. ................................. 70
Figure 5.9. Variation of nitrate concentration in wastewater with time under various initial phosphorus concentrations, \((\text{PO}_4)_i\) experiments. \([\text{PO}_4^{-}\text{P (mg/L)} (\bullet) 13.1; (\blacksquare) 26.21; (\blacktriangle) 52.42] \) and constant nitrate concentration \((\text{NO}_3^- \sim 183 \text{ mg/L})\) ................................ 74

Figure 5.10. Variation of phosphorus concentration in wastewater with time under various initial phosphorus concentrations, \((\text{PO}_4)_i\) experiments. \([\text{PO}_4^{-}\text{P (mg/L)} (\bullet) 13.1; (\blacksquare) 26.21; (\blacktriangle) 52.42] \) and constant nitrate concentration \((\text{NO}_3^- \sim 183 \text{ mg/L})\) .................. 74

Figure 5.11. Variation of phosphorus concentration in wastewater with time under various initial nitrate concentrations, \((\text{NO}_3)_i\) experiments. \([\text{NO}_3^{-}\text{ (mg/L)} (\bullet) 90.12; (\blacksquare) 182.68; (\blacktriangle) 362.69; (\blacklozenge) 741.56] \) and constant phosphorus concentration \((\text{PO}_4^{-}\text{P } \sim 52 \text{ mg/L})\) ................................................................. 77

Figure 5.12. Variation of nitrate concentration in wastewater with time under various initial nitrate concentrations, \((\text{NO}_3)_i\) experiments. \([\text{NO}_3^{-}\text{ (mg/L)} (\bullet) 90.12; (\blacksquare) 182.68; (\blacktriangle) 362.69; (\blacklozenge) 741.56] \) and constant phosphorus concentration \((\text{PO}_4^{-}\text{P } \sim 52 \text{ mg/L})\) ....... 77

Figure 5.13. Nitrate uptake rate as a function of initial nitrate concentration. ................. 78

Figure 5.14. Nitrate removal efficiency in synthetic wastewater for \((\text{NO}_3)_i\) experiments. ......................................................................................................................... 78

Figure 5.15. Comparison of lipid content of \(C. vulgaris\) grown in synthetic wastewater with different initial nitrate concentration in exponential and stationary phase. .......... 79

Figure 5.16. Nitrogen validation for \((\text{PO}_4)_1\) experiments. ............................................. 82

Figure 5.17. Nitrogen validation for \((\text{PO}_4)_2\) experiment.................................................... 82

Figure 5.18. Nitrogen validation for \((\text{PO}_4)_3\) experiment.................................................. 83

Figure 5.19. Nitrogen validation for \((\text{NO}_3)_1\) experiment................................................. 83

Figure 5.20. Nitrogen validation for \((\text{NO}_3)_2\) experiment.................................................. 84
Figure 5.21. Nitrogen validation for (NO$_3^-$)$_3$ experiment .......................................................... 84
Figure 5.22. Nitrogen validation for (NO$_3^-$)$_4$ experiment .......................................................... 85
Figure 5.23. Effect of initial NO$_3$-N concentration on specific NO$_3$-N removal rate for
(NO$_3^-$)$_i$ set of experiments ........................................................................................................ 87
Figure 5.24. Effect of initial PO$_4$-P concentration on specific PO$_4$-P removal rate for
(PO$_4^-$)$_i$ set of experiments ........................................................................................................ 88
Figure 5.25. Effect of temperature on algal biomass productivity ...................................................... 89
Figure 5.26. Effect of temperature on nitrate uptake of C. vulgaris .................................................. 91
Figure 5.27. Effect of temperature on phosphorus uptake of C. vulgaris ........................................... 91
Figure 5.28. Growth of C. vulgaris under different nitrogen sources ............................................... 93
Figure 5.29. Biomass productivity of C. vulgaris under different nitrogen sources ....................... 93
Figure 5.30. Variation of nitrogen concentration with time in synthetic wastewater containing four different nitrogen sources ........................................................................................................ 95
Figure 5.31. Variation of nitrogen removal efficiency with time in synthetic wastewater containing four different nitrogen sources ........................................................................................................ 95
Figure 5.32. Nitrogen uptake by C. vulgaris from different nitrogen sources ................................. 96
Figure 5.33. Growth curve of Pleasant hill Lake species at different phosphorus concentrations (P1= ~10 ppm, P2= ~20 ppm, P3= ~30 ppm) ................................................................. 97
Figure 5.34. Growth curve of Delaware Lake species at different phosphorus concentrations (P4= ~10 ppm, P5= ~20 ppm, P6= ~30 ppm) ................................................................. 97
Figure 5.35. Phosphorus removal from Pleasant hill lake species (P1= ~10 ppm, P2= ~20 ppm, P3= ~30 ppm) ........................................................................................................ 98
Figure 5.36. Phosphorus removal from Delaware lake species (P4= ~10 ppm, P5= ~20 ppm, P6= ~30 ppm). ................................................................................................................. 98

Figure 5.37. Phosphorus removal efficiency of Plesant hill lake species (P1= ~10 ppm, P2= ~20 ppm, P3= ~30 ppm). ................................................................................................................. 99

Figure 5.38. Phosphorus efficiency of Delaware lake species (P4= ~10 ppm, P5= ~20 ppm, P6= ~30 ppm). ................................................................................................................. 99

Figure 5.39. Comparison of Phosphorus removal efficiency of Pleasant Hill Lake Delaware Lake and C. vulgaris............................................................................................................. 100

Figure 5.40. Comparison of Phosphate uptake per biomass by Pleasant Hill Lake Delaware Lake and C. vulgaris............................................................................................................. 101

Figure 5.41. Hexavalent chromium removal by C. vulgaris......................................................... 102

Figure 5.42. Hexavalent chromium removal by N. oleoabundans............................................. 103

Figure 5.43. Growth curves of C. vulgaris and N. oleoabundans during chromium removal process......................................................................................................................... 103

Figure 5.44. Variation of chromium concentration with time in control experiment..... 104

Figure 5.45. Cadmium removal by C. vulgaris. ........................................................................... 105

Figure 5.46. Cadmium removal by N. oleoabundans................................................................. 106

Figure 5.47. Growth curves of C. vulgaris and N. oleoabundans during cadmium removal process......................................................................................................................... 107

Figure 5.48. Variation of cadmium concentration with time in control experiment...... 107

Figure 5.49. Effect of initial biomass concentration on Cr$^{6+}$ removal
(pH 6.8; Cr(VI) = 30 mg L$^{-1}$; contact time = 240 min; temperature = 25 °C). ................. 108
Figure 5.50. Chromium percent removal for different initial biomass concentrations
(pH 6.8; Cr (VI) = 30 mg L\(^{-1}\); contact time = 120 hrs; temperature = 25 °C)......... 109
Figure 5.51. Effect of initial biomass concentration on Cd\(^{2+}\) removal
(pH = 6.8; Cd (II) = 100 mg L\(^{-1}\); contact time = 30 min; temperature = 25 °C).......... 110
Figure 5.52. Growth curves of \textit{C. vulgaris} during Cr (VI) removal at different initial biomass concentrations. .......................................................... 111
Figure 5.53. Growth curves of \textit{C. vulgaris} during Cd (II) removal at different initial biomass concentrations. .......................................................... 112
Figure 5.54. Effect of initial Cr (VI) concentration on Cr\(^{6+}\) removal (pH 6.8; algae concentration = ~1.45 g L\(^{-1}\); contact time =120 hrs; temperature = 25 °C)................... 113
Figure 5.55. Effect of initial Cd (II) concentration on Cd\(^{2+}\) removal (pH = 6.8;
algae concentration = ~1.53 g L\(^{-1}\); contact time =30 mins; temperature = 25 °C)........ 114
Figure 5.56. Effect of initial solution pH on Cr (VI) and Cd (II) ions adsorption by \textit{C. vulgaris} (chromium concentration = 30 mg L\(^{-1}\); cadmium concentration =150 mg L\(^{-1}\);
algae concentration = 1.5 g L\(^{-1}\); temperature = 25 °C).............................. 115
Figure 5.57. Effect of contact time on Cr (VI) metal uptake by \textit{C. vulgaris}
(pH = 6.5; algae concentration = ~1.5 g L\(^{-1}\); temperature = 25 °C)....................... 117
Figure 5.58. Effect of contact time on Cd (II) metal uptake by \textit{C. vulgaris}
(pH = 6.5; algae concentration = ~1.4 g L\(^{-1}\); temperature = 25°C)....................... 117
Figure 5.59. Effect of temperature on Cr (VI) and Cd (II) removal by \textit{C. vulgaris}
([Cr (VI) = 30 ppm; initial algae concentration = ~1.45 gL\(^{-1}\); pH = 6.5, contact time = 5 days]; [Cd (II) = 50 ppm; initial algae concentration = ~1.53 gL\(^{-1}\); pH = 6.5, contact time = 90 min]). .......................................................... 118
Figure 5.60. Plots for the pseudo first order kinetics of Cr (VI) biosorption on 
*C.vulgaris*................................................................. 121

Figure 5.61. Plots for the pseudo second order kinetics of Cr (VI) biosorption on 
*C.vulgaris*................................................................. 122

Figure 5.62. Plots for the pseudo second order kinetics of Cd (II) biosorption on 
*C.vulgaris*................................................................. 122

Figure 5.63. Chromium metal uptake by *C.vulgaris*: comparison of experimental metal 
uptake and normalized correlated values. (Good only within the range of pH: 2-8; 
temperature: 10-35 °C; initial metal ion concentration: 10-30 mg/L; initial algae 
concentration: 1.43-2.043 g)................................................................. 126

Figure 5.64. Cadmium metal uptake by *C.vulgaris*: comparison of experimental metal 
uptake and normalized correlated values. (Good only within the range of pH: 2-8; 
temperature: 10-35 °C; initial metal ion concentration: 50-150 mg/L; initial algae 
concentration: 1.45-2.15 g)................................................................. 127

Figure 5.65. Growth of *C.vulgaris* under mixotrophic and heterotrophic conditions with 
glycerol as organic carbon source.............................................. 130

Figure 5.66. Growth of *C.vulgaris* under mixotrophic and heterotrophic conditions with 
glucose as organic carbon source.............................................. 130

Figure 5.67. Growth of *C.vulgaris* under mixotrophic and phototrophic conditions.... 133

Figure 5.68. Chromium removal by mixotrophic and phototrophic cultures of 
*C.vulgaris*.................................................................................. 134

Figure 5.69. Growth of mixotrophic and heterotrophic cultures during chromium 
removal. .................................................................................. 135
Figure 5.70. Variation of Chromium percent removal with time by C.vulgaris. ........ 137

Figure 5.71. Comparison of chromium removal for two biomass treatment cycles
for 1 hour............................................................................................................................. 137

Figure 5.72. Cadmium removal by C.vulgaris. ................................................................. 138

Figure 5.73. Schematic representation of metal bounded algae gasification.............. 139

Figure 5.74. Flow chart of the design optimization strategy. ........................................... 144

Figure 5.75. Impeller ( D1/w = 5); D1 = 0.40 d. ................................................................. 145

Figure 5.76. Schematic representation of AWTS design.................................................. 151
LIST OF TABLES

Table 1.1. Sources of heavy metals from various industries ........................................ 6
Table 1.2. Health risks of various heavy metals .......................................................... 6
Table 1.3. Element requirements set by WHO ............................................................ 7
Table 1.4. Permissible limits of phosphate in water ....................................................... 8
Table 1.5. Industrial heavy metal discharge limits in US. .............................................. 8
Table 2.1. Nitrogen and phosphorus removal efficiencies. ........................................... 25
Table 2.2 Optimum pH values for sorption of Cr and Cd by algae.............................30
Table 4.1. Microalgae strains used in this study. .......................................................... 36
Table 4.2. TAP medium ......................................................................................... 38
Table 4.3. f/2 medium ............................................................................................. 39
Table 4.4. Experimental matrix for nitrate and phosphorus removal from synthetic
wastewater by Chlorella vulgaris .............................................................................. 44
Table 4.5. Experimental conditions for nitrate and phosphorus removal ....................44
Table 4.6 Experimental matrix for N removal from different nitrogen sources ..........45
Table 4.7. Experimental matrix for P removal by Lake Species (species acclimated to
high P environments) ............................................................................................. 47
Table 4.8 Biomass concentrations for preliminary experiments of Cr$^{6+}$ and Cd$^{2+}$ ....49
removal. .................................................................................................................... 49
Table 4.9 Experimental matrix for Cr$^{6+}$ removal by Chlorella. .................................................. 52
Table 4.10 Experimental matrix for Cd$^{2+}$ removal by Chlorella. ................................. 53
Table 4.11 Initial organic carbon and biomass concentrations for mixotrophic and heterotrophic growth of Chlorella vulgaris ................................................................. 56
Table 5.1. Specific growth rates of C. vulgaris. ................................................................. 71
Table 5.2. Effect of nitrate concentration on lipid content of C. vulgaris. .................... 80
Table 5.3. Sorption rate expression for different metal concentrations. ....................... 121
Table 5.4. Comparison of adsorption capacities of various adsorbents used for Cr (VI) and Cd (II) removal. .................................................................................................. 123
Table 5.5 Growth rates of mixotrophic .............................................................................. 132
Table 5.6. Operation parameters for contaminant removal ........................................ 152
Table 5.7: Pond construction cost ................................................................................. 154
Table 5.8. Other costs involved in AWTS design ......................................................... 155
Table 5.9. Installation cost for equipment as a percentage of the purchased equipment cost. ............................................................................................................. 156
Table 5.10. Total cost estimation for AWTS design ...................................................... 157
Table 5.11. Conventional process vs AWTS for treating metal laden water ............. 158
Table 5.12. Conventional process vs. AWTS for treating municipal wastewater. ....... 159
LIST OF SYMBOLS, NOTATIONS, AND DEFINITIONS

Abbreviation Key

\begin{itemize}
  \item \textbf{CaCl}_2 \cdot 2\text{H}_2\text{O} \quad \text{Calcium chloride dihydrate}
  \item \textbf{Co (NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O} \quad \text{Cobalt nitrate hexahydrate}
  \item \textbf{CoCl}_2 \cdot 6\text{H}_2\text{O} \quad \text{Cobalt dichloride hexahydrate}
  \item \textbf{KH}_2\text{PO}_4 \quad \text{Potassium phosphate}
  \item \textbf{FeSO}_4 \cdot 7\text{H}_2\text{O} \quad \text{Iron sulfate heptahydrate}
  \item \textbf{H}_3\text{BO}_3 \quad \text{Boric acid}
  \item \textbf{MgSO}_4 \cdot 7\text{H}_2\text{O} \quad \text{Magnesium sulfate heptahydrate}
  \item \textbf{MnCl}_2 \cdot 4\text{H}_2\text{O} \quad \text{Manganese chloride tetrahydrate}
  \item \textbf{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} \quad \text{Sodium dihydrogen phosphate}
  \item \textbf{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O} \quad \text{Sodium molybdate dehydrate}
  \item \textbf{NaNO}_3 \quad \text{Sodium nitrate}
  \item \textbf{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O} \quad \text{Sodium metasilicate pentahydrate}
  \item \textbf{NH}_4\text{Cl} \quad \text{Ammonium Chloride}
  \item \textbf{NH}_2\text{CONH}_2 \quad \text{Urea}
  \item (\textbf{NH}_4\text{)}_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O} \quad \text{Ammonium molybdate tetrahydrate}
  \item \textbf{ZnSO}_4 \cdot 7\text{H}_2\text{O} \quad \text{Zinc sulfate heptahydrate}
  \item \textbf{GC/MS} \quad \text{Gas chromatography/ Mass spectrometry}
  \item \textbf{ICP-MS} \quad \text{Inductively coupled plasma mass spectrometry}
\end{itemize}
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TAG</td>
<td>Triacylglyceride</td>
</tr>
<tr>
<td>AWTS</td>
<td>Algae based Wastewater Treatment System</td>
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CHAPTER 1

INTRODUCTION

Despite the fact that the world is two-thirds water, most of it is not potable. Population expansion and industrial development has deteriorated the quality of freshwater reservoirs around the world and has caused freshwater shortages in certain areas. According to U.S. geological survey, it is projected that by the year 2025 global water demand will exceed supply by 56% [1]. To solve this water crisis and to meet the growing population’s demand for freshwater, wastewater treatment techniques aimed at remediating wastewater should be employed.

Wastewater is any type of water that has been negatively affected in quality by anthropogenic influence. There are mainly two kinds of wastewater i.e. municipal wastewater and industrial wastewater. Municipal wastewater consists of domestic wastewaters originated from households, sewer overflows and storm drains. On average, each American uses about 150 gallons of water a day– most of it in the home and produces 80 gallons of wastewater per day [2]. As reported by Ramanarayanan et.al, the total domestic wastewater generation rate in the US is about $31 \times 10^9$ million gallons per day [3]. Remediating at least half volume of the wastewater generated daily will provide a solution for water crisis.
Different industries produce various kinds of industrial wastewater based on their own specific combination of pollutants. Among those, metal working industry serves as an important pollution distributor discharging heavy metal waste (toxic waste) such as nickel, lead, chromium, cadmium, zinc and iron contaminated wastewater into the environment. This indiscriminate disposal of industrial and domestic wastes leads to heavy pollution of water, threatening all kinds of inhabiting organisms [4]. Therefore, it is necessary to focus on the treatment techniques and ways to partially remove contaminants (nitrates, phosphates and heavy metals) from wastewater before discharging them into waterways.

1.1 Conventional Wastewater Treatment

Most of the wastewater treatment plants employ conventional wastewater treatment methods to reduce the contaminant concentration and to improve the quality of wastewater effluent before it discharges to groundwater or re-enters water bodies [5]. This method is employed only for treating municipal wastewater and is not designed and equipped for handling toxic industrial waste. Toxic waste is pretreated at the source of generation (industries) bringing down the concentration level and is sent to wastewater treatment plant as industrial wastewater. Figure 1.1 shows the schematic representation of conventional wastewater treatment plant.
In brief, municipal and industrial wastewater is initially collected and subjected to two main treatment processes: primary and secondary treatment. During primary treatment, pretreatment is performed which involves the usage of bar screens to remove large solids from the wastewater. The water is then pumped to pre-aeration tanks where grit (sand, eggshells, etc.) is removed. Next, the wastewater is fed to a primary clarifier where suspended solids are allowed to settle (primary settlement). After the primary clarifier, the wastewater still contains high amount of organic content and is sent to secondary treatment. During the secondary treatment, the organic content of the wastewater is degraded by microorganisms within the aeration tanks, and waste sludge is allowed to settle in the secondary clarifier. Sludge that settles on the bottom of the primary and secondary clarifiers is pumped to a digester via a sludge thickener to produce methane and carbon dioxide [7]. However, the water leaving the secondary clarifier could still contains significant amounts of nitrogen and phosphorus. Also in this process, there is no special method employed during the treatment cycle to remove heavy metals from wastewater.
1.2 Nitrogen, Phosphorus and Heavy Metal Contamination

1.2.1 Nitrogen and Phosphorus Contamination

(a) Sources

Conventional wastewater treatment method can only reduce nitrogen and phosphorus concentrations in wastewater to a limited extent [8]. Discharge of this partially treated water leads to high release of nitrates and phosphates into the environment. In addition, agricultural activities also contribute significantly for the release of nitrates and phosphates to the groundwater in most parts of the United States. They contribute about 50% of the nitrogen and phosphorus entering surface waters. Livestock and dairy farm operations also contribute their share to nitrate and phosphate loading of soils and contamination of groundwater due to the mobility of nitrates and phosphates through the soil. Storm water run-off and chemical pollution (automotive products, paint, cleaners, and pesticides) are the other sources that release nitrogen and phosphorus into the environment.

(b) Effect on environment and human health

Discharge of water having high amounts of nitrate and phosphates into the environment leads to eutrophication in rivers and lakes. Eutrophication has been recognized as major water pollution in North American lakes and reservoirs [9]. Phosphorus has been identified as a key grow-limiting nutrient for algae in lakes and reservoirs [10]. Excess discharge of phosphorus causes eutrophication in freshwater ecosystems [11] and results in serious issues like water discoloration and foaming, decreased water transparency, increased algal biomass and depletion of dissolved oxygen [12]. Coastal eutrophication is caused due to excess nitrogen discharge and can cause
excessive production of algal biomass, blooms of toxic algal species, increased mortality rate in aquatic species and increase in sedimentation of organic particles [13]. Hence, eutrophication related water quality concerns could have very significant negative economic effects.

Excess nitrate concentration in drinking water is a possible health risk to human health. Nitrate itself does not pose a health threat; however, nitrate reductase readily reduces nitrate to nitrite (NO$_2^-$) which is carcinogenic and can cause methaemoglobinemia (blue-baby syndrome) [14].

1.2.2 Heavy Metal Contamination

(a) Sources

Industries such as leather processing, printed board manufacturing, metal finishing and plating, welding units, textile dyes, etc. are mainly responsible for release of heavy metals into the environment. In addition, street runoffs also contribute to the heavy metal contamination in wastewater. Table 1.1 gives the list of industries that release heavy metals into water bodies.

(b) Effect on environment and human health

Heavy metal contaminants mainly impact the environment in two aspects. Firstly, they have an ability to exist in natural ecosystems for a long period. Secondly they have the tendency to accumulate in biological chains, thereby causing chronic and acute disorders [15]. Some of the common health risks of heavy metals are listed in Table 1.2.
Table 1.1. Sources of heavy metals from various industries [16].

<table>
<thead>
<tr>
<th>Metal</th>
<th>Source (Industries)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>Leather, pulp, petroleum and Electroplating</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Paint, plastics, pesticides, copper refineries.</td>
</tr>
<tr>
<td>Lead</td>
<td>Fertilizers, vehicle, aircraft plating</td>
</tr>
<tr>
<td>Copper</td>
<td>Steel works, paper and pulp</td>
</tr>
<tr>
<td>Zinc</td>
<td>Rubber industries, alkalis, detergents</td>
</tr>
<tr>
<td>Mercury</td>
<td>Light bulb, leather, ointment, adhesives</td>
</tr>
<tr>
<td>Arsenic</td>
<td>Automobile, dyes</td>
</tr>
<tr>
<td>Iron</td>
<td>Metal refining</td>
</tr>
</tbody>
</table>

Table 1.2. Health risks of various heavy metals [17].

<table>
<thead>
<tr>
<th>Metal</th>
<th>Health Risks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>Carcinogen, kidney and liver damage (long term exposure), tissue damage.</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Loss of hair, emphysema, cancer and damage to immune system</td>
</tr>
<tr>
<td>Lead</td>
<td>Memory loss and nerve damage</td>
</tr>
<tr>
<td>Copper</td>
<td>Tumor, leukemia, high blood pressure</td>
</tr>
<tr>
<td>Zinc</td>
<td>Vomiting and nausea</td>
</tr>
<tr>
<td>Mercury</td>
<td>Fatigue, depression and anxiety</td>
</tr>
<tr>
<td>Arsenic</td>
<td>Coma, pigmentation, muscle cramps</td>
</tr>
</tbody>
</table>
1.3 Regulations

Nowadays, it is evidently clear that nitrogen, phosphorus and heavy metal related problems are one of the serious concerns among different environmental issues in the society. Environmental laws have been increasingly stricter over these issues [6].

The World Health Organization sets up the limitation of nitrogen, phosphorus and other heavy metals in drinking water [18]. The NPDES sets up the limitation of nitrogen and phosphorus discharged from domestic wastewater treatment plants, which are typically 10 mg/l and 0.1 mg/l respectively [19]. Permissible limits of heavy metals, nitrate and phosphate levels in different water bodies are presented in Table 1.3 and Table 1.4 respectively. In addition to these regulations, some states such as Arizona, Minnesota, California and Texas passed state pollution laws that include source reduction and waste minimization plan [20]. The clean water act allows some qualified Publicly Owned Treatment Works (POTW) to regulate industrial discharges into sewer. Table 1.5 summarizes the industrial heavy metal discharge limits in United States.

<table>
<thead>
<tr>
<th>Element</th>
<th>Health based guidelines by the WHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate and nitrite</td>
<td>50 mg/l total nitrogen</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.003 mg/l</td>
</tr>
<tr>
<td>Lead</td>
<td>0.01 mg/l</td>
</tr>
<tr>
<td>Copper</td>
<td>2 mg/l</td>
</tr>
<tr>
<td>Zinc</td>
<td>3 mg/l</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.001 mg/l</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0.01 mg/l</td>
</tr>
</tbody>
</table>
Table 1.4. Permissible limits of phosphate in water [21].

<table>
<thead>
<tr>
<th>Designated use</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streams/rivers</td>
<td>0.1mg/l</td>
</tr>
<tr>
<td>Lakes/reservoirs</td>
<td>0.025mg/l</td>
</tr>
<tr>
<td>Streams entering lakes</td>
<td>0.05mg/l</td>
</tr>
</tbody>
</table>

Table 1.5. Industrial heavy metal discharge limits in US [20].

<table>
<thead>
<tr>
<th>Metal</th>
<th>Maximum total concentration (mg/L) USA (EPA, 1990 (a) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>0.26</td>
</tr>
<tr>
<td>Chromium</td>
<td>1.71</td>
</tr>
<tr>
<td>Copper</td>
<td>2.07</td>
</tr>
<tr>
<td>Lead</td>
<td>0.43</td>
</tr>
<tr>
<td>Nickel</td>
<td>2.38</td>
</tr>
<tr>
<td>Zinc</td>
<td>1.48</td>
</tr>
</tbody>
</table>

1.4 Conventional Removal Technologies

Partial removal of nitrogen and phosphorus from municipal wastewater and inability to remove heavy metals from industrial wastewater via conventional wastewater treatment technique clearly calls for an additional treatment methods. Thus, to complete the water purification process an additional tertiary treatment, known as advanced treatment is performed. The methods of advance treatment range from various physical/chemical separation methods to biological treatments.
1.4.1 Nitrogen Removal Technology

Nitrogen removal in advanced treatment comprises two steps: nitrification followed by denitrification. During nitrification, ammonia NH$_4^+$ is converted to nitrite (NO$_2^-$) and this nitrite is converted to nitrate (NO$_3^-$) by autotrophic bacteria *Nitrosomonas* and *Nitrobacter* respectively within the aerobic zone. Then, denitrification takes place where nitrate is reduced to gaseous nitrogen by heterotrophic bacteria such as *Flavobacterium Alcaligenes, Pseudomonas* etc. Nitrifying bacteria are very sensitive and susceptible to a variety of conditions. Organic substrates such as glucose or acetic acid should be supplemented to heterotrophic denitrifying bacteria to support the process (reaction).

1.4.2 Phosphorus Removal Technology

Tertiary treatment is employed as an advance treatment for removing phosphorus from wastewater. During tertiary treatment, most of the wastewater treatment plants around the world use chemical precipitation method to remove phosphorus from wastewater. In this method, phosphate is retained as insoluble metal phosphate when iron or aluminum salt is added to wastewater. Few other technologies such as crystallization, bacteria phosphorus removal and other sludge base methods are also employed to recover phosphorus from wastewater [22].

1.4.3 Heavy Metal Removal Technology

Several physico-chemical technologies like chemical precipitation, ion-exchange, adsorption, filtration, phytoremediation etc. are mainly implemented for stripping toxic heavy metals from wastewaters [23, 24, and 25]. Brief description of each method is described below.
(a) Chemical Precipitation

In this process, under controlled pH, precipitation of metals is achieved by the addition of flocculation agents such as iron chloride, aluminum hydroxide, lime and other organic polymers.

(b) Ion Exchange

During this process, exchange of metal ions from dilute solutions takes place between two electrolytes.

(c) Adsorption

A fluidized bed formed using granular iron hydroxide or manganese dioxide is used to retain heavy metals from wastewater.

(d) Filtration

This technique is used if heavy metals are present in the form of suspended matter or colloidal particles. This is a conventional membrane filtration technique where porous membranes are used to remove heavy metals from wastewater.

1.5 Need for Novel Technology

Despite the fact that the above described nitrogen, phosphorus and heavy metal removal methods are effective, all the methods have their own disadvantages. These methods consume significant amounts of energy, usage of additional chemicals and therefore are expensive. Furthermore, toxic sludge is generated as a by-product in chemical treatment methods [26, 27] and these techniques may be ineffective when concentration of metal in wastewater is in the range between 10–100 mg l⁻¹ [28].

To overcome these problems, there is a need for economic and energy efficient nitrogen, phosphorus and heavy metal removal technology. By introducing an alternative,
biological method (bioremediation) for the treatment of metal enriched wastewaters containing high amounts of nitrates and phosphates, most of these issues can be mitigated and will provide a means for cost-effective removal of contaminants in wastewater. Bioremediation is a process in which microorganisms are employed to return water altered by contaminants to its original condition.

1.6 Microalgae

Microalgae are phototrophic unicellular organisms found individually or in groups that consume carbon dioxide, nitrogen and phosphorus, and release oxygen. Algae have an affinity for polyvalent metals and are very effective in removing heavy metals, nitrates and phosphates present in wastewater. Therefore, use of algae for bioremediation of wastewater offers potential advantages over other techniques in use. In recent times, algae has become of great interest since it provides several advantages such as biofuel production, carbon dioxide mitigation, wastewater treatment, food production and so on. Typically, algae are grown in inorganic nutrient media but the increasing cost of inorganic fertilizers and emergence of serious problems of pollution during the production of such fertilizers [29] has led researchers to search for an alternative source, i.e. the use of organic materials of different origin. Animal wastes have a long history that shows their use as a source of phosphorus, nitrogen and carbon for microalgal growth and the production of natural food [30].

To provide an alternative method to the conventional wastewater treatment techniques, the present study focuses on the potential of algae to treat wastewater and to remove heavy metals ions from metal laden wastewater.
CHAPTER 2
LITERATURE

2.1 Wastewater Treatment with Microalgae

Microalgae have a great potential to solve energy and environmental challenges around the world. Wastewater treatment with microalgae is a more environmental sound approach to reduce nitrogen and phosphorus and to remove heavy metals from wastewater. Microalgae can absorb significant amount of nutrients because they need large amounts of nitrogen and phosphorus for proteins (45-60% microalgae dry weight) and metals as micronutrients for their growth. William Oswald (1957) first developed the idea of treating wastewater in using microalgae and performed photosynthesis in sewage treatment [31]. Figure 2.1 briefly depicts the process involved in high rate algal pond in which algae plays a dual role by assimilating nutrients from wastewater and supplying oxygen to bacteria. The bacteria take up the oxygen and degrade organic material in the wastewater, the same process which is used in activated sludge treatment [31].
The selection of algae strain to be used in wastewater treatment is determined by
their robustness against wastewater and by their ability to grow in and to assimilate
nutrients from wastewater [32]. *Chlorella, Scenedesmus, Neochloris* and *Spirulina* are
the widely used algae species in experimental studies of wastewater treatment. The major
advantages of using microalgae over conventional methods as summarized by De la Noüé
(1992) [33] are: (a) nutrients can be removed more efficiently; (b) no generation of toxic
by-product (sludge) (c) biofuels can be produced from biomass harvested (energy
efficient); (d) cost–effective.

### 2.2 Mechanisms of Nutrient and Heavy Metal Removal

Nutrient removal by algae involves various metabolic pathways of the algal cell.
Carbon, nitrogen, phosphorous and sulfur are the main components responsible for algal
growth. Metals such as potassium, sodium, calcium, iron and magnesium serve as
micronutrients for the growth of algae. Emphasis is usually put on nitrogen and
phosphorus uptake but importance is also given to the technological aspects of heavy
metal removal by algal biomass [34].
2.2.1 Nitrogen

Organic nitrogen is the key element in biological substances like enzymes, peptides, proteins, chlorophylls and energy transfer molecules such as ADP (adenosine diphosphate) and ATP (adenosine-5'-triphosphate) [35]. Organic nitrogen is derived from inorganic sources including nitrite (NO$_2^-$), nitrate (NO$_3^-$), nitric acid (HNO$_3$), ammonia (NH$_3$), ammonium (NH$_4^+$), and nitrogen gas (N$_2$). Microalgae has an ability to convert inorganic nitrogen only in the forms of nitrite, nitrate and ammonium to organic nitrogen through a process called assimilation. Only eukaryotic algae can perform assimilation of inorganic nitrogen [34]. Figure 2.2 describes the assimilation process of inorganic nitrogen. As shown in the figure 2.2, translocation of inorganic nitrogen takes place across the plasma membrane where reduction of nitrate takes place followed by the incorporation of ammonium into amino acids and glutamine. Initially nitrate is reduced to nitrite by a “NADH-dependent” nitrate reductase and the nitrite reduced to ammonium by “NADPH-linked” nitrite reductase present within the algae. The resulting ammonium is assimilated to form amino acids by glutamine and glutamate synthase within the intracellular fluid using adenosine triphosphate (ATP), glutamate (Glu) and glutamine synthase. Thus, all inorganic forms of nitrogen are finally reduced to organic form (amino acids) [34].

![Diagram of assimilation process](image_url)

Figure 2.2 Conversion of inorganic nitrogen to organic nitrogen (assimilation).
2.2.2 Phosphorus

Phosphorus is found in lipids, proteins and nucleic acids. It plays a crucial role in cell growth and metabolism of algae. During algae metabolism, phosphorus mainly in the forms of $\text{H}_2\text{PO}_4^-$ and $\text{HPO}_4^{2-}$ is incorporated into organic compounds through a process called phosphorylation. Phosphorylation is an active process and it requires energy. This energy comes from the oxidation of respiratory substrates, the electron transport system of the mitochondria, or from light. Generation of ATP from ADP takes place during phosphorylation and microalgae is able to assimilate and store phosphorus in excess within the cell in the form of volutin granules. These reserves will be available during the growth cycle in the absence of phosphorus in the media. The growth rate of algae may therefore not respond at once to changes in the external concentration of phosphorus, as opposed to the immediate responses to temperature and light [36].

2.2.3 Heavy Metals

Microalgae also require metals for their biological functions. Selected microalgae are cultivated for bio removal of certain metals. They have a potential to accumulate high concentrations of metals from the contaminated aquatic systems. Metal accumulation in algae involves two processes: an initial rapid (passive) uptake followed by a much slower (active) uptake [37]. During the passive uptake, physical adsorption takes place in which the metal ions are adsorbed over the cell surface very quickly just in a few seconds or minutes and this process is metabolism-independent. Then chemisorption, a process which is metabolism-dependent takes place in which the ions are transported slowly across the cell membrane into the cytoplasm.
The algal cell wall has an anionic surface and contains many functional groups, such as hydroxyl (-OH), carboxyl (-COOH), amino (-NH₂), sulphydryl (-SH), etc. Since metal ions in water are generally in the cationic form, they are adsorbed onto the cell surface [38, 39]. Polyphosphate bodies are present in algae and they provide a storage pool for metals. Several researchers have established that metals such as Cd, Co, Hg, Ni, Cu, Ti, Pb, Mg, Zn, are sequestered in polyphosphate bodies in green algae [40]. Once metal accumulates inside the cell, metal ions may be preferentially located within specific organelles and bound to proteins such as a metallothionein [41, 42] as shown in the figure 2.3.

Figure 2.3. Metal binding sites of a typical algal cell [28].

2.3 Logic of Strain Selection

Selection of algal strain is a key consideration during bioremediation process. Algae are diverse photosynthetic organisms that have an estimated 10 million species on the earth. Most algal strains are photoautotrophs, but some have an ability to grow in heterotrophic and mixotrophic conditions. Extensive research has been conducted since
the 1980’s to identify the algal strain suitable for wastewater treatment [43].

Complimenting this, current research work is focused on some of the species which have been found to grow well in wastewater. In addition, the chosen algae should accumulate Cd and Cr to a high concentration without suffering toxic symptoms. Chlorella vulgaris, Scenedesmus dimorphous, Neochloris oleoabundans, Nannochloroposis, Spirulina, Botrycoccus braunii, Dunaliella salina etc. are commonly used species for treating wastewater and removing heavy metals [44, 45, and 46].

Among these species, Chlorella vulgaris was selected for the present study as it is very robust compared to other species and can perform well in wastewater and have an ability to remove heavy metals. It can also tolerate a wide range of nutrient concentrations, temperature, and pH, making it versatile for wastewater remediation. In addition, chlorella can grow in all three growth conditions (autotrophic, heterotrophic and mixotrophic) and contains high lipid content (28.82 %) [47] which can be further processed for biofuel production.

2.4 Growth Conditions of Chlorella Vulgaris

Chlorella vulgaris can be grown in autotrophic [48], heterotrophic [49] and mixotrophic [50] conditions. During autotrophic growth, algae (Chlorella) utilizes energy from sun or external source, nutrients (nitrogen and phosphorus) from wastewater and carbon dioxide to increase its algal density. The major advantage of autotrophic growth condition is that it can reduce carbon dioxide to make useful organic compounds. However, the limitation is that light penetration is inversely proportional to the algal density. As the density of algae increases, the light exposure to algae cell decreases resulting in limitation of nutrient removal from wastewater.
During heterotrophic growth, no light is required and organic carbon source such as glucose or glycerol acts as source of carbon. Algae uses this organic carbon for growth. High algal densities can be obtained in heterotrophic growth as the growth is not limited by the light. Mixotrophic growth is a combination of heterotrophic and autotrophic growth, where carbon dioxide and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operates concurrently [51, 52].

2.5 Growth Controlling Factors of Microalgae

Carbon, nitrogen and phosphorus are the three main factors that influence the growth of any microalgae [53, 54]. Further, they also depend on complex interactions among physical factors such as light intensity [55, 56], pH [57] and temperature [58].

2.5.1 Carbon

The average stoichiometric formula of algae is \( C_{106}H_{181}O_{45}N_{16}P \) [59]. More than fifty percent of algae composition constitutes to carbon content. During photosynthesis, microalgae assimilate inorganic carbon (CO\(_2\)) and convert it to starch and oils. Atmospheric carbon dioxide may be provided to algal cultures by means of aeration [60]. However, the concentration of CO\(_2\) in air is very low (0.033 %) and is not enough for optimum algal growth. Therefore, supply of extra carbon dioxide up to 1 to 5 % CO\(_2\) (feasibility of obtaining from digestor gas) along with air [61] will increase the algal density. Beside this, algae can also use organic carbon sources such as glucose, glycerol or acetate during heterotrophic growth [61, 62, and 63]. Carbon is the key nutrient for getting algae to pump out more oil.
2.5.2 Nitrogen and Phosphorus

Nitrogen is the second most important nutrient to microalgae and exists in many forms such as \( \text{NH}_4^+ \), \( \text{NO}_3^- \) and \( \text{NO}_2^- \) etc. Algae prefer ammonium and when this is available, no other form of nitrogen sources will be assimilated [64]. Urea can also be used as a nitrogen source. Phosphorus is another macro-nutrient and algae take up inorganic ortho-phosphate (\( \text{PO}_4^{3-} \)) for its growth. The mechanism of nitrate uptake and phosphate uptake of algae was mentioned in the section 2.2.1 and 2.2.2 respectively. Literature suggests that typically below the N: P of 30, algae growth was determined solely by N limitation and above 30, by P limitation [65].

2.5.3 Light Intensity

Microalgae are phototrophs and they utilize energy from light. However, some microalgae can grow in the dark using organic compounds as energy. Light intensity affects directly the photosynthesis and growth rate of microalgae. During photosynthesis, microalgae need light for a photochemical phase to produce ATP and NADPH [66]. Insufficient light may lead to photo oxidation resulting in growth limitation and too much light may also cause photo inhibition lowering the photosynthetic effectivity [55, 56]. Many researchers have studied the effect of light on algae growth and concluded that light is the most often limiting factor of algal growth. In dense cultures, algae themselves decrease the light availability due to self-shading. When algae is cultivated in wastewater, presence of high amounts of particulate matter further aggravates the shading effect. To avoid this problem, proper mixing is essential as it exposes algae cells to light uniformly.
2.5.4 Temperature

Increase in temperature increases the algal growth until an optimum temperature is reached [60, 62, and 67]. Increase beyond optimum temperature leads to a rapid decline in growth rate. The optimum temperature range 15–25 °C is favorable for most algal species, even those which are adapted to growth at colder temperatures. At low temperatures, microalgae easily get photo inhibited by high light intensities. Algae sensitivity to light at low temperatures may pose an operational constraint to outdoor wastewater treatment in cold climate. At optimum growth temperatures, microalgae can better tolerate high light intensities before getting inhibited [64].

2.5.5 pH

Growth of algae species may also be affected by pH. Different species have different optimum pH levels. pH usually increases due to carbon dioxide assimilation and sometimes it reaches to 10 when no CO₂ is supplied [62, 67]. It can reach 11 or more if bicarbonate is used as a carbon source [61]. Nitrate and phosphate absorption by algae affects the pH. Nitrate ion assimilation will raise the pH and ammonium ion assimilation will decrease the pH as low as 3 [61, 68]. High pH may lead to phosphate precipitation in the medium forming calcium phosphates [62, 67] and also result in algae flocculation, thereby reducing nutrient uptake and growth.

2.6 Metal Chemistry

The toxicity of a particular metal is mainly governed by its electron distribution and local pH [69].
2.6.1 Electron Distribution

Metals can be classified based on their co-ordination chemistry, a property which is influenced by electron environment of the element. Metal ions are divided into three categories.

2. Soft Cations- Non-essential and very toxic eg. Cd²⁺, Cr³⁺, Cu²⁺, Pb²⁺ and Hg²⁺.
3. Borderline ions – Biologically essential and toxic eg. Fe³⁺, Fe²⁺, and Mn²⁺

The individual element characteristics are influenced by ionic potential and the number of electrons.

(i) Ionic potential: It is defined as charge to radius ratio and metals with high charge density metals (Cr and Cd) form a ligand whereas low charge density metals such as alkali metals do not bind well.

(ii) Number of electrons: Soft cations eg. Cr (4s¹ 3d⁵) Cd (4d¹⁰) ions with multiple electron shells are more easily polarized and form stable complexes with biochemically active bases such as thiol groups. Hard cations are not easily polarized, and are readily displaced from their binding site by competing soft cations.

So when algae comes in contact with wastewater containing these metal ions, alkali metal ions will be taken by algae for performing biological functions and toxic metal ions form complex with anionic surface of algae. This way any toxic metals present in the wastewater can be removed [69].
2.6.2 Effect of pH

The pH of the environment regulates the metal uptake and the amount of metal bound to the surface. When pH drops, cell membrane proton responds to maintain an internal negative charge leading to depolarization of membrane and a change in the metal affinity of the cell surface [70].

2.7 Nutrients and Heavy Metal (Cd, Cr) Removal (current knowledge)

2.7.1 Nutrients (nitrogen and phosphorus)

To use microalgae for wastewater treatment is an old idea, and it was first by proposed by Oswald and Gotaas in 1957 [31]. Since then several researchers have developed techniques for exploiting the algae’s nutrient removal capacity and its growth rate in wastewater. Numerous laboratory and pilot scale studies of wastewater treatment with algae have been conducted [6, 71, and 72].

Algae wastewater treatment systems have traditionally been employed as tertiary treatment systems [59, 73, and 74] and some researchers proposed them as potential secondary treatment systems [75]. Many studies have demonstrated successful wastewater treatment with microalgae [76, 77]. In most cases, species of the family of Chlorella, such as Chlorella vulgaris and Chlorella pyrenoidosa [78, 79, 80, 81, and 82] have been employed for wastewater treatment. The most widely studied microalgae species for nutrient removal are Chlorella [83, 84], Scenedesmus [6], Spirulina [85], Nannochloris sp. [86], Botryococcus braunii [87] etc. Few species of Scenedesmus and Chlorella completely remove ammonia, nitrate and total P from secondary treated wastewater [88, 89, and 90]. Most of algae based wastewater treatment experiments were performed at lab scale under batch culture conditions with the microalgae showing high
growth rates over the batch growth period. Table 2.1 shows the nitrogen and phosphorus removal efficiencies from wastewater from several sources by microalgae [91].

Wang et.al used green algae Chlorella sp. culture to remove nutrients from the wastewater samples collected from four different locations, i.e. before primary treatment (#1), after primary treatment (#2), after activated sludge treatment (#3) and centrate (#4) which is the wastewater generated from sludge centrifuge. From this study, it was concluded that Chlorella sp. successfully grew in all the samples and 82.4%, 74.7%, and 78.3% of nitrate was removed from the samples 1, 2 and 4 respectively. Also, 90% of phosphorus was removed from samples 1, 2 and 4 and N/P ratio dropped from 52.3 to 20.8 making the algae culture P-limited species to N-limited species. However, only 4.7% of phosphorus and 62.5% nitrate were removed from sample #3. The low removal of N,P from sample #3 was probably due to the excretion of small photosynthetic organic molecules by algae. [92].

Arbib et al. [93] studied the algal growth rate and nutrient removal rate of the species Chlorella stigmatophor and Scenedesmus obliquus in urban wastewater at different nitrogen and phosphorus ratios, ranging from 1:1 to 35:1. It was reported in this study that nitrogen to phosphorus ratios ranging between 9 and 13 achieved best biomass productivity. Renuka et al. [94] used Calthrix sp to remove nitrates and phosphates from sewage wastewater. They found that 44–91% PO$_4$-P and 57–58% NO$_3$-N can be removed and a highest dry cell weight of 0.97 mg/L can be attained in sewage wastewater using this species. Kim et al. treated swine wastewater with Chlorella vulgaris and reported 96 and 95.3% removal of phosphorus and nitrogen, respectively [95]. Travieso et al. reported removal of 90.2, 84.1 and 85.5% organic nitrogen, ammonia, and total
phosphorus, respectively from distillery wastewater with an anaerobic fixed-bed reactor in a microalgae pond [96].

Mohamed et. al [97] reported that Scenedesmus sp. is very common species that is available in fresh water bodies and plays a significant role in purifying eutrophic waters. Colak and Kaya (1988) reported removal of nitrogen (50.2%) and phosphorus (85.7%) in industrial wastewater treatment and elimination of phosphorus (97.8%) in domestic wastewater treated by algae. [98]. Min-kyu et.al used Scenedesmus obliquus to remove nitrogen and phosphorus from piggery wastewater. The authors reported 23 to 58% removal of TN and 48 to 69% removal of TP [99].

Bhatnagar et. al identified a species Chlorella minutissima in wastewater treatment oxidation ponds in India [100]. On characterizing the species, it was found that it was a eukaryotic species which belongs to Chlorellaceae family. They cultivated Chlorella minutissima in high concentrations of raw sewage (municipal wastewater) and standard BG 11 medium. The results showed that growth of Chlorella minutissima in municipal wastewater is 146% more than the growth in standard BG 11 medium [100]. Further it was found that this species can grow well under heterotrophic and mixotrophic conditions over a wide pH range (4-10).

Few studies have been done to investigate the influence of culture conditions on nutrient removal efficiency and growth rate of algae. Lodi et al. (2003) studied the effect of temperature on nutrient uptake of Spirulina platensis. He conducted experiments over a wide range of temperatures (23-40°C) and observed the highest removal rate occurred at 30 °C; however, the removal efficiencies were not satisfactory [101].
Table 2.1. Nitrogen and phosphorus removal efficiencies.

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Waste water</th>
<th>Removal (%)</th>
<th>Experimental Set-up</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botryococcus braunii</td>
<td>Domestic wastewater</td>
<td>79.6 %</td>
<td>Fermenter, V = 9 l, T = 25 °C, LI = 3,500 lx, CT = 14 days</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Botryococcus braunii</td>
<td>Secondary treated wastewater</td>
<td>80 %</td>
<td>Column bioreactors, V = 500 ml, T = 25 °C, LI = 100 μEm−2s−1, CT = 12 days</td>
<td>103</td>
</tr>
<tr>
<td>Chlorella kessleri</td>
<td>Synthetic wastewater</td>
<td>19 %</td>
<td>Conic bioreactors, V = 100 ml, T = 30 °C, LI = 45 μmol m−2s−1, CT = 72 h</td>
<td>104</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>Soyabean processing wastewater</td>
<td>89.1 %</td>
<td>Conic bioreactors, V = 500 ml, T = 27 ± 1 °C, LI = 40 μmol m−2s−1, CT = 5 days</td>
<td>105</td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>Agro-industrial wastewater</td>
<td>95 %</td>
<td>Cylindri.bioreactors, V = 2 l, T = 20 ± 2 °C, LI = 60 μmol m−2s−1, CT = 9 days</td>
<td>106</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>Synthetic wastewater</td>
<td>97 %</td>
<td>Column bioreactors, V = 2 l, T = 30 °C</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table

<table>
<thead>
<tr>
<th>Algal Species</th>
<th>Treatment</th>
<th>Light Intensity</th>
<th>Contact Time</th>
<th>Notes</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematococcus pluvialis</td>
<td>Primary treated wastewater</td>
<td>LI = 3,000 lx</td>
<td>CT = 14 days</td>
<td>Conic bioreactors</td>
<td>100% 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V = 130 ml T = 23 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LI = 50 μmol m⁻²s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT = 5 days</td>
<td></td>
</tr>
<tr>
<td>Neochloris oleoabundas</td>
<td>Synthetic wastewater</td>
<td>LI = 1,280 lumens.</td>
<td>CT = 7 days</td>
<td>Cylindrical bioreactors</td>
<td>99% 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V = 400 ml T = 30 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LI = 1,280 lumens</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT = 7 days</td>
<td></td>
</tr>
<tr>
<td>Spirulina</td>
<td>Pig wastewater</td>
<td>LI = 50 μmol m⁻²s⁻¹</td>
<td>CT = 7 days</td>
<td>Raceway ponds of 6 and 24 m²</td>
<td>84-96% 72-87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>outdoor conditions</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT = 7 days</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.7.2 Cd and Cr Removal

Limited efforts have been made to use algal biomass for removing toxic heavy metals such as Cr and Cd from aqueous solutions. Out of thousand algal species, only few have an ability to remove toxic heavy metals (Cr, Cd) from wastewaters. The commonly used species for removing Cr and Cd from wastewater are fresh water green algae like Chlorella spp, Neochloris oleoabundans, Cladophora spp., Scenedesmus spp., Chlamydomonas reinhardtii and blue green algae like Microcystis aeruginosa and Oscillatoria. The mechanism of algae metal removal ability which involves metal sorption is described in section 2.2.3. Metal sorption is defined as metal sorbed per unit biomass. During metal sorption process, metal will first bind to the cell surface and then to intracellular ligands. Several factors such as temperature, initial metal ion concentration, pH, initial biomass concentration and contact time affect the metal sorption process.
sorption ability of algae. Several researchers have performed various experiments to study the effect of these parameters on the algae ability to remove Cr and Cd from wastewater.

(a) Initial metal ion concentration

Sorption and heavy metal removal ability of algae largely depend on the initial concentration of metals in the solution. An increase in metal concentration in the solution increases metal sorption (metal sorbed per unit biomass) increases and becomes saturated after a certain concentration of metal in the solution [110, 111, 112]. In contrast to the metal sorption, the metal removal capacity decreases with the increase in metal concentration in the solution [24, 112].

(b) Biomass concentration

Biomass concentration affects the amount of heavy metal ion removed from a solution. Roy et. al showed that increase in biomass concentration of Chlorella minutissima decrease Cd binding per unit cell mass. Their results showed that a decrease of 91% in Cd binding per unit cell of C. minutissima for a 12 fold increase in biomass concentration [113]. Mehta et. al tested the effect of biomass concentration on metals by C. vulgaris at different metal concentrations. They found that metal sorption was maximal at the lowest tested biomass concentration [112]. Hamdy has performed experiments with four different algae species to remove metals such as Cr, Co, Ni, Cu and Cd from wastewater. He found that an increase in biomass concentration decreased the metal sorption capacity of all four species [114]. Similarly, Gong et al. (2005) also found that metal sorption capacity of Spirulina maxima decreased with increase in biomass concentration [115].
A few researchers explained that inverse proportional relationship between biomass concentration and sorption may be due to limited availability of metal, increased electrostatic interactions, interference between binding sites and reduced mixing at higher biomass concentrations [116, 117]. However, there is no straightforward relationship between and metal removal and biomass concentration.

(c) Temperature

Literature review reported contrasting results regarding the effect of temperature on heavy metal sorption of algae. Few researchers reported an increase in algae metal sorption with increase in temperature [118, 119]. The increase in temperature causes bond rupture and this increases the number of active sites involved in metal sorption. Aksu performed experiments with Chlorella vulgaris and found that with an increase in temperature from 15 °C to 45 °C there is an increase in metal sorption from 48.1 mg/g to 60.2 mg/g which suggests that algae metal biosorption is an endothermic process [120]. On the contrary, some studies indicate the exothermic nature of metal sorption by algae. For instance, Cruz et al. reported that an increase in ambient temperature decreased the Cd$^{2+}$ sorption by Sargassum sp. biomass. Few other studies also reported the same conclusion [121, 122].

Interestingly, there are reports stating that temperature has no impact on metal sorption of algae [123, 124]. Cossich et. al conducted experiments to study the temperature effect on Cr sorption by Sargassum sp. at different pH. They reported that the effect of temperature was not as significant as that of pH [125]. Likewise, Norris et.al and Gadd et. al have shown that heavy metal sorption of algae is relatively unaffected over a moderate range of temperature [123, 126].
(d) pH

As reported in the literature, sorption of metal ions is mainly affected by pH of the media or the wastewater. Most of the algae species have optimum pH and various studies have been conducted to find the optimum pH value for maximal removal of heavy metals. Cossich et. al reported that maximum Cr (III) sorption on Sargassum sp. can be achieved at pH 4 [125]. Sheng et. al found that the optimum pH for Cr (VI) and Cd sorption by Padina sp. and Sargassum sp was at pH 2 [127]. Zhou et. al showed that the sorption of Cd on Sargassum *kjellmanianum* was optimum at pH between 4 and 5 [45].

In general, pH range of 3 to 5 (acidic) is best optimum pH for the sorption of metal ions. This is because most of the metal binding groups of algae are acidic. At acidic pH, these groups cause electrostatic interactions between cationic species and generate a negative charge at the cell surface on which all metals ions are adsorbed. At Low pH (< 2), high concentration of H⁺ ions are present and they will prevent metals from binding to ligands on the cell surface [70, 128, 129, 130]. Some studies proved this phenomenon and reported that algae possess low metal sorption ability at extremely acidic conditions (pH < 2) [39, 112, and 131]. Table 2.2 shows optimum pH values for sorption of Cr and Cd by algae.
Table 2.2 Optimum pH values for sorption of Cr and Cd by algae.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Algae</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr (III)</td>
<td><em>Ulva lactuca</em></td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td><em>Padina pavonica</em></td>
<td>4</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Sargassum <em>sp.</em></td>
<td>4</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td><em>C. prolifera</em></td>
<td>3</td>
<td>132</td>
</tr>
<tr>
<td>Cr (VI)</td>
<td><em>Chlorella vulgaris</em></td>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td><em>Padina sp.</em></td>
<td>2</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Sargassum <em>sp.</em></td>
<td>2</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Cladophora <em>crispate</em></td>
<td>1</td>
<td>131</td>
</tr>
<tr>
<td>Cd</td>
<td><em>Laminaria japonica</em></td>
<td>4.0–5.0</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td><em>Sargassum kjellmanium</em></td>
<td>4–5</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td><em>Cladophora prolifera</em></td>
<td>6</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td><em>Padina pavonica</em></td>
<td>6</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td><em>Fucus vesiculosus</em></td>
<td>3.5–6.8</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td><em>Pilayella littoralis</em></td>
<td>5</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td><em>Lyngbya taylorii</em></td>
<td>3–7</td>
<td>134</td>
</tr>
</tbody>
</table>
(e) Contact time

As mentioned in the literature, the metal sorption is very fast, and typically complete in less than one hour. Sorption is usually achieved in 5-10 minutes for metabolism independent uptake by algae [132]. Binding of metal ions to cell walls is considered to be a spontaneous chemical reaction, with an equilibrium time depending only on the mass transfer resistance [20].
CHAPTER 3
RESEARCH OBJECTIVES

Traditional wastewater and metal removal treatments are prohibitively expensive. This has necessitated research into developing other alternative and cost-effective methods. The primary objective of this study was focused on evaluating and demonstrating potential of microalgae for bioremediation of wastewater laden with nitrogen (N) in the form of nitrates, phosphorous (P) in the form of phosphates, chromium (Cr(VI)) and cadmium (Cd(II)). The secondary objective was to design a commercial system based on the experimental results that can be easily customized and is suitable for integration into existing wastewater treatment facilities.

3.1 Specific Objectives

- To determine the rate of nitrate and phosphorus removal by different algal species and select the alga that is optimal in removing nitrates and phosphorus from simulated and actual waste water (Dayton Water Reclamation Plant).
- To evaluate the growth of selected strain and to study the effect of initial nitrate concentration on phosphorus removal by selected strain and vice versa.
- To determine the bio kinetic coefficients of nitrate and phosphate removal.
- Determine the requirements of incorporating an algal treatment system within a conventional wastewater treatment plant.
• Investigate the capability of microalgae to remove heavy metals from wastewater and determine the removal mechanism.

• Study the effect of various parameters such as temperature, pH, light intensity, initial biomass concentration, and metal concentration on algae metal removal capacity.

• Investigate and compare the biomass productivities of selected algae grown under phototrophic, heterotrophic and mixotrophic conditions.

• Investigate the capability of heterotrophically and mixotrophically grown algae to remove heavy metals from wastewater.

• Propose and design a commercial algae based wastewater treatment technology (AWTS) which mainly focuses on providing a technology that is cost effective, requires low maintenance, and has minimal operator requirements for treating industrial wastewater containing high amounts of nitrates, phosphates and heavy metals.

• Perform life cycle cost analysis for AWTS and compare the wastewater treatment cost with conventional process.
CHAPTER 4
MATERIALS AND METHODS

4.1 Microalgae

A total of 8 microalgae strains were used in this study. The details of strains, source, class, and growth medium are listed in Table 4.1. Among them, 6 species (No. 1-6, Table 4.1) were used in the preliminary experiments performed to identify suitable microalgae for wastewater treatment. Two other species (No. 7, 8, Table 4.1) were used to investigate their ability to remove phosphorus from wastewater.

The following algal cultures were obtained from the Culture Collection of Algae at the University of Texas at Austin: Botryococcus braunii (572), Neochloris oleoabundans (1185), Scenedesmus dimorphus (1237), Chlamydomonas reinhardtii (2337) and Phaeodactylum tricornutum (646). Chlorella vulgaris (152075) was purchased from Carolina Biological Supply (Burlington, NC, USA). In addition, two species were collected from Pleasant Hill Lake, Perrysville, Ohio and Delaware Lake, North Delaware, Ohio. Figure 4.1 shows the microscopic photos of some of these species.
Chlorella *vulgaris*  
Botryoccus *braunii*

Neochloris *oleoabundans*  
Scenedesmus *dimorphus*

Figure 4.1. Microscopic photos of algae species [135].

All the stock cultures were received in agar slants from the source; they were stored at room temperature (22 °C) and illuminated at 40-50 μE m$^{-2}$ s$^{-1}$.

In the case of species collected from lakes, strains were enriched in Bolds Basal Medium and incubated in Petri-dishes for 5 days at 22 °C with illumination about 40-50 μE m$^{-2}$ s$^{-1}$. 

35
Table 4.1. Microalgae strains used in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain</th>
<th>Medium</th>
<th>Class</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Botryococcus braunii</td>
<td>BBM</td>
<td>Trebouxiophyceae</td>
<td>UTEX 572</td>
</tr>
<tr>
<td>2</td>
<td>Chlamydomonas reinhardtii</td>
<td>TAP</td>
<td>Chlorophyceae</td>
<td>UTEX 2337</td>
</tr>
<tr>
<td>3</td>
<td>Scenedesmus dimorphus</td>
<td>BBM</td>
<td>Chlorophyceae</td>
<td>UTEX 1237</td>
</tr>
<tr>
<td>4</td>
<td>Neochloris oleoabundans</td>
<td>BBM</td>
<td>Chlorophyceae</td>
<td>UTEX 1185</td>
</tr>
<tr>
<td>5</td>
<td>Phaeodactylum tricornutum</td>
<td>F/2</td>
<td>Bacillariophyceae</td>
<td>UTEX 646</td>
</tr>
<tr>
<td>6</td>
<td>Chlorella vulgaris</td>
<td>BBM</td>
<td>Trebouxiophyceae</td>
<td>Carolina biological supply 152075</td>
</tr>
<tr>
<td>7</td>
<td>Pleasant Hill Lake</td>
<td>BBM</td>
<td>-</td>
<td>Pleasant Hill, Perrysville, Ohio</td>
</tr>
<tr>
<td>8</td>
<td>Delaware Lake</td>
<td>BBM</td>
<td>-</td>
<td>North Delaware, Ohio</td>
</tr>
</tbody>
</table>

4.2 Growth Medium

Three different growth media were used in the study. They were: BBM (Bold’s Basal Medium), TAP-medium (Tris-Acetate-Phosphate) and f/2 medium.

4.2.1 BBM

The composition of BBM (per L) is as follows: KNO₃, 1210 mg; CaCl₂·2H₂O, 35 mg; MgSO₄·7H₂O, 70 mg; KH₂PO₄, 230 mg; EDTA, 50 mg; ZnSO₄·7H₂O, 0.882 mg;
MnCl₂.4H₂O, 0.144 mg; MoO₃, 0.07 mg; CuSO₄.5H₂O, 0.157 mg; and Co(NO₃)₂.6H₂O, 0.049 mg. Solution must be autoclaved at 121 °C for 15 mins.

4.2.2 TAP Medium

Stock solutions of 1-3 (Table 4.2) were prepared and stored at 4 °C. TAP medium was prepared by mixing 2.42 g Tris, 25 ml of solution #1, 0.375 ml of solution #2, 1 ml of solution #3 and 1 ml of glacial acetic acid. The solution was brought to 1L by adding water and autoclaved at 121 °C for 15 mins.

4.2.3 f/2 Medium

This medium is a slight modification of the original f medium of Guillard and Ryther (1962). It is designated as f2 or f/2. To prepare this medium, 0.5 ml of each stock solution (1-5) (Table 4.3) was added to 1 liter of seawater and autoclaved at 121 °C for 15 mins.
Table 4.2. TAP medium.

<table>
<thead>
<tr>
<th>Solution no.</th>
<th>Stock Solutions</th>
<th>Per L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tap salts</td>
<td>NH₄Cl</td>
<td>15 g</td>
</tr>
<tr>
<td></td>
<td>MgSO₄·7H₂O</td>
<td>4g</td>
</tr>
<tr>
<td></td>
<td>CaCl₂·2H₂O</td>
<td>2g</td>
</tr>
<tr>
<td>2. Phosphate solution</td>
<td>K₂HPO₄</td>
<td>2.88 g</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>1.44 g</td>
</tr>
<tr>
<td>3. Hutner’s trace elements</td>
<td>EDTA disodium salt</td>
<td>50 g/250 ml water</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄·7H₂O</td>
<td>22 g/100 ml water</td>
</tr>
<tr>
<td></td>
<td>H₃BO₃</td>
<td>11.4 g/200 ml water</td>
</tr>
<tr>
<td></td>
<td>MnCl₂·4H₂O</td>
<td>5.06 g/50 ml water</td>
</tr>
<tr>
<td></td>
<td>CoCl₂·6H₂O</td>
<td>1.61 g/50 ml water</td>
</tr>
<tr>
<td></td>
<td>CuSO₄·5H₂O</td>
<td>1.57 g/50 ml water</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>1.10 g/50 ml water</td>
</tr>
<tr>
<td></td>
<td>FeSO₄·7H₂O</td>
<td>4.99 g/50 ml water</td>
</tr>
</tbody>
</table>
Table 4.3. f/2 medium.

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Per L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. NaNO₃</strong></td>
<td>150.0 g</td>
</tr>
<tr>
<td><strong>2. Trace metals</strong></td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>19.6 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>44.0 mg</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>22.0 mg</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>360.0 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>12.6 mg</td>
</tr>
<tr>
<td><strong>3. Na₂SiO₃·5H₂O</strong></td>
<td>22.7 g</td>
</tr>
<tr>
<td><strong>4. Fe citrate:</strong></td>
<td></td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>9.0 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>9.0 g</td>
</tr>
<tr>
<td><strong>5. Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1.0 ml primary stock</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>1.0 ml primary stock</td>
</tr>
<tr>
<td>Thiamine HCL</td>
<td>20.0 mg</td>
</tr>
<tr>
<td><strong>Primary Stocks:</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>10.0 mg /100 ml dH₂O</td>
</tr>
<tr>
<td>Biotin</td>
<td>10.0 mg /100 ml dH₂O</td>
</tr>
<tr>
<td><strong>6. NaH₂PO₄·2H₂O</strong></td>
<td>11.3 g</td>
</tr>
<tr>
<td><strong>7. Na₂EDTA·2H₂O</strong></td>
<td>30.0 g</td>
</tr>
</tbody>
</table>
4.3 Culture Conditions

To produce sufficient material (algae) for use in wastewater and heavy metal treatment experiments, stock cultures were transferred and initially grown in 250 ml Erlenmeyer flasks containing 100 ml of respective medium at $22 \pm 2 \, ^\circ C$ with cool white fluorescent lamps giving a continuous irradiance of $40-50 \, \mu \text{molm}^{-2}\text{sec}^{-1}$. CO$_2$ and air was bubbled through each culture at a flow rate of 100 ml/min and 250 ml/min, respectively. After 2-3 weeks, when algae reaches the exponential growth phase, part of it was harvested by centrifugation at 3000 rpm (Dynac centrifuge, serial no 103094) for 10 min and was used in wastewater and heavy metal experimental studies.

4.4 Glassware Cleaning

Before the start of an experiment all glass apparatus were washed with phosphate free detergent i.e. super concentrated non-foaming liquid cleanser (Decon Laboratories Ltd, Hove), scrubbed with a nylon brush to remove stains and then rinsed with deionized water. Glass apparatus used for heavy metal removal experiments were washed with 4% HNO$_3$ solution to remove all traces of bound metal and then repeatedly rinsed with deionized water.

4.5 Experimental Setup

The same experimental set up was used for all the experiments conducted in this study. Experiments were performed in batch photo bioreactors on a laboratory scale by using 2000 ml cylindrical borosilicate pyrex bottles. Three $\frac{3}{4}$” holes were drilled into the caps of the cylindrical bioreactors providing two inlets and one outlet. Carbon dioxide and air lines were secured in the two inlet openings, and the third outlet opening was left unobstructed to prevent oxygen and pressure buildup in the reactor. The gas flow rate
into the bioreactors was maintained by means of two Rota meters. The cultures were subjected to illumination by four fluorescent lamps (Philips F40T12/DX 40 Watts) placed horizontally and parallel to the front and back of the cylindrical photo bioreactor (Figure 4.2a).

4.6 Wastewater Experiments (Nitrate and Phosphorus Removal)

This section gives the details of the experiments performed for removing nitrate and phosphorus from wastewater. It is divided into four sub sections. Preliminary experiments performed for selection of strain are described in section 4.6.1. The details of the experiments performed to investigate the nitrate and phosphorus removal rates of the selected strain are discussed in section 4.6.2. Section 4.6.3 gives the details of the experiments performed to test the selected strain’s ability to remove nitrogen from different nitrogen sources. Phosphorus removal experiments were listed in section 4.6.4.

4.6.1 Preliminary Experiments (selection of microalgae)

Preliminary experiments were performed using 6 different algae strains to select the best microalga species for nitrate and phosphate removal studies. The experimental bioremediation system consisted of six 2 L cylindrical bioreactors placed in parallel as shown in Figure 4.2b. The cultures were subjected to illumination by fluorescent lamps placed vertically at the back of the reactor. The wastewater treated in this experimental study was obtained from the Dayton Water Reclamation Plant. Samples were collected from the treatment plant’s effluent stream which is discharged into the Great Miami River. Initial nitrate and phosphorus content in the wastewater was measured and found to be 7.5 mg/L and 1.56 mg/L respectively. According to NPDES, phosphorus content
was above the permissible limit and nitrate content was within the limit in the wastewater sample.

Figure 4.2. Bioremediation experimental set up.

All six species were initially grown in 250 ml Erlenmeyer flasks as explained in section 4.3. Each of the six bioreactors was filled with 1 L of wastewater, and each of the six microalgal species was inoculated in different bottles. The initial absorbance of algae in each wastewater sample was maintained at ~0.5 by transferring 0.6 g of the wet algal pellet (equivalent to 0.18 g of dry mass) to each of the six cylindrical bioreactors containing 1 L of Dayton wastewater. Experiments were performed at room temperature i.e. 23±2 °C and were subjected to continuous fluorescent illumination at an irradiance of 45 µmolm^-2sec^-1. The pH value of wastewater measured as 7 at the beginning of the experiment. The inlet flow rate of air was sustained at 1 L/min and that of CO₂ at 0.1 L/min. A 2 ml sample was collected on alternate days from each of the six cylindrical bioreactors. One half of the collected sample was used to measure the cell growth by means of absorbance and other half was subjected to nitrate and phosphate analysis.
4.6.2 Nitrate and Phosphorus Removal Experiments

Based on the preliminary experimental results, Chlorella *vulgaris* was found to be the ideal species for removing nitrates and phosphates. A detail discussion of the preliminary results will be presented in the next chapter. After the strain selection study, nitrate and phosphorus removal rates of Chlorella *vulgaris* at various temperatures were investigated. Synthetic wastewater was used instead of actual wastewater and the concentrations of nitrate and phosphorus in actual wastewater were replicated in the synthetic wastewater. KNO$_3$ and KH$_2$PO$_4$ served as nitrate and phosphorus sources respectively. Other nutrients in the BBM (micronutrients) required for growth of algae are also added to the synthetic wastewater. The concentration of micronutrients is mentioned in section 4.2.1.

The experimental set up described in section 4.5 was used and bioreactors of volume 2 L containing 1 L of synthetic wastewater (4.2.1) were used. A culture grown under autotrophic growth conditions was used as initial inoculum. Different sets of experiments as shown in table 4.4 were performed varying the concentrations of nitrate and phosphate in synthetic wastewater. Ratios of nitrogen/phosphorus varied from 0.78 to 14 similar to ratios found in the effluents from wastewater treatment plants [136]. A nitrate (NO$_3^-$) concentration between 90.12 to 741.56 mg/L and phosphorus (P-PO$_4^{3-}$) concentration between 13.12 to 52.42 mg/L was studied. The experimental conditions are shown in table 4.5.

Samples were taken daily in 5 ml volumes for 8 days from each reactor and analyzed for dry cell weight and the nitrate and phosphate content. The dissolved oxygen, pH, and temperature were monitored on a regular basis during the experiments.
Table 4.4. Experimental matrix for nitrate and phosphorus removal from synthetic wastewater by Chlorella vulgaris.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(P-PO_{4}^{3-}) mg/L</th>
<th>(NO_{3}^{-}) mg/L</th>
<th>Initial biomass (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PO_{4})_1</td>
<td>13.1</td>
<td>183.56</td>
<td>0.206</td>
</tr>
<tr>
<td>(PO_{4})_2</td>
<td>26.21</td>
<td>187.78</td>
<td>0.2</td>
</tr>
<tr>
<td>(PO_{4})_3</td>
<td>52.42</td>
<td>182.68</td>
<td>0.212</td>
</tr>
<tr>
<td>(NO_{3})_1</td>
<td>52.42</td>
<td>90.12</td>
<td>0.242</td>
</tr>
<tr>
<td>(NO_{3})_2</td>
<td>52.36</td>
<td>182.68</td>
<td>0.252</td>
</tr>
<tr>
<td>(NO_{3})_3</td>
<td>51.87</td>
<td>362.69</td>
<td>0.25</td>
</tr>
<tr>
<td>(NO_{3})_4</td>
<td>52.41</td>
<td>741.56</td>
<td>0.256</td>
</tr>
</tbody>
</table>

Table 4.5. Experimental conditions for nitrate and phosphorus removal.

<table>
<thead>
<tr>
<th>Volume of synthetic wastewater</th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 °C</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
<tr>
<td>Light intensity</td>
<td>45 μmolm^{-2}sec^{-1}</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.1 L/min</td>
</tr>
<tr>
<td>Air</td>
<td>1 L/min</td>
</tr>
</tbody>
</table>

Further, to investigate the effect of temperature on nitrate and phosphorus removal additional experiments were performed at the same experimental conditions but
at four different temperatures i.e. 10°C, 20°C, 27°C and 35°C keeping initial nitrate and phosphorus concentrations at ~182 ppm and ~54 ppm mg/L respectively.

4.6.3 Effect of nitrogen source on growth of Chlorella vulgaris

An experimental matrix was designed using the same experimental set up and conditions as in the previous study (section 4.6.2) in order to investigate the effect of nitrogen source on the growth of C. vulgaris. Four forms of nitrogen i.e. potassium nitrate, urea, potassium nitrite and ammonium chloride were used as nitrogen sources and KH₂PO₄ was used as phosphorus source along with other nutrients in BBM (section 4.2.1) to prepare synthetic wastewater. The concentration of phosphorus and other micronutrients used in this study was described in section 4.2.1. The initial nitrogen and biomass concentration and experimental conditions are shown in Table 4.6.

Table 4.6 Experimental matrix for N removal from different nitrogen sources.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source</th>
<th>N (mg/L)</th>
<th>(P-PO₄³⁻) mg/L</th>
<th>Initial dry biomass concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₁</td>
<td>KNO₃</td>
<td>41.25</td>
<td>26.21</td>
<td>0.185</td>
</tr>
<tr>
<td>N₂</td>
<td>Urea</td>
<td>41.34</td>
<td>26.32</td>
<td>0.186</td>
</tr>
<tr>
<td>N₃</td>
<td>(NH₄)₂SO₄</td>
<td>41.81</td>
<td>26.29</td>
<td>0.185</td>
</tr>
<tr>
<td>N₄</td>
<td>KNO₂</td>
<td>41.84</td>
<td>26.28</td>
<td>0.187</td>
</tr>
</tbody>
</table>

Each experiment was run for 10 days, and a sample of 5 ml was taken daily to determine algae cell dry weight and total nitrogen content in synthetic wastewater. Dissolved oxygen, pH, and temperature were monitored on a regular basis during the experiments.
4.6.4 Phosphorus Removal by Species Acclimated to High P Environments

The results of experimental section 4.6.2 showed that Chlorella vulgaris can remove phosphates only to a certain extent (normal uptake). To enhance the removal of phosphate from wastewater, two species known to grow well in a high P environment were selected to determine their ability to remove phosphates from wastewater. Two species were collected from Pleasant Hill Lake, Perrysville, Ohio and Delaware Lake, North Delaware, Ohio. To remove more amounts of phosphorus from wastewater, in this study, these two species were tested to investigate their ability to remove phosphorus from synthetic wastewater. KH$_2$PO$_4$ and KNO$_3$ were used as phosphorus and nitrate sources in synthetic wastewater along with other nutrients in BBM (section 4.2.1). The experimental matrix is listed in Table 4.7 and experimental conditions are same as listed in Table 4.5. Each experiment was run for 10 days, and a sample of 5 ml was taken daily to determine algae cell dry weight and phosphorus content in synthetic wastewater.
Table 4.7. Experimental matrix for P removal by Lake Species (species acclimated to high P environments)

<table>
<thead>
<tr>
<th>Species</th>
<th>Experiment</th>
<th>(P-PO$_4^{3-}$) mg/L</th>
<th>(N-NO$_3^-$) mg/L</th>
<th>Initial dry biomass concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleasant Hill lake species</td>
<td>P1</td>
<td>10.484</td>
<td>36.47</td>
<td>0.349</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>20.968</td>
<td>37.96</td>
<td>0.385</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>31.452</td>
<td>35.47</td>
<td>0.367</td>
</tr>
<tr>
<td>Delaware lake species</td>
<td>P4</td>
<td>10.3684</td>
<td>32.457</td>
<td>0.338</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>20.774</td>
<td>35.89</td>
<td>0.327</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>31.308</td>
<td>36.81</td>
<td>0.3404</td>
</tr>
</tbody>
</table>
4.7 Heavy Metal (Cr, Cd) Removal Experiments.

4.7.1 Preliminary Studies

The primary objective of the preliminary studies is to verify the capability of species to remove Cr and Cd from synthetic wastewater. Preliminary experiments were conducted using two microalgae species i.e. Chlorella Vulgaris and Neochloris Oleoabundans with Cd and Cr metal concentrations in solution ranging from 50 to 350 mg/L. Both microalgae cultures used were cultivated batch wise in 250 ml Erlenmeyer flasks as described in section 4.3. After reaching the exponential growth phase, part of it was harvested and transferred to heavy metal containing synthetic wastewater.

Stock metal solutions of 1000 mg/L of Cd$^{2+}$ and Cr$^{6+}$ were prepared by dissolving 1.857 g of CdSO$_4$, and 2.8289 g of K$_2$Cr$_2$O$_7$ in 1000 ml of D.I water. The stock solutions were diluted to obtain test solutions of desired concentration. All chemicals used in this study were analytical grade.

Metal removal experiments were performed in 2 L bioreactor (see section 4.5) containing 1 L of metal solution of desired concentration. Nutrients required for the growth of algae were also added to the desired test solution. Synthetic wastewater each containing metal concentrations of 350 mg/L and 250 mg/L of Cr$^{6+}$ and Cd$^{2+}$ were treated with N. oleoabundans and a wastewater containing 350 mg/L of Cr$^{6+}$ and 250 mg/l of Cd$^{2+}$ was treated using Chlorella. Pre cultured algae was added to the metal solution. The initial biomass concentrations for these preliminary experiments are listed in Table 4.8. Experiments were performed at room temperature i.e. 23±2 °C and were subjected to continuous fluorescent illumination at an irradiance of 45 μmolm$^{-2}$sec$^{-1}$. The pH was
monitored at 7 at the beginning of the experiment. Air and CO₂ were passed into the reactor at a flow rate of 1 L/min and 0.1 L/min respectively.

Table 4.8 Biomass concentrations for preliminary experiments of Cr⁶⁺ and Cd²⁺ removal.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cr⁶⁺ (mg/L)</th>
<th>Initial dry biomass concentration (g/L)</th>
<th>Cd²⁺ (mg/L)</th>
<th>Initial dry biomass concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neochloris</td>
<td>350</td>
<td>2.34</td>
<td>250</td>
<td>1.93</td>
</tr>
<tr>
<td>Chlorella</td>
<td>350</td>
<td>2.314</td>
<td>250</td>
<td>1.932</td>
</tr>
</tbody>
</table>

Blank controls (containing only medium plus metal) were also carried out for each concentration tested. The blanks were meant to confirm that the supernatant Cd and Cr concentration initially provided was not affected by any factor other than those conveyed by the microalgae during the time frame of the experiments. Metal removal experiments using Neochloris and Chlorella were run for 72 hours and 148 hours respectively. Sample volumes of 5 ml were collected and analyzed for Cd and Cr content.

4.7.1.1 EDTA Washing

An interesting observation was made from the study in 4.7.1. Rapid removal of Cd²⁺ took place when synthetic wastewater was treated with C.vulgaris. Almost 90% of the Cd was removed during the first 10 minutes of experimental time. Cadmium removal by algae is a metabolism-independent process, in which Cd will be adsorbed on to the algae surface. So, 90% of Cd removed from the aqueous solution during the experiment
should be adsorbed on the surface. To verify this, 1ml of algae solution was taken in a plastic cuvette and was centrifuged in a micro centrifuge (Accuspin Micro 17, serial no 41055477) for 2 mins. The supernatant was drained off and the remaining algae paste was treated (washed) with 1 ml of 20 mM Ethylene diamine tetra acetic acid (EDTA) for 5 mins. EDTA has a property to remove surface-bound metal, leaving behind the intracellular fractions of the algal cell [6]. The EDTA washed algae was then centrifuged and supernatant (EDTA) was analyzed for Cd content. The results showed that Cd content present in the EDTA is same as the Cd that is removed from the aqueous solution. Same procedure was employed to detect on the Cr content on algae surface.

To economize the usage of EDTA and to know the minimum concentration and minimum volume of EDTA required to remove Cd from algae surface, series of experiments were performed varying the concentration and volume of EDTA. 5mM, 10Mm, 20 mM concentrations of EDTA each of volume 0.25, 0.5 ml and 1 ml were tested to remove Cd from the surface of algae paste resulted from the centrifugation of 1 ml of algae solution.

4.7.2 Cr and Cd Removal Experiments

Based on the preliminary tests, we found that Chlorella is very efficient in removing Cr$^{6+}$ and Cd$^{2+}$ from synthetic wastewater. The details of the preliminary results are discussed in section 5.2.2. After the preliminary experiments, effect of various parameters such as initial metal concentration, contact time, initial biomass concentration, pH and temperature on the metal removal ability of Chlorella vulgaris were investigated.
The experimental setup is same as in the preliminary experimental study. Experimental setup is discussed in detail in section 4.5. Bioreactors in which experiments were performed are subjected to continuous fluorescent illumination at an irradiance of 45 µmolm\(^{-2}\)sec\(^{-1}\). Air and CO\(_2\) were passed into the reactor at a flow rate of 1 L/min and 0.1 L/min respectively. Table 4.9 and 4.10 comprises the experimental matrix designed for removing Cr and Cd from wastewater varying the parameters such as pH, initial metal concentration, biomass dosage and temperature. The pH was adjusted using 1 M NaOH or 1 M HCl. As the cadmium removal is very rapid, samples were collected at 0, 5, 10, 20, 30, 60, and 90 min for Cd removal experiments and as the chromium removal was mainly based on physical absorption, samples were collected at 0, 4, 12, 18, 24, 36, 42, 60, and 120 hours for Cr removal experiments. The collected samples were analyzed for algae cell dry weight and for Cd and Cr content.
Table 4.9 Experimental matrix for Cr\(^{6+}\) removal by Chlorella.

<table>
<thead>
<tr>
<th>Set</th>
<th>pH</th>
<th>Initial metal concentration (mg/L)</th>
<th>Initial dry biomass concentration (mg/L)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8</td>
<td>30</td>
<td>1.45</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>20</td>
<td>1.43</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>10</td>
<td>1.364</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
<td>30</td>
<td>1.43</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>30</td>
<td>1.76</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>30</td>
<td>2.03</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>30</td>
<td>2.38</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>30</td>
<td>2.042</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30</td>
<td>2.045</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30</td>
<td>2.043</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30</td>
<td>2.04</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>30</td>
<td>2.041</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>30</td>
<td>1.45</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>30</td>
<td>1.45</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 4.10 Experimental matrix for Cd\(^{2+}\) removal by Chlorella.

<table>
<thead>
<tr>
<th>Set</th>
<th>pH</th>
<th>Initial metal concentration (mg/L)</th>
<th>Initial dry biomass concentration (mg/L)</th>
<th>Temperature ((^{\circ})C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8</td>
<td>50</td>
<td>1.545</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>100</td>
<td>1.53</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>150</td>
<td>1.5364</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>6.8</td>
<td>100</td>
<td>1.45</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>100</td>
<td>1.87</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>100</td>
<td>2.15</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>150</td>
<td>1.503</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>150</td>
<td>1.504</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>50</td>
<td>1.538</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>50</td>
<td>1.545</td>
<td>25</td>
</tr>
</tbody>
</table>
4.7.3 Heavy Metal Removal by Mixotrophically and Heterotrophically Grown Algae

The results from the study in 4.7.2 indicated that the metal uptake from the aqueous solution will depend on the initial biomass concentration. It was observed in the study that a larger percentage of metal was removed at higher biomass concentration. This is because, an increase in biomass concentration increases algae cell surface and therefore a larger amount of metal will be adsorbed on the surface.

It is known that the growth rate of mixotrophically and heterotrophically grown algae are higher than that of phototrophically grown algae [137,138]. So, a higher biomass concentration can be achieved in a short time via mixotrophic and heterotrophic conditions. Time to remove metal ions from aqueous solutions can be reduced if we can treat synthetic wastewater with heterotrophically and mixotrophically grown algae. Therefore, experiments were performed to investigate the capability of heterotrophically and mixotrophically grown algae to remove Cr$^{6+}$ and Cd$^{2+}$ from synthetic wastewater.

Initially, the experiments were conducted to determine the capability of *C. vulgaris* to grow under heterotrophic and mixotrophic conditions. The experimental set up used for these studies was described in section 4.5. Algae cells were grown in 2 L bottles containing 1 L of BBM with an organic carbon source. A culture grown under autotrophic growth conditions served as initial inoculum (section 4.3). Glucose and glycerol were used as organic carbon sources. The autotrophic control group was cultivated in BBM with illumination. The details of the initial concentration of organic carbon and initial biomass concentration used for mixotrophic and heterotrophic growth are listed in Table 4.11. An air flow rate of 1 L/min and a CO$_2$ flow rate of 0.1 L/min
were provided to mixotrophically grown species along with a light irradiance of 45 
µmolm⁻²sec⁻¹. Only air was supplied at a flow rate of 1 L/min to heterotrophically grown 
species without light and CO₂. Temperature of 23 °C and pH of 7.1 was maintained for 
both conditions. Samples were collected daily for 4 days, and analyzed for algal biomass 
concentration and lipid content.

The results of the initial experiments confirmed that C. vulgaris can grow 
effectively under mixotrophic and heterotrophic conditions generating large amount of 
biomass in short interval of time. Therefore, in the second phase of study, algae that was 
grown under mixotrophic and heterotrophic conditions was used to treat metal 
contaminated water having a concentration of 60 ppm of Cr⁶⁺ and Cd²⁺ ions. Samples 
were taken daily for six days to measure biomass concentration and the metal removal 
capability of C. vulgaris under mixotrophic and heterotrophic conditions. Lipids were 
analyzed to determine potential revenue to offset costs for algae biomass generation to 
remove heavy metals.
Table 4.11 Initial organic carbon and biomass concentrations for mixotrophic and heterotrophic growth of Chlorella vulgaris.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Glycerol (ml)</th>
<th>Initial dry biomass concentration (g/L)</th>
<th>Glucose (ml)</th>
<th>Initial dry biomass concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixotrophic</td>
<td>10</td>
<td>3.042</td>
<td>10</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.145</td>
<td>20</td>
<td>1.1802</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.921</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterotrophic</td>
<td>10</td>
<td>1.604</td>
<td>10</td>
<td>1.164</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.7014</td>
<td>20</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.804</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.8 Analysis

4.8.1 Absorbance

Absorbance is the primary measurement used to determine the cell growth by means of culture’s optical density. In the present study, cell absorbance was measured at a wavelength 550 nm using spectrophotometer (Perkin-Elmer spectrophotometer, Model # Lambda-3B, S/N - 69430). Due to the presence of photosynthetic pigments, it is important to conduct the measurement outside of the range of wavelengths where these pigments absorb. For this reason, as Becker (1994) suggests, an absorbance wavelength of 550 nm was used to measure absorbance [139].
4.8.2 Algae Cell Growth

Turbidity and Dry cell weight estimations are two of the most prominent methods used by researchers to determine growth rate of algae. As Becker showed [68] biomass concentration can be correlated to algae absorbance. The amount of light that passes through the algae suspension will be inversely proportional to the algae concentration, in accordance with Beer’s Law relating absorption to concentration. Considering the wavelengths where Chlorophyll-a and -b absorb, an absorbance at 550 nm is recommended and will be used to construct the standard curve [68]. Use of the standard curve yields a linear equation that compares absorbance of the suspension at 550 nm to the cell concentration at that particular time. The equation, in its generic form, will appear as follows:

\[ y = Ax \ldots \text{Eq. 4.1} \]

where \( A \) is the linear regression line fit variable, \( x \) is the A550 reading and \( y \) is the cell concentration of the suspension.

A volume of 0.5 ml was used to measure the cell dry weight via filtration. Prior to the filtration, 0.2 μm Whatman GF/C filters were initially placed in a temperature chamber (micro oven) at 85 °C for 24 hours to remove moisture. After 24 hours, filters were taken out of the oven, weighed and then 0.5 ml algae solution was filtered on pre-weighed 0.2 μm Whatman GF/C filters. Then, the filters were again dried for 24 hours at 85 °C (optimum temperature for drying the wet algae), and re-weighed using a microbalance (Radwag microbalance, Model no MYA31/24 error – 1 μg). The difference
between empty filter weight and filter with algae gives the dry cell biomass concentration.

4.8.3 Nitrate Analysis

Nitrate analysis was done using Hach DR-890 colorimeter. A 2ml of algae solution collected from the experiment was centrifuged for 5 min at 700xg in micro centrifuge to separate the algae from the media. The clear supernatant was used to measure nitrate content by cadmium reduction method (Hach Method 8049). Nitrogen uptake was measured as nitrate. A Hach Nitra Ver 5 Nitrate reagent powder pillow was added to the appropriate dilution of the supernatant. Final volume of the testing sample should be 10ml. The sample was shaken for one minute and allowed to rest for five minutes permitting the reaction to complete.

A blank was also prepared by adding Nitra Ver 5 Nitrate reagent powder pillow to 10 ml of distilled water. This blank was used to zero the colorimeter prior to testing of samples. The cadmium metal reduces nitrates present in the sample to nitrite. This nitrite form an amber-colored product by reacting in acidic medium with sulfanilic acid. This amber color indicates the presence of nitrate and intensity is converted to \((\text{NO}_3^-)\) mg/L.

4.8.4 Phosphorus Analysis

Again, as described in nitrate analysis, a 2ml of algae solution collected from the experiment was centrifuged for 5 min at 700xg in micro centrifuge to separate the algae from the media. The clear supernatant was measured for phosphate using the Hach DR-890 colorimeter and the molybdate-ascorbic acid method (Hach Method 8507). Phosphate reagent powder pillow was added to the test sample that is diluted to a total volume of 10 ml by adding D.I water. The sample mixture was shaken for 15 seconds and
left to sit for two minutes for the reaction to complete. A blank was prepared by dissolving reagent powder pillow in 10 ml of distilled water. This blank was used to zero the colorimeter prior to testing of samples. Orthophosphate present in the sample reacts with molybdate in the reagent in the acid medium to produce a phospho-molybdate complex. Ascorbic acid reduces this complex and forms a blue color. This blue color indicates the presence of phosphate and the intensity is converted to \((P-PO_4^{3-})\) mg/L.

4.8.5 Total Nitrogen Analysis

Total N analysis was done using DR-890 colorimeter by per sulfate digestion method (method 10072). Sample preparation and volume of sample is same as nitrate and phosphate analysis. An alkaline per sulfate digestion converts all forms of nitrogen to nitrate. Sodium metabisulfite added after the digestion will eliminate halogen oxide interferences. Under acidic conditions, nitrate reacts with chromotropic acid to form a yellow complex. The intensity of this yellow complex will determine the total N concentration in mg/L.

4.8.6 Metal Analysis

The amount of metal present in the supernatant and the amount incorporated into the cells were both analyzed in the samples via inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS is a relatively new method for determining multi-element analysis and ideal for water, since the vast majority of target compounds can be detected below 0.1 mg/l [141, 142]. It consists of Meinhart nebulizer and silica cyclonic spray chamber with continuous nebulization. The operating conditions are listed below: Nebulizer Gas flow rates: 0.95 l/min; Auxiliary Gas Flow: 1.2 l/min; Plasma Gas Flow: 15 l/min; Lens Voltage: 7.25 V; ICP RF Power: 1100 W;
The concentration of metal removed was calculated as follows: difference between the initial metal concentration in the solution and the remaining metal concentration in the supernatant, at each sampling time;

Percent of metal removed by algae was determined according to:

\[ R \% = \left( \frac{C_0 - C_t}{C_0} \right) \times 100 \] .......Eq.4.2

Where R is the percentage of metal removed by algae, \( C_0 \) is the initial metal concentration in mg/L and \( C_t \) is the remaining concentration at time t in mg/L.

Metal uptake by biomass was determined according to:

\[ q = V \frac{C_0 - C_t}{x} \] ................. Eq.4.3.

where q is the metal uptake in mg (metal) g\(^{-1}\) biomass, V is the volume of metal containing solution, \( C_0 \) is the initial metal concentration in mg/L and \( C_t \) is the remaining concentration at time t in mg/L [143].

4.8.7 Lipid Extraction

Extraction of lipid was performed by Bligh and Dyer (1959) method. Initially algae cells were harvested by centrifugation at 3,000 rpm for 10 min. The cells were washed once with distilled water and re centrifuged. The pellet was dried in oven at 80°C. For 1 g of algal biomass, 1 ml of chloroform and 2 mL of methanol was added and the mixture was agitated in vortex for 2 min. 1 mL of chloroform was again added and the mixture was shaken vigorously for 1 min. After that, 1 ml of distilled water was added and the mixture was mixed in a vortex again for 2 min. The layers were separated by centrifugation for 10 min at 3000 rpm. The lower layer was separated and the procedure was again repeated with the pellet. The two supernatants collected were allowed to stand for 2 h. Lower organic layer with the lipids was transferred to a clean pre-weighed vial
(W_1). Evaporation was carried out and the weight of the vial was again recorded (W_2). Lipid content was calculated by subtracting W_1 from W_2 and was expressed as % dry cell weight [140].
This chapter discusses the results of the experiments performed for removing nitrate, phosphorus and heavy metals from wastewater using microalgae. It is mainly divided into two sections. The first section gives the details of the results of the experiments conducted to remove nitrate and phosphorus from wastewater and the second section discusses the results of heavy metal removal experiments. The first section is again divided into four sub-sections. Preliminary results of the experiments performed for selection of strain are described in section 5.1.1. The results of the main experiments performed to investigate the nitrate and phosphorus removal rates of the selected strain are discussed in section 5.1.2. Effect of temperature on nitrate and phosphorus uptake from synthetic wastewater by selected species was discussed in section 5.1.3. Section 5.1.4 gives the details of the results of experiments performed to test the selected strain’s ability to remove nitrogen from different nitrogen sources. Results of the experiments conducted using local algal strains to remove high amount of phosphorus from wastewater are discussed in Section 5.1.5.
5.1 Nitrate and Phosphorus Removal

5.1.1. Preliminary Experiments (selection of microalgae)

(a) Nitrate removal

Five of the six microalgal species tested efficiently removed nitrate from the wastewater. The rate and degree of nitrate consumption of six species is shown in Figure 5.1. Figure 5.2 shows the nitrate uptake per unit biomass over the experimental time investigated. Phaeodactylum *tricornutum* is a cosmopolitan marine pennate diatom which grows well in nitrogen depleted media and hence consumed fewer nitrates when compared to freshwater species. But nitrate uptake per unit biomass is very high for Phaeodactylum as initial biomass of the marine species is very low. Discounting Phaeodactylum *tricornutum* the remaining algal species removed nitrate efficiently from wastewater. The algal species reduced nitrate concentration from 7.5 mg/L to 0.8 mg/L within the first 10 days. The nitrate uptake efficiency of the five algal species was found to be 90% by the end of two week period.

![Figure 5.1. Nitrate removal from wastewater.](image-url)
b) Phosphorus removal

All six of the microalgal species efficiently removed phosphorus from the wastewater. The rate and degree of phosphorus uptake from the wastewater by each of the six species is presented in Figure 5.3. Figure 5.4 shows the phosphate uptake per unit biomass over the experimental time investigated. In all six of the bioreactors, prior to treatment with algae, the initial phosphorus content was 1.563 mg/L. Within two weeks, the phosphorus concentration was reduced to 0.16 mg/L (below the permissible limit) in all of the reactors. Phosphorus uptake by Neochloris was faster than other species for the first two days of the experimental period. Approximately 71% of initial phosphorus content was consumed by Neochloris during first two days. It was then followed by Phaeodactylum tricornutum (68%), Scenedesmus dimorphus (66%), Botryococcus braunii (56.06%), Chlamydomonas reinhardtii (34.8%) and Chlorella vulgaris (13.5%)
during this 2-day period. Regardless of the algal specie, by the end of the two week period 80-85% of the initial wastewater phosphorus content was removed by the algal species in each of the reactors.

The uptake efficiency was calculated as

\[ E_i = \frac{(C_o - C_i)}{C_o} \times 100 \text{…………………Eq.5.1} \]

where \( C_o \) and \( C_i \) were the concentrations of contaminant at the start of experiment (day 0) and day \( i \), respectively.

Figure 5.3. Phosphorus removal from wastewater.
After inoculating the algae in the wastewater, absorbance measurements were taken in regular intervals. The initial measured absorbance for five of the six species were found to be around 0.5 A. The absorbance for Phaeodactylum *tricornutum* was 0.0132.

Since nitrate and phosphorus are the primary nutrients required for algal photoautotrophic growth, all six of the algal species exhibited growth over the two week period. The growth curve for all six species over seventeen day period is shown in Figure 5.5. For most of the strains, a 200% increase in absorbance was observed at the end of seventeenth day. Chlorella *vulgaris* exhibited exponential growth, and an 800% increase in absorbance was attained by the end of the seventeen day period. Among the six species, Phaeodactylum *tricornutum* exhibited the slowest and most limited growth while Chlorella *vulgaris* exhibited the fastest growth with the greatest biomass yield.
The main finding of the preliminary experiments was chlorella vulgaris. Of all species investigated, C. vulgaris was found to be the ideal species for treating wastewater as it efficiently removed nitrate and phosphorus from wastewater and its unique robust nature helped the species to grow well in wastewater compared to other species. The ability of C. vulgaris to grow faster in wastewater would be an added advantage for production of algal biomass for biofuel along with other value added products. Using C. vulgaris, experimental matrix was designed in such a way that the results of these experiments would give sufficient input to design and incorporate an algae based wastewater treatment systems into conventional treatment process such as Dayton Water Reclamation Plant.

5.1.2 Nitrate and Phosphorus Removal Experiments

To study the nitrate and phosphorus removal rates of Chlorella vulgaris and to determine its growth rate, different sets of experiments as shown in table 4.4 were performed varying the concentrations of nitrate and phosphorus in synthetic wastewater. Ratios of nitrogen/phosphorus varied from 0.78 to 14 similar to the ratios found in the

Figure 5.5. Growth of species in wastewater.
effluents from wastewater treatment plants [136]. Experiments (PO₄)₁, (PO₄)₂ and (PO₄)₃ have different levels of phosphorus i.e. p₁ = 13.1 mg/L, p₂ = 26.21 mg/L, p₃ = 52.42 mg/L in synthetic wastewater containing nitrate concentration of 183.56 mg/L, 187.78 mg/L and 182.68 mg/L respectively. The nitrate concentration is kept constant (almost same) in these set of experiments. These experiments were performed to study the effect of phosphorus level on the nitrate removal rate and the growth of C. vulgaris.

Experiments (NO₃)₁, (NO₃)₂, (NO₃)₃ and (NO₃)₄ have different levels of nitrate i.e. n₁ = 90.12 mg/L, n₂ = 182.68 mg/L, n₃ = 362.69 mg/L and n₄ = 741.56 mg/L in synthetic wastewater containing phosphorus concentration of 52.42 mg/L, 52.36 mg/L, 51.87 mg/L and 52.41 mg/L respectively. The phosphorus concentration is kept constant (almost same) in these set of experiments. These experiments were performed to study the effect of nitrate level on the phosphorus removal rate and the growth of C. vulgaris.

5.1.2.1 Effect of Nitrate and Phosphorus on Growth

Figure 5.6 and 5.7 shows the time course biomass profile of C. vulgaris grown in synthetic wastewater with different concentrations of phosphorus and nitrate respectively. As shown in the Figures 5.6 and 5.7, C. vulgaris grew in the wastewater to different extents depending on the nitrate and phosphorus loading, ranging the final dry biomass concentration between 0.735 g/L [(NO₃)₁] and 1.77 g/L [(NO₃)₄]. The starting biomass concentration in all these experiments was ~0.2 g/L, which is typically a biomass concentration in growth phase. Due to this, no lag phase was observed in all these experiments. Growth curves exhibit an exponential phase followed by a stationary phase. In some experiments [(NO₃)₃, (NO₃)₄], no stationary phase was observed because the duration of the experiments was not enough to reach the cell stationary phase.
Figure 5.6. Growth curves of C. vulgaris in synthetic wastewater at different P loading, (PO$_4$)$_3$ experiments [PO$_4$-P (mg/L) (♦) 13.1; (■) 26.21; (▲) 52.42]; [NO$_3$- ~ 183 mg/L].

Figure 5.7. Growth curves of C. vulgaris in synthetic wastewater at different nitrate loading, (NO$_3$)$_3$ experiments [NO$_3$- (mg/L) (♦) 90.12; (■) 182.68; (▲) 362.69; (●) 741.56]; [PO$_4$-P ~ 52 mg/L].
Table 5.1 shows the specific growth ($\mu$) of *C. vulgaris* for all the experiments performed at different nitrate and phosphorus concentration. Specific growth is defined as the increase in cell mass per unit time. It is commonly given by the symbol, $\mu$, and units are in reciprocal days (day$^{-1}$). It is determined empirically by

Growth rate; $K' = \ln\left(\frac{N_2}{N_1}\right) / (t_2 - t_1)$ …………………Eq 5.2 [144]

where $N_1$ = biomass at time ($t_1$)

$N_2$ = biomass at time ($t_2$)

Figure 5.8. General pattern of microalgal growth in batch cultures.
Once the growth curve has been plotted (time on x-axis and biomass on y-axis), careful determination of the exponential (straight line) phase of growth is needed. Two point, \(N_1\) and \(N_2\), at the extremes of this linear phase as shown in Fig. 5.8 are taken and substituted into the equation 5.2 [145].

Table 5.1. Specific growth rates of \(C. vulgaris\).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific growth rate (day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PO(_4))(_1)</td>
<td>0.14817</td>
</tr>
<tr>
<td>(PO(_4))(_2)</td>
<td>0.14742</td>
</tr>
<tr>
<td>(PO(_4))(_3)</td>
<td>0.1498</td>
</tr>
<tr>
<td>(NO(_3))(_1)</td>
<td>0.135</td>
</tr>
<tr>
<td>(NO(_3))(_2)</td>
<td>0.158</td>
</tr>
<tr>
<td>(NO(_3))(_3)</td>
<td>0.163</td>
</tr>
<tr>
<td>(NO(_3))(_4)</td>
<td>0.194</td>
</tr>
</tbody>
</table>

As regards to the set of experiments (PO\(_4\))\(_i\), where the nitrate concentration is constant and the phosphorus concentration is varied, all growth curves are very similar (Figure 5.6). Table 5.1 shows that the regardless of the phosphorus level in the media, the specific growth rate of \(C. vulgaris\) was similar in all three experiments indicating that levels of phosphorus in the media does not impact the algae growth. This observation was supported by B. Tubea et al. (1981) who found that different levels of P resulted in no
significant differences in algal growth [146]. Michael DiLorenzo (2007) also reported that phosphorus levels does not the affect micro- and macro algae growth on coral reefs [147].

Similarly Figure 5.7 and Table 5.1 show that the growth rate of algae increased with the increase in the nitrate concentration in the media. It can be seen from the Table 5.1 there is almost 1.5 fold increase in specific growth rate when nitrate concentration increased was from 90.12 ppm ((NO$_3$)$_1$) to 741.56 ppm ((NO$_3$)$_4$). This indicates that the initial nitrate content present in the media significantly affect the growth of algae. This result was supported by the results reported by many researchers who have performed experiments to investigate the effect of nitrate on biomass production. Chittra and Benjamas (2011) reported a decrease in biomass when Botrycocccus spp. was exposed to nitrogen deficient conditions [148]. Hanhua and Gao have also reported decreased growth pattern in Nannochloropsis sp, under nitrogen deficient conditions [149]. On contrary, the results of our study were contradicted by Pietilainen and Niinioja (2001), who found that initial nitrate levels had no significant effect on algal growth. However, their samples were exposed to a light and dark cycle ratio of 15:9 whereas our samples experienced twenty four hours of light each day [150].

5.1.2.2 Effect of Initial Phosphorus Concentration on Nitrate Removal

The variation of nitrate and phosphorus concentrations with time for 8 days of batch operation for (PO$_4$)$_1$ set of experiments is shown in Figures 5.9 and 5.10 respectively. The experimental data of the set of experiments (PO$_4$)$_1$ confirm the hypothesis discussed previously about the algae growth dependency on nitrate and phosphorus concentration. Phosphorus concentration hardly changed in the wastewater.
during the tests (PO$_4$)$_i$, while nitrate was completely removed from the synthetic wastewater in 7 days in all three experiments [(PO$_4$)$_1$, (PO$_4$)$_2$ and (PO$_4$)$_3$]. Regardless of the initial phosphorus concentration in the media, removal rate of nitrate appears to be identical and 80% of nitrate content was removed within first 5 days of the experiment indicating that initial phosphorus content has no impact in removing nitrates from wastewater. A total amount of ~182 mg/L of nitrate was removed in all three experiments in 8 days by C.vulgaris having an initial biomass concentration of ~0.2 g/L. However, the phosphorus efficiency was around 29% for (PO$_4$)$_1$ and (PO$_4$)$_2$ experiments and 62% for (PO$_4$)$_3$ experiment. A total amount of 8.14,7.65 and 14.16 mg/L of phosphorus was removed from (PO$_4$)$_1$, (PO$_4$)$_2$ experiments and (PO$_4$)$_3$ experiments respectively. From this study it was found that C.vulgaris can only remove less amount of phosphorus from wastewater. Low removal of phosphorus was also observed in another green alga Botryococcus braunii. A study conducted by Sawayama et al., (1992) on the growth of B. braunii in secondarily treated piggery wastewater showed that the species B. braunii could remove only 0.29 ppm of phosphorus in 5 days [151]. This is because green algae species like C. vulgaris are capable of absorbing phosphorus only to a limited extent. The absorbed phosphorus is usually stored as polyphosphate granules and will be available for the growth cycle of algae. The growth rate of algae may therefore not respond at once to changes in the external concentration of phosphorus. Our total phosphorus removal rate was higher than that of Sreesai and Pakpain (2007), who reported a removal of approximately 6 ppm of total phosphorus from septage effluent wastewater by Chlorella vulgaris [152].
Figure 5.9. Variation of nitrate concentration in wastewater with time under various initial phosphorus concentrations, (PO₄)ᵢ experiments. [PO₄-P (mg/L) (*) 13.1; (■) 26.21; (♦) 52.42] and constant nitrate concentration (NO₃⁻ ~ 183 mg/L).

Figure 5.10. Variation of phosphorus concentration in wastewater with time under various initial phosphorus concentrations, (PO₄)ᵢ experiments. [PO₄-P (mg/L) (*) 13.1; (■) 26.21; (♦) 52.42] and constant nitrate concentration (NO₃⁻ ~ 183 mg/L).
5.1.2.3 Effect of Initial Nitrate Concentration on Phosphorus Removal

The results of the set of experiments $(\text{NO}_3)_i$ gives the details of the nitrate removal rate of $C. vulgaris$ and describes the effect of initial nitrate concentration on phosphorus removal by $C. vulgaris$. Figure 5.11 shows the variation of phosphorus concentration with time for $(\text{NO}_3)_i$ set of experiments. The removal rate of phosphorus appears to be identical in all the experiments (Figure 5.11). The phosphorus removal efficiency was around 28% for $(\text{NO}_3)_i$ experiments and was constant irrespective of initial nitrate content in the wastewater. A total of ~14 mg of phosphorus was removed in 8 days and there is no removal of phosphorus by $C. vulgaris$ when wastewater is out of nitrates. Hence, the phosphorus removal ability of $C. vulgaris$ does not depend on the initial nitrate content in wastewater but the presence of nitrate is required for the uptake of phosphorus from wastewater.

The nitrate removal rate and nitrate uptake of $C. vulgaris$ are presented in Figure 5.13 and 5.14. The nitrate was completely removed from wastewater within first 6 days in $(\text{NO}_3)_1$, $(\text{NO}_3)_2$ and $(\text{NO}_3)_3$ experiments. A total amount of 88.76 mg/L, 181.33 mg/L, 360.8 mg/L and 520.71 mg/L of nitrate was removed from $(\text{NO}_3)_1$, $(\text{NO}_3)_2$ $(\text{NO}_3)_3$ and $(\text{NO}_3)_4$ experiments respectively. Figure 5.12 indicates that there was a difference in the kinetic curve decrease for $(\text{NO}_3)_1$ experiment compared to the other experiments. For the nitrate concentration of 90.12 ppm $[(\text{NO}_3)_1]$ in wastewater, the decrease rate curve appears to be linear and for other concentrations it appears to have a hyperbolic shape (Fig. 5.12). This is because at high nitrate concentrations, the nitrate removal rate will be rapid during the first 24 h of cultivation. Hao et al also reported same observation in a study performed to remove nitrate and ammonium ions from groundwater by a hybrid
system of zero-valent iron combined with adsorbents [153]. The removal rate of NO$_3^-$ with 100 mg L$^{-1}$ initial NO$_3^-$ concentration was higher than that of 50 and 25 mg L$^{-1}$ during the first 24 h of contact time. The nitrate uptake rate increased with increase in nitrate concentration (Fig.5.11) and a maximum uptake rate of 10.59 mg nitrate h$^{-1}$ g$^{-1}$ was observed for *C. vulgaris* at an initial nitrate concentration of 741.56 mg/L.

The nitrate removal efficiencies for (NO$_3$)$_i$ experiments are shown in Figure 5.14. The nitrate removal efficiency increased with time and remained nearly constant after equilibrium time (6 days). For all concentrations tested, 90% of the nitrate was removed within first 6 days except for wastewater having a nitrate concentration of 741.56 ppm [(NO$_3$)$_4$]. At higher nitrate concentrations, the uptake ability of algae will be higher with lower percent removal rate. Growth rates of *C. vulgaris* reached saturation phase after 8 days in (NO$_3$)$_1$ and (NO$_3$)$_2$ experiments (Fig 5.7) as they ran out of nitrates in the media (Fig 5.12). In experiment (NO$_3$)$_3$, *C. vulgaris* appeared to be in growth phase (Fig 5.7) despite the presence of nitrates. Nutrients necessary for the growth at this stage were hypothetically supported by the consumption of intracellular nitrogen pools such as chlorophyll molecules present in microalgae [154]. *Chlorella vulgaris* grown in high nitrate concentration [experiment (NO$_3$)$_4$] continued to be growth phase as there is an availability of nitrate in the media.
Figure 5.11. Variation of phosphorus concentration in wastewater with time under various initial nitrate concentrations, (NO$_3$)$_i$ experiments. [NO$_3^-$ (mg/L) (♦) 90.12; (■) 182.68; (♦) 362.69; (●) 741.56] and constant phosphorus concentration (PO$_4^-$-P ~ 52 mg/L).

Figure 5.12. Variation of nitrate concentration in wastewater with time under various initial nitrate concentrations, (NO$_3$)$_i$ experiments.[ NO$_3^-$ (mg/L) (♦) 90.12; (■) 182.68; (♦) 362.69; (●) 741.56] and constant phosphorus concentration (PO$_4^-$-P ~ 52 mg/L).
5.1.2.4 Effect of Nitrate Concentration on Lipid Content

Lipid analysis was performed to the algae that were grown under different nitrate concentrations. Table 5.2 and Figure 5.15 shows the lipid content of *C. vulgaris* in different nitrate concentrations both in exponential and stationary phase. A decreasing trend is observed in lipid content in both phases as the nitrate concentration increased.
When the concentration of nitrate was increased from 362.69 to 741.56 ppm (doubled), the lipid content decreased from 16.29 % to 11.53 % in saturation phase and decreased from 14.12% to 10.77 % in exponential phase. Moreover, when the nitrate concentration was decreased from 362.69 to 182.68 ppm (halved), the lipid content increased from 16.29 % to 19.24 % in saturation phase and increased from 14.12 % to 16.06 % in exponential phase. A sharp rise in lipid accumulation of 26% in saturation phase and 22.85 % in exponential phase was recorded when the cultures grown in initial nitrate concentration of 90.12 ppm (1/4th of 362.69 ppm).

![Lipid productivity graph](image)

Figure 5.15. Comparison of lipid content of *C.vulgaris* grown in synthetic wastewater with different initial nitrate concentration in exponential and stationary phase.
Table 5.2. Effect of nitrate concentration on lipid content of *C. vulgaris*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nitrate concentration ( ppm)</th>
<th>Lipid content (% dry cell weight)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stationary</td>
<td>Exponential</td>
</tr>
<tr>
<td>(NO₃)₁</td>
<td>90.12</td>
<td>26</td>
<td>22.85</td>
</tr>
<tr>
<td>(NO₃)₂</td>
<td>182.68</td>
<td>19.24</td>
<td>16.06</td>
</tr>
<tr>
<td>(NO₃)₃</td>
<td>362.69</td>
<td>16.29</td>
<td>14.12</td>
</tr>
<tr>
<td>(NO₃)₄</td>
<td>741.56</td>
<td>11.53</td>
<td>10.77</td>
</tr>
</tbody>
</table>

Our results are in accordance with the results reported in the literature. Yeesang and Cheirsilp observed an increase in lipid content with the decrease in the nitrate concentration in the media and they reported that under nitrogen starvation conditions, algal cells accumulate carbon metabolites as lipids [148]. Hanhua and Gao has reported a four-fold increase in lipid content in *Nannochloropsis* spp. when subjected to low nitrogen source conditions [149]. Nitrate starvation has shown to trigger lipid accumulation in freshwater algae like *C. vulgaris* and *Neochloris oleoabundans*. The highest total lipid content was reported in *Neochloris oleoabundans* (25-37% of DW), while the highest triacylglyceride (TAG) content was found in *C. vulgaris* (11-14% of DW) [155]. Yasemin et al. (2011) have recorded nearly three-fold increase in lipid in *C. vulgaris*, when grown in nitrogen-free medium [156]. Enhancement in lipid production up to 43% (dry cell weight) has been obtained when cultures of *Scenedesmus obliquus* were transferred to media deficient in nitrate for 7 days [157].
Also, our results indicated that at the same concentrations of nitrate, stationary phase cultures showed higher lipid accumulation than exponential phase. This is expected because nitrate levels are significantly lower in stationary phase compared to the exponential phase. Zhu et al., (1997) reported the same trend in lipid accumulation during exponential and stationary phase [158]. They reported that under saturated growth phase, more carbon is incorporated into carbohydrates and lipids which imply that lipid content should be higher in saturation phase than in exponential phase.

5.1.2.5 Nitrogen Uptake Validation

Nitrogen uptake validation was performed for (PO₄)ᵢ and (NO₃)ᵢ experiments. The stoichiometric formula of algae is C₁₀₆H₁₈₁O₄₅N₁₆P [61]. According to this formula, algae contain 8-9 % of nitrogen. From the experimental data, the growth rate and nitrogen uptake of C. vulgaris on daily basis was known. Theoretically, amount of nitrogen removed from wastewater by algae should be equal to the amount of nitrogen present in algae. For example, if we see in Fig 5.9, in (PO₄)ᵢ experiment, nitrate concentration decreased from 84.55 ppm to 61.28 ppm from Day 3 to Day 4, which means 23.27 ppm of nitrate i.e.5.23 mg of nitrogen was removed from wastewater. In the same way, if we see in Fig 5.6. in the experiment (PO₄)ᵢ, biomass of C. vulgaris increased from 0.464 g to 0.537 g from Day 3 to Day 4 resulting in a biomass increase of 0.073g. Assuming 8% of nitrogen present in algae, then amount of nitrogen present in 0.073g of algae would be 5.84 mg which is close to the value of nitrogen that is removed from wastewater.

Figures 5.16 – 5.18 and Figures 5.19-5.21 compares the nitrogen present in increased biomass algae (assuming 8% is nitrogen) and nitrogen removed from wastewater for (PO₄)ᵢ and (NO₃)ᵢ set of experiments respectively.
Figure 5.16. Nitrogen validation for (PO$_4$)$_1$ experiments.

Figure 5.17. Nitrogen validation for (PO$_4$)$_2$ experiment.
Figure 5.18. Nitrogen validation for (PO₄)₃ experiment.

Figure 5.19. Nitrogen validation for (NO₃)₁ experiment.
Figure 5.20. Nitrogen validation for (NO$_3$)$_2$ experiment.

Figure 5.21. Nitrogen validation for (NO$_3$)$_3$ experiment.
Figure 5.2. Nitrogen validation for (NO$_3$)$_4$ experiment.

From the Figures 5.1 to 5.2 it can be seen that, during the exponential phase of *C. vulgaris* (i.e. Day 2, Day 3, Day 4, and Day 5), the nitrogen removed from wastewater is approximately equal to the amount of nitrogen present in increased algae biomass during that phase. After Day 5, the nitrogen removed from the wastewater and nitrogen present in algae is not the same (see Fig 5.1-5.2). This is because there is no significant amount of nitrate present in wastewater to remove after Day 5 (see Fig 5.9 and 5.12) but algae is still in growth phase and about to reach saturation phase (see Fig 5.6 and 5.7). Nutrients necessary for the growth at this stage were hypothetically supported by the consumption of intracellular pools in microalgae [154]. But in the case of (NO$_3$)$_4$ experiment, nitrogen removed from wastewater and nitrogen present in algae are approximately same on Day 6, Day 7 and Day 8 (see Fig 5.22) and this is because even after Day 5, wastewater is still rich in nitrates.
5.1.2.6 Bio Kinetic Coefficients of Nitrate and Phosphorus Removal

The Michaelis-Menten kinetic relationship is used to determine saturation constants and reaction rate coefficients, \( K_m \) and \( k \). This logistic model was found to give a more consistent and accurate description of algal growth [159]. Aslan and Kapdan (2006) outline this method and equations are used as presented by them for algae substrate (nutrient) utilization [136].

\[
R = \frac{R_{\text{max}}S}{K_m + S} \quad \text{Eq 5.3}
\]

where \( R \) = Substrate removal rate or velocity of the reaction

\( R_{\text{max}} \) = maximum substrate removal rate

\( S \) = effluent substrate concentration.

The initial substrate concentrations and the initial substrate removal rates are considered in batch operation. Therefore, the Eq. (5.3) takes the following form:

\[
R_{S_0} = \frac{R_{\text{mo}}S_0}{K_m + S_0} \quad \text{Eq 5.4}
\]

Where \( R_{\text{mo}} = k \cdot X_0 \) is the maximum initial rate of substrate removal. So, Eq. 5.4 can be written as;

\[
R_{S_0} = \frac{kX_0S_0}{K_m + S_0} \quad \text{Eq 5.5}
\]

Where, “\( k \)” is the reaction rate constant (time\(^{-1}\)), \( X_0 \) is the initial biomass concentration of algae. The specific rate of substrate removal (\( R_{Xi} \)) can be calculated by dividing both term of Eq. 5.6 to the initial biomass concentration

\[
R_{S_0} = \frac{R_{S_0}}{X_0} = \frac{kS_0}{K_m + S_0} \quad \text{Eq 5.6}
\]
Eq. 5.6 can be linearized in double reciprocal form as in Eq. 5.6 and a plot of $1/R_{Xi}$ versus $1/S_0$ yields a linear line with a slope of $K_m/k$ and y-axis intercept of $1/k$.

$$
\frac{1}{R_{Xi}} = \frac{1}{K_m} + \frac{K_m}{k} \frac{1}{S_0} \quad \text{..........................Eq 5.7}
$$

Experimental data given in Fig 5.12 and 5.8 were plotted in form of $1/R$ versus $1/ (\text{Initial concen})_0$ as shown in Fig 5.23 and Fig 5.24 respectively.

![Graph](image-url)

Figure 5.23. Effect of initial NO$_3$-N concentration on specific NO$_3$-N removal rate for (NO$_3$)$_i$ set of experiments.
Figure 5.2. Effect of initial PO$_4$-P concentration on specific PO$_4$-P removal rate for (PO$_4$)$_i$ set of experiments.

From the slope and intercept of best fit line of the plot Fig 5.23, kinetic coefficients of NO$_3$-N removal by C. *vulgaris* were determined as $k = 16.66$ mg NO$_3$-N/g biomass/day and $K_m = 47.06$ mg/L ($R^2 = 0.99$). However data from PO$_4$-P removal rates did not present a fitted regression line using the procedures outlined above. This suggests that phosphate removal rate does not depend on initial phosphorus concentration and PO$_4$-P level was above saturation in all experiments.

5.1.3. Effect of Temperature on Nitrate and Phosphorus Removal

To investigate the effect of temperature on nitrate and phosphorus removal, *C. vulgaris* was grown in synthetic wastewater containing initial nitrate concentration of ~182 ppm (~41 ppm NO$_3$-N) and phosphorus concentration (PO$_4$-P) of ~54 ppm at four different temperatures i.e. 10°C, 20°C, 27°C and 35°C. Figure 5.25 shows the biomass productivity of *C. vulgaris* at various temperatures. The biomass productivity levels
increased with the increase in the temperature. Low growth rates were observed at low temperatures and approximately a 50% increase in biomass productivity was reported at 35 °C compared to 10 °C. This was expected because the reactions which make up the process of photosynthesis in algae can be divided into two reactions i.e. photochemical reactions and biochemical reactions. Photochemical reactions do not alter with the change in temperature whereas biochemical reactions of photosynthesis changes with the change in temperature. Biochemical reactions have a temperature coefficient (Q₁₀) of about 2 and similar to all enzyme controlled reactions, they will have an optimum temperature above which the enzymes begin to lose their structure and the growth starts to decline. In addition to this, temperature also has an effect on the rate of the other biochemical reactions which synthesize the components of Chlorella cells to enable them to grow. Temperature will also increase the rate of respiration and if the Q₁₀ of this process is different to the Q₁₀ for photosynthesis, then the net effect of temperature on growth may be influenced by the balance between photosynthesis and respiration.

Figure 5.25. Effect of temperature on algal biomass productivity.
Similar observations were reported in the literature. Krauss and Sorokin (1962) reported that algae growth was low at colder temperatures [160]. Converti et al. (2009) and Bajguz (2009) reported that the optimum growth for C.vulgaris would be around 30°C [161,162]. As per the literature notes, C.vulgaris had the best growth rates at temperatures 27°C and 35°C.

The nitrate and phosphorus uptake rates of C.vulgaris at different temperatures are given in Figure 5.26 and 5.27 respectively. In the presence of C.vulgaris, the nitrate concentration in the wastewater showed significant decreases at the end of treatment period (8 days). After 8 days of treatment period, the nitrate concentration in wastewater decreased from an initial value of ~182.85 ppm to 108.37 ppm, 73.542 ppm, 1.35 ppm and 0.32 ppm, at 10°C, 20°C, 27°C and 35°C respectively (Figure 5.26). Nitrate was almost completely removed from the synthetic wastewater at the end of 8 th day at 27°C and 35°C whereas only ~42% and ~60% of nitrate was removed at 10°C and 20°C respectively. This suggests that higher temperatures are favorable for nitrate removal by C.vulgaris. The present study revealed the optimum temperature range for C.vulgaris to remove nitrate from wastewater to be 27°C-35°C. It indicates that in biological nutrient removal process where eukaryotic algae perform nitrogen assimilation, translocation of inorganic nitrogen across the plasma membrane occurs at a faster rate at optimum temperatures (27°C-35°C) but slows down at colder temperatures. Previous reports have reported that at low temperatures of between 10-12°C the nitrate removal rate of algae diminished by 80% [163].
In the presence of \textit{C.vulgaris}, the phosphorus concentration in the wastewater showed average decreases after 8 days of treatment time. After 8 days, the concentration of phosphorus at different temperatures were found to change from an initial value of ~52.85 ppm to final concentrations of 38.49 ppm, 38.98 ppm, 38.26 ppm and 32.57 ppm, at temperatures of 10°C, 20°C, 27°C and 35°C respectively (Figure 5.25).

Figure 5.26. Effect of temperature on nitrate uptake of \textit{C.vulgaris}.

Figure 5.27. Effect of temperature on phosphorus uptake of \textit{C.vulgaris}.
Phosphorus levels in the wastewater after 8 days of treatment at temperatures 10°C, 20°C and 27°C were found to be similar and slightly higher than level at 35°C. This suggests that phosphorus removal by C.vulgaris is almost independent of temperature. But there are few studies in the literature which conflicts the effect of temperature on phosphorus removal. Some studies indicated that biological phosphorus removal efficiency improved at temperatures of 20 °C to 37 °C. Some other studies have reported that lower temperatures of 5°C – 15°C favor better phosphorus removal. Some studies have reported that phosphate accumulating organisms are found to be lower-range mesophiles or perhaps psychrophiles at predominantly 20°C or possibly lower [164,165]. Therefore based on the reports in the literature and on the experimental results of this study, the effect of temperature on phosphorus removal was inconclusive.

5.1.4. Nitrogen Removal from Different Nitrogen Sources

The growth rate and nitrogen removal capability of C.vulgaris with different nitrogen sources was studied using synthetic wastewater containing potassium phosphate (~26.32 ppm) along with nitrogen (~ 41.5 ppm) from four different nitrogen sources. The growth curve of C.vulgaris with different nitrogen sources is shown in Figure 5.28. The C.vulgaris was grown fastest in potassium nitrate followed by urea and ammonium sulfate. The algae grew slowest in presence of potassium nitrite. The maximum cell concentration of 0.992 g/L and maximum specific growth rate of 0.213 day⁻¹ were found in algae grown in potassium nitrate. Figure 5.29 shows the biomass productivities of C.vulgaris grown in four different nitrogen sources. The order of biomass productivity and specific growth rate of C.vulgaris with different nitrogen sources were NO₂⁻ < NH₄⁺ < urea < NO₃⁻. This order was similar to the growth rate order of Scenedesmus sp.
after 13 days of cultivation with nitrate, ammonium and urea as nitrogen sources [166].

Yadavalli et.al also reported that \textit{C.pyrenoidosa} prefers nitrate as nitrogen source than urea for obtaining maximum biomass productivity when grown photoautotrophically in a customized lab scale photo bioreactor [167].

![Figure 5.28. Growth of \textit{C.vulgaris} under different nitrogen sources.](image)

![Figure 5.29. Biomass productivity of \textit{C.vulgaris} under different nitrogen sources.](image)
The removal amounts of nitrogen and efficiencies depending on the nitrogen source when C. vulgaris was cultured in phototrophic conditions are illustrated in Fig. 5.30 and Fig. 5.31. Figure 5.32 shows nitrogen uptake from different nitrogen sources. As shown in Fig. 5.30, the nitrogen was completely removed from the media containing potassium nitrate and urea in 6 days and removal rate appears to be identical. A total amount of ~42 ppm of nitrogen was removed from the synthetic wastewater containing potassium nitrate and urea by C. vulgaris having an initial biomass concentration of ~0.185 g/L. After 8 days of cultivation with nitrate, urea, ammonium and nitrite as nitrogen sources, total nitrogen removal efficiencies of C. vulgaris was significantly high with nitrate and urea nitrogen. The nitrogen removal efficiency increased with time and a maximum of ~100% removal efficiency was achieved in the synthetic wastewater containing nitrate and urea. The nitrogen removal efficiency was only around 54% and 33.5% for the media containing ammonium sulfate and potassium nitrite respectively (Fig. 5.31.). However, when compared to the values reported in the literature, the nutrient (nitrogen) removal efficiency of C. vulgaris found in this study was higher than Chlorella spp. and Phormidium bohneri [104, 168 and 169]. Aslan and Kapdan (2006) reported that the NH$_4^+$ was completely removed from the media by C. vulgaris when the initial concentration was between 13.2 and 21.2 mg L$^{-1}$ [136]. Further, the removal efficiency of NH4$^+$ was decreased to less than 24% when the NH$_4^+$ concentration was higher than 129 mg L$^{-1}$. In the present study, the removal efficiency was 54% when the initial NH$_4^+$ concentration was 70 mg L$^{-1}$. Based on the results of this study, it can be concluded that C. vulgaris prefers nitrogen in nitrate form
and when nitrate and urea were used as a nitrogen source, removal rate of nitrogen was higher than those with ammonia and urea as nitrogen source.

Figure 5.30. Variation of nitrogen concentration with time in synthetic wastewater containing four different nitrogen sources.

Figure 5.31. Variation of nitrogen removal efficiency with time in synthetic wastewater containing four different nitrogen sources.
5.1.5. Phosphorus Removal by Species Acclimated to High P Environments

Two local species known to grow well in high P environment were selected to determine their ability to remove phosphorus from synthetic wastewater. Three different phosphorus concentrations i.e. ~10, 20 and 30 ppm were selected to investigate the phosphorus removal rates of these local species at constant nitrate concentration of ~36 ppm.

Experiments P1, P2, P3 represent Pleasant Hill Lake species having phosphorus concentrations of 10, 20 and 30 ppm respectively. Experiments P4, P5, P6 represent Delaware lake species having phosphorus concentrations of 10, 20 and 30 ppm respectively. Fig 5.33 and 5.34 represent the growth curves of Pleasant hill Lake species and Delaware lake species. Growth rates are similar for these two species at different phosphorus concentrations which indicate that growth does not depend on the initial phosphorus content. This was expected as the nitrate concentration was maintained constant at 36 ppm in all these experiments. A maximum growth of 1.162 g/L and 1.1172 g/L was observed in Pleasant Hill and Delaware Lake species respectively over a period.
of 10 days. After 10 days, growth reached the saturation phase as the media ran out of nitrates.

Figure 5.33. Growth curve of Pleasant hill Lake species at different phosphorus concentrations (P1= ~10 ppm, P2= ~20 ppm, P3= ~30 ppm).

Figure 5.34. Growth curve of Delaware Lake species at different phosphorus concentrations (P4= ~10 ppm, P5= ~20 ppm, P6= ~30 ppm).

Figure 5.35 and 5.36 shows the phosphorus removal rates of Pleasant hill and Delaware lake species respectively and Figure 5.37 and 5.38 shows the removal efficiencies of these species with respect to time.
Figure 5.35. Phosphorus removal from Pleasant hill lake species (P1= ~10 ppm, P2= ~20 ppm, P3= ~30 ppm).

Figure 5.36. Phosphorus removal from Delaware lake species (P4= ~10 ppm, P5= ~20 ppm, P6= ~30 ppm).
From the Figures 5.37 and 5.38 it can be observed that a maximum percent removal of 90% and 97.97% was observed at an initial phosphorus concentration of 10 ppm for Pleasant hill and Delaware lake species respectively over a period of 10 days. A low percentage of 65.43% and 58.96% were reported at higher phosphorus concentration.
i.e. at 30 ppm for pleasant hill and Delaware lake species respectively. The removal efficiencies of both of these species were compared with *C. vulgaris* and Figure 5.39 shows the graphical representation of the removal efficiencies of three species at three different phosphorus concentrations over a period of 10 days. Figure 5.40 compares the phosphate uptake per biomass for three species at three different phosphorus concentrations. It was observed from the Figure 5.39 that the phosphorus removal efficiencies of Pleasant hill and Delaware lake species are significantly higher than (more than double) that of *C. vulgaris*.

![Bar chart showing phosphorus removal efficiency](image_url)

Figure 5.39. Comparison of Phosphorus removal efficiency of Pleasant Hill Lake Delaware Lake and *C. vulgaris*. 
5.2. Heavy Metal (Cr, Cd) Removal Experiments.

5.2.1 Preliminary Studies

Two species i.e. *C. vulgaris* and *Neochloris oleoabundans* were tested to investigate their capability to remove Cr and Cd from synthetic wastewater. Two set of experiments were conducted during the preliminary phase. In the first set, synthetic wastewater containing metal concentration of 350 mg/L of Cr$^{6+}$ was treated using *C. vulgaris* and *N. oleoabundans* and in the second set 350 mg/L of Cr$^{6+}$ was replaced with 250 mg/l of Cd$^{2+}$. The Cr and Cd content were measured both in water and on algae surface. The technique to measure Cd and Cr content on algae surface was described in section 4.7.1.1. Blank controls (containing only medium plus metal) were also carried out for each concentration tested.

Figure 5.41 and 5.42 shows the removal of hexavalent chromium by chlorella and Neochloris respectively from synthetic wastewater. It was observed that chlorella removed 43.57% of Cr$^{6+}$ i.e. 153.9 mg/l in 128 hours whereas Neochloris removed
31.76% of Cr$^{6+}$ i.e. 110.84 mg/l in 148 hours from the metal contaminated wastewater. Very less amount of Cr (~6 mg/l) was detected on the surface of algae. Figure 5.43 shows the growth curves of C.vulgaris and N.oleoabundans during hexavalent chromium removal. Significant growth inhibition was observed and there is no growth during the first 5 days of chromium removal and after 5 days a loss of biomass was observed in both the species. This is because the initial algae concentration of species used to treat chromium contaminated wastewater was in growth phase and was high enough to withstand the chromium concentration for first 5 days but after 5 days as more chromium was taken by the algae, the biomass started to decrease.

![Graph showing chromium concentration and biomass over time](image)

Figure 5.41. Hexavalent chromium removal by C.vulgaris.
Figure 5.42. Hexavalent chromium removal by *N. oleoabundans*.

Figure 5.43. Growth curves of *C. vulgaris* and *N. oleoabundans* during chromium removal process.

Figure 5.44 shows the variation of chromium concentration with time in control experiment which contains only medium (BBM) and potassium dichromate (Cr\textsuperscript{6+}). It can be seen from Figure 5.44 that chromium concentration was uniform throughout the
experimental period confirming that no other external factor was responsible reducing the chromium concentration when wastewater was treated with algae.

The first set of experimental results conclude that *C. vulgaris* was able to remove $\text{Cr}^{6+}$ from wastewater and was also effective compared to *N. oleoabundans*. The capability to remove $\text{Cd}^{2+}$ from wastewater was tested in the second set of experiments. Figure 5.45 and 5.46 shows the removal of cadmium by *C. vulgaris* and *N. oleoabundans* respectively from synthetic metal contaminated wastewater. Rapid removal of $\text{Cd}^{2+}$ took place when wastewater was treated with algae species. Both the species have almost removed 100% of the $\text{Cd}^{2+}$ from the wastewater during the first 10 minutes of experimental time. The amount of cadmium that was removed from wastewater was found on the algae surface when the surface was washed with EDTA. The cadmium that is present on the algae surface eventually enters inside the algae cell (cytoplasm). When cadmium enters the cytoplasm of the algae species, significant growth inhibition will occur and this was seen during the experiment. Figure 5.47 shows the growth curves of
C. *vulgaris* and *N. oleoabundans* during the cadmium removal process. A decline in biomass was observed after 36 hours and 52 hours for *C. vulgaris* and *N. oleoabundans* respectively as cadmium entered inside the algaecell. Figures 5.45 and 5.46 confirm that there is decrease in cadmium content on algae surface after 36 hours and 52 hours respectively as cadmium entered into the algae cell from the surface. Control blank experiments were conducted on samples containing only the Cd$^{2+}$ metal solution and BBM with no algae in it. The experimental data of the control blank experiment is shown in Figure 5.48 and it showed good reproducibility and metal measurement errors are within the acceptable limits.

![Figure 5.45. Cadmium removal by C. vulgaris.](image-url)
Heavy metal removal in algae involves two processes: an initial rapid (passive) uptake followed by a much slower (active) uptake. During the passive uptake, physical adsorption takes place in which the metal ions are adsorbed over the cell surface very quickly just in a few seconds or minutes and this process is metabolism-independent. Then, chemisorption a process which is metabolism-dependent takes place in which the ions are transported slowly across the cell membrane into the cytoplasm. The cadmium removal undergoes passive uptake i.e. physical adsorption which is metabolism independent and cadmium was removed from wastewater and adsorbed on cell surface within minutes. It was also seen in our experiments when algae surface was washed with EDTA entire cadmium present on the algae surface was recovered in EDTA. On contrary, most of chromium removal undergoes active uptake i.e. chemisorption which is metabolism dependent. This was also seen in our experiments where a dip in
Biomass was seen when chromium was removed from the wastewater (because chromium enters the cytoplasm).

Figure 5.47. Growth curves of C. vulgaris and N. oleoabundans during cadmium removal process.

Figure 5.48. Variation of cadmium concentration with time in control experiment.
5.2.2 Biosorption of Chromium and Cadmium from Synthetic Wastewater by Chlorella vulgaris.

Preliminary studies concluded that C. vulgaris was effective in removing Cr and Cd from synthetic wastewater. Later, experiments were conducted to study the nature of the biosorption process, kinetics of the metal removal process and to investigate the effect of various parameters such as initial metal ion concentration, contact time, initial biomass concentration, pH and temperature on the metal removal ability of Chlorella vulgaris.

(a) Effect of initial biomass concentration

Different initial biomass concentrations ranged from 1.43 to 2.38 g/L was applied to study the effect of biomass dosage on the biosorption of Cr$^{6+}$ and Cd$^{2+}$. Figure 5.49 shows the effect of initial algae biomass concentration on chromium metal uptake and chromium percent removal.

![Figure 5.49](image)

The Cr (VI) removal percentage (R%) improved significantly with the increase in initial algae concentration. The percent removal of Cr (VI) increased from 70.41% to
85.58% with an increase in biomass concentration from 1.43 to 2.38 g L\(^{-1}\). This can be attributed to the increase in surface area resulting from the increase in algae biomass concentration (biosorbent) which in turn increases the number of active binding sites. However, the metal uptake \((q)\) i.e. the amount of Cr (VI) adsorbed per unit mass of biomass decreased with increase in biomass dosage. This is expected because an increase in initial algae biomass concentration keeping initial metal ion concentration constant will reduce adsorbate/adsorbent ratio which in turn reduces metal uptake. Similar behavior for the effect of adsorbent concentrations on Cr (VI) adsorption capacity has been reported for other types of adsorbents [170, 171]. Figure 5.50 shows the chromium percent removal for different biomass concentrations over the experimental time investigated i.e. 120 hours. Equilibrium was attained after 60 hours and at equilibrium chromium removal percentage of ~95% was achieved in all four biomass concentrations tested.

Figure 5.50. Chromium percent removal for different initial biomass concentrations (pH 6.8; Cr (VI) = 30 mg L\(^{-1}\); contact time = 120 hrs; temperature = 25 °C).
Figure 5.51 shows the effect of initial algae biomass concentration on cadmium metal uptake and cadmium percent removal. The cadmium percent removal data from the Figure 5.51 revealed that the biosorption efficiency of Cd (II) was not significantly affected by the increased dose of algae concentration. In other words, the biosorption of Cd (II) was slightly increased with subsequent increase in the initial algae biomass concentration and almost became constant at higher concentration than 1.8712 g/L. This is because alga having a concentration of 1.8712 g/L was enough to adsorb 100 mg/L of Cd (II) on its surface. Over the experimental time investigated, i.e. 30 mins, the cadmium percent removal of 98.9%, 100% and 100% was achieved with initial algae biomass concentrations of 1.45, 1.8712 and 2.154 respectively. Metal uptake decreased with an increase in biomass dosage from 1.45 to 2.154 g/L. This decrease can be attributed to the concentration gradient between the sorbent and the sorbate; an increase in biomass concentration causes a decrease in the amount of metal sorbed onto a unit weight of the algae. Similar observations were reported for lead removal using S. cumini [172].

Figure 5.51. Effect of initial biomass concentration on Cd$^{2+}$ removal (pH = 6.8; Cd (II) = 100 mg L$^{-1}$; contact time = 30 min; temperature = 25 $^\circ$C).
Growth:

Significant growth inhibitions were observed in all treatments. Figures 5.52 and 5.53 show the growth curves of *C. vulgaris* during Cr (VI) and Cd (II) removal. Cultures with Cr (VI) concentrations of 30 mg/L and having initial biomass concentrations of 1.43, 1.76, 2.03 and 2.38 g/L grew initially until chromium was present on their surface. Growth started to decline when chromium entered into the cytoplasm i.e. after 36 hours. In case of cadmium, as the time spent to remove Cd (II) (experimental time) was in minutes growth remained almost constant throughout the removal period.

![Growth curves of *C. vulgaris* during Cr (VI) removal at different initial biomass concentrations.](image)

Figure 5.52. Growth curves of *C. vulgaris* during Cr (VI) removal at different initial biomass concentrations.
(a) Effect of initial metal ion concentration

Effect of initial metal ion concentration on Cr (VI) and Cd (II) removal was studied by performing the experiments at different initial concentrations of Cr (10, 20 and 30 mg L\(^{-1}\)) and Cd (50, 100 and 150 mg L\(^{-1}\)) at optimum pH, temperature and optimum biomass dosage. Figure 5.54 shows the effect of initial Cr (VI) concentration on chromium metal uptake and chromium percent removal. Similarly, Figure 5.55 shows the effect of initial Cd (II) concentration on cadmium metal uptake and cadmium percent removal. The results presented in Figures 5.54 and 5.55 indicated that the metal percent removal decreases with an increase in the initial metal ion concentration. The percentage removal of Cd (II) decreased from 98.52% to 66.66% as the initial Cd (II) concentration increased from 50 to 150 mg L\(^{-1}\). Similarly, the percentage removal of Cr (VI) decreased from 95.42% to 91.96% as the initial Cr (VI) concentration increased from 10 to 30 mg L\(^{-1}\). This is because, at low initial metal ion concentrations, sufficient adsorption sites are available on the surface of algae for adsorption of heavy metal ions. But, at higher
concentrations, the number heavy metal ions are relatively higher compared to 
availability of adsorption sites. Therefore, metal percent removal depends on initial metal ion concentration and decreases with increase in initial metal ion concentration.

However, metal uptake increases with increasing metal ion concentration. This can be attributed to the fact that an increase in initial ion concentration provides a larger driving force to overcome all mass transfer resistance between solid and aqueous phase, thus resulting in higher metal ion adsorption [173]. In other words, at low concentration, the ratio of available surface to the initial Cr (VI) concentration is larger, so the removal may become higher. However, in case of higher concentration, this ratio becomes low, so the percentage removal may become lesser. Few studies in the literature reported similar trend in other metals [174]. Meena et.al observed the similar trend i.e. decrease in metal percent removal and increase in metal uptake with an increase in metal ion concentration during the removal of heavy metal ions such as Cd(II), Pb(II), Hg(II), Cu(II), Ni(II), Mn(II) and Zn(II) from aqueous solutions by using carbon aerogel as an absorbent [175].

Figure 5.54. Effect of initial Cr (VI) concentration on Cr\textsuperscript{VI} removal (pH 6.8; algae concentration = \(~1.45\) g L\textsuperscript{-1}; contact time =120 hrs; temperature = 25 °C).
Effect of initial Cd (II) concentration on Cd\(^{+2}\) removal (pH = 6.8; algae concentration = ~1.53 g L\(^{-1}\); contact time = 30 mins; temperature = 25 °C).

(b) Effect of pH

The pH plays an important role in the biosorption of metals as it influences both the speciation of metal ions in the aqueous solution and the binding sites availability on the surface of alga (biosorbent). Figure 5.56 shows the effect of pH on Cr (VI) and Cd (II) biosorption by algal biomass. There is a sharp decline in chromium percent removal with increase in pH. This is because in acid media (i.e. at low pH), chromium exists in the form of HCrO\(_4\)^\(-\), Cr\(_2\)O\(_7\)^{2\,-\} and H\(_2\)CrO\(_4\) whereas in basic solutions it exists in the form of CrO\(_4\)^{2\,-\}. The lower pH value can cause the surface of the algae to be protonated to a higher extent. This results in a stronger attraction for negatively charged Cr (VI) complex ions in the solution. As pH value increases, the proton concentration decreases and the biomass surface is more negatively charged. Adsorption of Cr (VI) at lower pH shows the bind of the negatively charged chromium species (HCrO\(_4\)^\(-\)) through electrostatic attraction to the positively charged (due to more H\(^+\) ions) surface functional
groups of the algae which results in the high percent removal. For pH > 6.0, surface is negatively charged and Cr exists in CrO$_4^{2-}$ form and because it is repulsed by high negative surface there is decrease in percent removal [176, 177].

But in the case Cd (II) removal, cadmium percent removal increased with the increase in pH [Fig. 5.56]. This is because cadmium always exists in Cd$^{+2}$ form in aqueous solutions at all pH values. At lower pH, the positive charge on the sites of biomass surface restricts the approach of Cd (II) to the surface of biomass because of charge repulsion which results in the low percentage removal. But as the pH increases, the biomass surface is more negatively charged and biosorption of Cd (II) increases till it reaches its maximum biosorption around pH 6. After pH 6, Cd (II) percent removal again decreased and this can be attributed to the formation of soluble hydroxilated complexes of the metal ions (Cd (OH)$_2$).

Figure 5.56. Effect of initial solution pH on Cr (VI) and Cd (II) ions adsorption by C.vulgaris (chromium concentration = 30 mg L$^{-1}$; cadmium concentration = 150 mg L$^{-1}$; algae concentration = 1.5 g L$^{-1}$; temperature = 25 °C).
(c) Effect of contact time

Contact time plays a significant role in the metal biosorption process. Figure 5.57 and Figure 5.58 shows the effect of contact time on the metal uptake of Cr (VI) and Cd (II) ions using C. vulgaris. From the experimental data represented in Figures 5.57, more than 80% Cr (VI) was removed in first 4 hours at all concentrations tested. The process of Cr (VI) metal uptake was almost constant after the first 4 hours. The reaction rate is rather fast at first and thereafter it proceeds at a lower rate and finally no further significant metal uptake was observed. Figure 5.58 shows the effect of contact time on the biosorption of Cd (II) using C. vulgaris. The results shown in Fig. 5.58 indicated that for all the concentrations tested, biosorption of Cd (II) was rapid in the first 20 min then was gradually increased till the equilibrium attained at 60 min, and the biosorption became almost constant thereafter. The uptake of Cd (II) occur in two steps; a very rapid surface biosorption followed by a slow intercellular diffusion. Similar behavior was reported by other researchers. Chen et al., observed same trend during the biosorption of Ni and Cu on treated alga Undaria pinnatifida [174] and the biosorption of Cd by biomass fungal biosorbent [178].
Figure 5.57. Effect of contact time on Cr (VI) metal uptake by C. vulgaris. (pH = 6.5; algae concentration = ~1.5 g L$^{-1}$; temperature = 25 °C).

Figure 5.58. Effect of contact time on Cd (II) metal uptake by C. vulgaris. (pH = 6.5; algae concentration = ~1.4 g L$^{-1}$; temperature = 25 °C).
(d) Effect of temperature

The test for observing effects of temperature on metal biosorption by *C. vulgaris* was performed by varying the temperatures between 10 °C – 35 °C. The results are shown in the Figure 5.59. The highest Cr (VI) and Cd (II) removal was obtained at temperature of 35 °C, while the lowest of that was found at 10 °C. In general, the results showed that the higher the temperatures were, the higher the percentages of metal removal were, which indicated that metal sorption processes occurred in this trial were endothermic reactions [179]. Similar results were reported by Bai and Abhraham on biosorption of Cr (VI) by *Rhizopus nigricans* showing the increase of metal adsorption with increasing temperature [180]. The increase in metal percent removal with the rise in temperature may be due to the bond breaking of functional groups on the surface of algae and there could be increase in the number of adsorption sites on the algae surface, which will increase the extent of adsorption with the rise in temperatures.

Figure 5.59. Effect of temperature on Cr (VI) and Cd (II) removal by *C. vulgaris*. ([Cr (VI) = 30 ppm; initial algae concentration = ~1.45 gL⁻¹; pH = 6.5, contact time = 5 days]; [Cd (II) = 50 ppm; initial algae concentration = ~1.53 gL⁻¹; pH = 6.5, contact time = 90 min]).
5.2.3 Kinetics Studies

The kinetics of metal sorption described by the relationship between contact time and metal uptake by \textit{C. vulgaris} for three initial Cr (VI) concentrations (10, 20 and 30 mg L\(^{-1}\)) and three initial Cd (II) concentrations (50, 100 and 150 mg L\(^{-1}\)) are discussed below.

The kinetics was demonstrated to understand the sorption behavior of the \textit{C. vulgaris} and to find out the rate expressions. The kinetics of adsorption was studied by using two kinetic models: pseudo first order and pseudo second order models.

**Pseudo first order model:** The model of the pseudo first order used is that of Lagergren given by the Eq.

\[
\frac{dq}{dt} = k_1 (q_e - q_t) \text{ Eq.5.8}
\]

Integration of Eq.5.8 in the conditions (t = 0, q\(_t\) = 0) and (t = t, q = q\(_t\)) gives:

\[
\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t \text{ Eq.5.9}
\]

where, \(k_1\) is rate constant for adsorption (1/min) and \(q_e\) and \(q_t\) are amounts of adsorbed metal (mg/g) at equilibrium and time t respectively.

If straight line obtains after plotting \(\log(q_e - q_t)\) vs t, then it indicates that metal adsorption is a pseudo first order. But, our experimental values didn’t follow a straight line when data was plotted between \(\log(q_e - q_t)\) vs t as shown in Figure 5.60. Then, equations of Lagergren, a pseudo-second order mechanism was used for mathematical description of adsorption of Cr (VI) and Cd (II). Adsorption constants were determined from the equation to find out the adsorption capacity of \textit{C. vulgaris}. Lagergren pseudo-second order model kinetic equation defined as follows:
\[ \frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad \text{Eq. 5.10} \]

where \( q_t \) (mg g\(^{-1}\)) is the amount of metal uptake by \( C. vulgaris \) at time \( t \)

and \( k_2 \) (g mg\(^{-1}\) min\(^{-1}\)) is the rate constant of second-order adsorption. The adsorption rate constant \( k_2 \) was calculated from the slope of the linear plot of \( t/q_t \) vs \( t \).

Plots for the pseudo second order kinetics of Cr (VI) and Cd (II) biosorption on \( C. vulgaris \) are shown in Figures 5.61 and 5.62. Straight lines were obtained after plotting \( t \) and \( t/q_t \) for both Cr (VI) and Cd (II) biosorption indicating the degree of fitness of metal sorption to pseudo second order kinetic model. The better fit with pseudo-second-order model also implies that the metal adsorption process is interaction controlled with the involvement of chemisorption mechanism. But at the same time, the initial rapid phase within the first 20 min may involve physical adsorption or ion exchange at absorbent surface. Therefore, there are more than one mechanisms involved in the metal removal process. Similar mechanisms were observed in the adsorption of Cr (VI) onto activated carbons derived from agricultural waste materials [181] and wheat-residue derived black carbon [182].

To quantify the applicability of this model, the correlation coefficient, \( R^2 \), was calculated from these plots. \( R^2 \) value is greater than 0.99 for biosorption of Cr (VI) at all concentrations whereas for biosorption of Cd (II) \( R^2 \) is in the range of 0.8535 – 0.9871. Table 5.3 gives the model constants and correlation coefficients for adsorption of Cr (VI) and Cd (II).
Table 5.3. Sorption rate expression for different metal concentrations.

<table>
<thead>
<tr>
<th>Initial concentration (mg/L)</th>
<th>Pseudo second order</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( q_e ) (mg/g)</td>
<td>( k_2 ) (g/ mg min)</td>
</tr>
<tr>
<td>Cr (10 mg/L)</td>
<td>7.027</td>
<td>0.001066</td>
</tr>
<tr>
<td>Cr (20 mg/L)</td>
<td>13.1752</td>
<td>0.000633</td>
</tr>
<tr>
<td>Cr (30 mg/L)</td>
<td>19.047</td>
<td>0.000607</td>
</tr>
<tr>
<td>Cd (50 mg/L)</td>
<td>37.174</td>
<td>0.003502</td>
</tr>
<tr>
<td>Cd (100 mg/L)</td>
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<td>0.00023</td>
</tr>
<tr>
<td>Cd (150 mg/L)</td>
<td>156.25</td>
<td>0.000141</td>
</tr>
</tbody>
</table>

Figure 5.60. Plots for the pseudo first order kinetics of Cr (VI) biosorption on C.vulgaris.
Comparison with other adsorbents:

The comparison between the metal uptake capacities of Cr (VI) and Cd (II) of C. vulgaris and other adsorbents reported in the literature are given in the below Table 5.4. The results show that the range of adsorption capacities obtained for Cr (VI) i.e. 7.027-19.047
mg Cr (VI) /g and for Cd i.e. 37.174-156.25 mg Cd (II) /g are comparable to the values reported for other adsorbents, which ranges from 10.61 to 473.91 mg Cr (VI)/g and 6.1942 to 416 mg Cd (II) /g. Thus, the green algae *C.vulgaris* can be used as an efficient biosorbent for the uptake of Cr (VI) and Cd (II) ions from the metal laden wastewater.

Table 5.4. Comparison of adsorption capacities of various adsorbents used for Cr (VI) and Cd (II) removal.

<table>
<thead>
<tr>
<th>Adsorbents (Algal biomass)</th>
<th>Adsorption capacity (mg/g)</th>
<th>Initial Cr (VI) concentration (mg/L)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Spirulina platensis</em></td>
<td>188.68</td>
<td>250</td>
<td>183</td>
</tr>
<tr>
<td>Filamentous algae <em>Spirogyra</em> species</td>
<td>14.7</td>
<td>5</td>
<td>184</td>
</tr>
<tr>
<td>Green alga <em>Oedogonium Hatei</em></td>
<td>31</td>
<td>50-100</td>
<td>171</td>
</tr>
<tr>
<td>Green alga <em>Ulva lactuca</em></td>
<td>10.61</td>
<td>5-50</td>
<td>185</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adsorbents (Microorganisms)</th>
<th>Adsorption capacity (mg/g)</th>
<th>Initial Cd (II) concentration (mg/L)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>122</td>
<td>120</td>
<td>186</td>
</tr>
<tr>
<td>Algae, marine, dead biomass</td>
<td>80</td>
<td>250</td>
<td>187</td>
</tr>
<tr>
<td>Alginate Carriers</td>
<td>220</td>
<td>50</td>
<td>188</td>
</tr>
<tr>
<td><em>Ascophyllum nodosum</em></td>
<td>38</td>
<td>250</td>
<td>189</td>
</tr>
<tr>
<td><em>Aspergillus niger</em>, living</td>
<td>15.50</td>
<td>75</td>
<td>190</td>
</tr>
<tr>
<td>Water hyacinth</td>
<td>2.44</td>
<td>4</td>
<td>191</td>
</tr>
</tbody>
</table>
5.2.4 Chromium and Cadmium Metal Uptake Correlations:

Biosorption capacity of algae varies greatly with parameters such as initial pH, algae biomass concentration, initial metal ion concentration, contact time and temperature. Numerous studies have been conducted by the researchers to study the effect of these parameters on the metal uptake of algae. But, no study gave a correlation that will relate metal uptake as a function of these parameters. Moreover, the efforts to generate a correlation have not been attempted so far in the literature. One of the aims of the present work is to provide correlations for Cr (VI) and Cd (II) metal sorption by C.vulgaris. The key parameters that would affect the metal sorption rates of C.vulgaris were considered while developing the correlations. Using these correlations, a commercial system can be designed to treat metal laden wastewater at large scale. The design details of the commercial scale algal wastewater treatment technology (AWTS) are discussed in section 5.5.

In the present study, experiments were conducted for heavy metal sorption over pH range of 2-8, covering a temperature range of 10-35°C, Cr (VI) metal ion concentration of 10-30 ppm, Cd (II) metal ion concentration ranging from 50-150 ppm and biomass concentration varying from 1.43 to 2.15 g. Regression analysis was performed to the Cr (VI) and Cd (II) experimental data to obtain the correlations which are applicable only within the range of parameter values given above. The correlation expression for Cr and Cd uptake by C.vulgaris is given in Eq. 5.11 and 5.12 respectively. Metal uptake = f (time, algae conc, pH, temperature, initial metal conc).

For Cr (VI),

\[ q_{cr} = e^{-6.6184 \text{(time)^{0.0862}} \text{(algae conc)^{0.093}} \text{(pH)^{-0.26}} \text{(T)^{2.070}} \text{(initial metal conc)^{0.945}}} \]  ...Eq. 5.11
For Cd (II),

\[ q_{cd} = e^{-4.035 \text{(time)}^{0.476831} \text{(algae conc)}^{1.09} \text{(pH)}^{0.885} \text{(T)}^{0.49} \text{(initial metal conc)}^{0.945}} \]  

…Eq. 5.12

where the metal uptake, \( q \), was in mg/g, algae concentrations in g/L, initial metal concentration in mg/L, time in hours and temperature is in °C. The chromium metal uptake correlation predicted a very strong dependency on pH, i.e. the increase in pH would result in low Cr metal uptake. Dependency on algae concentration was relatively less compared to the temperature effect on chromium metal uptake by \( C. vulgaris \). In contrast to the Cr metal uptake, Cd metal uptake of \( C. vulgaris \) strongly depend on algae concentration and metal uptake increases with the increase in pH to certain extent (upto pH 6).

Figure 5.63 and 5.64 shows the comparison of experimental values and correlated values for Cr (VI) and Cd (II) metal uptake respectively. The observed experimental metal uptake values show little variance from the correlated values. To quantify the correlation, the correlation coefficient, \( R^2 \), was calculated from these plots. \( R^2 \) is greater than 0.99 for biosorption of Cd (II) and is 0.9653 for biosorption of Cr (VI) indicating that the derived correlations are good fit for the experimental data.
Figure 5.63. Chromium metal uptake by C.vulgaris: comparison of experimental metal uptake and normalized correlated values. (Good only within the range of pH: 2-8; temperature: 10-35 °C; initial metal ion concentration: 10-30 mg/L; initial algae concentration: 1.43-2.043 g).
Figure 5.64. Cadmium metal uptake by C. vulgaris: comparison of experimental metal uptake and normalized correlated values. (Good only within the range of pH: 2-8; temperature: 10-35 °C; initial metal ion concentration: 50-150 mg/L; initial algae concentration: 1.45-2.15 g).

5.2.5 Heavy Metal Removal Under Mixotrophic and Heterotrophic Conditions

5.2.5.1 Mixotrophic and Heterotrophic Growth

The results from the study in section 5.2.2 and the C. vulgaris metal uptake correlations indicated that more amount of metal can be removed with high initial algae concentration. To enhance the algae cell growth rate in shorter interval of time, C. vulgaris was grown under mixotrophic and heterotrophic conditions. Glucose and glycerol were used as organic carbon sources. Different concentrations of glycerol solution (1%, 2% and 3% on volume basis) and glucose solution (1% and 2% on volume basis) were supplemented to the BBM medium and cultured under dark and light (heterotrophic and mixotrophic ) conditions. Carbon dioxide was supplied to the
mixotrophically grown algae along with the organic carbon source. The autotrophic control group was also cultivated in BBM with illumination.

(a) Growth curves

Results presented in Fig. 5.65 and Fig. 5.66 demonstrates the growth of *C. vulgaris* under mixotrophic and heterotrophic conditions with glycerol and glucose as organic carbon sources. The samples supplied with organic carbon sources were superior in their growth compared to the autotrophic control. Growth rates of *C. vulgaris* calculated by Eq.5.1. for different culture conditions were given in Table 5.5. The growth rate of heterotrophic microalgae was higher followed by mixotrophic and phototrophic microalgae. Algae grown heterotrophically with 3% glycerol as organic carbon source achieved a growth rate of 0.86 day\(^{-1}\) whereas algae grown mixotrophically (with 3% glycerol as organic carbon source) and phototrophically achieved growth rates of 0.7508 day\(^{-1}\) and 0.137 day\(^{-1}\) respectively. The heterotrophic microalgae had more than 6 times higher growth rate than phototrophic algae. This is because the most significant parameter for the growth of phototrophic microalgae is light intensity, while organic carbon as a sole carbon source significantly affects to the growth of heterotrophic microalgae, as heterotrophic microalgae grow under lightless condition [66]. Thus, the reason for the slower growth rate of phototrophic microalgae observed in this study is suspected to result from photo inhibition due to high microalgae cell density. In several studies, heterotrophic microalgae presented more than 3-4 times higher growth rate than phototrophic microalgae [192,193].

In mixotrophic conditions, more than five times higher microalgae growth was observed compared to that in phototrophic condition. Similar observation was reported by
Bhatnagar et al. when *C. minutissima* was cultured in autotrophic, mixotrophic and heterotrophic conditions; the growth rate in mixotrophic condition was more than 2 times and 7 times higher than that in heterotrophic and autotrophic conditions respectively [194]. However, when microalgae growth rate was compared between heterotrophic and mixotrophic conditions in this study, the growth rate in mixotrophic conditions was less than in heterotrophic condition. The low growth rate in mixotrophic culture compared to heterotrophic culture might be due to low light intensity supplied to the mixotrophic culture. According to Chojnacka and Noworyta, when microalgae growth rate was compared between mixotrophic and heterotrophic conditions, the growth rate in mixotrophic conditions with light intensity of 33 W m$^{-2}$ was higher than in heterotrophic conditions while the growth rate in mixotrophic conditions with light intensity of 17 W m$^{-2}$ was less than in heterotrophic conditions [195]. Therefore, light intensity is an important factor for mixotrophic cultivation affecting to microalgae growth, and the slower growth rate in mixotrophic conditions than in heterotrophic conditions observed in this study is probably due to reduced light intensity.
Figure 5.65. Growth of *C. vulgaris* under mixotrophic and heterotrophic conditions with glycerol as organic carbon source.

Figure 5.66. Growth of *C. vulgaris* under mixotrophic and heterotrophic conditions with glucose as organic carbon source.

In addition, the growth rates of mixotrophic culture supplied with 1% and 2% glycerol are 0.382 and 0.406 day$^{-1}$ respectively and growth rates of heterotrophic culture supplied with 1% and 2% glycerol are 0.666 and 0.697 day$^{-1}$ respectively. Similarly, the growth rates of mixotrophic culture supplied with 1% and 2% glucose are 0.599 and
0.579 day\(^{-1}\) respectively and growth rates of heterotrophic culture supplied with 1% and 2% glycerol are 0.516 and 0.581 respectively. There was no significant difference in the growth rate in culture mediums supplied with 1% and 2% organic carbon under mixotrophic and heterotrophic conditions. This is because the growth dynamics parameters (specific growth rate, biomass content and productivity) of the algae may not be affected by the amount of organic carbon content in the medium until certain concentration (i.e. 2% of organic carbon). The same observation was reported by Chojnacka et.al in his study. No change in growth was observed when 5 and 10 g/L of glycerol (1% and 2% of organic carbon) were added to the medium containing Spirulina sp. [195]. But after certain organic carbon concentration, growth parameters would affected directly by amount of carbon present in the medium. This is because at high concentration of organic carbon under mixotrophic conditions, the adenosine triphosphate formed in the photochemical reactions accelerates the organic carbon anabolism in the mixotrophic culture of eukaryotic cells [196]. This might be the reason why the growth of eukaryotic cell “C.vulgaris” increased significantly when organic carbon concentration was increased from 2% to 3% glycerol under mixotrophic conditions.
<table>
<thead>
<tr>
<th>Mixotrophic condition</th>
<th>Organic carbon source</th>
<th>Growth rate (day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycerol (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.382</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.406</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.7508</td>
</tr>
<tr>
<td></td>
<td>Glucose (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.579</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterotrophic condition</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Organic carbon source</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.666</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.697</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Glucose (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.516</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.581</td>
</tr>
<tr>
<td></td>
<td>Phototrophic condition</td>
<td>0.137</td>
</tr>
</tbody>
</table>
5.2.5.2 Heavy Metal Removal

The previous section results showed that algae biomass concentration can be increased by growing *C. vulgaris* under mixotrophic or heterotrophic conditions. To investigate the efficiency of mixotrophically grown algae to remove heavy metals, algae that was grown under mixotrophic conditions was used to treat metal contaminated water having a concentration of 60 ppm of Cr$^{6+}$ ions. Figure 5.67 compares the growth of *C. vulgaris* under mixotrophic and phototrophic conditions. Glucose (1 g/L) was added as an organic carbon source to mixotrophic culture along with carbon dioxide. After 168 hours of cultivation, mixotrophic culture reached a biomass concentration of ~3g/L whereas phototrophic culture almost reached its saturation phase. At this point (after 168 hours) 60 ppm of Cr$^{6+}$ ions was introduced into both the mixotrophic and phototrophic cultures. Then, samples were collected on daily basis and analyzed for Cr ions to compare the chromium metal removal capabilities of both cultures.

![Graph showing the growth of *C. vulgaris* under mixotrophic and phototrophic conditions.](image)

Figure 5.67. Growth of *C. vulgaris* under mixotrophic and phototrophic conditions.
Figure 5.68 compares the removal of hexavalent chromium by mixotrophic and phototrophic culture of *C. vulgaris*. It was observed that mixotrophic culture removed 61.92% of Cr\(^{6+}\) i.e. 37.15 mg/l in 144 hours whereas phototrophic culture removed 52.24% of Cr\(^{6+}\) i.e. 31.34 mg/l in 144 hours. The percentage removal of chromium increased with time in both the species. Figure 5.63 shows the growth curves of mixotrophic and phototrophic cultures during hexavalent chromium removal. Initially, during the first 24 hours, an increase in growth was observed in both the cultures. This is because the chromium that was removed from the wastewater during the first 24 hours stays on the algae surface without inhibiting the cell growth. But after 24 hours, the chromium enters into the cytoplasm of the algae cell and therefore decreases the growth of algae as shown in Figure 5.69.

Figure 5.68. Chromium removal by mixotrophic and phototrophic cultures of *C. vulgaris*. 
No studies were performed so far in the literature to treat heavy metal contaminated water with mixotrophically grown algae. The results of this experiment prove that mixotrophically grown algae can be used to remove heavy metals from the wastewaters.

5.3 Application of Algal Bioremediation for Wastewater Treatment at Industrial Complexes

To apply algae based bioremediation for industrial wastewater cleanup at industrial complexes for both commercial and air force freight industry, UDRI contacted the wastewater treatment plant manager at Hill Air Force Base to determine plant wastewater conditions. During those communications, it was mentioned that a high concentration of Cr (as high as ~ 50000 ppm) can be present in the wastewater at Hill AFB. Therefore, taking Hill AFB as a potential model wastewater treatment plant to demonstrate concept, experiments were conducted at lab scale to verify the capability of *C. vulgaris* to clean metal laden wastewater having a high concentration of Cr.
High concentration Cr (VI) removal experiments were performed in falcon centrifuge tubes of volume 50 ml containing 30 ml of Cr (VI) stock solution at ~35000 ppm and 6 g of wet biomass. Metal-bearing suspensions were kept at 25 °C on a vortexer for 24 hours. At regular time intervals, 2 mL samples were collected from the bottles, filtered and analyzed for residual Cr (VI) concentration and total chromium concentration in solution. Figure 5.70 shows the variation of chromium percent removal with time for C.vulgaris. It can be observed from the Figure 5 that the maximum chromium removal (~30%) was achieved within the first 60 minutes. After 1 hour (60 minutes), no significant change in the percentage removal was observed. A total of 10959 ppm of Cr (31%) was removed at the end of 24 hours. After 24 hours, algae metal suspension was centrifuged to separate algae from metal solution. The separated metal solution having a Cr concentration of 23,744 ppm was again treated with 6 g of fresh wet algae paste for 24 hours. A sample of 2 ml was taken at the end of 24 hours to see the chromium removal efficiency of C.vulgaris. Results of the analysis showed that a total of 9,129 ppm of Cr (38%) was removed from the metal contaminated water. Figure 5.71 compares the chromium removal of C.vulgaris for two biomass treatment cycles for 1 hour. Similarly, an experiment was performed to investigate the capability of C.vulgaris to remove a high concentration of cadmium from wastewater. Figure 5.72 shows the cadmium removal capability of C.vulgaris. A total amount of 13,093 ppm of Cd was removed from the wastewater in 1 hour. The results of these experiments proved that C.vulgaris was capable of removing high concentrations of Cd and Cr from wastewater.
Figure 5.70. Variation of Chromium percent removal with time by *C. vulgaris*.

Figure 5.71. Comparison of chromium removal for two biomass treatment cycles for 1 hour.
The release of live microalgae with heavy metals binding on their surface into the environment has the possibility to cause greater harm than good. Algae having heavy metals binding on their surface would accelerate the biogeochemical cycling of heavy metals and their accumulation in food chains. Therefore, necessary measurements should be taken to preclude the release of metal bonded algae in any bioremediation application.

Chemical extraction techniques can be used to recover metals from algae surface. Some of the chemical extractions are: (a) easily soluble metals are soluble in water or MgCl$_2$ at pH 7 (b) metals associated with carbonate phases can be recovered using a weak acid with pH ranging between 2 to 5 (c) EDTA can be used to recover heavy metals such as Cd and Cr from algae surface as discussed in the previous sections (d) Iron and manganese compounds can be recovered using hydroxylamine hydrochloride. In addition to these techniques, biomass (algae) gasification can also be performed to the metal
bounded algae to produce methane. Figure 5.73 shows the schematic representation of algae gasification to produce methane [207].

Figure 5.73. Schematic representation of metal bounded algae gasification.

During this process, the metal bounded algae slurry is initially fed into a preheater where it absorbs heat from the effluent leaving from gasification and methanation reactor. At this stage, the algae slurry will act as a compressible liquid. It was then fed into a superheater and heated to 400 °C where it absorbs heat from natural gas fired heater. At this point, the fluid is supercritical as it has passed the critical point of water, 374 °C.

Two changes occur at this stage; firstly, the dissolved nutrient salts will precipitate out.
Secondly, the algae begin to degrade into a syngas. Salts are removed and the desalted algae slurry is fed into gasification and methanation reactor where it interacts with a Ru/C catalyst. The reaction converts the incoming algae material to a mixture of gasses such as carbon monoxide, carbon dioxide, methane, hydrogen etc. (4-9). This reaction is exothermic and heat produced during this reaction is used to heat the incoming algae slurry. The gas mixture was then passed through a carbon dioxide scrubber to separate carbon dioxide and was fed into methane purification chamber to obtain natural gas. A secondary product hydrogen is also produced during this process. The metals bounded to the algae surface will be present in the gasification ash and this ash can be used as an ingredient during cement production.

5.5 Design of Algae-based Wastewater Treatment Technology (AWTS)

This report addresses major considerations for designing commercial algae based wastewater treatment system (AWTS) to treat industrial wastewater containing high amounts of nitrates, phosphates and heavy metals. AWTS is a technology that is cost effective, requires low maintenance, and has minimal operator requirements. This design is divided into two sections: the first section being devoted to algae growth under heterotrophic conditions and other for treating industrial wastewater. Both these sections are discussed in more detail below.

In this design open raceway pond was used for treating wastewater, a technology already used in commercial microalgae production plants and some pilot scale biofuels projects. The design of this report differs from existing commercial designs in cultivating the algae heterotrophically in a continuous stirred tank reactor prior to treating wastewater. It was demonstrated in our lab scale system and studies conducted by Bashan.
et.al [197] shows that heterotrophically grown algae can efficiently clean wastewater containing nitrates, phosphates and heavy metals (Cr, Cd). A correlation was developed from the experimental studies for metal uptake of algae (*chlorella vulgaris*) as a function of biomass concentration, initial metal concentration, time, pH, temperature and light intensity. Using this correlation, for a given contaminant (nitrates, phosphates, metals) concentration in the raceway pond, contact time and initial biomass needed to treat wastewater can be known. The results of the lab scale experiments conducted at UDRI proved that cost and time to clean wastewater can be reduced considerably by growing algae heterotrophically initially as lag phase of algae can be avoided in the raceway pond. Also, known concentration of algae can be introduced directly into the pond depending on the contaminant present in the wastewater.

5.5.1 Technology Assumptions

This report assumes that a piping network or some other collection system (e.g., tank trucks) is in place that carries wastewater from individual sources to the AWTS plant. It also assumes that carbon dioxide from flue gas was separated and stored near treatment facility to feed the algae. Paddlewheels, on a 2-hp, 1-phase, 230V/60 Hz motor approximately 2.5m wide were used to mix the pond. Paddle wheel rotates providing a current of 25cm/s around the pond to degasify dissolve oxygen in the pond and to ensure that carbon dioxide is uniformly distributed over the pond.

Volume of wastewater treated in AWTS design was assumed to be 200,000 liters. To meet this volume, a heterotrophic growth chamber of volume 25 m³ and a race way pond of 1000 m² area were designed. Race way pond is 77m in length, 15 m wide, and 20 cm in depth corresponding to a volume of 200,000 liters. Growth chamber is assumed to
be cylindrical having a diameter of 4m and height of 2m. For each batch of wastewater (200,000 liters) treated in the pond, glucose concentration of 5 g/L will be used as an organic carbon source for heterotrophic cell growth in the growth chamber containing 40,000 liters of algae solution. A pilot scale microfiltration system followed by centrifugation was installed at the harvest station of the race way pond.

5.5.2 Site Location and Climatic Conditions

Generally, tropical or semi-tropical areas are the preferable locations for treating wastewater with this technology. But to implement this design in cold climatic locations, geothermal systems are to be installed near the pond surface to maintain the temperature shown in Table 5.6 for algae to remove contaminants from wastewater. The following parameters are to be considered while selecting location for wastewater treatment.

(a) Evaporation: Evaporation is a major problem in dry tropical areas. A high evaporation rate increases salt concentration in wastewater and pumping cost due to water loss.

(b) Humidity: With high relative humidity and no winds the water may heat up (even to 40 °C). With low humidity, high rates of evaporation occur that can have a cooling effect on the medium. The best humidity is at an average humidity of 40%.

(c) Location: Site close to the wastewater treatment plant and heavy metal industry would be ideal as there won’t be too much cost (long piping can be avoided) involved for transporting waste effluent to the AWTS facility.
5.5.3 Design Theory

A schematic of the AWTS bioreactor set-up that is being designed for treating wastewater is shown in Figure 5.7.6. It mainly consists of the heterotrophic growth chamber (CSTR) of volume 25 m$^3$ and an open race way pond of area 1000 m$^2$. Both these sections are discussed in detail below.

(a) Heterotrophic growth chamber

The components that make up the heterotrophic growth chamber include the bioreactor, compressor and mixer. Reactor was jacketed to maintain constant temperature throughout the process. All sensors (temperature, level, pH, pressure and dissolved oxygen) are installed within the bioreactor. An impeller driven by a motor was installed inside the reactor to mix the feed and to keep algae solution homogenous. Compressed air was passed through the air filter and introduced at the bottom of the bioreactor through a sparger. Bioreactor was equipped with air outflow and safety pressure relief valve protected from contamination.

To design a cost effective bioreactor, the starting point of the design optimization is to choose a bioreactor size, with specific algae of known biomass concentration (0.1 g/L). A flowchart of the entire design optimization strategy used is given in Figure 5.7.4 [198]. The details of each step, with accompanying equations, constraints, and references follow. The main focus of the strategy is to minimize the cost of the CSTR. The total cost includes both the capital cost of the equipment and the operating cost.
Figure 5.74. Flow chart of the design optimization strategy.
**Flow chart 1:** A Bioreactor volume of 60 m$^3$ was designed in this process.

**Flow chart 2:** To scale up a process from the laboratory scale to a large scale at which it is commercially feasible, aspect ratio should be kept constant. Aspect ratio for lab scale reactor is 2. \(\frac{h}{d} = \text{height of the bioreactor/diameter of the bioreactor} = 24 \text{ cm} / 12 \text{ cm} = 2\).

The assumed values for large scale bioreactor are given below.

Height of the growth chamber (bioreactor) = 6.6 m

Diameter of the growth chamber (bioreactor) = 3.3 m

Volume of the growth chamber = \(\pi r^2 h = 60 \text{ m}^3\)

**Flow chart 3:** A working volume of 40 m$^3$ was assumed initially to design the bioreactor. Working volume would be 2/3$^{rd}$ of tank height to achieve uniform mixing throughout the reactor.

**Flow chart 4:** Scale up parameters should be defined for effective operation of the bioreactor.

**Flow chart 5:** Mixing time and selection of impeller are agitation based parameters.

**Flow chart 5.1:** The impeller selected was a standard six blade radial flow design used most often in bioreactors as shown in Figure 5.75. It creates a turbulent flow pattern that improves mixing and uniformity.

![Diagram](image)

Figure 5.75. Impeller \(D_1/w = 5\); \(D_1 = 0.40 \text{ d}\).
Where \( D_1 \) = diameter of the impeller

\[ d = \text{diameter of bioreactor} \]

\[ w = \text{width of the impeller} \]

*Flow chart 5.2.*: The minimum agitation rate is required to create a homogeneous environment. Theoretically, the minimum stirring (for stirring the liquid media alone) determined from [199]

\[
N_{MIN} = \left(1.22 + 1.25 \frac{d}{D_1}\right) \left(\frac{\sigma g}{\rho_L}\right)^{0.25} \frac{1}{D_1} \quad \text{Eq. 5.13}
\]

\[
d/ D_1 = 1/0.4 = 2.5
\]

\[
g = 980 \text{ cm/s}^2
\]

\[
\sigma = \text{surface tension of water} = 72.8 \text{ dyne/cm}
\]

\[
\rho_L = \text{density of liquid} = 1 \text{ g/cc}
\]

If we substitute these values in eqn (1),

\[
N_{min} = 53.79 \text{ rpm}
\]

Based on scale up factor agitation rate was given by

\[
N_2 = N_1 \left(\frac{D_1}{D_2}\right)^{1/4}
\]

\( N_2 \) agitation speed in scale-up

\( N_1 \) agitation speed in scale-down

\( D_1 \) impeller diameter of scale-down

\( D_2 \) impeller diameter of scale-up

If we substitute lab scale values (scale-down),

\[
N_2 = 125 \left(0.12 / 3.3\right)^{0.25} = 54 \text{ rpm}
\]
Hence experimental agitation rate validate the theoretical value and this is the minimum agitation rate that has to be maintained for homogeneity.

Flow chart 6.1.: The aeration rate is optimized to meet the oxygen demand of cultured cells and to take out the dissolved carbon dioxide from the reactor. It is usually represented in vvm which means the vessel volumes per minute. A low initial guess of 0.1 vvm is assumed so,

\[ Q = \left( \frac{0.1}{60} \right) V_W, \text{ m}^3/\text{sec} \] \[ \text{Eq.5.14} \]

This is the starting point for the mathematical optimization of aeration rate, Q, for cost minimization during the CSTB design procedure. The superficial gas velocity is then given by:

Superficial velocity = \( v_s = \frac{Q}{\pi d^2/4} \), m/s

Flow chart 7: The required compressor power needed for aeration is determined by the

\[ W_C = \frac{2 \times R \times 298 \gamma_{\text{AIR}}}{M_{\text{AIR}}(\gamma-1)} \left( \frac{\gamma-1}{\gamma} - 1 \right) \] \[ \text{Eq.5.15} \]

\( W_C \) = compressor power, kW
\( \gamma \) = ratio of specific heats
\( M_{\text{AIR}} \) = molecular weight, air, g/mol

Flow chart 8: Power needed for stirring the aerated reactor, [202]

\[ P_G = \frac{0.80}{(1-\varepsilon_G)N_A^{0.38}N_{\text{WE}}^{0.25}} P_0 \] \[ \text{Eq.5.16} \] (valid for 0 < N_A < 1200)

\( P_0 \) = power of unaerated reactor = \( N_P \rho_L N^3 D_1^5 \)

where \( N_P \) = power number = 2.5737 \( N_{\text{RE}}^{0.0614} \) (valid for 66 < \( N_{\text{RE}} \) < 50,000)

\[ N_P = 13.131 \times 10^{-0.2775} \] (valid for conditions 1 < \( N_{\text{RE}} \) < 100,000; \( N_{\text{RE}} > 100,000 \) )
\[ N_{RE} = \frac{\rho L N D_1^2}{\mu} \]

\[ N_A = \text{aeration number} = \frac{Q}{N D_1^2} \quad \text{and} \quad N_{WE} = \text{weber number} = \frac{N^2 \rho L D_1^3}{\sigma} \]

\[ \varepsilon_G = \text{gas holdup} = 0.113 \left( \frac{QN^2}{\sigma} \right)^{0.57} \] \[ [203] \]

and \( N \) = agitation speed, rev/s

\( Q \) = aeration rate, m\(^3\)/s

\( \sigma \) = surface tension of water

\( \rho_L \) = density of liquid

\( D_1 \) = diameter of the impeller

*Flow chart 9:* The total external power required by the CSTB is the sum of power used by compressor and mixer.

\[ P_{EXT} = \eta_c W_c + \eta_M (P_d/1000) \]

\( \eta_c \) = number of compressors

\( \eta_M \) = number of mixers

*Flow chart 10 & 11:* Cost of the bioreactor, mixer and compressor was given in the following section based on the present market price.

Usually the bioreactor capital cost is assumed based on six-tenths-factor rule. According to this rule, if the cost of given unit at one capacity is known, the cost of a similar unit with \( X \) times the capacity of the first is approximately \((X)^{0.6}\) times the cost of initial unit \[200\].

Cost of bioreactor to be scaled =

\[ \text{(Known bio reactor cost) } \times \text{(Capac. of scaled bioreactor/Capac. of known bioreactor)}^{0.6} \]
(b) Open race way pond

The 'raceway pond' is a large open water raceway track where algae and wastewater are pumped around by a motorized paddle. The depth of race way pond used in this design is 20 cm corresponding to a volume of 200,000 liters. The pond is 15x77m, plastic lined with a central wooden divider. Usually open ponds records an average water loss of 6.2 mm per day due to evaporation. The evaporation rate is a function of wind velocity, temperature and solar radiation over the pond. To avoid water losses, race way ponds in the present study are covered with a hoop-structure (plastic hoop greenhouses) that will prevent water losses to a greater extent.

Sensor equipment is installed within the pond for monitoring conductivity, pH, algal density, temperature, dissolved oxygen and dissolved carbon dioxide. Biosensors are also installed to detect the concentration of nitrate, phosphate and heavy metal in wastewater. Pond will be constantly mixed with paddle wheel to maintain cells in suspension, to provide good contact between algae and wastewater. Ample amount of carbon dioxide was infused into the pond all the time. The CO$_2$ is injected into the algae pond in the form of bubbles and the bubblers are spaced around the pond so that the CO$_2$ is evenly dispersed throughout the pond. A pilot scale microfiltration followed by centrifugation is installed at the harvesting station to make dewatering process economical.

5.5.4 Design Principle

This section is a step by step walk through of the Algae based Wastewater Treatment System (AWTS). Figure 5.76 shows the schematic representation of entire process. Initially, growth media (BBM) of volume 30 m$^3$ will be fed into the bioreactor
along with algae solution of volume 10 m\(^3\) having a concentration of 0.1g/L. Glucose will be supplemented to growth media at a concentration of 5 g/L. The culture will be stirred by continuous bubbling of sterile air. Microalgae are known to grow rapidly in presence of organic carbon source under heterotrophic conditions and the algae will metabolize glucose in the dark with a doubling time of 12 to 16 hours (experimental observation). The time that algae will grow heterotrophically will depend on the contaminant that is treated in the race way pond as the initial biomass concentration is different for different contaminants (nitrates, phosphates and heavy metals) in wastewater. This heterotrophically grown algae will be pumped to raceway pond to inoculate a volume of 200,000 liters. The inoculate volume also depends on the type of contaminant present in the wastewater. Table 5.6 presents the pH, temperature, residence time and initial biomass concentration values required for a given contaminant concentration.

Once the algae reaches required concentration in the growth chamber, 80% of the volume will be transferred to the pond containing the wastewater. For a given contaminant concentration in wastewater, residence time can be known from the Table 5.6 and algae will be grown in wastewater for that residence time with uniform mixing and constant supply of CO\(_2\). The contaminant concentration in wastewater will be monitored on regular basis and once it reaches below permissible limit, the algae suspension from the pond is fed into the pilot microfiltration system across the membrane maintaining a positive pressure at the permeate.
Figure 5.76. Schematic representation of AWTS
Table 5.6. Operation parameters for contaminant removal

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Initial concen. in the influent (ppm)</th>
<th>Initial dry biomass concen. (g/L)</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>Residence time in the pond (days)</th>
<th>Final concen. in the effluent (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>187</td>
<td>0.2</td>
<td>6.7</td>
<td>27</td>
<td>7</td>
<td>&lt; 2 ppm</td>
</tr>
<tr>
<td>Phosphate</td>
<td>31</td>
<td>0.327</td>
<td>6.7</td>
<td>27</td>
<td>7</td>
<td>20 ppm</td>
</tr>
<tr>
<td>Chromium</td>
<td>30</td>
<td>1.45</td>
<td>6.7</td>
<td>27</td>
<td>2</td>
<td>&lt; 2 ppm</td>
</tr>
<tr>
<td>Cadmium</td>
<td>150</td>
<td>1.53</td>
<td>6.7</td>
<td>27</td>
<td>1</td>
<td>&lt; 4 ppm</td>
</tr>
</tbody>
</table>

The dewatering process (filtration process) will be operated in a closed loop with pond. A portion of the feed (algae suspension) which is smaller than the membrane pore size (< 0.2 µ) passes through the membrane as permeate (usually clean water); everything else is retained on the feed side as concentrate and is recycled into the pond. The process is upheld till a major volume of the pond is drained off as clean water. Then, the remaining concentrated algae in the pond will be pumped into a large centrifuge. By doing filtration followed by centrifugation, lot of energy can be saved during harvesting process as the volume of algae sent to the centrifuge is reduced considerably.
During centrifugation, the water was forced out and algae will be settled to the bottom of a flask in the form of a paste at about 25% algae. Half of the water coming out from the centrifuge is recycled into the heterotrophic growth chamber to grow algae and provide inoculant volume for the next cycle and remaining half will be discharged out as clean water. The harvested algae paste will be processed for oil extraction or gasification.

5.5.5 Cost Estimation

Described below are the design, construction, operations, and estimated costs of the AWTS system that uses a paddle-wheel mixed race way pond to treat wastewater. The detail calculations are given in Appendix A. Construction costs include cost of land, impeller, turbine, pumps, flow meters, lining material, paddlewheel, sensors, and installation of harvesting system. Some of the key components in a race way pond system design are discussed in detail below. The cost for pond construction [204] is given in Table 5.7. The cost of other components used in the design, installation costs of equipment and total cost involved in AWTS design is given in Table 5.8, 5.9 and 5.10 respectively.
<table>
<thead>
<tr>
<th>Item Description</th>
<th>Details</th>
<th>Item Description</th>
<th>Description</th>
<th>Qty</th>
<th>Unit Quote</th>
<th>Extended Quote</th>
<th>Total Quotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>~15M x 75M Liner Material</td>
<td>A</td>
<td>Rein. Poly Prop Pond</td>
<td>Materials</td>
<td>1</td>
<td>$32,200</td>
<td>$32,200</td>
<td>$32,200</td>
</tr>
<tr>
<td>PVC sparger</td>
<td>B</td>
<td>Paddle Wheel Assy</td>
<td>8</td>
<td>$210</td>
<td>$1,680</td>
<td>$1,680</td>
<td></td>
</tr>
<tr>
<td>Paddle Wheel Stand</td>
<td>C</td>
<td>Paddle Wheel Assy</td>
<td>1</td>
<td>$22,400</td>
<td>$22,400</td>
<td>$22,400</td>
<td></td>
</tr>
<tr>
<td>Curve Baffles--small For Stable flow</td>
<td>D</td>
<td>Paddle Wheel Stand</td>
<td>1</td>
<td>$3,640</td>
<td>$3,640</td>
<td>$3,640</td>
<td></td>
</tr>
<tr>
<td>Curve Baffles--large For stable flow</td>
<td>E</td>
<td>Curve Baffles--small</td>
<td>5</td>
<td>$231</td>
<td>$1,155</td>
<td>$1,155</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>Curve Baffles--large</td>
<td>2</td>
<td>$315</td>
<td>$630</td>
<td>$630</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$58,996</td>
<td>$61,705</td>
<td></td>
</tr>
<tr>
<td>Mech. Engineer</td>
<td>H</td>
<td>Engineering/Design</td>
<td>Std Engineering Design, Analysis</td>
<td>52</td>
<td>$125</td>
<td>$6,500</td>
<td>$6,500</td>
</tr>
<tr>
<td>Designer</td>
<td></td>
<td>Std Engineering Design, Drafting</td>
<td>60</td>
<td>$85</td>
<td>$5,100</td>
<td>$5,100</td>
<td></td>
</tr>
<tr>
<td>Install Engineers</td>
<td></td>
<td>Installation based on Travel and Time, Lodging etc.</td>
<td>1</td>
<td>$11,900</td>
<td>$11,900</td>
<td>$11,900</td>
<td></td>
</tr>
<tr>
<td>Mech. Engineer w/grading, site improvement requirements</td>
<td>III</td>
<td>Documentation</td>
<td>32</td>
<td>$163</td>
<td>$5,200</td>
<td>$5,200</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.8. Other costs involved in AWTS design

<table>
<thead>
<tr>
<th>Component</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotrophic growth chamber (assuming 30,000 liters)</td>
<td>$280,000</td>
</tr>
<tr>
<td>Impeller</td>
<td>$5000</td>
</tr>
<tr>
<td>Agitator</td>
<td>$2500</td>
</tr>
<tr>
<td>Paddle wheel</td>
<td>$1251.53</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>$500,000</td>
</tr>
<tr>
<td>Sensors</td>
<td>$3,000</td>
</tr>
<tr>
<td>Flow meters</td>
<td>$800</td>
</tr>
<tr>
<td>Type of equipment</td>
<td>Installation cost, %</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Pumps</td>
<td>25-60</td>
</tr>
<tr>
<td>Gasifiers</td>
<td></td>
</tr>
<tr>
<td>Pellet mill</td>
<td></td>
</tr>
<tr>
<td>Organic carbon source, (glucose) / ton</td>
<td></td>
</tr>
<tr>
<td>Chemicals,(Sodium hydroxide)/ ton 33</td>
<td></td>
</tr>
<tr>
<td>Plastic hoop (1000 m²)</td>
<td></td>
</tr>
<tr>
<td>Land cost (1 acre)*</td>
<td></td>
</tr>
</tbody>
</table>

*Dayton real estate

Table 5.9. Installation cost for equipment as a percentage of the purchased-equipment cost.
Table 5.10. Total cost estimation for AWTS design

<table>
<thead>
<tr>
<th>Component</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Land cost (1 acre)*</td>
<td>$37,550</td>
</tr>
<tr>
<td>Geo thermal systems</td>
<td>$27,500</td>
</tr>
<tr>
<td>Pond installation cost</td>
<td>$95,815</td>
</tr>
<tr>
<td>Installation and other cost</td>
<td>$1,378,020</td>
</tr>
<tr>
<td><strong>Total cost</strong></td>
<td><strong>$1,538,885</strong></td>
</tr>
</tbody>
</table>

*Dayton Real Estate Est.

**Class 2 estimate (±25%)

5.5.6 Conventional Process vs AWTS for Treating Metal Laden Water.

This section compares the cost between the conventional process and AWTS in treating industrial wastewater containing Cr as heavy metal pollutant. Consider treating a wastewater containing Cr at a concentration of 60 ppm at a facility generating 30,000 GPD and 10 million gallons per year. Table 5.11 compares the cost of conventional process vs. the AWTS.
Table 5.11. Conventional process vs AWTS for treating metal laden water.

<table>
<thead>
<tr>
<th>Cost description</th>
<th>Conventional process [205]</th>
<th>AWTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Capital cost</strong></td>
<td>$1,219,205</td>
<td>$1,538,885</td>
</tr>
<tr>
<td><strong>Annual operating costs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct materials</td>
<td>$8,850</td>
<td>$21,030</td>
</tr>
<tr>
<td>Utilities</td>
<td>$55,650</td>
<td>$9,800</td>
</tr>
<tr>
<td>Direct labor</td>
<td>$163,390</td>
<td>$48,180</td>
</tr>
<tr>
<td>Waste Management</td>
<td>$67,000</td>
<td>$0</td>
</tr>
<tr>
<td>Regulatory compliance</td>
<td>$27,500</td>
<td>$0</td>
</tr>
<tr>
<td>Revenues (By-products)</td>
<td>$0</td>
<td>$31,943</td>
</tr>
<tr>
<td><strong>Net Annual operating costs</strong></td>
<td>$322,390</td>
<td>$47,067</td>
</tr>
</tbody>
</table>

From the Table 5.11 it can be observed that metal laden wastewater annual clean-up cost can be reduced by 7 times using AWTS design. Return on investment is 4.7 years and an estimated amount of over 20 million can be saved over a period of 5 years if we install AWTS in 14 DOD locations.

Also, a cost assessment was performed for treating municipal wastewater. Consider treating a total of 10 MGD wastewater with a nitrogen concentration of 8 ppm and phosphorus concentration of 10 ppm. The conventional process uses two separate systems to remove N, P whereas in AWTS both N, P can be removed together in the same
system. Table 5.12 compares the cost of the conventional process vs. AWTS for treating municipal wastewater.

Table 5.12. Conventional process vs. AWTS for treating municipal wastewater.

<table>
<thead>
<tr>
<th>Cost description</th>
<th>Conventional process [206]</th>
<th>AWTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capital cost (for N removal)</td>
<td>$4,927,000</td>
<td>$1,538,885</td>
</tr>
<tr>
<td>Capital cost (for P removal)</td>
<td>$6,969,000</td>
<td></td>
</tr>
<tr>
<td><strong>Total capital cost</strong></td>
<td><strong>$11,896,000</strong></td>
<td><strong>$1,538,885</strong></td>
</tr>
<tr>
<td>O &amp; M Cost (for N removal)</td>
<td>$157,469</td>
<td>$79,010</td>
</tr>
<tr>
<td>O &amp; M Cost (for P removal)</td>
<td>$1,095,000</td>
<td></td>
</tr>
<tr>
<td>Revenues (By-products)</td>
<td>$0</td>
<td>$31,943</td>
</tr>
<tr>
<td><strong>Net Annual operating costs</strong></td>
<td><strong>$1,252,469</strong></td>
<td><strong>$47,067</strong></td>
</tr>
</tbody>
</table>

From Table 5.12 it can be observed that the capital cost of the AWTS system is approximately 3 times less than the conventional process and wastewater clean-up cost can be reduced by twenty six times. The return of investment is less than a year and considering 10 different DOD locations, an estimated amount of 60 million can be saved if we replace the conventional process with AWTS. If we install algae ponds near water reclamation plants, wastewater coming from the secondary clarifier in the conventional process can be routed to the algae pond batch wise for the removal of nitrogen and phosphorus from wastewater. However, more experiments have to be conducted with real municipal and industrial wastewater at larger scale in outdoor conditions in order to
evaluate real concerns that could hinder implementing algae based wastewater treatment systems.
6.1. Summary

This study focused on evaluating and demonstrating potential of microalgae for bioremediation of wastewater laden with nitrogen (N) in the form of nitrates, phosphorous (P) in the form of phosphates, chromium (Cr(VI)) and cadmium (Cd(II)). After screening several microalgae, Chlorella vulgaris and algae taken from Pleasant Hill Lake were chosen as candidate species for this study. The viability of the process was demonstrated in laboratory bioreactors and key parameters that would affect remediation rates were studied and correlations were developed to enable customizing and designing a commercial AWTS. A commercial AWTS system that can be easily customized and is suitable for integration into existing wastewater treatment facilities was developed, and capital cost estimates for system including installation and annual operating costs were determined.

The work concludes that algal bioremediation is a viable alternate technology for treating wastewater in an economical and sustainable way when compared to conventional treatment processes. The annual wastewater treatment costs to remove N, P is ~26x lower and to remove Cr, Cd is 7x lower than conventional treatment processes. The cost benefit analysis performed shows that if this technology is implemented at Air
Force and other Department of Defense installations with wastewater treatment plants, it could lead to millions of dollars in savings that could be repurposed for meeting the warfighters needs.

### 6.2 Future Work

- It is recommended to conduct experiments to assess removal of other toxic heavy metals such as Arsenic (As) and Mercury (Hg) from industrial wastewater using Chlorella vulgaris.

- It is recommended to conduct experiments with the combination of various toxic heavy metals in wastewater to investigate the synergistic/antagonistic effect on algae metal removal efficiency.

- It is recommended to conduct experiments to remove heavy metals from wastewater using combination of algae strains and compare its metal removal efficiency with single strain.

- It is also recommended to conduct experiments with real metal laden wastewater in outdoor conditions at larger scale in order to evaluate the efficiency of the algae bioremediation at commercial scale.

- It is recommended to perform chemical extraction techniques as well as the gasification experiments for metal bounded algae to investigate the percentage of metal recovery from algae surface.

- It is recommended that long term experiments with the integrated system combining the AWTS with the existing wastewater treatment plants are conducted to assess its performance limit.
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47. Illman AM, Scragg AH and Shales SW. 2000. Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology. 27 (8): 631-635.


75. Tam NFY and Wong YS. 1995. Wastewater treatment with microorganisms. The commercial Press (H.K.) Ltd. 2D Finnie St. Quarry Bay, Hong Kong.


treatment condition Korean Journal of Applied Microbiology and Biotechnology. 26: 76-82.


204. Personal Communication from NanoVoltaics Inc: Algae, Nanomaterials, Solar.


APPENDIX A

Cost Estimation

A1. Total capital cost estimation for algae based wastewater system

<table>
<thead>
<tr>
<th>Component</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Land cost (1 acre)*</td>
<td>$37,550</td>
</tr>
<tr>
<td>Geo thermal systems</td>
<td>$27,500</td>
</tr>
<tr>
<td>Pond installation cost</td>
<td>$95,815</td>
</tr>
<tr>
<td>Other costs</td>
<td>$1,378,020</td>
</tr>
<tr>
<td><strong>Total cost</strong></td>
<td><strong>$1,538,885</strong></td>
</tr>
</tbody>
</table>

A2. Other costs

<table>
<thead>
<tr>
<th>Component</th>
<th>Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotrophic growth chamber (assuming 30,000 liters)</td>
<td>$280,000</td>
</tr>
<tr>
<td>Impeller</td>
<td>$5,000</td>
</tr>
<tr>
<td>Agitator</td>
<td>$2,500</td>
</tr>
<tr>
<td>Paddle wheel</td>
<td>$1,251.53</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>$500,000</td>
</tr>
<tr>
<td>Sensors</td>
<td>$3,000</td>
</tr>
<tr>
<td>Flow meters</td>
<td>$800</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Pumps</td>
<td>$2,500</td>
</tr>
<tr>
<td>Gasifiers</td>
<td>$150,000</td>
</tr>
<tr>
<td>Pellet mill</td>
<td>$17,550</td>
</tr>
<tr>
<td>Organic carbon source, (glucose) / ton</td>
<td>$500</td>
</tr>
<tr>
<td>Chemicals,(Sodium hydroxide)/ ton</td>
<td>$530</td>
</tr>
<tr>
<td>Plastic hoop (1000 m²)</td>
<td>$1,076</td>
</tr>
<tr>
<td>Installation costs</td>
<td>$413,312</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>$1,378,020</strong></td>
</tr>
</tbody>
</table>

A3. Annual operating cost for AWTS

<table>
<thead>
<tr>
<th>Cost description</th>
<th>AWTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Annual operating costs</strong></td>
<td></td>
</tr>
<tr>
<td>Direct materials</td>
<td>$21,030</td>
</tr>
<tr>
<td>Utilities</td>
<td>$9,800</td>
</tr>
<tr>
<td>Direct labor</td>
<td>$48,180</td>
</tr>
<tr>
<td><strong>Total Operating cost</strong></td>
<td><strong>$79,010</strong></td>
</tr>
<tr>
<td>Revenues (By-products)</td>
<td>$31,943</td>
</tr>
<tr>
<td><strong>Net Annual operating costs</strong></td>
<td><strong>$47,067</strong></td>
</tr>
</tbody>
</table>

Direct materials cost includes centrifuge membrane replacement cost i.e. ~$20,000/year along with glucose ($500) and sodium hydroxide ($530). Utilities cost include the power consumed by the centrifuge per year.
Revenue cost:

In the present study the cost estimation was done for treating 10 mega gallons of wastewater. 10 mega gallons constitutes to 37854400 liters of water and if we consider 3g of dry algae was produced per liter of wastewater during the treatment process, then the total amount of algae produced will be 113563200 g.

Theoretically 1g of algae produces 0.012055298 MJ i.e. 0.002344 KWH.

<table>
<thead>
<tr>
<th>g of algae</th>
<th>KWH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.002344</td>
</tr>
<tr>
<td>113563200</td>
<td>266192.1408</td>
</tr>
</tbody>
</table>

Assuming 12 cents per 1 KWH, the revenue produced = 266192.1408 x 0.12

= $31.943.06.